# Evaluation of Antioxidant Activity of *Spilanthes calva, Solanum virginianum, Stevia rebaudiana, Ruellia tuberosa* and Phytochemical Investigation of *Mikania cordata*


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## CERTIFICATE BY THE SUPERVISOR

This is to certify that the dissertation entitled "**Evaluation of Antioxidant Activity of *Spilanthes calva, Solanum virginianum, Stevia rebaudiana, Ruellia tuberosa* and Phytochemical Investigation of *Mikania cordata***" is a bona-fide research work done by Md. Moshiur Rahman**,** ID: 2011-3-70-015 in partial fulfillment of the requirements for the Bachelor of Pharmacy Degree.

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## DECLARATION BY THE CANDIDATE

I hereby declare that this dissertation entitled "**Evaluation of Antioxidant Activity of *Spilanthes calva, Solanum virginianum, Stevia rebaudiana & Ruellia tuberosa* and Phytochemical Investigation of *Mikania cordata***" is an authentic and genuine research work carried out by me under the guidance of **Dr. Shamsun Nahar Khan,** Associate Professor & Chairperson, Department of Pharmacy, East West University, Dhaka, Bangladesh.


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**CERTIFICATE**

This is to certify that, the research work on "**Evaluation of Antioxidant Activity of *Spilanthes calva, Solanum virginianum, Stevia rebaudiana, Ruellia tuberosa* and Phytochemical Investigation of *Mikania cordata***" submitted to the department of pharmacy, East West University, Dhaka, Bangladesh, in partial fulfillment of the requirement for the Bachelor of Pharmacy Degree (B.Pharm) was carried out by Md. Moshiur Rahman**,** ID: 2011-3-70-015, under our guidance and supervision and that no part of the thesis has been submitted for any other degree. We further certify that all the resources of the information in this connection are duly acknowledged.

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**ACKNOWLEDGMENT**

Above all, I express my gratitude to Almighty Allah for giving me the strength and energy to carry out this research work.

I would like to express my gratitude and admiration to **Dr. Shamsun Nahar Khan**, Associate Professor & Chairperson, Department of Pharmacy, East West University, for her suggestion, careful guidance, sincere help, constructive criticism and valuable time without which I would not have been able to complete this work. She had been very enthusiastic and supportive in my research.

Moreover, I am grateful to my administration as they provide the facilities to use the laboratory for research work.

I owe my deepest gratitude to all of my **Respected Teachers** of the Department of Pharmacy, East West University, Dhaka for their continuous support, affection and sincere advice to complete my investigation.

I also acknowledge the support and help of **Laboratory assistants** and **Employees** of the Department of Pharmacy, East West University, Dhaka in doing my research work.

I am so much grateful to Md. Farhan Ahmed, GTA of the Department of Pharmacy, East West University, Mohammad Ali who is the research student of our chairperson, and my classmates for their continuous support during the laboratory work.

Lastly my special regards and gratitude go to **my Parents, my Family Members and my Friends** for their encouragement, appreciation and support throughout my work.

**Md. Moshiur Rahman**

**Dedicated to My Parents for their Continuous Support and Encouragement**

**ABSTRACT**

Antioxidant assay is widely used by the pharmaceutical industries to screen for antioxidant activity in compound libraries. In present study five plant samples were selected to conduct the antioxidant activity; these are ***Spilanthes calva, Solanum virginianum, Stevia rebaudiana, Ruellia tuberosa*** and ***Mikania cordata.*** In total phenolic content assay *Stevia rebaudiana* has the highest amount of phenolic content than other extracts. *Spilanthes calva* has the least amount of phenolic content among all extracts. DPPH radical activity of methanolic extract of *Stevia rebaudiana* is good after which is methanolic fraction of *Mikania cordata*. In Nitric oxide scavenging assay *Solanum virginianum* is good after which is methanolic extract of *Stevia rebaudiana.* In reducing power assay *Solanum virginianum* showed highest level of reducing power where as *Stevia rebaudiana and Ruellia tuberosa* showed moderate reducing power activity. Among all different fractions of *Mikania cordata* methanolic fraction showed the highest antioxidant activity. In Column chromatography, I used an open column to purify individual chemical compounds from mixtures of compounds. The methanolic extract of *Mikania cordata* was subjected to open column and different fractions were collected using four different solvents. Lastly I experimented these fractions to identify their antioxidant property.

**Keywords:** *Spilanthes calva, Solanum virginianum, Stevia rebaudiana, Ruellia tuberosa, Mikania cordata,* Total phenolic content assay, DPPH radical scavenging assay.

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# 1. Introduction

The study of disease and their treatment have been existing since the beginning of human civilization. Fossil records date human use of plants as medicines at least to the Middle Paleolithic age some 60,000 years ago (Solecki et al, 1975). From that point of traditional medicine systems incorporating plants as a means of therapy can be traced back only as far as recorded documents of their likeness. However the value of these systems is much more than a significant anthropologic or archeologist fact. Their value is as a methodology of medicinal agents, which according to the World Health Organization (WHO), almost 65% of the world's population have incorporated into their primary modality of health care (Farnsworth et al, 1985).

Since the ancient times, in search for rescue for their disease, the people looked for drugs in nature. The beginnings of the medicinal plants‟ use were instinctive, as is the case with animals (Stojanoski, 1999). In view of the fact that at the time there was not sufficient information either concerning the reasons for the illnesses or concerning which plant and how it could be utilized as a cure, everything was based on experience. Norman R. Farnsworth of the University of Illinois declared that, for every disease that affect mankind there is a treatment and cure occurring naturally on the earth. Plant kingdom is one of the major search areas for effective works of recent days. The goals of using plants as sources of therapeutic agent are –

1. To isolate bioactive compounds for direct use of drugs, e.g. Digoxin, Digitoxin, Morphine, Reserpine, Taxol, Vinblastin, Vincristine.

2. To produce bioactive compounds of novel or known structures as lead compounds for semisynthesis to produce patentable entities of higher activity.

3. To use agents as pharmacological tools, e.g. lysergic acid diethylamide, mescaline, yohimbina, and

4. To use the whole plant or part of it as a herbal remedy, e.g. cranberry, echinaceae, feverfew, garlinc, ginko biloba, St. John‟s wort, saw palmetto.

The importance of plants in search of new drugs is increasing with the advancements of medical sciences.

In time, the reasons for the usage of specific medicinal plants for treatment of certain diseases were being discovered; thus, the medicinal plants‟ usage gradually abandoned the empiric framework and

became founded on explicatory facts. Until the advent of iatrochemistry (i.e. Chemical Medicine) in 16th century, plants had been the source of treatment and prophylaxis (Kelly, 2009). Nonetheless, the decreasing efficacy of synthetic drugs and the increasing contraindications of their usage make the usage of natural drugs topical again.

Medicines, the core of health care, cure diseases (such as antibiotics), relieve symptoms (such as analgesics), are preventive (such as anti-hypertension drugs) or substitute for endogenous compounds (such as insulin). The search for medicines, which undoubtedly began in prehistorical times, has led to compounds such as morphine, atropine, tubocurarine, quinine and digoxin. Indeed, many of the present day medicines in the western world have been developed on the basis of traditional medicines with receptors and mechanisms of action identified only recently. This identification of receptors has opened ways of screening for novel, bioactive compounds and to design and subsequently synthesize similar structures (R.J. Bogers et al, 2014).

Ayurvedic medicine is a system of healing that relies heavily on herbs and other plants including oils and common spices. Currently, more than 600 herbal formulas and 250 single plant drugs are included in the "pharmacy" of Ayurvedic treatments. Historically, Ayurvedic medicine has grouped plant compounds into categories according to their effects (for example, healing, promoting vitality, or relieving pain). Ayurvedic medicine (also called Ayurveda) is one of the world‟s oldest medical systems. It originated in India and has evolved there over thousands of years. In the United States, Ayurvedic medicine is considered complementary and alternative medicine (CAM) more specifically, a CAM whole medical system. Many therapies used in Ayurvedic medicine are also used on their own as CAM for example, herbs, massage, and specialized diets.

Since time immemorial people have tried to find medications to alleviate pain and cure different illnesses. In every period, every successive century from the development of humankind and advanced civilizations, the healing properties of certain medicinal plants were identified, noted and conveyed to the successive generations. The benefits of one society were passed on to another, which upgraded the old properties, discovered new ones, till present days. The continuous and perpetual people's interest in medicinal plants has brought about today's modern and sophisticated fashion of their processing and usage (Biljana, Bauer & Petrovska, 2012).

## 1.1 Medicinal Plants

A considerable number of definitions have been proposed for medicinal plants. According to the WHO, "A medicinal plant is any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes, or which are precursors for chemo-pharmaceutical semi-synthesis." When a plant is designated as "medicinal", it is implied that the said plant is useful as a drug or therapeutic agent or an active ingredient of a medicinal preparation.

"Medicinal plants may therefore be defined as a group of plants that possess some special properties or virtues that qualify them as articles of drugs and therapeutic agents, and are used for medicinal purposes" (Ghani,1998).

As therapeutic use of plants continued with the progress of civilization and development of human knowledge, scientists endeavored to isolate different chemical constituents from plants, put them to biological and pharmacological tests and identify therapeutically active natural compounds, which have eventually used to prepare modern medicines. In course of time synthetic analogues and derivatives of the natural compounds were also prepared. In this way, ancient uses of Datura plants have led to the isolation of hyoscinic, hyoscyamine, atropine and tigloidine, Cinchona barks to quinine and quinidine, *Rauwolfia serpentina* root to reserpine and rescinnamine, *Digitalis purpurea* to digitoxin and digoxin; Opium to morphine and codeine, Ergot to ergotamine and ergametrine, Sena to sennosides, *Catharanthus roseus* to vincristine and vinblastine to mention a few (Ghani, 1998).

In addition to these, there are many other plant-derived chemical substances of known structures that are used as drugs or necessary components of many modern medicinal preparations. These include camphor, capsaicin, eucalyptol, menthol, minor cardiac glycosides, various volatile oils, etc.

Facilitated by the rapid development of technology of isolation and characterization process, particularly chromatography and spectroscopic methods a large number of therapeutically active plant constituents have been isolate during the last two decades.

Simultaneous advancement in the fields of medical, botany, chemistry, biochemistry, pharmacognosy and pharmacology has tremendously helped the discovery, isolation, characterization, structure elucidation and synthesis of new drugs plants, with the development of further newer techniques and methods of plant analysis and with the tremendous increase in man's knowledge of chemistry and pharmacology more and more medicinal compounds are likely to be discovered from plants.

How prominently plant-derived drugs still feature in modern medicine can be accessed from the following facts: (Ghani, 1998).

1. A recent survey by the United Nations Commissions for Trade and Development (UNCTAD) indicated that about 33% of drugs, produced in the development countries, are derived from plants (UNCTAD/GATT 1974) and that if microbes are added 60% of medicinal products are of natural origin (Sofowara, 1982).

2. According to some sources almost 80% of present-day of medicines are directly or indirectly derived from plants (Meyers, 1892).

3. More than 47% of all drugs used in Russia, are obtained from botanical Sources (Ampofo, 1979).

4. From *Stephania cepharantha and Stephania sasabi* (Jap. Journ. Exp. Med. 1949, 1:69). In the United States, in 1980 alone, the consumer paid 8 billion dollars for prescription drugs in which the active ingredients are still alive from plants (Sofowara, 1982).

5. 47% of some 300 million new prescriptions written by physicians in America in 1961, contained as one as more active ingredients, a drug of natural origin (Fams-worth, 1966).

6. In 1960 47% of drugs, prescribed by physicians in the United States, of America, were from natural sources (Bingel *et al,* 1660).

7. In 1967 25% of the products, which appeared in 1.05 billion prescriptions filled in the United States, contained one or more ingredients derived from higher plants (Karolkovans *et al*, 1966).

8. Even today 80% of the rural population of most developing countries of the world depends as herbal medicine for maintaining its health and well being (Ghani, 1987).

9. The consumption of medicinal plants in increasing in many developed countries, where 35% of drugs contain active principles from natural origin (Irvine, 1995).

10. The North America used 170 drugs from different plants, which are as official in the USP or NF. Surprisingly this large quantity of modern drugs comes from less than 15% of the plants, which are known to have been investigated pharmacologically, out of an estimated 250000 to 500000 species of higher plants growing on earth (Farmsworth *et al*, 1985).

## 1.2 Medicinal plants in Bangladesh:

The total numbers of plants with medicinal properties in the subcontinent are present stands at about 2000. About 450 to 500 of such medicinal plants names so far has been enlisted as growing or available in Bangladesh.

In traditional systems indigenous knowledge (IK) plays a central role in discuses diagnosis and health care practices. Rapid westernization and introduction of modern medicine in many places has affected not only the traditional system but also associated with it. Especially in areas with rapid urbanizations the traditional medicinal system and IK have become seriously vulnerable. The risk is not so great for well documented Ayurvedic, Hekimi and Unani ones but many undocumented system of folk medicine which have been handed down form one generations to the next by word of month are in example of the indigenous knowledge based folk medicine which has been used and appreciated since prehistoric times.

IK based folk medicine constitutes a very diverse steam in Bangladesh and is ecosystem and ethic community specific different localities have different characteristics. The rich heritage if indigenous knowledge associated with herbal medicine is considered as the root of all systems of traditional remedies in Bangladesh. During the course of development in the remote past, a particular remedy discovered become widespread and subsequently listed refined revised improved upon and gradually incorporated in the traditional codified system among the practitioners. (Shengji, 2002)

## 1.3 Phytochemistry

Phytochemistry can be defined as the branch of biochemistry dealing with plants and plant processes. These are chemicals derived from plants. In a narrower sense the terms are often used to describe the large number of secondary metabolic compounds found in plants. Many of these are known to provide protection against insect attacks and plant disease. They also exhibit a number of protective functions for human consumers. Phytochemistry also deals with the identification, biosynthesis and metabolism of chemical constituents of plants. It is the early class of organic chemistry.

## 1.4 Primary Metabolites

Primary metabolites are involved in growth, development and reproduction of the organism. The primary metabolite is typically a key component in maintaining normal physiological processes; thus, it is often referred to as a central metabolite. Primary metabolites are typically formed during the growth phase as a result of energy metabolism, and are deemed essential for proper growth. Primary metabolites

consist of various kinds of organic compounds, like carbohydrates, lipids, proteins and nucleic acids. Without primary metabolites key cellular cycles such as glycolysis, the Krebs cycle and the Calvin cycle is not possible, for that every plant kingdom contained those substances. This substance helps plants to take part in synthesis, assimilation and degradation of organic substances, sucrose and starch, structural components such as cellulose, information molecules such as DNA and RNA and pigments, such as chlorophyll are the main primary metabolite contained by the plants. Although these substances are key for the plant survival they also acts as precursors for the synthesis of secondary metabolites sometimes.

### 1.5 Secondary Metabolites

Secondary metabolites are substances which are produced by plants as defense chemicals. Their absence does not cause bad effects to the plants. They include alkaloids, phenolics, steroids, essential oils, lignins, resins and tannina etc.

Secondary metabolites are compounds bio synthetically derived from primary metabolites. Secondary metabolites or Secondary compounds are compounds that are not required for normal growth and development and are not made through metabolic pathways common to all plants. In plant kingdom they are limited to occurrence and may be restricted to a particular taxonomic group genus, species or family. Secondary metabolites are accumulated by plant cells in smaller qualities than primary metabolites. Secondary metabolites are synthesized in specialized cells at particular developmental stages making extraction and purification difficult. (Farmsworth, 1985).

### 1.6 Phytochemistry and Medicinal Plants

The beneficial medicinal effects of plant materials typically result from the combinations of secondary products present in the plant. That the medicinal actions of plants are unique to particular plant species or groups is consistent with this concept as the combinations of secondary products in a particular plant are often taxonomically distinct. This is in contrast to primary products, such as carbohydrates, lipids, proteins, heme, chlorophyll, and nucleic acids, which are common to all plants and are involved in the primary metabolic processes of building and maintaining plant cells (Kaufman et al., 1999; Wink, 1999).

### 1.7 Concept of Free Radicals and Disease

The knowledge of free radicals and reactive oxygen species (ROS) in biology is producing a medical revolution that promises a new age of health and disease management (Aruoma OI, 2003). It is ironic that oxygen, an element indispensable for life, (Mohammed AA & Ibrahim AA, 2004) under certain

situations has deleterious effects on the human body. Most of the potentially harmful effects of oxygen are due to the formation and activity of a number of chemical compounds, known as ROS, which have a tendency to donate oxygen to other substances. Free radicals and antioxidants have become commonly used terms in modern discussions of disease mechanisms (Aruoma OI, 2003).

### 1.8 Free Radicals

A free radical can be defined as any molecular species capable of independent existence that contains an unpaired electron in an atomic orbital. The presence of an unpaired electron results in certain common properties that are shared by most radicals. Many radicals are unstable and highly reactive. They can either donate an electron to or accept an electron from other molecules, therefore behaving as oxidants or reductants (Cheeseman KH & Slater TF, 1993). The most important oxygen-containing free radicals in many disease states are hydroxyl radical, superoxide anion radical, hydrogen peroxide, oxygen singlet, hypochlorite, nitric oxide radical and peroxynitrite radical. These are highly reactive species, capable in the nucleus, and in the membranes of cells of damaging biologically relevant molecules such as DNA, proteins, carbohydrates, and lipids.

### 1.9 Production of Free Radicals in the Human Body

Free radicals and other ROS are derived either from normal essential metabolic processes in the human body or from external sources such as exposure to X-rays, ozone, cigarette smoking, air pollutants and industrial chemicals (Bagchi K & Puri, 1998). Free radical formation occurs continuously in the cells as a consequence of both enzymatic and nonenzymatic reactions.

Enzymatic reactions, which serve as source of free radicals, include those involved in the respiratory chain, in phagocytosis, in prostaglandin synthesis and in the cytochrome P-450 system (Liu T et al, 1999). Free radicals can also be formed in nonenzymatic reactions of oxygen with organic compounds as well as those initiated by ionizing reactions.

### 1.10 Consequences of free radicals and ROS mediated oxidation of biomolecules

Free radicals, ROS and RNS cause irreversible damage to biomolecules such as fatty acid, amino acid, and DNA. Oxidative modification / damage of these molecules lead to the onset of several degenerative diseases such as cancer, vascular diseases, diabetes, inflammation etc.

### 1.10.1 Cancer:

The possible role of free radicals in cancer has been discussed based on the discovery of excess production of free radicals in tumor cells (Saprin et al., 1965). Further, the discovery of superoxide in biological system and superoxide dismutase attracted much attention towards the association of free radicals and carcinogenesis. Studies in expression level of SOD gene in normal and cancer cells pointed out this association because, the tumor cells express low levels of SOD (Oberley, 1982). In addition, Mn SOD is not expressed at all in cancer cells even at elevated level of superoxide (Oberley and Oberley, 1988). Consequently, elevated superoxide cause DNA damage and thus initiate carcinogenesis (Nakamura et al, 1988). During initiation process, the involvement of free radicals were emphasized based on the fact that organic peroxides promotes carcinogenesis (Floyd, 1990). Further, ROS and RNS react with guanine and forms 8-OHdG. The role of 8-OHdG in the process of carcinogenesis is well established (Floyd, 1990;) by its potential to mutate a few cancer related genes and transformation of proto-oncogenes to oncogenes (Cerutti, 1994).

### 1.10.2 Diabetes:

The involvement of free radicals in the development of diabetes is a core research area in the epidemiological studies of diabetes. Much study has been done on the free radicals in biology and diabetes independently. However, the number of comprehensive studies to understand the involvement of free radicals in the etiology of diabetes is very few. Type 1 diabetes is caused by destruction of pancreatic beta cells responsible for the production of insulin. In human the diabetogenic process is caused by immune destruction of beta cells; part of this process is apparently by white cell production of ROS. Wellestablished evidence is the experimental diabetic inducing agents; alloxan and streptozotocin. Though the mechanism of action of these two compounds are different, both results in the production of ROS. The presence of ROS scavengers effectively inhibited the development of diabetes in these compound induced diabetic models (Oberley, 1988).

### 1.10.3 Vascular diseases:

Vascular diseases such as atherosclerosis, peripheral artery disease, hypertension, peripheral vascular disease etc. are caused by xenobiotics, physical inactivity, unhealthy diet etc. However the free radicals and ROS in the vascular system promote the onset as well as progression. The role of free radicals in the etiology of a few vascular diseases are presented in the following sections

### 1.10.4 Ischemic reperfusion injury:

Hypoxia and reoxygenation generally causes injury to cells. The major cause of circulatory shook, myocardial ischemia, and stroke are believed to be reoxygenation. During reoxygenation, large amount of ROS especially superoxide and hydroxyl radicals (Werns et al., 1985) are formed and recognized as the cause of reoxygenation injury. Formation of ROS under this pathological conditions were established by several studies Werns et al., 1985).

### 1.10.5 Atherosclerosis:

A large number of reports emphasize that excess superoxide play an important role in the onset of atherosclerosis and hence promote endothelial dysfunction. Moreover the oxidized proteins, lipids, LDL and nucleic acids as a result of plasma oxidative stress also promote the progression of vascular tissue damage (Beckman and Koppenol, 1996) and atherosclerotic plaque formation.Promotion of atherosclerosis as a result of reduced expression of extracellular SOD and mutation in endothelial is an important evidence for the role of ROS in vascular diseases (Faraci and Didion, 2004; Fukai et al., 2002).

### 1.10.6 Hypertension:

The possible role of free radicals in the pathogenesis of atherosclerosis and hypertension has been suspected for long time. This was evidenced by low serum antioxidant capacity and hypertension (Salonen et al., 1988), high serum antioxidant capacity and low level of atherogenic protein. correlation between antioxidant supplement and normotension etc. (Salonen et al, 1994)

### 1.10.7 Inflammation:

Under chronic inflammatory condition, a large number of ROS are produced. Superoxide thus produced stimulate the release of IL-1 from blood monocytes. IL-1 act as feedback booster and in turn increases the formation of excess ROS in the vicinity. The excess ROS thus produced oxidize lipoprotein, lipids, protein etc. and accelerate atherogenic processes in the vascular system, induce carcinogenesis (Coussens and Werb, 2002), neurodegeneration (Akiyama et al, 2000)

### 1.10.8 Life expectancy:

Aging is the progressive accumulation of changes with time that are associated with or responsible for the ever-increasing susceptibility to disease and death which accompanies advancing age. Among the

several ageing theories proposed, the "free radical theory of aging" (Harman, 1956; Harman, 1992) has gained universal acceptance and is supported by the fact that the sum of the deleterious free radical reactions going on continuously throughout the cells and tissues constitutes the aging process (Sohal and Weindruch, 1996). The free radial theory is supported by the "rate of living" hypothesis, which links metabolic rate and subsequent free radical production with the short lifespan of organisms (Ku et al., 1993). Under vigorous metabolism, free radicals and ROS are produced and can damage proteins, DNA and lipids and this oxidation process accelerate aging process (Barja and Herrero, 2000)

### 1.11 Oxidative Stress

Oxidative stress, defined as a disturbance in the balance between the production of reactive oxygen species (free radicals) and antioxidant defenses. It is associated with damage to a wide range of molecular species including lipids, proteins and nucleic acids (Mc Cord JM, 2000). Short-term oxidative stress may occur in tissues injured by trauma, infection, heat injury, hypertoxia, toxins and excessive exercise. These injured tissues produce increased radical generating enzymes (e.g. xanthine oxidase, lipogenase, cyclooxygenase) activation of phagocytes, release of free iron, copper ions, or a disruption of the electron transport chains of oxidative phosphorylation, producing excess ROS.

### 1.12 Mechanism of Oxidative Stress

Free radicals are species containing one or more unpaired electrons in their outer atomic orbital. This electron imbalance renders them highly reactive and capable of widespread oxidation of lipids, proteins, DNA and carbohydrates. This eventually causes disruption of cell membranes, leading to release of cell contents and death (Halliwell B, Gutteridge JMC, 1989). Free radicals are formed by several exogenous processes such as radiation and tobacco smoke, and are the endogenous natural by-products of cellular metabolism (Halliwell B., 1984).

When oxygen is reduced in the electron transport chain, oxygen-derived free-radical intermediates are formed. The superoxide radical ($O_2^-$) and hydrogen peroxide ($H_2O_2$) intermediates can escape from the system, and in the presence of transition metal ions (e.g. $Fe^{2+}$, $Cu^{2+}$) form the far more damaging hydroxyl radical ($OH^-$) (Halliwell B & Gutteridge JMC, 1989). One example of this oxidative damage is lipid peroxidation (Gutteridge JMC, 1995). Free radicals may attack polyunsaturated fatty acids within membranes, forming peroxyl radicals. These newly-formed free radicals can then attack adjacent fatty acids within membranes causing a chain reaction of lipid peroxidation.

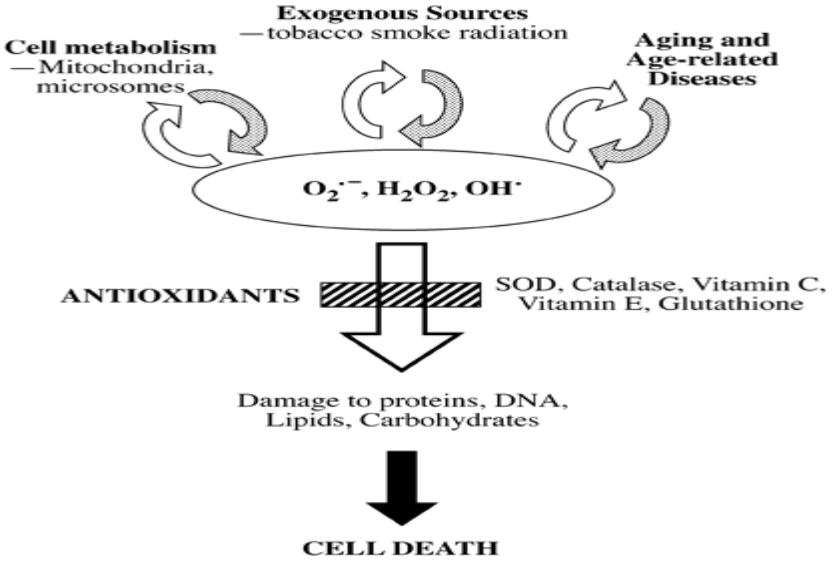



**Figure 1.12:** Mechanisms of oxidative stress.

### 1.13 Oxidative Stress and Human Diseases

A role of oxidative stress has been postulated in many conditions, including anthersclerosis, inflammatory condition, certain cancers, and the process of aging. Oxidative stress is now thought to make a significant contribution to all inflammatory diseases (arthritis, vasculitis, glomerulonephritis, lupus erythematous, adult respiratory diseases syndrome), ischemic diseases (heart diseases, stroke, intestinal ischema), hemochromatosis, acquired immunodeficiency syndrome, emphysema, organ transplantation, gastric ulcers, hypertension and preeclampsia, neurological disorder (Alzheimer's disease, Parkinson's disease, muscular dystrophy), alcoholism, smoking-related diseases and many others. An excess of oxidative stress can lead to the oxidation of lipids and proteins, which is associated with changes in their structure and functions. (Sohal and Weindruch, 1996).

### 1.14 Antioxidant

An antioxidant is a molecule stable enough to donate an electron to a rampaging free radical and neutralize it, thus reducing its capacity to damage. These antioxidants delay or inhibit cellular damage mainly through their free radical scavenging property (Halliwell B., 1995).

These low-molecular-weight antioxidants can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged. Some of such antioxidants, including glutathione, ubiquinol and uric acid, are produced during normal metabolism in the body (Shi HL., 1999). Other lighter antioxidants are found in the diet. Although there is several enzymes system within the body that

scavenges free radicals, the principle micronutrient (vitamins) antioxidants are vitamin E (α-tocopherol), vitamin C (ascorbic acid) and B-carotene (Levine M. et al., 1991). The body cannot manufacture these micronutrients, so they must be supplied in the diet.

### 1.15 Antioxidant Defense System

Antioxidants act as radical scavenger, hydrogen donor, electron donor, peroxide decomposer, singlet oxygen quencher, enzyme inhibitor, synergist and metal-chelating agents. Both enzymatic and nonenzymatic antioxidants exist in the intracellular and extracellular environment to detoxify ROS (Frie B. et al, 1988).

### 1.16 Mechanism of Action of Antioxidants

Two principle mechanisms of action have been proposed for antioxidants (Rice-Evans CA, Diplock AT., 1993). The first is a chain- breaking mechanism by which the primary antioxidant donates an electron to the free radical present in the systems. The second mechanism involves removal of ROS/reactive nitrogen species initiators (secondary antioxidants) by quenching chain-initiating catalyst. Antioxidants may exert their effect on biological systems by different mechanisms including electron donation, metal ion chelation, co-antioxidants, or by gene expression regulation (Krinsky NI., 1992).

**Chain reactions of free radicals**

**Initiation stage**

(1) $RH \rightarrow R^{\cdot} + H^{\cdot}$

(2) $R^{\cdot} \rightarrow R^{\cdot} + O2 \rightarrow ROO^{\cdot}$

(3) $2ROOH \rightarrow ROO^{\cdot} + RO^{\cdot} + H2O$

**Propagation stage**

(1) $R^{\cdot} + O2 \rightarrow ROO^{\cdot}$

(2) $ROO^{\cdot} + RH \rightarrow ROOH + R^{\cdot}$

(3) $RO^{\cdot} + RH \rightarrow ROH + R^{\cdot}$

**Termination stage**

(1) $R^{\cdot} + R^{\cdot} \rightarrow R - R$

(2) $R^{\cdot} + ROO^{\cdot} \rightarrow ROOR$

(3) $ROO^{\cdot} + ROO^{\cdot} \rightarrow ROOR + O2$

(4) Antioxidants + O2 _ oxidized antioxidants (Borek, 1991

**1.17 Antioxidant's classification**

**1.17.1 Endogenous antioxidants**

ROS contribute to the formation of various pathological conditions. To counteract the effects of ROS, the body is endowed with a protective mechanism consisting of enzymatic and non-enzymatic endogenous antioxidants. Up regulation of enzymatic antioxidants have been reported to minimize free radical production and oxidative stress mediated tissue damage and hence the onset and progression of degenerative disease (Li et al, 2006).

Endogenous antioxidants are capable of different activities and work synergistically with exogenous antioxidants contributing the overall protective effect to the individuals by preventing or delaying the onset and progression of various free radical contributed degenerative disease (Serafini, 2006).Following section presents about various endogenous antioxidant and their importance.

**1.17.2 Superoxide dismutase:**

Superoxide dismutase is the first antioxidant enzyme involved in the antioxidant defense system found in higher organism and microbes (Fridovich, 1983; Fridovich, 1986) and the major function is the removal of superoxide radicals formed by various reasons as presented in section.

**1.17.3 Catalase:**

Catalase is a common peroxidase enzyme found virtually in all aerobic organisms that breakdown hydrogen peroxide which is produced by superoxide dismutase enzyme. Hydrogen peroxide is an important starting molecule for the production of hydroxyl radicals by Fenton's reaction. Catalase decomposes hydrogen peroxide into water and oxygen and thereby prevents the damaging effect caused by hydroxyl radicals (Deisseroth and Dounce, 1970).

### 1.17.4 Glutathione peroxidase:

Glutathione peroxidase (GPX) is ubiquitous selenium containing antioxidant enzyme in all higher organisms that catalyze the decomposition of hydrogen peroxide to water by utilizing reduced glutathione as hydrogen atom source. There are several isozymes differentially located in various organs. The GPX enzyme, which is more abundant in extracellular fluid, especially plasma, is isozyme-3. isozyme-4 also expressed in all cells in less abundance whose preferred substrate is lipid hydroperoxids (Muller et al, 2007).

### 1.17.5 Glutathione reductase:

Glutathione reductase (GR) catalyzes the reduction of oxidized glutathione. Oxidation of glutathione by GPX forms two glutathione molecules, which are linked to form glutathione disulfide (GSSG), a stable molecule. The reduction of glutathione disulfide catalyzed by GR produces two molecules of GSH, which is one important substrate for GPX for decomposing hydrogen peroxide to water. The substrate, GSH, used from the pool during the detoxification of hydrogen peroxide is maintained by the glutathione reductase.

### 1.17.6 Thiols:

Thiols contain highly active SH group and therefore having antioxidant property. The most studied endogenous antioxidant thiols are lipoic acid and glutathione. Lipoic acid and dihydrolipoic acids are present in most kind of cells. Properties and therapeutic effects of LA and DHLA are well reviewed (Fuchs et al, 1997). DHLA is an efficient scavenger of all oxygen radicals; however, LA is active only in the reaction with highly reactive hydroxyl radicals.

### 1.17.7 Ubiquinones:

Ubiquinones are essential electron carriers in the mitochondrial electron transport chain. They shuttle electron from NADH and succinate dehydrogenase to the cytochrome b-c1 complex. There are two types of redox interaction, in which ubiquinone can manifest their antioxidant activity: the reaction with quinone and hydroquinone formation. The antioxidant activities of ubiquinone has been demonstrated in vitro and in vivo studies (Filipe et al, 2001)

### 1.17.8 Uric acid:

Uric acid is another physiologically important antioxidant. Uric acid contains two active hydroxyl groups in the purine heterocycle. The physiological level of uric acid protects erythrocyte against free radical damage (Ames et al., 1981). It is also a major antioxidant in human airway mucosal surface (Peden et al, 1990).

### 1.17.9 Melatonin:

Melatonin is a pineal hormone, which is synthesized from tryptophan. Melatonin is an effective scavenger of hydroxyl radicals, nitric oxide and peroxy nitrite (Reiter et al, 2000). It is an effective inhibitor of ironinitiated peroxidation of brain phospholipids liposome (Marshall et al, 1996).

## 1.18 Exogenous antioxidants.

Similar to endogenous antioxidants, some exogenous dietary compounds can neutralize the free radicals as well as enhance the activities of endogenous antioxidants. When the system is under oxidative stress and the endogenous antioxidants are not sufficient enough to scavenge the free radicals and ROS, the dietary antioxidants may be required to maintain optimal cellular functions (Rahman, 2007). Some important dietary antioxidants are presented below.

### 1.18.1 α-Tocopherol:

α-Tocopherol is a lipid soluble phenolic antioxidant with an active hydroxyl group. Several authors reported the high antioxidant and antiradical activities of α-tocopherol. However similar to many other antioxidants, α-tocopherol also shows pro-oxidant action under certain conditions (Terao and Matsushota, 1986).

### 1.18.2 Vitamin C:

Ascorbic acid is a highly active free radical scavenger and strong reducing agent. Oxidation and reduction reactions of ascorbic acid with numerous oxidants and reductants are well studied (Afanas'ev, 1989). Other than its antioxidant properties pro-oxidant activities also well studied. It is known that the competition between antioxidant and pro-oxidant activities of ascorbic acid depends on the rate of reaction (Afanas'ev et al, 1987). Ascorbic acid at lower concentration enhanced lipid peroxidation but inhibited at higher concentration (Afanas'ev et al, 1989).

### 1.18.3 Carotenoids:

Hundreds of carotenoids are found in nature but relatively a few are found in human tissues, the five main carotenoids are; β-carotene, lutein, lycopene, β-cryptoxanthin, and α-carotene (Bendich and Olson, 1989; Rock et al., 1996; Thurnham, 1994). The antioxidant properties of the carotenoids closely relate to the extended system of conjugated double bonds, which occupies the central part of carotenoid molecules, and to the various functional groups on the terminal ring structures (Mathews-Roth, 1974; Stryker, 1988; Thurnham, 1997). The reactive oxygen species scavenged by carotenoids are singlet oxygen and peroxyl radicals (Foote and Denny, 1968; Palozza and Krinsky, 1992). In this process the carotenoid absorbs the excess energy from singlet oxygen and then releases it as heat.

### 1.18.4 Flavonoids:

Flavonoids are naturally occurring low molecular weight phenolic compounds widely distributed in plant Kingdom. Huge amount of literature is available on the antioxidant activates of flavonoids. Flavonoids are reported to have multiple biological activities such as anti-inflammatory, antidiabetic, antiallergic, antiviral, anticancer etc. (Critchfield et al., 1996; Havsteen, 1983).

Since they are polyphenols, their antioxidant activities depend on the hydroxyl groups. Flavonoids are generally good scavengers of peroxyl radicals, hydroxyl radical and superoxide radicals (Denisov and Afanas′ev, 2005).

### 1.18.5 Steroids:

Some steroid molecules such as estrone, estradiol, and estriol has phenolic hydroxyl group and therefore are able to react with free radicals. All the above said compounds are reported to inhibit liposomal lipid peroxidation (Nakano et al., 1987; Sugioka et al., 1987). The role of phenolic hydroxyl group in the steroid molecules have been studied using various steroids. Only phenolic hydroxyl group containing steroids inhibited lipid peroxidation (Huber et al., 1990).

## 1.19 Antioxidants and disease prevention

In vitro as well as in vivo cell culture studies showed that intra cellular and extracellular antioxidants may prolong the onset or progression of degenerative diseases such as diabetes, cancer, and cardiovascular diseases etc. the literature evidences showing role of antioxidants in a few degenerative disease are presented below.

**1.20 Cancer:**

In vitro and in vivo research indicated that some dietary antioxidants show anticancer activity. Strong antioxidants such as pyrrolidine dithiocarbamate (PDTC) and N-acetyl cysteine (NAC) inhibited growth of human colorectal cell in culture and when fed to mice with implanted tumors (Chinery et al., 1997). Another similar study showed that consumption of antioxidant rich tea reduced the risk of breast cancer several fold (Hirvonen et al., 2006). Though the mechanism of action of antioxidants and low cancer risks are not clear, there are some indications about the activation of tumor suppressor genes which are inactivated in almost one half of human tumors (Chinery et al., 1997). It was shown that the possible first step in the activation of tumor suppressor genes is by antioxidant-induced activation of protein kinase A. Antioxidants are also reported to induce apoptosis by the activation of nuclear factor *k*B (NF*k*B).

**1.21 Diabetes:**

Pancreatic β-cell dysfunction together with insulin resistance is associated with the development of type 2 diabetes. Various authors have shown the significance of hyperglycemia as a direct cause of β-cell glucose toxicity in vivo (Zangen et al, 1997) and in vitro (Olson et al, 1993). At the onset of insulin resistance and hyperglycemia, β-cell function progressively deteriorates and subsequently glucose induced insulin secretion becomes further impaired and β-cells number decreases as a result of degranulation . One important reason attributed for the hyperglycemia induced dysfunction is through hyperglycemia mediated production of free radicals and ROS by the glycation of biomolecules (Hunt et al, 1991). Although the induction of the glycation reaction in diabetes was originally found in neural cells and the lens crystalline, which are known targets of diabetic complications, another target that accelerate the progression of DM was shown to be the β-cell (Ihara et al., 1999; Kaneto et al., 1996). Moreover the ROS thus produced also play significant role in the development of other complications related to diabetes (Baynes, 1991). In vitro studies that simulated β-cells in hyperglycemic condition showed several glycosylation end products as well as oxidative stress markers (Ihara et al., 1999). (Tiedg et al., 1997).

### 1.22 Cardio vascular disease:

Quite a lot of recent studies have demonstrated that altered oxygen utilization and/or increased formation of ROS contribute to atherogenesis and CVD progression. Several sources of oxygen/nitrogen species do occur in CVD. Intracellular ROS are formed during mitochondrial electron transport chain and are controlled by antioxidant defense system. However several studies suggested that oxidative stress as well as polymorphic variations in endogenous antioxidants are linked to increased risk for atherosclerosis and CVD (Hiroi et al., 1999). Immediate targets of ROS are long-chain free fatty acids in the cytosolic compartment and membrane-bound lipids however chemically vulnerable substrates to ROS are the polyunsaturated fatty acids in the lipoproteins. Free radicals attack plasma low density lipoprotein (LDL) that is oxidatively modified to oxidized low density lipoprotein (oxLDL) leading to the attraction of blood monocytes beneath the endothelium. Based on the 'oxidation theory' for atherosclerosis, dietary antioxidants have attracted considerable attention as preventive and therapeutic agents.

### 1.23 Plants as a Source of Antioxidants

Synthetic and natural food antioxidants are used routinely in foods and medicine especially those containing oils and fats to protect the food against oxidation. There are a number of synthetic phenolic antioxidants, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) being prominent examples. These compounds have been widely uses as antioxidants in food industry, cosmetics and therapeutic industry. However, some physical properties of BHT and BHA such as their high volatility and instability at elevated temperature, strict legislation on the use of synthetic food additives, carcinogenic nature of some synthetic antioxidants, and consumer preferences have shifted the attention of manufacturers from synthetic to natural antioxidants (Papas AM, 1993).

It has been reported that there is an inverse relationship between the dietary intake of antioxidant-rich food and medicinal plants and incidence of human diseases. The use of natural antioxidants in food, cosmetic and therapeutic industry would be promising alternative for synthetic antioxidants in respect of low cost, highly compatible with dietary intake and no harmful effects inside the human body. Many antioxidant compounds, naturally occurring in plant sources have been identified as free radical or active oxygen scavengers (Brown JE, 1998). Attempts have been made to study the antioxidant potential of a wide variety of vegetables like potato, spinach, tomatoes, and legumes (Furuta S, 1997). There are several reports showing antioxidant potential of fruits (Wang H, 1996). Strong antioxidants activities have been found in berries, cherries, citrus, prunes, and olives etc.

# 2. Objective of the study

Plants have formed the basis for traditional medicine systems which have been used for thousands of years in many countries of the world. These plant-based systems continue to play an essential role in health care, and it has been estimated by the World Health Organization that approximately 80% of the world's inhabitants mainly on traditional medicines for their primary health care.

For doing a pharmacological study on a suitable plant is a very essential and decisive step. Mainly the plants are selected on basis of their traditional use, chemical content, toxicity, randomized selection or a combination of several criteria. The most common strategy is careful observation of the use of natural resources in folk medicine in different cultures; which is usually known as ethnobotany or ethnopharmacology. Keeping these selection criteria in mind, I selected four medicinally important plants for my research work.

My five plants are- *Spilanthes calva, Solanum virginianum, Stevia rebaudiana, Ruellia tuberosa and Mikania cordata.* These plants are very commonly distributed in Bangladesh. Ethnopharmacological data reveals that these plants are very widely used by various ethnic groups to treat various health problems. So these plants could be very good source of discovering novel compounds with medicinal properties.

Column chromatography in chemistry is a method used to purify individual chemical compounds from mixtures of compounds. It is often used for preparative applications on scales from micrograms up to kilograms. The main advantage of column chromatography is the relatively low cost and disposability of the stationary phase used in the process. Another advantage of column chromatography is that it can usually be scaled to the project at hand. This is especially useful if one is trying to separate and purify a reaction mixture preparing an intermediate in a sequence of reactions.

Encouraged by these ethnobotanical data on the traditional uses of these plants, I wanted to explore some pharmacological effects of various plant samples and column chromatography of plant sample which were previously collected as a part of the on-going research project conducted and supervised by Dr. Shamsun Nahar Khan. In my study I mainly focused to determine the antioxidant activity of five plant samples and the different fractions of *Mikania cordata.*

# 3. Introduction to the plant

### 3.1 *Spilanthes Calva*

*Spilanthes calva* is a medicinal herb, belongs to Compositae family. It is a well-known antitoothache plant with high medicinal usages, has been recognized as an important medicinal plant and has an increasingly high demand worldwide.

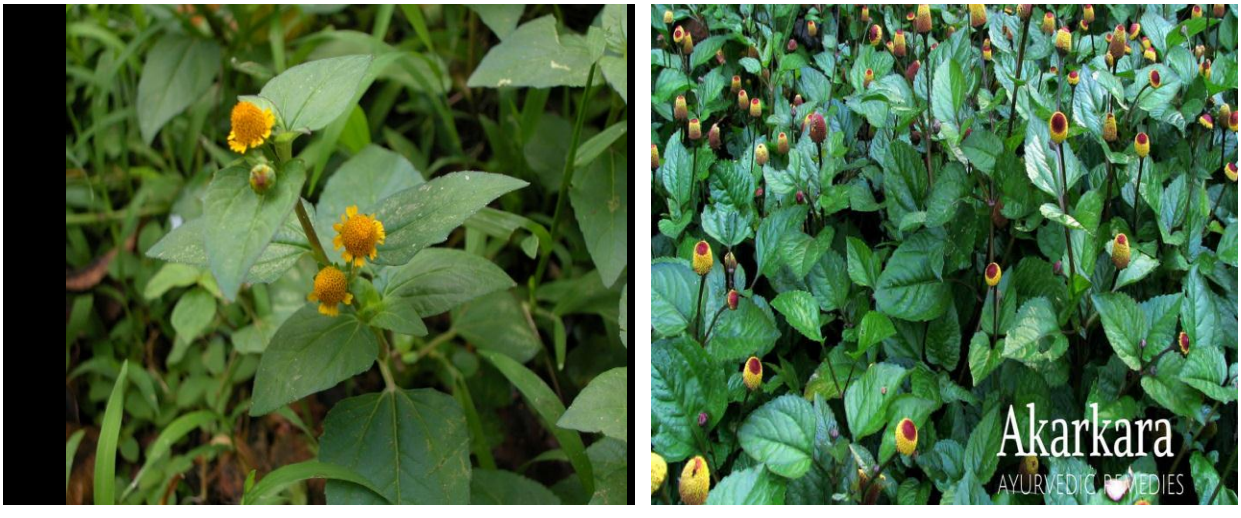



Figure 3.1. : *Spilanthes Calva*

The traditional uses of *Spilanthes calva* in health care and food, extensive phytochemical studies have been reported. Spilanthes (Compositae or Asteraceae) is a genus comprising of over 60 species that are widely distributed in tropical and subtropical regions of the world, such as Africa, America, Borneo, India, Sri Lanka and Asia (Sahu et al., 2011).

Its flowers and leaves have pungent taste and when touched it is accompanied by tingling sensation and numbness (Wongsawatkul et al., 2008). The plant species has been used commonly as a folk remedy, e.g. for toothache, rheumatic and fever (Wongsawatkul et al., 2008), as fresh vegetable as well as spice for Japanese appetizer (Leng et al, 2004).

#### 3.1.1 Distribution

Spilanthes Calva is native to India and cultivated all throughout the Indian Peninsula for ornamental and medicinal plant

### 3.1.2 Taxonomy

Domain : Eukaryota

Kingdom : Plantae

Subkingdom : Tracheobionta

Superdivision : Spermatophyta

Division : Magnoliophyta

Class : Magnoliopsida

Subclass : Asteridae

Order : Asterales

Family : Asteraceae

Genus *: Spilanthes*

Species *: Spilanthes calva*

### 3.1.3 Traditional Uses:

Flowers head is stimulant and sialagogue; given in toothache, affections of throat and gums and paralysis of the tongue. It is a powerful mosquito larvicide. The decoction of the plant is diuretic and lithontriptic; useful in dysentery and is employed as a bath for rheumatism and as a lotion in scabies and psoriasis. Crushed plant is used as fish poison. It is prescribed for few days to cure glossitis. Juice of the plant and flower head is rubbed in scabies to cure. Roots are used as a purgative.

### 3.1.4 Chemical Constituents:

Aerial parts contain palmitic and stearic acids, tetra-triacontanoic acid, sitosterol, stigmasterol and sitosterol glucoside. Flower heads contain a local anaesthetic amide, spilanthol and three other amides, a sterol, a non-reducing polysaccharide (Ghani, 2003).

### 3.2 *Solanum virginianum*

*Solanum virginianum* is a very prickly, diffuse herb, somewhat woody at the base; stem somewhat zigzag. Prickles compressed, straight, yellow, often exceeding 1.3 cm long. Leaves 5-10 cm long, ovate or elliptic, sinuate or subpinnatifid, obtuse or subacute, armed on the midrib and nerves with long yellow sharp prickls. Flowers in extra axillary few-flowered cymes; corolla purple, 2 cm long. Berry 1.3-2 cm diam., yellow or white with green veins, surrounded by the enlarged calyx.

It is also called Surattense nightshade, yellow-fruit nightshade, yellow-berried nightshade, Thai green eggplant, Thai striped eggplant (from the unripe fruit).

**Figure 3.2:** *Solanum virginianum*

#### 3.2.1 Taxonomy :

Kingdom : Plantae

Subkingdom : Viridiplantae

Superdivision : Embryophyta

Division : Tracheophyta

Class : Magnoliopsida

Superorder : Asteranae

Order : Solanales

Family : Solanaceae

Genus : Solanum

Species : *Solanum virginianum*

### 3.2.2 Distribution :

It is a species of nightshade native to Asia (Saudi Arabia, Yemen, Afghanistan, Iran, China, Bangladesh, India, Nepal, Pakistan, Sri Lanka, Myanmar, Thailand, Vietnam, Indonesia, Malaysia), and is adventive in Egypt. It is a medicinal plant used in India, but the fruit is poisonous.

In Bangladesh it is found in Chuadanga, Khulna, Kustia, Nawabganj, Rajshahi, in fallow lands.

### 3.2.3 Traditional Use

Roots are diuretic and expectorant; employed in cough, asthma, chest pain and catarrhal fever. Fruit juice is useful in sore throat and rheumatism. Stem, flowers and fruits are carminative. Paste of the leaves is applied on painful joints to relieve pains. Seeds are given as an expectorant in asthma and cough. Decoction of the plant is useful in gonorrhoea. The plant also possesses cardioactive and antipyretic acivities.Crude plant extract caused hypotension which has been attributed to release of histamine by some constituents (Rastogi & Mehrotra, 1990).

### 3.2.4 Chemical Constituent:

The plant contains sterols, alkaloids and glycosides. The plant also contains quercetin glycoside, apigenin, sitosterol and carpesterol. Fruits contain steroidal glycoalkaloids, solasonine, solamargine, solasurine, solanocarpine, solanine-S and alkaloidal bases, solanidine-S and solasodine. Seeds contain solanocarpine. (Ghani, 2003). Dry fruits contain traces of isochlorogenic, neochlorogenic, chlorogenic and, caffeic acid. Quercetin-3-O-β-D-glucopyranosyl-(1→ 4)-mannopyranoside, apigenin and sitosterol have also been isolated from dry fruits (Rastogi & Mehrotra, 1993).

### 3.3 *Stevia rebaudiana*

Stevia rebaudiana is a plant species in the genus Stevia of the sunflower family (Asteraceae), commonly known as sweetleaf, sweet leaf, sugarleaf. It is a tender perennial native to parts of Brazil and Paraguay that favors humid, wet environments, although the root does not tolerate standing water.

Stevia is widely grown for its sweet leaves, which are the source of sweetener products known generically as stevia and sold under various trade names. The active compounds are various steviol glycosides (mainly stevioside and rebaudioside), which have 250–300 times the sweetness of sugar.The leaves can be eaten fresh, or put in teas and foods.

**Figure 3.3:** *Stevia rebaudiana*

#### 3.3.1 Taxonomy :

Kingdom : Plantae

Superdivision : Embryophyta

Division :Tracheophyta

Subdivision : Spermatophytina

Class : Magnoliopsida

Superorder : Asteranae

Order : Asterales

Family : Asteraceae

Genus : Stevia

Species : Stevia rebaudiana

### 3.3.2 Distribution:

Stevia rebaudiana has been grown on an experimental basis in Ontario, Canada since 1987 to determine the feasibility of commercial cultivation. Duke University researchers developed a strategic plan to assist farmers and exporters in Paraguay to compete in the global market for stevia.

Today, Stevia rebaudiana is cultivated and used to sweeten food elsewhere in East Asia including China (since 1984), Korea, Taiwan, Thailand, and Malaysia. It can also be found in Saint Kitts and Nevis, Brazil, Colombia, Peru, Paraguay, Uruguay, and Israel.

### 3.3.3 Traditional Uses:

For hundreds of years, indigenous peoples in Brazil and Paraguay have used the leaves of stevia as a sweetener. The Guarani Indians of Paraguay call it *kaa jheé* and have used it to sweeten their yerba mate tea for centuries. They have also used stevia to sweeten other teas and foods and have used it medicinally as a cardiotonic, for obesity, hypertension, and heartburn, and to help lower uric acid levels.

In addition to being a sweetener, stevia is considered (in Brazilian herbal medicine) to be hypoglycemic, hypotensive, diuretic, cardiotonic, and tonic. The leaf is used for diabetes, obesity, cavities, hypertension, fatigue, depression, sweet cravings, and infections. The leaf is employed in traditional medical systems in Paraguay for the same purposes as in Brazil. (Sohal and Weindruch, 1996).

### 3.3.4 Plant Chemicals

Over 100 phytochemicals have been discovered in stevia since. It is rich in terpenes and flavonoids. The constituents responsible for stevia's sweetness were documented in 1931, when eight novel plant chemicals called glycosides were discovered and named. The main plant chemicals in stevia include: apigenin, austroinulin, avicularin, beta-sitosterol, caffeic acid, campesterol, caryophyllene, centaureidin,

chlorogenic acid, chlorophyll, cosmosiin, cynaroside, daucosterol, diterpene glycosides, dulcosides A-B, foeniculin, formic acid, gibberellic acid, gibberellin, indole-3-acetonitrile, isoquercitrin, isosteviol, jhanol, kaempferol, kaurene, lupeol, luteolin, polystachoside, quercetin, quercitrin, rebaudioside A-F, scopoletin, stigmasterol, umbelliferone, and xanthophylls.

### 3.4 *Ruellia tuberosa:*

*Ruellia tuberosa* L. also known as *Ruellia clandestine*. Its common name is minnieroot, iron root, feverroot, poping pod, bluebell, cracker plant etc. Ruellia tuberosa is an erect, suberect, or diffuse perennial herb up to 60–70 cm tall herb and belongs duction to to family Acanthaceae, a native of Central America, introduced into Indian garden as ornament. The simple leaves are opposite and elliptic, the stem is 4 sided. The plant only flowers after the start of the rainy season. It has thick finger like roots. The ripe fruits with 7-8 seeds, burst open with a bang. It is used medicinally in West Indies, Central America, Guiana, and Peru. In Siddha system of medicine, leaves are given with liquid copal as remedy for gonorrhea and ear diseases, used in stomach cancer. Dried and ground roots can cause abortion and also used in sore eyes. The herb also exhibits emetic activity and employed substitute of ipecac, also used in bladder stones and decoction of leaves used in treatment of Bronchitis. (Chothani et. al, 2012)

**Fig 3.4:** *Ruellia tuberosa*

### 3.4.1 Distribution:

Ruellia tuberosa originates from tropical America, but is naturalized in southeast asia (Thailand, Peninsular Malaysia, Java) and elsewhere in the tropics (India, Srilanka, Africa), as an escape from cultivation and as an ornament.

### 3.4.2 Taxonomy:

Kingdom: Plantae

Subkingdom: Viridiplantae

Infrakingdom: Streptophyta

Superdivision: Embryophyta

Division: Tracheophyta

Subdivision: Spermatophytina

Class: Magnoliopsida

Order: Lamiales

Family: Acanthaceae

Genus: Ruellia

Species: *Ruellia tuberosa*

Scientific name: *Ruellia tuberosa*

### 3.4.3 Traditional uses:

In Suriname's traditional medicine system, it is used as anthelmintic and also in management of joint pain and strained muscles. In folk medicine, it has been used as diuretic, antipyretic, antidiabetic, antidotal, thirst-quenching agent and analgesic and anti-hypertensive activity. Ruellia tuberosa is used as cooling in urinary problem, uterine fi- broids. It has been experimentally proved to possess antioxidant, antimicrobial, anticancer, gastroprotective activity, antinociceptive, and anti-inflammatory activity. It is reported that it contains flavonoids, steroids, and triterpenoids and alkaloid.

### 3.5 *Mikania cordata*

*Mikania cordata* is a tropical plant in the Asteraceae; known as bitter vine, climbing hemp vine, or American rope. It is also sometimes called mile-a-minute vine. It is a creeping woody perennial from the family Asteracea. A climber to 8 m or more of regeneration on old farms and in secondary jungle and waste places generally, widespread throughout the Region; probably of S American origin, but now pantropical.

Figure 3.5.: *Mikania cordata*

A fast growing, creeping or twining, perennial vine; stems branched, pubescent to glabrous, ribbed, from 3 to 6 m long; leaves opposite, cordate or triangular-ovate, blade 3 to 12 cm long, 2 to 6 cm wide, on a slender petiole 1 to 8 cm long, base broadly cordate, tip acuminate, margins crenate, dentate, or entire, surfaces nearly glabrous, three- to seven-veined from base; flowers in small heads in open, nearly flat-topped (corymbose) panicles; axillary and terminal heads 6 to 9 mm long. (H. A. Kadir et al, 1989).

#### 3.5.1 Distribution

This plant is widely distributed in tropical region including Southeast Asia and Eastern Africa, but currently invasive in many parts of the world.

#### 3.5.2 Taxonomy

Domain : Eukaryota

Kingdom : Plantae

Subkingdom : Trachoeobionta

Division : Magnoliophyta

Class : Magnoliopsida

Subclass : Asteridae

Order : Asteales

Family : Asteriaceae

Genus : *Mikania*

Species: *Mikania cordata*

Scientific Name: *Mikania cordata*

### 3.5.3 Characteristics

It Grows most frequently in places receiving high rainfall, probably 1,500 mm or more; prefers rich, damp soil; rarely grows in dry areas; and thrives in open, disturbed places. For that reason it is common in young secondary forests, in forest clearings, in plantation tree crops, fallow or neglected lands and along rivers and streams, waste areas, steep hillsides and even mountainsides from whence winds probably spread the seeds to new areas. The species will grow in partial shade, but cannot tolerate dense shade. Large amounts of seed transported by the wind or by adhering to human clothing or the hair of animals.

### 3.5.4 Traditional Uses

The plant is used as a cover crop to prevent erosion and the leaves are used in some places as a soup vegetable, and can be used as cattle fodder. In southern Nigeria a decoction is given for coughs, and the leaf-juice is a remedy for sore eyes. In Portuguese East Africa, the Tongas use the plant as a remedy for snake and scorpion bite. An infusion of the plant is given in affections of the stomach and intestines.

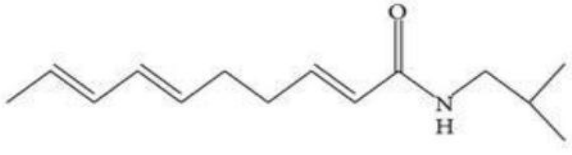
# 4. Literature review:

## 4.1 *Spilanthes calva*

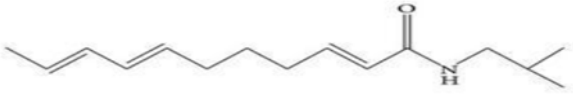
### 4.1.1 Bioactive Molecules

It is necessary to explore the phytochemical constituents of any medicinal plant to establish a relation between pharmacology and chemistry of the plant. Many studies have been carried out for chemical analysis and structural determination of pungent alkamides from Spilanthes calva. The major pungent constituent reported in this plant S. calva is "spilanthol," which is an isobutylamide and is well known for its insecticidal properties (I. J. O. Jondiko, 1986; H. A. Kadir et al., 1989). The flower head and root part of the plant have been reported to be the rich source of active principles. Triterpenoids have also been found in the plant (D. K. Mukharya et al., 1987) Spilanthol is chemically N-isobutylamide which is bitter in taste and could stimulate salivation. The structure of the compounds are below.

**Structures**



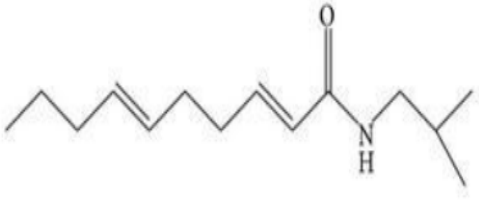
Spilanthol



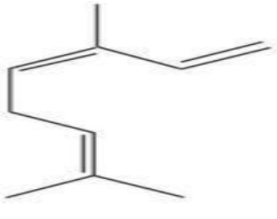
Undeca-2E,7Z,9E-trienoic acid isobutylamide

Undeca-2E-en-8,10-diyonicacid isobutylamide

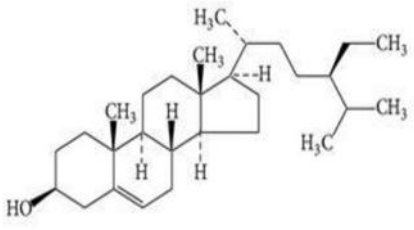
2E-N-(2-Methylbutyl)-2-undecene-8,10-diynamide



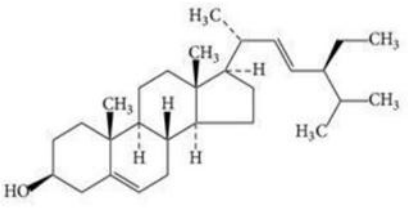
2E,7Z-N-Isobutyl-2,7-tridecadiene-10,12-diynamide



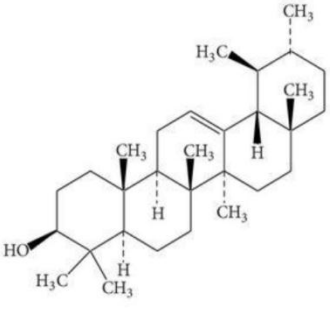
(Z)-β-Ocimene



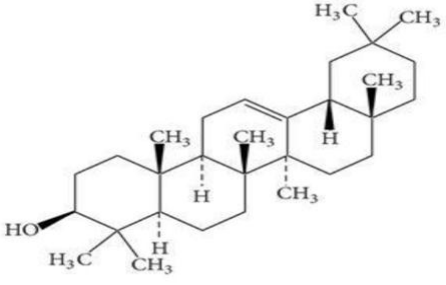
β-Sitosterol



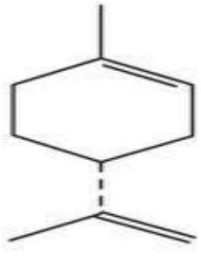
Stigmasterol



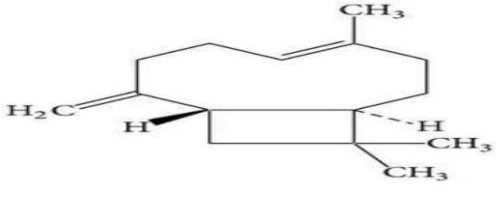
α-Amyrin



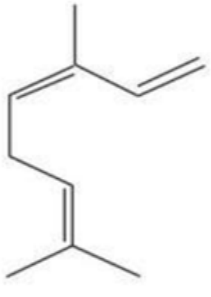
β-Amyrin



Limonene



Caryophylene



(Z)-β-Ocimene

Germacrene-D

### 4.1.2 Pharmacological Studies

The Spilanthes genera have been used for the treatment of various disorders including life-threatening diseases. Diverse pharmacological activities of this plant species were previously reported (Sahu et al., 2011). Selected bioactivities of S. acmella are summarized below. Antipyretic Activity Chakraborty et al. (2010) studied the antipyretic activity of Spilanthes acmella which was carried out by yeast induced method as yeast is commonly used for the induction of pyrexia. The dose varies accordingly in various studies.

#### 4.1.2.1 Anti-inflammatory Activity

EtOH extract from the leaves of S. acmella exhibited significant antiinflammatory activity against acute (carragenan induced rat paw edema method), sub-acute (granuloma pouch method) and chronic (adjuvant arthritis method) inflammation (Barman et al., 2009) but has been shown to be less than that of aspirin. The observed anti-inflammatory activity originates from the inherent flavonoids that are found in the plant extracts (Chakraborty et al, 2010).

#### 4.1.2.2 Analgesic Activity

A number of antitoothache plants has been recognized, S. acmella is one of these plants that has been used in pain relief. The studies showed that S. acmella EtOH leaves extracts exerted significant centrally (e.g. tail flick method) and peripherally (e.g. Writhing test) analgesic activities (D' Armour and Smith, 1941; Witkin et al., 1961). The mechanism of action was possibly due to the presence of flavonoids in the plant extract (Chakraborty et al., 2010) which decreases prostaglandins, PGE2 and PGF2 that are known to be involved in pain perception (Jyothi et al., 2008). In addition, cold aqueous extract of S. acmella flowers also displayed antinociceptive activity against persistent pain and antihyperalgesic activity. The mechanism of action was possibly through inhibition of prostaglandins by spilanthol-containing extract (Ratnasooriya and Pieris, 2005).

#### 4.1.2.3 Local Anesthetic Activity

S. acmella aerial aqueous extract exhibited significant activity that could be due to the presence of alkamides (Chakraborty et al., 2010). However, its onset of action was slower than that of xylocaine, the standard drug. The well-recognized local anesthetics are comprised of mostly amide compounds such as xylocaine (lidocaine). Its mechanism of action involves the blockage of voltage-gated Na+ channels.

#### 4.1.2.4 Antimicrobial Activity

Ethyl acetate (EtOAc) and methanol (MeOH) extracts from the leaves of S. calva exhibited the strongest antimicrobial activity among the tested extracts using the well diffusion method against Klebsiella pneumoniae (Arora et al., 2011). The EtOAc extract had two-fold higher activity than that of doxycycline, the standard drug, whereas the MeOH extract showed comparable activity with doxycycline. On the other hand, aerial parts of EtOAc and MeOH extracts from S. acmella tested by the agar dilution method were shown to be inactive antimicrobials whereas its chloroform (CHCl3) extract displayed antimicrobial activity against Streptococcus pyogenes with MIC of 256 μg/mL.

#### 4.1.2.5 Antifungal Activity

Several parts of S. calva were tested for antifungal activity and the studies showed that S. calva leaves (EtOAc and aqueous) extracts exhibited better antifungal activity than the standard drug (fluconazole) against Rhizopus arrhigus and Rhizopus stolonifer (Arora et al., 2011).The leaves extract also displayed weak activity against Aspergillus niger and Penicillium chrysogenum (Arora et al., 2011). The whole plant CHCl3 extract was shown to be active antifungal against opportunistic fungal infection (e.g. Microsporum gypseum and Cryptococcus neoformans) in AIDS patients.

#### 4.1.2.6 Antimalarial Activity

S. calva is a traditional medicine used in Africa and India for the treatment of malaria (Spelman et al. 2011). Spilanthol and acetylenic alkamide isolated from the root EtOH extract of S. acmella, displayed antimalarial activity against two strains of Plasmodium falciparum. Both compounds had a reported antimalarial activity with IC50 in the range of 5.8-41.4 μg/mL in which the spilanthol was the most potent compound. Semi-purified compounds of S. acmella, isolated by centrifugal partition chromatography (CPC) and showed significantly higher antiplasmodial activity as indicated by the lower IC50 value.

#### 4.1.2.7 Antioxidant Activity

Antioxidant activity of S. calva extracts obtained from polar and nonpolar solvents were investigated. It was found that S. acmella flower EtOAc extract displayed the highest free radical scavenging activity when compared to the other tested extracts (Wu et al., 2008). On the other hand, leaves and flowers of S. acmella MeOH extracts showed weak antioxidant activity (Nanasombat and Teckchuen, 2009). The aerial parts of S. calva were also investigated.

#### 4.1.2.8 Vasorelaxant Activity

S. calva extracts were studied for their vascular effects using rat thoracic aorta (Wongsawatkul et al., 2008). The results showed that the tested extracts exhibited vasorelaxant activity via partial endothelium- induced NO and PGI2 in dose dependent manner. EtOAc extract displayed immediate vasorelaxant and the most potent antioxidant (DPPH) activities. Similar vasorelaxant and antioxidant (SOD) activities were also observed in the CHCl3 extract of the plant species (Prachayasittikul et al., 2009b; Wongsawatkul et al., 2008). These bioactivities can be attributed to the presence of phenolic and triterpenoids (Prachayasittikul et al, 2009).

#### 4.1.2.9 Diuretic Activity

Naturally occurring diuretics such as caffeine are known to be present in coffee, tea and cola. So far in Ayurvedic practice, many indigenous drugs have been claimed to have diuretic effect. The study of S. acmella EtOH leaves extract revealed diuretic effect possibly arising from tannin, steroid and carotenoid (Vanamala et al., 2012). In addition, flower cold aqueous extract of the plant species exhibited strong diuretic activity. The effect may be attributed to its alkaloids. It was suggested that the extract acted as a loop diuretic, which is the most powerful of all diuretics (Ratnasooriya et al., 2004). However, several other diuretic plants from different families have been reported to contain triterpenoids, steroids, saponins, alkaloids, flavonoids, phenolics, glycosides and bis-benzylisoquinolines (Vanamala et al, 2012).

#### 4.1.2.10 Immunostimulant Activity

S. calva leaves have been used traditionally as tonic, treatment of rheumatism, gout and sialogogue as well as being claimed to possess immunostimulant activity (Savadi et al., 2010). The investigation was performed using various experimental models. The EtOH leaves extract showed significant immunomodulatory activity by increasing macrophage count with the maximum number of cells on the 15th day (Savadi et al., 2010). The leaves of S. acmella contained various compounds such as alkamides, pungent amides, carbohydrates, tannins, steroids, carotenoids, essential oils, sesquiterpenes and amino acids (Amal and Sudhendu 2014).

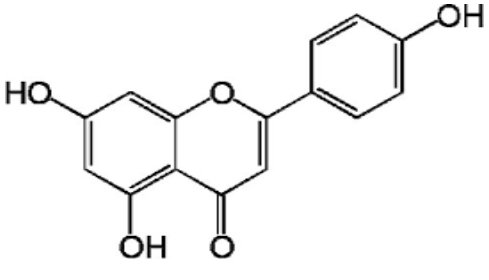
### 4.2 *Solanum virginianum:*

#### 4.2.1 Phytochemistry:

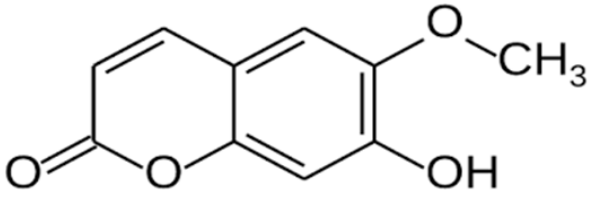
Different classes of compounds were previously isolated from various *Solanum virginianum*, which can be associated to this plant's pharmacological activities. The main groups are glycoalkaloid, solasonine. From the non alkaloidal portion, a glycoside of β-sitosterol with galactose as a sugar moiety has been obtained along with two phenolic substances, which could be identified as methyl caffeate and caffeic acid. The fruits are reported to contain several steroidal alkaloids like solanacarpine and solamargine . Other constituents like caffeic acid coumarins like aesculetin and aesculin. steroids carpesterol, diosgenin, campesterol, daucosterol and triterpenes like cycloartanol and cycloartenol were reported from the fruits.

The fruit of *Solanum virginianum* contains alkaloid saponins which can be extracted in alcohol and have a heartstimulating function .The detailed study on this plant resulted in the isolation of solasonine and solasodine ,ß-sitosterol , and carpesterol . The fruits contained 20.71% of dry seeds, 4.62 of pericarp and 74.67 percent of moisture. The powdered seeds were extracted with benzene and yielded 19% of greenish-yellow oil which did not contain nitrogen or sulphur. The composition of the oil was calculated as oleic acid, 42.93; linolic acid, 36.18; palmitic acid, 5.37; steric acid, 9.77; arachidic acid, 0.35, and unsaponifiable matter, 1.2 percent. ( H. A. Kadir et al., 2014).

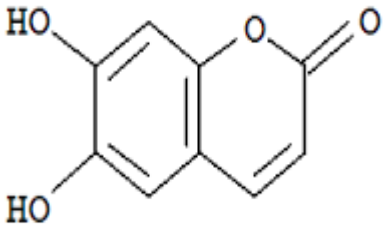
**Flavones, Phenolics & Coumarin**



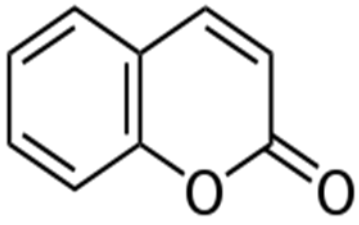

Apigenin



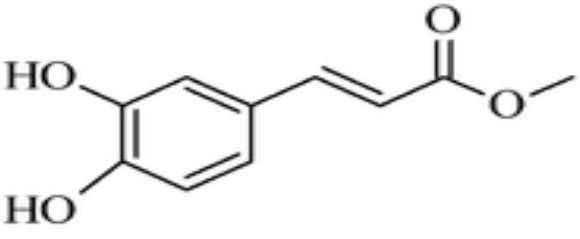

Scopoletin



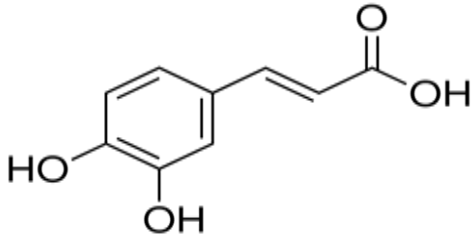

Esculetin




Coumarin

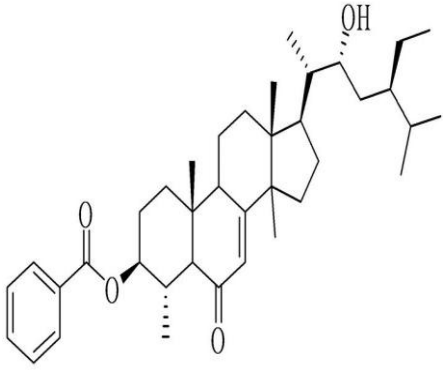



Methyl Caffeate

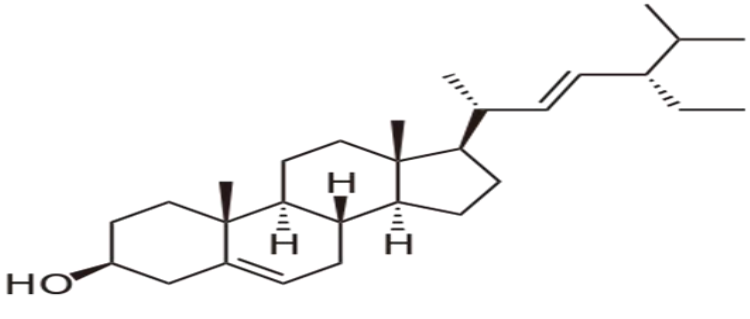



caffeic acid

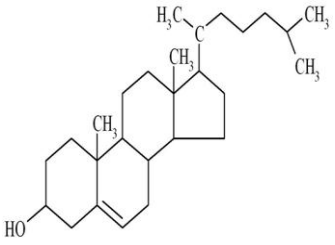
**Steroids and triterpenoid**



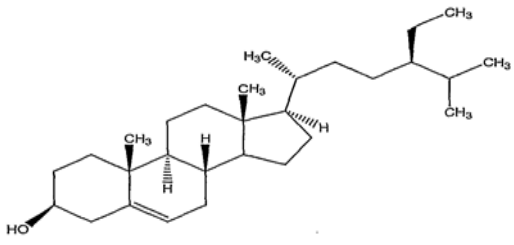

Carpesterol



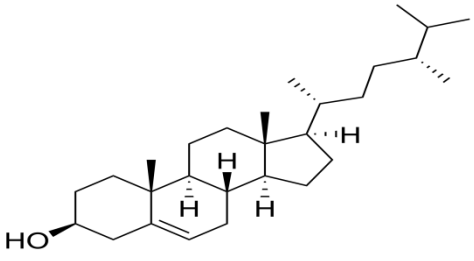

Stigmasterol



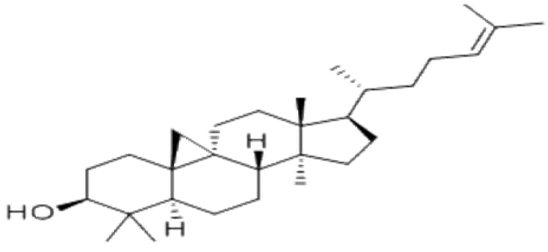
Cholesterol



Sitosterol

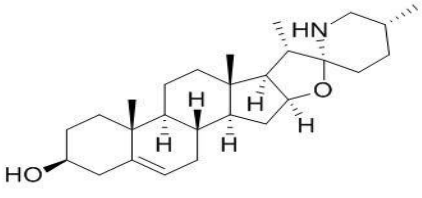


Campesterol

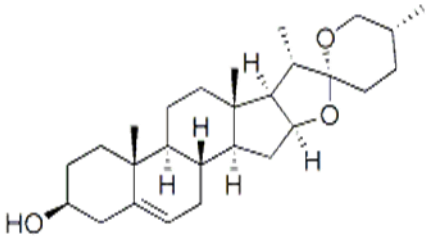


Cycloartenol

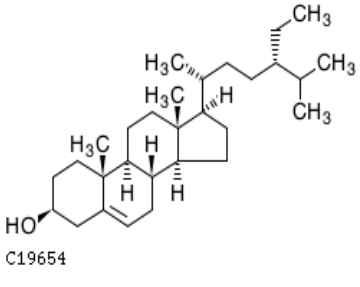
**Steroidal alkaloids, glycoalkaloids**



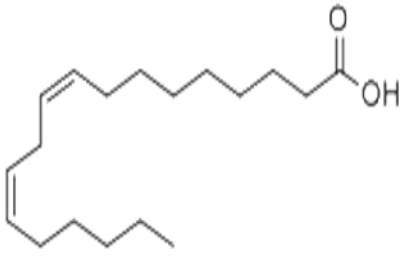
Solasonin



Diosgenin




Tomatidenol



Linoleic acid

### 4.2.2 Biological Activity

#### 4.2.2.1 Antifertility Activity

Solasodine and alkaloid of *S. viginianum* possesses antispermatogenic activity. Chronic administration of solasodine (20 mg/kg alternate day for 30 days) caused testicular lesions resulting in a severe impairment of spermatogenic elements. Total protein, sialic acid and glycogen contents of testis and epididymis were reduced significantly whereas testicular cholesterol was elevated. Acid phosphatase enzyme activity of testes was low after solasodine treatment. Serum enzymes(SGPT, alkaline phosphatase), serum protein,triglycerides, non-esterified fatty acid levels were in normal range when compared with their controls. (Jeppesen et al, 2013)

#### 4.2.2.2 Pyretic Body Temperature Effect

Action of solasodine was studied on normal and pyretic body temperature of rats and mice. In rats, a single dosage (3 mg/kg) depressed by an average of -1.5+/-0.3°C normal temperature for 24 h. With larger dosages or longer treatment, effect could not be intensified and tolerance was also not observed. In mice, temperature decrease was even more explicit;-2.0+/-0.2°C lasting 48 h. Effect was reproducible by repeated treatment. In mice, fever provoked by a suspension of killed bacteria, Pyrago or by 2, 4-dinitro-phenol (DNP) could be counteracted with solasodine(1 mmol/kg). Body temperature depressed with solasodine was not raised by Pyrago but became higher after administration of DNP, in part of animals even comparable to normal initial averages.

#### 4.2.2.3 Anticancer Effect

Lupeol, apigenin and solamargine exhibited anticancer property. Appearance in solamargine-treated cells of chromatin condensation, DNA fragmentation, and a sub-G1 peak in a DNA histogram suggested that solamargine induced cell death by apoptosis. Maximum number of dead Hep3B cells was detected within 2 h of incubation with constant concentrations of solamargine, and no further cell death was observed after an extended incubation with solamargine, indicating that action of solamargine was irreversible. To determine susceptibility of cell phases to solamargine-mediated apoptosis, Hep3B cells were synchronized at defined cell cycles by cyclosporin A, colchicine, and genistein, followed by values of solamargine for control, G0/G1-,M-, and G2/M-synchronized Hep3B cells were 5.0, 10,3.7, and 3.1 ìg/ml, implying that cells in G2/M phases are relatively susceptible to solamargine-mediated apoptosis. (Jeppesen et al., 2013)

#### 4.2.2.4 Inhibition of Fungal Growth

Fewell studied inhibition of mycelium development in Phoma medicaginis and Rhizoctoniasolani by solamargine and solasonine, which generally increased with increasing pH. P. medicaginis was more susceptible and solamargine more potent compound. Solasonine was inactive against R. solani over tested pH range (5-8). Dose-response curves confirmed these differential effects. Solamargine caused 50% growth inhibition in P. medicaginis at 60 μM (pH 7), where as no other treatment achieved this effect at 100 μM. Combinations of 50 μM of each glycoalkaloid produced synergistic effects against both fungi, especially Rhizooctonia solani, which was essentially unaffected by either compound, but significantly inhibited by a 1:1mixture of the two.

#### 4.2.2.5 Hypoglycemic activity

The Kondh tribes of Dhen kanal district of Orissa, India use the hot aqueous extract of the matured fruits as a traditional medicine for the treatment of diabetes mellitus. The aqueous extract showed significant hypoglycemic effect in both normal and streptozotocin induced diabetic rats at dose of 100 and 200 mg/kg. The activity showed by aqueous extract was comparable to that of standard oral hypoglycemic agent glibenclamide. The experimental results indicated that it exhibited a potent blood glucose lowering property both in normal and streptozotocin induced diabetic rats. The LD50 of the extract was found to be high indicating high margin of safety.

#### 4.2.2.6 Hepatoprotective activity

Jigrine is a poly pharmaceutical herbal formulation containing aqueous extracts of 14 medicinal plants including *solanum virginianum* and used for liver ailments. A. K. Najmi investigated the DPPH-free radical scavenging activity, hepatoprotective and antioxidant activity of Jigrine against galactos amine induced hepatotoxicity in rats.

#### 4.2.2.7 Cardiovascular effects

Pasnani JS (1988) reported that Abana, a polyherbal formulation containing SV causes: (i) A direct sensitization of the atrium through an increase in permeability to Ca2+ and (ii) an effect similar to withdrawal of chronic ISO administration, i.e. down regulation of beta adrenoceptors. Lalit Mohan (2006) reported the larvicidal potential of crude extracts of SV and suggested its suitability as an ecofriendly, effective larvicide in the management of mosquito populations and in limiting the outbreak of various vector borne epidemics.

#### 4.2.2.8 Mosquito larvicidal effect

The plant has been used in the various fields of pest management but it is not exploited in vector control. The fruit extracts of SV revealed larvicidal activity against An. stephensi and Cx. Quinque fasciatus and one culicine species Ae. aegypti. Volatile oil obtained from SV exhibited repellency against mosquito at a very lower concentration than those of the plants studied earlier. The lethal concentrations of fruit extract at LC50 and LC90 levels against An. culicifacies, An. stephensi and Ae. aegypti were determined as 0.112 and 0. 258, 0.058 and 0.289 and 0.052 and 0.218% respectively .

#### 4.2.2.9 Snail-killing Activity

α-Solamargine from fruit of S. viginianum shows an excellent effect in killing (100% at 28 °C)Oncomelania snails in solution of alpha-solamarrgine(0.2 mg/l).

#### 4.2.2.10 Anti-allergy Activity

Apigenin has shown anti-allergic effect of apigenin in ovalbumin (OVA)-induced asthma modelmice. OVA-induced mice showed allergic airway reactions and included an increase in number of eosinophils in bronchoalveolar lavage (BAL) fluid, an increase in inflammatory cell infiltration into lung around blood vessels and airways, airway luminal narrowing, and development of airway hyper-responsiveness (AHR).

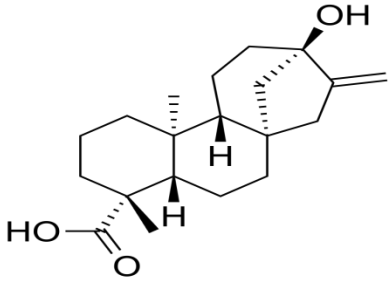
### 4.3 *Stevia rebaudiana*

#### 4.3.1 Phytochemistry:

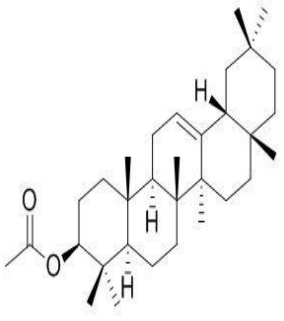
The sweet diterpene glycosides of stevia have been the subject of a number of reviews (Kinghorn and Soejarto 1985; Crammer and Ikan 1986; Hanson and De Oliveira 1993). Treatment of this substance with the digestive juice of a snail yielded 3 mol of glucose and 1 mol of steviol, while acid hydrolysis gave isosteviol (Bridel and Lavieille 1931b). Isosteviol was also obtained when steviol was heated in dilute sulfuric acid. Subsequent studies have led to the isolation of seven other sweet glycosides of steviol. Typical proportions, on a dry weight basis, for the four major glycosides found in the leaves of wild stevia plants is 0.3% dulcoside, 0.6% rebaudioside C, 3.8% rebaudioside A and 9.1% stevioside.

### 4.3.2 Structure of Steviol, Isosteviol and Stevioside

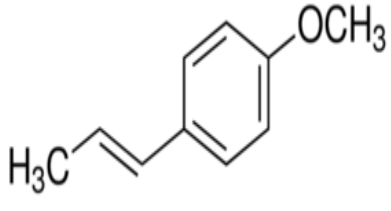
The structure, steriochemistry and absolute configuration of steviol and isosteviol were established, through a series of chemical reactions and correlations over 20 yr after the pioneering work of Bridel and Lavieille. Structures of these and other diterpenes and diterpene glucosides are presented in Fig.



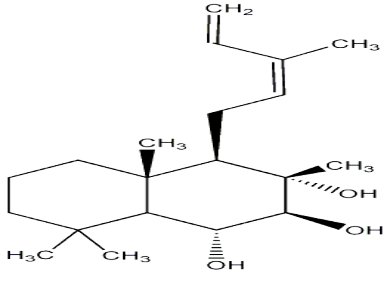

Steviol



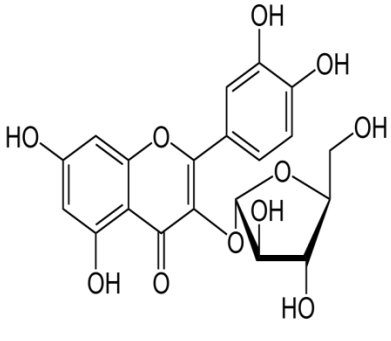

Amyrin, beta acetate

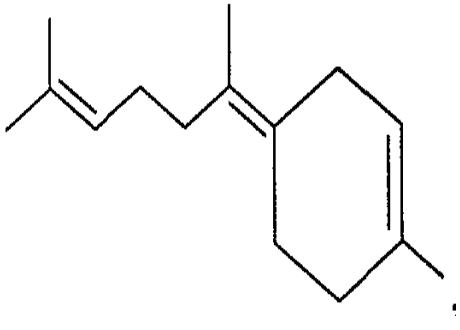



Anethole



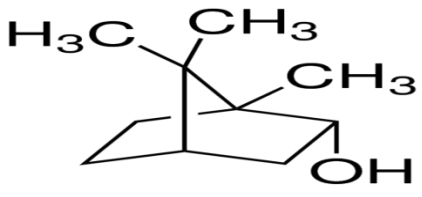

Austroinulin

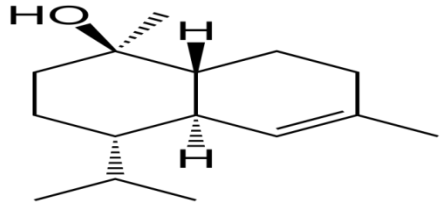


Avicularin

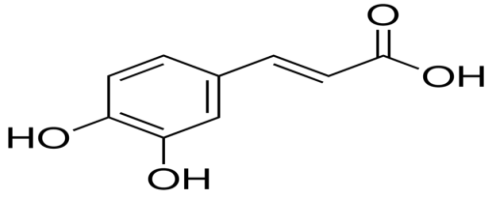
Bisabolene

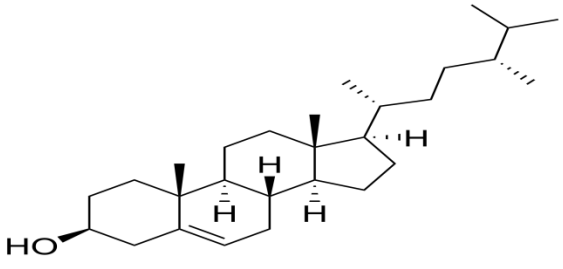



Borneol

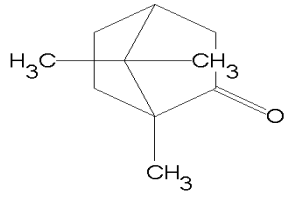
Cadinol, alpha

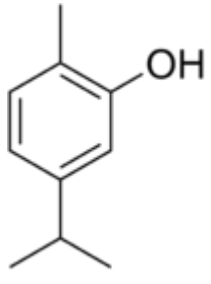



Caffeic acid

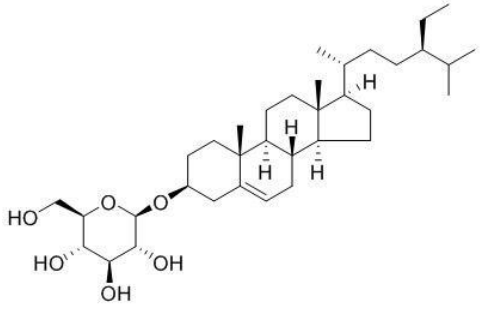
Campesterol

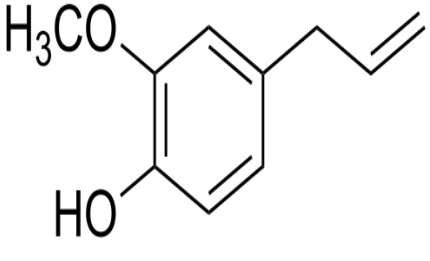



Camphor

Carvacrol




Daucosterol

Eugenol

### 4.3.3 Pharmacological activity of *Stevia rebaudiana*

#### 4.3.3.1 Diabetes

Diabetes mellitus (DM) is a group of diseases characterized by hyperglycemia and varying degrees of an insufficient insulin effect. Steviol glycosides have an enhancing effect on insulin secretion by directly acting on β-cells without altering the K+ - ATP channel activity and cAMP level in the islets, thus documenting stevioside and steviol as potent antihyperglycemic agents (Jeppesen et al., 2000). Stevioside regulate blood glucose levels by enhancing not only insulin secretion, but also insulin utilization in insulin-deficient rats; which was due to decreased phosphoenolpyruvate carboxykinase (PEPCK) gene expression in rat liver by stevioside's action of slowing down gluconeogenesis suggested by Chen et al. (2005). Study conducted in diabetic humans, where a single acute dose of stevioside (1,000 mg) was able to reduce the diseases. (Goyal et al, 2013).

#### 4.3.3.2 Blood pressure regulation

Essential hypertension‖ is defined as an increase in blood pressure above certain measured levels. The definition of high blood pressure begins at a systolic blood pressure of 140 mmHg and a diastolic blood pressure of 90 mmHg. *Stevia* can be used as a heart tonic to normalize blood pressure levels, to regulate heartbeat, and for other cardiopulmonary indications. In humans, a hot water extract of the leaf has been shown to lower both systolic and diastolic blood pressure. Studies on *Stevia* extracts, as well as its isolated glycosides, demonstrate its hypotensive and diuretic action. *Stevia* acts at the cell membrane level much in the same way as a type of medication known as a calcium channel blocking agent. Studies suggest that *Stevia* acts to relax arteries and lower blood pressure.

#### 4.3.3.3 Cancer

Cancer can be regarded as a disease of the body's cells. Its development involves damage to the DNA of the cells and this damage accumulates overtime. *Stevia* and its metabolites have been used for years throughout the world as a natural non-nutritive sweetening alternative to sucrose and other nutritive variants (Goyal et al., 2010). In addition, the toxicity of *Stevia* has been investigated extensively in both short- and long-term studies. Importantly, no serious toxic, genotoxic, or carcinogenic effects were detected in mammalian species and it is safe for human consumption (Aze et al., 1991; Toyoda et al., 1997). Labdane sclareol, compound present in leaf extract of *Stevia* has anti-tumorous and cytotoxic properties (Kaushik et al., 2010). Studies have demonstrated the inhibitory effects of *Stevia* leaf extracts and their polyphenolic constituents on tumor promotion and initiation.

#### 4.3.3.4 Antimicrobial activity of *Stevia rebaudiana*

The present investigation was evaluated for potential antimicrobial activity of Stevia rebaudiana leaf extracts, procured from Indian acidic and basic soil zones. Separately Stevia leaves were extracted with aqueous, methanol and ethanol solvents and their microbiocides were compared against few selected gram positive (Bacillus substilis and Staphylococcus aureus) and gram negative bacteria (Escherichia coli, Salmonella typhi) by disc diffusion technique. The study revealed the potential antimicrobial activity of different leaf extracts of *Stevia rebaudiana* , determined with zone of inhibition against standard amphicilin.

#### 4.3.3.5 Antioxidant Properties of *Stevia Rebaudiana* Leaf Extracts

DPPH• radical scavenging by stevia extracts was performed according to the Brand-Williams et al. method . Stevia extract or appropriate solvent (1 mL) was mixed with 25 mM DPPH• solution in 96% ethanol (1 mL). Following 40 min incubation at room temperature the absorbance of the sample was measured at $\lambda = 515$ nm using 96% ethanol as a blank sample. All samples were analyzed in triplicates. The percentage of DPPH• scavenging was calculated for each sample based on the equation:

$$\% \text{ of DPPH• scavenging} = [1 - (As/Ac)] \times 100\%$$

Where: As—absorbance of the sample; Ac—absorbance of the control sample (DPPH• solution). The IC50 value was defined as the amount of total phenols or flavonoids in each extracts from 1 g of stevia leaves that is required to scavenge 50% of DPPH• radical activity. (Goyal et al., 2013).

#### 4.3.3.6 Renal effects

The effects of administration of *Stevia rebaudiana* extracts for 20, 40 and 60 days on renal function and mean arterial pressure in normal Wistar rats were evaluated. Results showed that the *Stevia rebaudiana* treated rats group for 20 days did not significantly differ from the control group. Chronic administration of a crude extract for 40 and 60 days induced hypotension, diuresis and natriuresis with glomerular filtration rate (GFR) constant. The results suggests that oral administration to rats of an aqueous extract of *Stevia* dried leaves induce systemic and renal vasodilation, causing hypotension, diuresis and natriuresis.

#### 4.3.3.7 Effects of *Stevia rebaudiana* on rat liver mitochondria

The effects of several atural products extracted from the leaves of *Stevia rebaudiana* on rat liver mitochondria were investigated. The compounds used were stevioside (a non-caloric sweetener), steviolbioside, isosteviol and steviol. Total aqueous extracts of the leaves were also investigated. *S. rebaudiana* natural products inhibited oxidative phosphorylation, ATPase activity NADH-oxidase activity, succinate-oxidase activity, succinate dehydrogenase, and l-glutamate dehydrogenase. The ADP/O ratio was decreased. Substrate respiration (state II respiration) was increased at low concentrations (up to 0.5 mM) and inhibited at higher concentrations (1 mM or more).

#### 4.3.3.8 Anti-inflammatory Activity of Stevia rebaudiana

In addition to its low calorie sweet taste, SR appears to have other beneficial properties, such as hypotensive capabilities and inflammation reduction. To identify the bioactive natural constituents exerting anti-inflammatory activities, we examined the EtOAc fraction of SR. In the inflammatory mediator inhibitory assay from lipopolysaccharide (LPS)-activated macrophages, the EtOAc fraction significantly, and dose dependently, inhibited the enhanced production of nitric oxide (NO) and inducible nitric oxide synthase (iNOS) expression.

#### 4.3.3.9 Anthelmintic and Antifungal Activities of Leaf Extracts of *Stevia rebaudiana*

The alcohol and aqueous extracts were subjected to determine their *in vitro* anthelmintic and antifungal activities against *P. posthuma* and *A. galli* earthworms as well as plant pathogenic fungi *B. cinerea* and *F. oxysporum*, respectively. The alcoholic and aqueous extracts of *S. rebuadiana* contained steroids, glycosides, tannis, alkaloids and saponnis like phytochemicals. Both alcoholic and aqueous extracts (25-100 mg/ml) exhibited significant anthelmintic activity at the used concentrations involving determination of paralysis and death time of the tested worms.

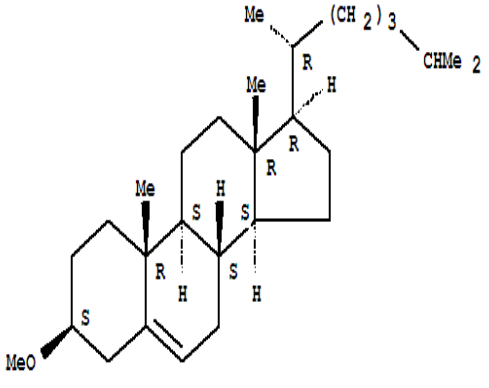
### 4.4. *Ruellia tuberosa*:

*Ruellia tuberosa* L. (Acanthaceae) is a tropical plant and widely distributed in Southeast Asia. In folk medicine, it has been used as antidiabetic, antipyretic, analgesic, anti hypertensive, thirst-quenching and antidotal agent. The GC-MS analysis of ethanol extract of tuber of *Ruellia tuberosa* has been evaluated to identify the compounds of therapeutic value. (Kumar et.al, 2014)

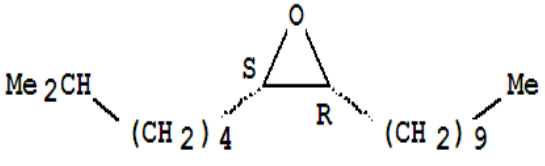
### 4.4.1. Bioactive molecules:

There are different compounds which were detected in ethanol extract of the *Ruellia tuberosa*. The results revealed that Lupeol (68.14%), Stigmasterol (8.89%), á- Sitosterol (3.99%), Sucrose (2.24%), Cholest- 5-ene, 3-bromo-, (3á- (2.24%), Octadecane, 2-methyl- (2.10%), Nonadecane, 2-methyl- (1.93%), Eicosane, 2-methyl- (1.79%) Heptacosane (1.43%) and Heptacosane (1.29%) were found as the major compounds in the ethanol extract of tuber of R. tuberosa plant. Among the identified phytochemicals, Tetradecanoic acid and n-Hexadecanoic acid have the property of antioxidant activity. (Sutha et.al, 2012)

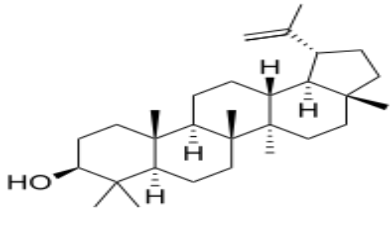
Squalene has the property of antioxidant. Recently squalene possesses chemopreventive activity against colon carcinogenesis. The results show that, reactive oxygen speciespromising novel class of pharmaceutical for the treatment of rheumatic arthritis and possibly other chronic inflammatory diseases. (Balamurugan et.al, 2012) (Jegadeeswari et.al, 2012).



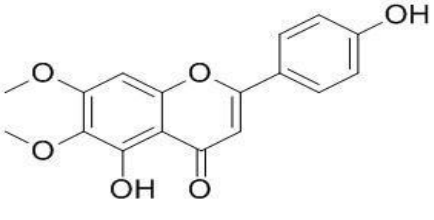

Cholest- 5-ene, 3-bromo



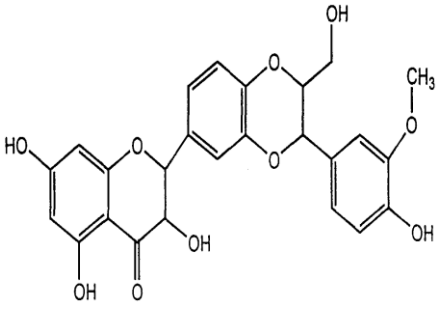

Octadecane, 2-methyl



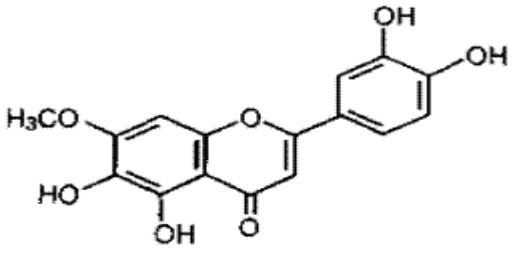

Lupeol



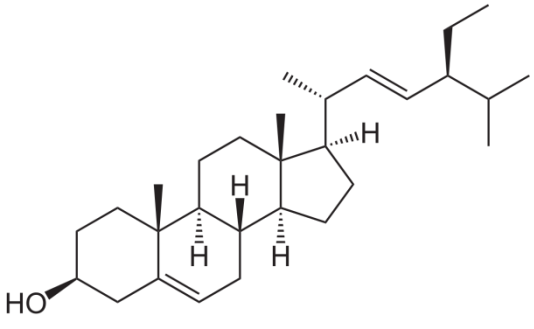

Cirsimaritin



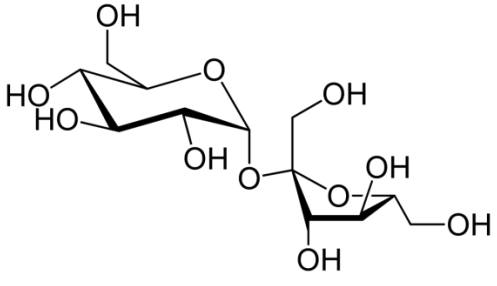
Cirsiliol 4′-glucoside



Pedalitin



Stigmasterol



Sucrose

Figure 4.4.1 : Structure of the *Ruellia tuberosa* compound

**4.4.2 Pharmacological activity:**

*Ruellia tuberosa* exhibits a number of pharmacological activity such as diuretic, emetic, antioxidant, analgesic etc. Some of activities are given below:

**4.4.2.1 Hepato protective activity:**

Arirudran et.al carried out a systematic investigation of the protective influences of the ethyl acetate and ethanolic extract of *R.tuberosa* against N-Nitrosodiethylamine-induced hepato-carcinogenesis in wistar albino rats. Liver function was assessed by the determination of AST, ALT and ALP levels along with histopathological examinations. The serum biochemical analysis suggests that the use of ethanolic extract of *R.tuberosa* exhibited significant protective effect from hepatic damage in N-Nitrosodiethylamine induced hepato-toxicity model. (Kumar et.al, 2014)

#### 4.4.2.1 Antinociceptive and anti-inflammatory properties

Alam M. A et.al studied the antinociceptive and anti-inflammatory properties of ethanol extract of *Ruellia tuberosa* L. in experimental mice and/or rat models. The maximum possible analgesia (MPH) was 1.93 for the extract dose 300 mg/kg, while that for diclofenac was 2.29 after 60 min of administration in the hot tail-flick method. The anti-inflammatory properties exhibited by the extract were comparable to that of indomethacin at a dose of 5 mg/kg (serotonin-induced edema 53.22; egg albumin- induced edema 57.01% inhibition after 4 h) (Alam et.al, 2009).

#### 4.4.2.2 Anti-carcinogenic activity:

Dey S. et. al studied the anti-carcinogenic & anti-oxidant property of Ruellia tuberosa L. (Acanthaceae) methanolic leaf extract (MERTL) on HepG2 cell line & RAW 264.7 murine macrophage cell lysate respectively. Cytotoxicity study of MERTL has been confirmed by MTT assay & the IC50 value is calculated to be 54.95µg/ml on HepG2 cell line. Agarose gel electrophoresis study showed fragmented DNA in the form of ladder.

#### 4.4.2.3 Antibacterial, antifungal and insecticidal activities:

Kader M.A et.al studied the activity of methanolic extract of *Ruellia tuberosa* against all the bacteria and fungi tested and showed significant antibacterial and antifungal properties with the zone of inhibition 9 to 23 mm for antibacterial screening and 8 to 15 mm for antifungal screening. The insecticidal assay by surface film activity test also revealed strong insecticidal activity with 80% mortality rate of *Tribolium castaneum* (Herbst) at a dose of 50 mg/ml in 48 hours. (Kader et al, 2012)

#### 4.4.2.4 Antidiabetic activity:

Rajan M. et.al studied induced diabetes in Albino rats by administration of alloxan monohydrate (150mg/kg, i.p). The methanol extract of *Ruellia tuberosa* linn leaves at a dose of 100 and 200mg/kg of body weight was administered at single dose per day to diabetes induced rats for a period of 14 days. The methanol extract of Ruellia tuberosa linn leaves elicited significant reductions of blood glucose ($P<0.05$). (Rajan et.al, 2012

#### 4.4.2.5 Hypolipidemic and anti-oxidant activity:

Krishna C.B studied the efficacy of Ruellia tuberosa ethanolic extract (RTEE2012) in reducing the cholesterol levels and as an antioxidant in hypercholesterolemic rats. Powdered form of RTEE2012 was

administered as feed supplement at 250, 500 and 1000 mg/kg dose levels to the hypercholesterolemic rats. Feed supplementation with 250, 500 and 1000 mg/kg of RTEE2012 resulted in a significant decline in plasma lipid profiles. The feed supplementation increased the concentration of catalase, SOD and HDL-c significantly in the experimental groups (250, 500 and 1000 mg/kg).

#### 4.4.2.6 Anti ulcerant activity:

SriKumar P.P studied the ulcer protective activity of ethyl acetate extract of *Ruellia tuberosa* L. (Acanthaceae) in male Wistar rats. Rantidine was used as standard drug (20mg/kg b.w., i.p). In pylorus ligation induced ulcer model, various parameters were studied (pH, total acidity, free acidity and ulcer index). The ethyl acetate extract showed significantly decrease in gastric volume, total acidity and free acidity. The value of ulcer index decreased in a dose dependent manner, when compared to control group. (Kumar et.al, 2014)

### 4.5 *Mikania cordata*

#### 4.5.1 Bioactive Compounds

From *Mikania cordata* different classes of compounds were previously isolated, which can be associated to this plant's pharmacological activities. The main groups are: coumarins and derivatives, sesquiterpenes, sesquiterpenes lactones, diterpenes, phytosterols or terpenoids and flavonoids.

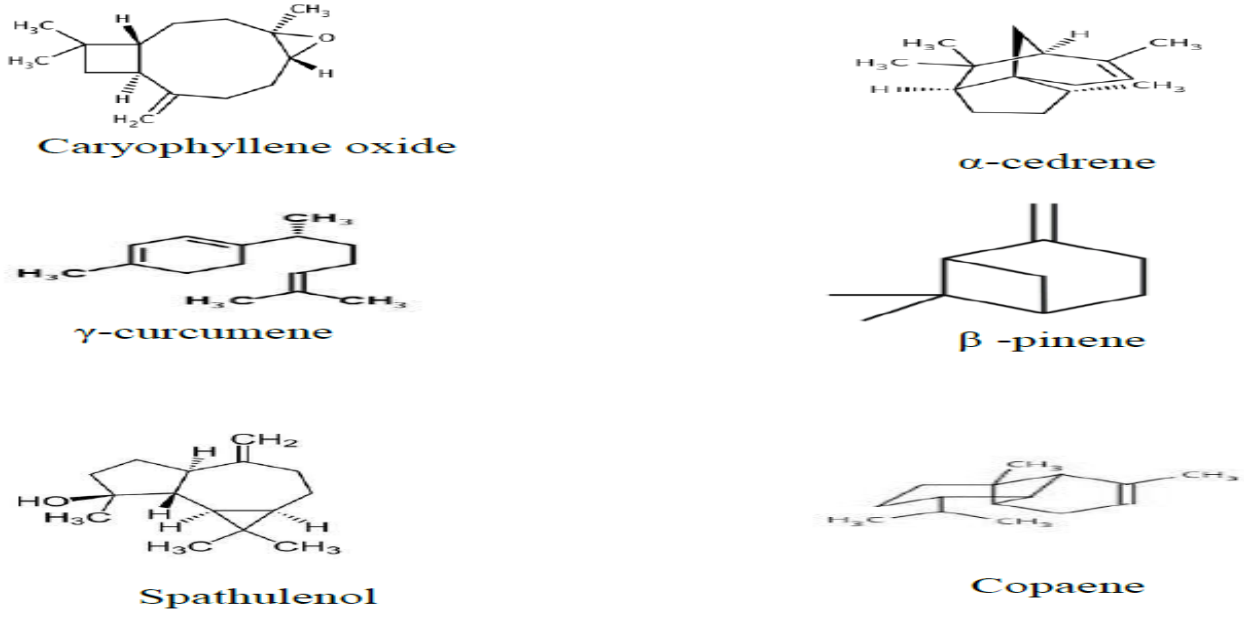



Figure 4.5.1: Sesquiterpenes in *Mikania cordata* in the part of leaves

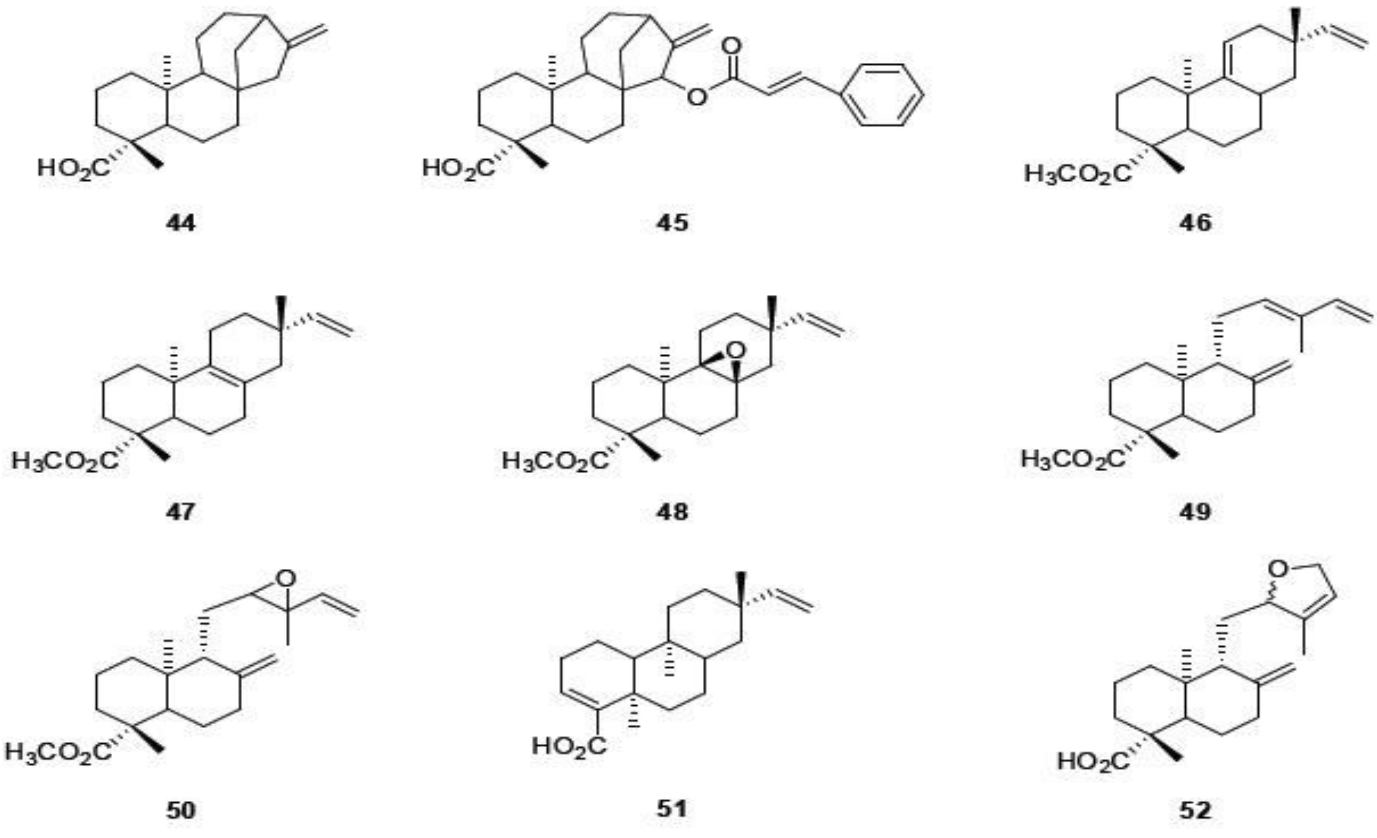



Figure 4.5.2: Structure of kaurenoic acid (44), cinnamoylgrandifloric acid (45), methyl-*ent*-pimara-9 (11),15-dien-19-oate (46), methyl-*ent*-pimara-8,15-dien-19-oate (47), methyl-8,9α-epoxy-*ent*-pimara-15-en-19-oate (48), labda-8(17),12,14-trien-19-oic methyl ester (49), pimara-9(11),15-dien-19-oic methyl ester, labda-8(17),13(16),14-trien-19-oic methyl ester, labda-12α-epoxy-8(17),14-dien-19-oic methyl ester, labda-12β-epoxy-8(17),14-dien-19-oic methyl ester (50), erythroxyla-3,15-dien-19-oic acid (51), labda-12,15-epoxy-8(17),13-dien-19-oic acid (52). (Kumar et.al, 2014)

### 4.5.2 Pharmacological Studies

#### 4.5.2.1 Analgesic Activity

In 2001 scientist M. Ahmed and his co worker studied on the analgesic activity of the crude extract of *Mikania cordata* and deoxymikanolide significantly inhibited acetic acid-induced writhing in mice. Three other sesquiterpene dilactones isolated from the same plant, namely mikanolide, dihydromikanolide and scandenolide, did not show significant analgesic activity.

#### 4.5.2.2 Anti Carcinogenic Activity

In 1994 A. Bishayee, and his co-worker studied on Anti carcinogenic biological response of *Mikania cordata*, at frist the study has been evaluated through its effects on Phase 1 and 2 of the hepatic drug-detoxifying enzyme system in rats. Although oral administration of a methanolic extract of this plant root (50, 100 or 150 mg/kg for 4, 8 or 12 weeks) has been found to have very little or no effect on hepatic microsomal cytochrome *P*-450 and cytochrome *b*5 contents as well as NADPH cytochrome *c* reductase activity, it afforded a marked induction of uridine.

The extract also significantly increased the activities of microsomal uridine diphosphoglucose dehydrogenase, reduced nicotinamide adenine dinucleotide (phosphate. Results of the study clearly indicate that the intracellular contents of active intermediates of various xenobiotics including chemical carcinogens would be reduced (Kumar et.al, 2014).

#### 4.5.2.3 Anti-stress Activity

In 1995 A. Bishayee, and his co-worker studied on the effects of *Mikania cordata* root extract where the extract were investigated on stress-induced alterations in central neurotransmitters, viz., adrenaline (Ad), noradrenaline (NA), dopamine (DA) and 5-hydroxytryptamine (5-HT), and enzymatic activities of monoamine oxidase (MAO) in brain and succinic dehydrogenase (SDH) in brain and liver of mice. Both 5 h swimming and 24 h immobilization stress elicited a decrease in the levels of Ad and NA whereas they increased those of DA and 5-HT. Pretreatment with M. cordata root extract for 15 days prevented the decrement in Ad and NA and increment in 5-HT while the level of DA was further increased. There was a marked inhibition in brain MAO and stimulation in brain and liver SDH activities following both types of stress.

#### 4.5.2.4 Anti-ulcer Activity

In 2006 scientist S. Pal and his co worker studied on the methanolic fraction of the root extract *M. cordata* where it was found to possess significant anti-ulcer activity in different experimental models. In preventive tests the extract demonstrated significant protective action in gastric lesions induced by acetylsalicylic acid, serotonin and indomethacin in experimental rats. Significant protection was observed with the extract in chemically-induced duodenal lesions. Significant enhancement of the healing process was also found to occur in acetic acid-induced chronic gastric lesions in experimental animals (Pal et al., 2006).

#### 4.5.2.5 The Antibacterial and Cytotoxic Properties

In 2011 scientist M. Sekendar and his co-worker studied on the antibacterial and cytotoxic properties of ethanol extract of leaves of *Mikania cordata* (Burm.f.) B.L. Robinson. To determine antibacterial activities, the extract was tested against four Gram positive and six Gram negative bacteria at three concentrations (500, 800, 1000 μg disc-1) through disc diffusion method. The extract showed moderate antibacterial actions and that was increased by increasing the concentration of the sample. Cytotoxic property of the sample was done using Brine shrimp lethality bioassay where it did not show noticeable toxicity. (Bhattacharya et al., 1988).

#### 4.5.2.6 Anti-inflammatory Activity

In 2006 scientist S. Bhattacharya and his co-worker studied on the antiinflammatory profile of *Mikania cordata* (Burm) B. L. The methanolic fraction of the root extract of Mikania cordata was found to possess an inhibitory effect on carrageenin and other mediator-induced oedema; there was a significant inhibition of protein exudation, an increase in peritoneal capillary permeability and leucocyte migration in inflammatory conditions. The extract significantly inhibited both cotton pellet and carrageenin-induced granuloma formation, was effective in experimentally induced arthritic conditions and turpentine-induced joint oedema. The extract also possessed inhibitory effects on sodium urate-induced experimental gout.

#### 4.5.2.7 Effect on Nervous System

*Mikania* extracts possesses some neuropharmacological properties confirmed. The studies with methanolic fraction of *M. cordata* root extract on experimental animals caused alterations in the general behavior pattern (*e.g.* reduction in spontaneous motility, analgesia, and suppression of aggressive behaviour), suppression of conditioned avoidance response and showed antagonism to amphetamine toxicity. The observations suggest that the root of *M. cordata* possesses a potent central nervous system-depressant action (Bhattacharya et al., 1988).

# 5. Materials and method

Five plant samples were selected to conduct this thesis work; these are *Spilanthes calva*, *Solanum virginianum Stevia rebaudiana* and *Mikania cordata.* All five sample plants are subjected to phytochemicals and antioxidant test. The following methods and materials were used to conduct this thesis work.

## 5.1 Materials

### 5.1.1 Lists of Glass Wares

1. Glass rod
2. Pipette
3. pasteur pipette
4. test tube
5. vial
6. conical flask
7. separating funnel
8. beaker (large, medium and small)
9. round bottomed flask
10. volumetric flask
11. funnels
12. reagent bottle
13. measuring cylinders
14. water tank/aquarium
15. watch glass
16. magnifying glass
17. petri dish.
18. flat bottomed flask

### 5.1.2 Lists of Other Material

1. Aluminium foil paper
2. Spatula
3. pipette pumper

4. micropipette tip
5. cotton wool cloth
6. gas burner
7. forceps
8. masking tape
9. filter paper
10. permanent marking pen
11. scissors
12. pH meter

### 5.1.3. Lists of Equipments

1. Rotary vaccum evaporator
2. UV-Visible Spectrophotometer
3. Hot air oven
4. centrifuge machine
5. lamp with bulb
6. Electric balance
7. Rough balance
8. Distilled water plant.

### 5.1.4 Lists of Solvents

1. Methanol
2. Benzene
3. Chloroform
4. cyclohexane
5. Ethyl acetate

### 5.1.5 Lists of Reagents

1. Folin-Ciocalteu reagent
2. Salicylic acid
3. Sodium carbonate
4. DPPH (2,2-diphenyl-2-picrylhydrazyl)
5. Ascorbic acid

6. Monobasic sodium phosphate
7. Dibasic sodium phosphate
8. Potassium ferricyanide
9. Trichloroacetic acid
10. Ferric chloride
11. Potassium dichromate

### 5.1.6 Lists of Plant Sample

1. *Spilanthes calva*
2. *Solanum virginianum*
3. *Stevia rebaudiana*
4. *Ruellia tuberosa*
5. *Mikania cordata*

## 5.2 Study Protocol

Our present study was designed to evaluate phytochemical and antioxidant property of crude extract *Spilanthes calva, Solanum virginianum Stevia rebaudiana, Ruellia tuberosa* and *Mikania cordata*

The study protocol consisted of the following steps ---

- Extraction of the powdered plant with methanol (*Spilanthes calva, Solanum virginianum and Stevia rebaudiana)* at room temperature.
- Filtration and solvent evaporation of the methanolic crude extract.
- Performing Biochemical investigation and phenolic content test of crude extract.
- Performing DPPH radical scavenging assay
- Reducing power assay of crude extract.
- Nitric oxide scavenging assay of crude extract

## 5.3 Phytochemical Investigation

The Phytochemical investigation of a plant can be divided roughly into the following major steps:

- Collection and proper identification of the plant material
- Preparation of plant sample
- Extraction of the plant material

### 5.3.1 Collection of Plant

The plants were collected from different places of Bangladesh. By using plant sample herbarium sheet was prepared and identified by Bangladesh National Herbarium. A voucher specimen had been deposited at the Bangladesh National Herbarium. The proper time of harvesting or collecting is particularly important because the nature and the quantity of constituents very gently in some species according to the season.

### 5.3.2. Cleaning and Drying

The collected plant was subjected to clean properly, separated from unwanted plant part and other unwanted material. The plant material is subjected to cut into small pieces to facilitate drying and grinding into coarse powder. The plant was dried in room temperature and drying was completed within 2 to 3 weeks. By drying the plant materials it removes sufficient moisture to ensure good keeping qualities and to prevent molding the action of enzyme, the action of bacteria and chemical or other possible changes. Proper and successful drying involved two main principles: control of temperature and regulation of air flow. The plant material can be dried by room temperature.

### 5.3.3. Grinding and Sieving

After complete drying the dried leaves were then ground in coarse powder using high capacity grinding machine. The coarse powder was then stored in air-tight container with marking for identification and kept in cool, dark and dry place for future use

### 5.3.4 Extraction of the Plant Material

About 500 gm of powdered plant material was taken in clean, round bottomed flask (2.5 liters) and macerated at room temperature in 2 liters of methanol for 3 days and everyday it was used to shake properly to ensure the maximum amount of constituents present in the grinded plant become soluble into methanol. After 3 days later, the mixture was filtered. For filtration, white cotton cloth was used. After filtration two parts were obtained. (Karolkovans *et al*, 1966).

- The residue portion over the filter
- The filtered part

The filtrated part, which contains the substance soluble in methanol, poured into a 1000ml round bottom flask, and then the flask was placed in a rotary evaporator. The evaporation was done at 40-50 degree

Celsius temperature. The number of rotation per minute was selected as 125 RPM. The pressure of vacuum pump machine was 6 bars. The water flow through the distillation chamber was also provided in a satisfactory flow rate. The whole mixture was then filtered through cotton followed by Whatman No.1 filter paper. The concentrated extract was then air dried to solid residue.

### 5.4 Total phenolic Content Assay

#### 5.4.1. Introduction

The antioxidative effect is mainly due to phenolic components, such as flavonoids, phenolic acids, and phenolic diterpenes. The antioxidant activity of phenolic compounds is mainly due to their redox properties, It has been reported that there is an inverse relationship between the antioxidative status occurrence of human diseases. In addition, antioxidant compounds which are responsible For Such antioxidants activity, could be isolated and then used as antioxidants for the prevention and treatment of free radical-related disorders.

#### 5.4.2. Principle

In the alkaline condition phenols ionize completely. When Folin-Ciocalteu reagent is used in this ionized phenolic solution the reagent will readily oxidize the phenols. Usual color of Folin-Ciocalteu reagent is yellow and after the oxidation process the solution become blue. The intensity of the color change is measured in a spectrophotometer at 700 nm. The absorbance value will reflect the total phenolic content of the compound.

#### 5.4.3 Materials & Methods

Total phenolic content of crude extracts was measured employing the method as described by Arpona Hira et al. (2013) involving Folin-Ciocalteu reagent as oxidizing agent and salicylic acid as standard.

**Composition of Folin-Ciocalteu Reagent**

| | |
|---|---|
| Water | 57.5ml |
| Lithium Sulfate | 15.0mg |
| Sodium Tungstate Dihydrate | 10.0mg |
| Hydrochloric Acid 25% | 10.0mg |

| | |
|---|---|
| Phosphoric Acid 85 % solution in water | 5.0mg |
| Molybdic Acid Sodium Dihydrate | 2.5mg |

### 5.4.4 Standard Curve Preparation

Salicylic acid was used here as standard. Different concentration of Salicylic acid solution were prepared having a concentration ranging from 10 mg/ml to 0.625 mg/ml. 5.0 ml of Folin-Ciocalteu reagent (1:10 v/v) and 4.0 ml of $Na_2CO_3$ (10.6 % w/v) solution was added to 100µl of Salicylic acid solution. The mixture was incubated for 1 hour at 25◦C or at room temperature. After 1 hour the absorbance was measured at 765 nm. After plotting the absorbance in ordinate against the concentration in abscissa a linear relationship was obtained which was used as a standard curve for the determination of the total phenolic content of the test samples. (Hills et al, 2006)

### 5.4.5 Sample Preparation

10 mg of crude extract was taken and dissolved in 1ml of methanol *Spilanthes calva, Solanum virginianum and Stevia rebaudiana* to get a sample concentration of 10mg/ml in every case.

### 5.4.6 Determination of total Phenolic Content of Samples

100 µl solution of crude extract mixed with 5.0 ml of Folin-Ciocalteu reagent (diluted 10 times with water) and 4.0 ml of Na2CO3 (10 % w/v) solution. The mixture was incubated for 1 hour at room temperature. After 1 hour the absorbance was measured at 765 nm. Using the absorbance of the sample, total phenolic content is measured by using following equation ---

$$T = \frac{C \times V}{M} \text{mg/gm}$$

Where,

T = Total phenolic content

C = x (Concentration from linear regresson equation)

V = Volume of samplein ml

M = Mass of sample in gm

## 5.5 DPPH Radical-Scavenging Assay

### 5.5.1 Introduction

There is considerable recent evidence that free radical induce oxidative damage to biomolecules. This damage causes cancer, aging, neurodegenerative diseases, atherosclerosis, malaria and several other pathological events in living organisms. Antioxidants which scavenge free radicals are known to posses an important role in preventing these free radical induced-diseases. There is an increasing interest in the antioxidants effects of compounds derived from plants, which could be relevant in relations to their nutritional incidence and their role in health and diseases. A number of reports on the isolation and testing of plant derived antioxidants have been described during the past decade.

Synthetic antioxidant such as *tert*-butyl-1-hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate (PG) and tert-butylhydroquinone (TBHQ) are widely used as food additives to increase shelf life, especially lipid and lipid containing products by retarding the process of lipid peroxidation. However, BHT and BHA are known to have not only toxic and carcinogenic effects and humans, but abnormal effects on enzyme systems. Arpona Hira et al. (2013)

### 5.5.2 Principle

The free radical scavenging activity of plant extracts against stable DPPH (1, 1-diphenyl-2-picrylhydrazyl) was determined spectrophotometrically. When DPPH reacts with an antioxidant compound, which can donate hydrogen, it was reduced. The changes in color (from deep violet to light yellow) were measured at 517 nm on a UV-visible light spectrophotometer.

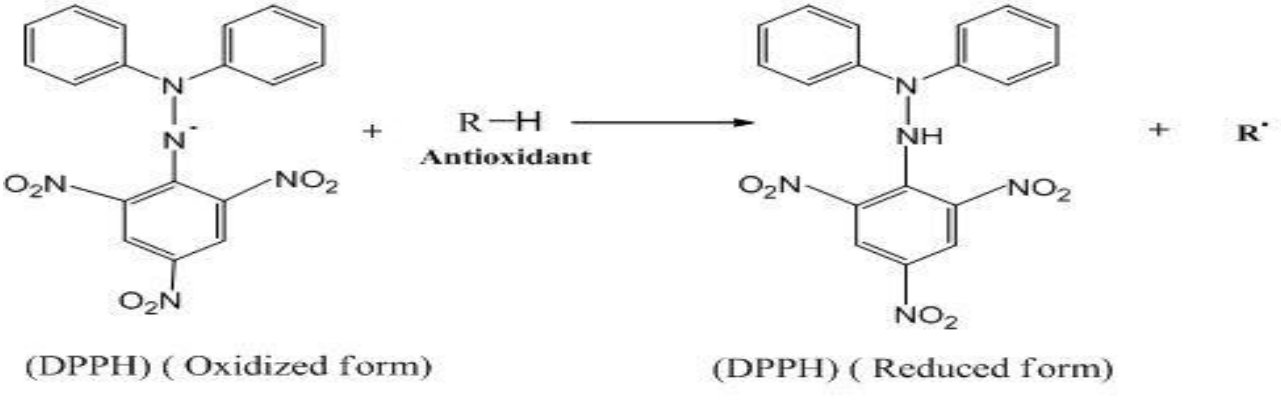



Figure 5.5.2.: Reaction of DPPH with antioxidant.

### 5.5.3 Material and Method

DPPH was used to evaluate the free radical scavenging activity of crude extracts was measured employing the slightly method described by Arpona Hira et al. (2013) involving DPPH as oxidizing agent and Ascorbic acid as standard.

#### 5.5.3.1. Preparation of DPPH Solution

A dry 250ml conical flask was cleaned and covered with an aluminium foil protect its contents from light. Accurately weighted 2mg of DPPH placed in conical flask and 50ml methanol was added to prepare 0.1mmol/L or 40µg/ml DPPH solution.

#### 5.5.3.2. Preparation of Sample Solution

Accurately weighted 10 mg of plant extract was taken into a vial and 1ml of methanol was added and the concentration of final solution is 10µg/µl ten test tube were taken and covered with an aluminium foil protect its contents from light and these test tubes were marked for different concentration. A serial dilution is carried out to prepare 10 different concentration of each sample and the final concentration ranging from 0.98 µg/ml to 500 µg/ml. The test was done three times.

#### 5.5.3.3. Preparation of Standard Solution

Accurately weighted 10 mg of ascorbic acid as standard was taken into a vial and 1 ml of distilled was added and the concentration of final solution is 10µg/µl ten test tube were taken and covered with an aluminium foil protect its contents from light and these test tubes were marked for different concentration. A serial dilution is carried out to prepare 10 different concentration of each sample and the final concentration ranging from 0.98 µg/ml to 500 µg/ml. The test was done three times.

#### 5.5.3.4 Measurement of DPPH Radical Scavenging Activity

2ml of a methanol solution of the extract at different concentration were mixed with 2ml of a DPPH methanol solution and this mixture was vigorously shacked and left at 250C for 30 minutes in the dark. After 30 minutes reaction period at room temperature in dark place the absorption was measured at 517nm of methanol as blank by UV spectrophotometer.

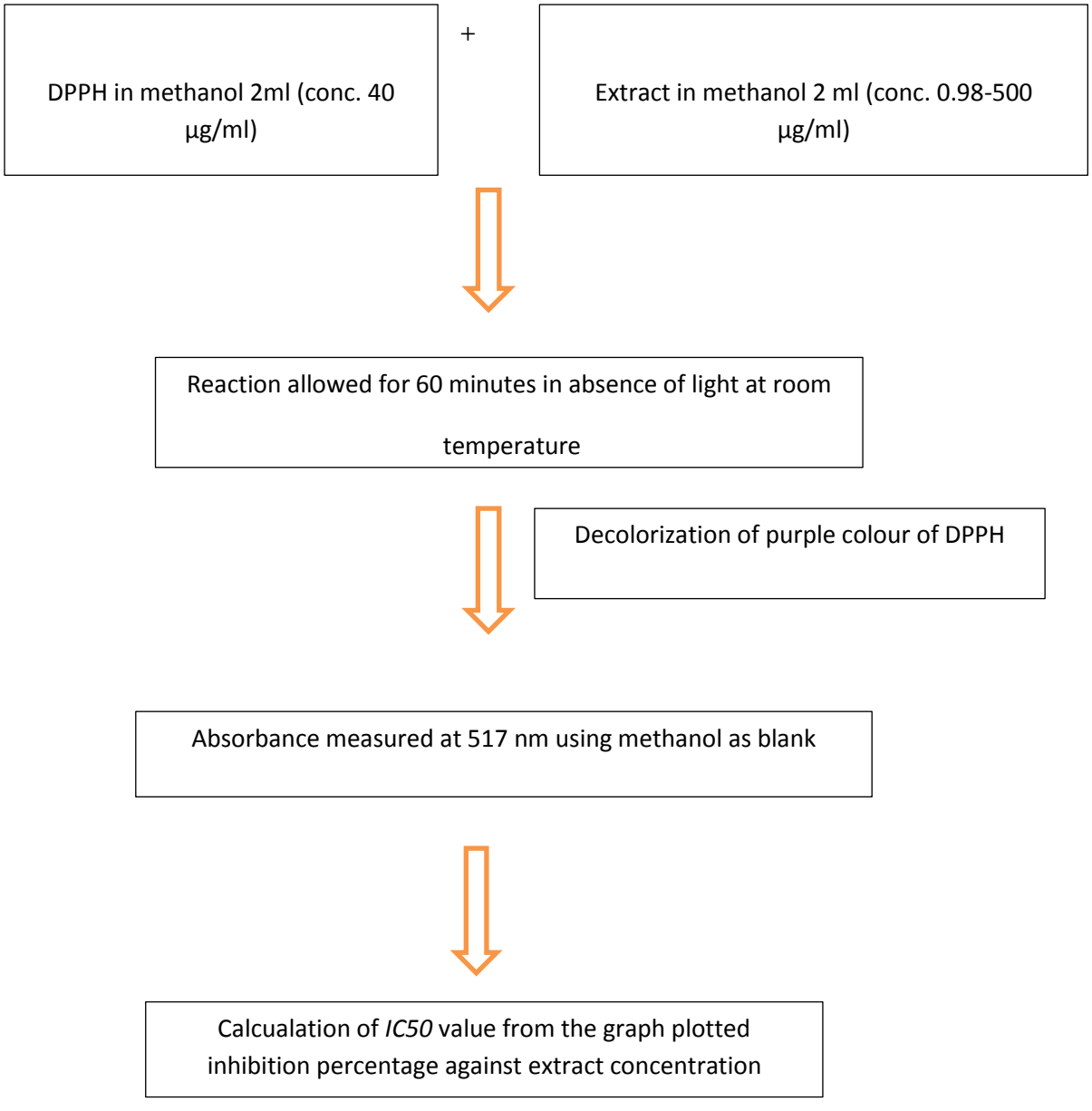



Figure 5.5.3.4: Schematic representation of the method of assaying free radical scavenging activity

## 5.6 Reducing Power Assay

### 5.6.1. Introduction

Free radicals are types of Reactive Oxygen Species (ROS), which include all highly reactive, oxygen-containing molecules. Types of ROS include the hydroxyl radical, the super oxide anion radical, hydrogen peroxide, nitric oxide radical, hypochlorite radical, and various lipid eroxides. These free radicals may either be produced by physiological or biochemical processes or by pollution and other endogenous sources. Antioxidants prevent the human system by neutralizing the free radicals interactively and synergistically. Plants are rich source of free radical scavenging molecules such as vitamins, terpenoids, phenolic acids etc.

### 5.6.2. Principle

Substances, which have reduction potential, react with potassium ferricyanide ($Fe^{3+}$) to form potassium ferrocyanide ($Fe^{2+}$), which then reacts with ferric chloride to form ferric ferrous complex that has an absorption maximum at 700 nm.

Antioxidant

Potassium ferricyanide + Ferric chloride $\longrightarrow$ Potassium ferrocyanide + ferrous chloride

### 5.6.3. Material and Method

Reducing power assay of crude extracts was measured employing the method as described by Arpona Hira et al. (2013) involving Phosphate Buffer (2.5ml, 0.2M, pH 6.6), 1% Potassium Ferricyanide [$K_3Fe(CN)_6$], 10% Trichloroacetic acid 0.1% $FeCl_3$and Ascorbic acid as standard.

#### 5.6.3.1. Preparation of Sample Solution

Accurately weighted 10 mg of plant extract was taken into a vial and 1ml of methanol was added and the concentration of final solution is 10µg/µl. Ten test tubes were taken and marked for different concentration. A serial dilution is carried out to prepare 10 different concentration of each sample and the final concentration ranging from 0.98 µg/ml to 500 µg/ml. The test was done triplicate.

### 5.6.3.2. Preparation of Standard Solution

Accurately weighted 10 mg of ascorbic acid as standard was taken into a vial and 1 ml of distilled was added and the concentration of final solution is 10µg/ml ten test tube were taken and marked for different concentration. A serial dilution is carried out to prepare 10 different concentration of each sample and the final concentration ranging from 0.98 µg/ml to 500 µg/ml. The test was done triplicate.

### 5.6.3.3. Procedure

1ml of stock mixture (concentration 0.98 µg/ml to 500 µg/ml) is mixed with 1ml of distilled water added with 2.5ml of Phosphate Buffer and 2.5ml of 1% Potassium Ferricyanide. The reaction mixture is incubated at $50^{o}$C for 20minute. After incubation 10% Trichloroacetic acid is added. The mixture is centrifuged for 10min at 3000rpm. After centrifugation Upper layer was taken (2.5ml) dissolved with 2.5ml distilled water and 0.5ml of $Fecl_3$. Absorbance was measured at 700nm.

## 5.7. Nitric oxide (NO) Scavenging Activity

### 5.7.1. INTRODUCTION:

Nitric oxide (NO) is an important chemical mediator generated by endothelial cells, macrophages, neurons and involved in the regulation of various physiological processes. Nitric Oxide (NO) is used in various types of disorders like AIDS, cancer, alzheimer's and arthritis by cytotoxic effects.( Sainani et al.,1997). DNA fragmentation, neuronal cell death and cell damage occur as the toxicity of overproduction of NO. Bioorganic macromolecules (DNA or proteins) not effected directly with the presence of NO. As in aerobic conditions NO is very unstable and producing intermediates ( NO2, N2O4, N3O4 ) reacts with oxygen. In this reaction the stable products nitrite and nitrate will also produce (Marcocci et aI.,1994a,b) and peroxynitrite will produce by reacting with superoxide . Antioxidant agents of natural origin have attracted special interest because they can protect human body from free radicals. (Arpona Hira et al. 2013)

### 5.7.2. Principle:

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions.

### 5.7.3. Preparation of Solution

#### 5.7.3.1. Preparation of Phosphate Buffered Saline (PBS Buffer)

8g of NaCl, 0.2g of KCl, 1.44g of $Na_2HPO_4$ and 0.24g of $KH_2PO_4$ was dissolved in 1 liter distilled $H_2O$. The pH was adjusted to 7.4 with HCl.

#### 5.7.3.2. Preparation of 5mmol Sodium Nitropruside in Phosphate Buffer Saline

0.130 gm of Sodium Nitroprusside was accurately weighed and dissolved in 100 ml Phosphate Buffered Saline (PBS Buffer).

#### 5.7.3.3. Preparation of Griess Reagent

Firstly, 1gm of Sulphanilamide was dissolved into 100ml distilled water. (Solution a). Then 0.1gm of naphthylethylenediamine dihydrochloride was dissolved into 100ml of 2% phosphoric acid. (solution b). 1 part of solution ‘a’ was mixed with 1 part of solution ‘b’. This reagent was cautiously put into amber glassed bottle to protect it from light as this reagent is very light sensitive.

#### 5.7.3.4. Preparation of Stock Solution ( sample)

Accurately weighted 20 mg of plant extract was taken into a vial and 2ml of methanol was added and the concentration of final solution is 10µg/µl ten test tubes were taken and marked for different concentration. A serial dilution is carried out to prepare 10 different concentration of each sample and the final concentration ranging from 0.98 µg/ml to 500 µg/ml. The test was done triplicate.

### 5.7.3.5. Preparation of Standard Solution

Accurately weighted 20 mg of ascorbic acid as standard was taken into a vial and 2 ml of distilled was added and the concentration of final solution is 10µg/µl ten test tube were taken and marked for different concentration. A serial dilution is carried out to prepare 10 different concentration of each sample and the final concentration ranging from 0.98 µg/ml to 500 µg/ml. The test was done triplicate.

### 5.7.3.6. Procedure:

2ml 5mmol Sodium Nitroprusside in Phosphate Buffer Saline (PBS Buffer) was mixed with different concentration (0.98µg/ml - 500µg/ml) of sample dissolved in 2ml of methanol. A control without the test compound but with an equivalent amount of methanol was taken and was incubated at 25$^{o}$C for 30minute. After 30min, 1.5mL of the incubation solution was removed and diluted with 1.5mL of Griess solution. The absorbance was measured at 546nm. The NO radical scavenging activity was expressed at the inhibition percentage (I%) and calculated as per,

I (%)=[($A_{blank}$ - $A_{sample}$)/$A_{blank}$]×100, Where $A_{blank}$ is the absorbance of the control reaction (containing all reagents except test compound). Finally $IC_{50}$ value is determined.

## 5.8 Column Chromatography

Column Chromatography is basically a type of adsorption chromatography techniques. Here the separation of components depends upon the extent of adsorption to stationary phase. Here the stationary phase is a solid material packed in a vertical column made of glass or metal.

### 5.8.1 Principle:

When a mixture of mobile phase and sample to be separated are introduced from top of the column, the individual components of mixture move with different rates. Those with lower affinity and adsorption to stationary phase move faster and eluted out first while those with greater adsorption affinity move or travel slower and get eluted out last.

### 5.8.2 Materials

1. Column

2. Thin Layer Chromatography (TLC) tank

3. Thin Layer Chromatographic (TLC) plate

4. Conical flask

5. Test tubes

6. Beakers

7. Pipettes

8. Pasture pipettes

9. Measuring cylinders

10. Funnels

### 5.8.3 Solvents

1. Methanol (MeOH)

2. Dichloromethane (DCM)

3. Cyclo-Hexane

4. Ethyl acetate (EtOAc)

5. Chloroform

### 5.8.4 Equipment

1. Rotary vacuum evaporator

2. Hot air oven

3. Dryer

4. Water bath

5. Electronic balance

6. Water distiller

### 5.8.5 Spray reagent

1. Charring solution (MeOH 90% and conc.H2SO4 10%)

### 5.8.6 Chromatographic Materials

Column grade silica gel

### 5.8.7 Column Packing

1. A glass column (90cm X 5cm) was cleaned and dried. Cotton pad was placed at the bottom of the column. The column was packed requied amount of column grade silica( mesh size was 70-230).
2. The silica, added to the column by making slurry with cyclohexane, was allowed to settle down of appropriate height and slowly keeping the column outlet closed.
3. When the desired height of adsorbend was obtained, the column was flashed with three column vcolumes of cyclohexane.
4. The crude methanolic extract was dissolved in small quantity of cyclohexane.
5. Then appropriate amount of silica was added to prepare the slurry of crude methanolic extract.
6. The slurry was kept overnight to be dried completely.
7. The dried silica was introduced into the column.
8. The applied sample was eluted by a mixture of solvents comprising of cyclohexane, dichloromethane, ethylacetate and methanol with increasing polarity..
9. The elutes were collected in conical flask.

### 5.8.8 Fractionation of sample

1. The eluted were collected in a beaker by a certain amount and concentrated through rotary evaporator and finally collected in vials.
2. At first, the column was run using cyclohexane as mobile phase. About 200ml was collected each time and fraction number was given numerically.
3. Then TLC was done. As the TLC shows that the compounds of similar chemical characteristics were coming for a long time, we increased the polarity of the solvent.
4. The composition of the mobile was changed into cyclohexane : dichloromethane =1:1
   This composition was running till the TLC shows any compound.
5. Again the solvent system was changed into 100% Dichloromethane and the componds which have the affinity to DCM was collected as fractions.
6. After that the solvent system was changed into Dichloromethane : Ethylacetate = 1:1
7. Then the composition of the mobile phase was changed into 100% Ethylacetae.
8. Finally, the column was washed by 100% methanol and collected the fractions.

### 5.8.9 Analysis of Column Chromatography fractions by TLC

All the column fractions were screened by TLC under UV light and by spraying with methanol sulphuric acid reagent.

#### 5.8.9.1 Thin Layer Chromatography (TLC)

#### 5.8.9.2 Principle

Thin layer chromatography (TLC) is a chromatographic technique used to separate the components of a mixture using a thin stationary phase supported by an inert backing. TLC functions on the same principle as all chromatography: a compound will have different affinities for the mobile and stationary phases, and this affects the speed at which it migrates. The goal of TLC is to obtain well defined, well separated spots.

They all have a stationary phase (a solid or a liquid supported on a solid) and a mobile phase (a liquid or a gas). The mobile phase flows through the stationary phase and carries the components of the mixture with it. Different components travel at different rates.

### 5.8.9.3 Procedure of TLC

The stationary phase is applied onto the plate uniformly and then allowed to dry and stabilize. These days, however, ready-made plates are preferred.

1. With a pencil, a thin mark is made at the bottom of the plate to apply the sample spots.
2. Then, samples solutions are applied on the spots marked on the line in equal distances.
3. The mobile phase is poured into the TLC chamber to a leveled few centimeters above the chamber bottom. A moistened filter paper in mobile phase is placed on the inner wall of the chamber to maintain equal humidity (and also thereby avoids edge effect this way).
4. Now, the plate prepared with sample spotting is placed in TLC chamber so that the side of the plate with the sample line is facing the mobile phase. Then the chamber is closed with a lid.
5. The plate is then immersed, such that the sample spots are well above the level of mobile phase (but not immersed in the solvent — as shown in the picture) for development.
6. Allow sufficient time for the development of spots. Then remove the plates and allow them to dry. The sample spots can now be seen in a suitable UV light chamber.

### 5.8.9.4 Retention Factor

After a separation is complete, individual compounds appear as spots separated vertically. Each spot has a retention factor (Rf) which is equal to the distance migrated over the total distance covered by the solvent. The Rf formula is

Rf = distance traveled by sample / distance traveled by solvent

The Rf value can be used to identify compounds due to their uniqueness to each compound. When comparing two different compounds under the same conditions, the compound with the larger Rf value is less polar because it does not stick to the stationary phase as long as the polar compound, which would have a lower Rf value.

# 6. Results and Discussion

## 6.1 Total Phenolic Content Assay

The Methanolic extract of *Spilanthes calva*, *Solanum virginianum*, *Stevia rebaudiana* and *Ruellia tuberosa* and different fractions of *Mikania cordata* were subjected to Total Phenolic Content Assay. Here, Total Phenolic Content was measured as Salicylic acid equivalence.

**Table 6.1.1.: Standard Curve Preparation by Using Salicylic Acid**

| SL | Concentration mg/ml | Absorbance | Regression Equation | $R^2$ |
|---|---|---|---|---|
| 1 | 0.625 | 0.431 | Y= 0.1169x+0.4168 | 0.991 |
| 2 | 1.25 | 0.585 | | |
| 3 | 2.5 | 0.764 | | |
| 4 | 5 | 0.990 | | |
| 5 | 10 | 1.578 | | |

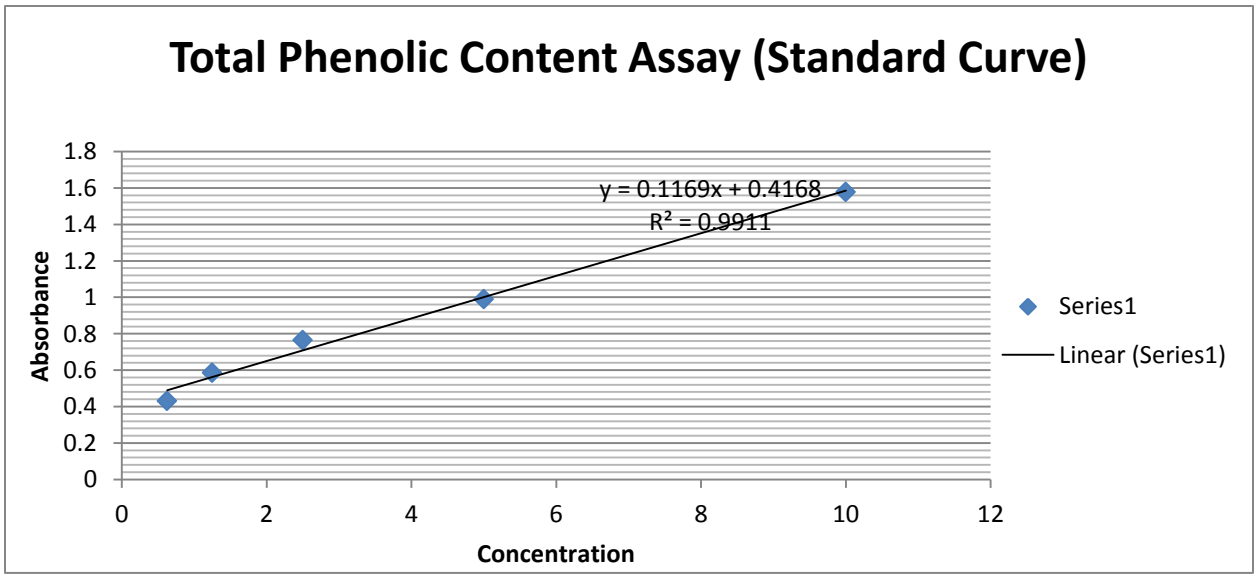



**Figure 6.1.1.: Total Phenolic Content (Standard Curve)**

**Table 6.1.2.: Total Phenolic Content of *Spilanthes calva***

| Absorbance | mgSAE/g | Mean |
|---|---|---|
| 0.834 | 35.69 | 35.62 |
| 0.835 | 35.74 | |
| 0.831 | 35.43 | |

**Table 6.1.3.: Total Phenolic Content of *Solanum virginianum***

| Absorbance | mgSAE/g | Mean |
|---|---|---|
| 1.183 | 65.54 | 65.06 |
| 1.173 | 64.69 | |
| 1.176 | 64.94 | |

**Table 6.1.4.: Total Phenolic Content of *Stevia rebaudiana***

| Absorbance | mgSAE/g | Mean |
|---|---|---|
| 1.751 | 122.2 | 122.12 |
| 1.757 | 122.8 | |
| 1.759 | 123.02 | |

**Table 6.1.5.: Total Phenolic Content of *Ruellia tuberosa***

| Absorbance | mgSAE/g | Mean |
|---|---|---|
| 0.868 | 41.28 | 54 |
| 1.090 | 61.65 | |
| 1.062 | 59.08 | |

**Table 6.1.6.: Total Phenolic Content of *Mikania cordata* (Cyclohexane fraction)**

| Absorbance | mgSAE/g | Mean |
|---|---|---|
| 1.112 | 59.46 | 59.26 |
| 1.102 | 58.61 | |
| 1.115 | 59.72 | |

**Table 6.1.7.: Total Phenolic Content of *Mikania cordata* (Dichloromethane fraction)**

| Absorbance | mgSAE/g | Mean |
|---|---|---|
| 1.456 | 88.9 | 86.73 |
| 1.321 | 77.35 | |
| 1.759 | 93.94 | |

**Table 6.1.8.: Total Phenolic Content of *Mikania cordata* (Methanol fraction)**

| Absorbance | mgSAE/g | Mean |
|---|---|---|
| 1.652 | 113.21 | 111.47 |
| 1.632 | 111.38 | |
| 1.615 | 109.82 | |

### 6.1.9 Comparison of Total Phenolic Content of Different plants

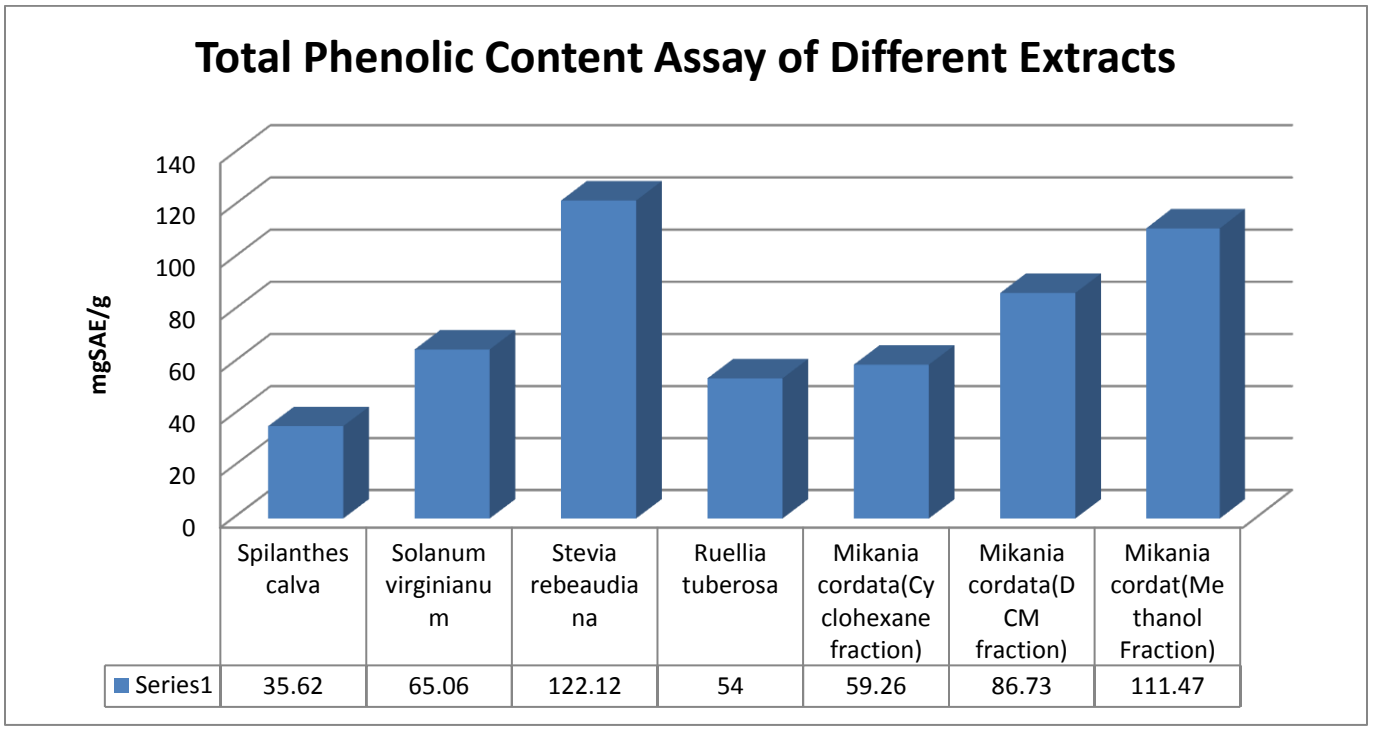



**Figure 6.1.9.: Comparison of Total Phenolic Content of Different plants**

**Discussion:**

From the above results we can see that among seven extracts *Stevia rebaudiana* (122.12mgSAE/g) has the highest amount of phenolic content than methanolic extract of *Spilanthes calva* (35.62 mgSAE/g), *Solanum virginianum* (65.06mgSAE/g), *Ruellia tuberosa* (54mgSAE/g), *Mikania cordata* Cyclohexane fraction (59.26 mgSAE/g) DCM fraction (86.73 mgSAE/g) and methanolic fraction (111.47 mgSAE/g). Dichloromethane fraction of *Mikania cordata* (86.73 mgSAE/g) and *Solanum virginianum* (65.06mgSAE/g) have moderate phenolic content . *Spilanthes calva* (35.62 mgSAE/g) has the least amount of phenolic content among all extracts. Among all the fractions of *Mikania cordata*, methanolic fraction (111.47 mgSAE/g) has the highest phenolic content than any other fractions.

From the above data it can be said that methanolic extract of *Stevia rebaudiana* has the highest amount of phenolic content (122.12mgSAE/g) and thus it can be a good source of phenol.

## 6.2 DPPH Radical Scavenging Assay

The methanolic extract of *Spilanthes calva*, *Solanum virginianum*, *Stevia rebaudiana* and *Ruellia tuberosa* and *Mikania cordata* fractions were subjected to DPPH Radical Scavenging Assay and ascorbic acid was used as reference standard in this experiment.

**Table 6.2.1.: DPPH Radical Scavenging Assay of Ascorbic Acid (Standard)**

| Absorbance of Blank | Concentration µg/ml | Log Concentration | Absorbance of the Sample | % of Inhibition | $IC_{50}$ (µg/ml) |
|---|---|---|---|---|---|
| 0.625 | 0.98 | - 0.009 | 0.535 | 14.4 | 4.21 |
| | 1.95 | 0.290 | 0.507 | 18.89 | |
| | 3.91 | 0.592 | 0.365 | 41.6 | |
| | 7.81 | 0.893 | 0.198 | 68.32 | |
| | 15.63 | 1.194 | 0.098 | 84.32 | |
| | 31.25 | 1.495 | 0.058 | 90.72 | |
| | 62.5 | 1.796 | 0.042 | 93.25 | |
| | 125 | 2.097 | 0.027 | 95.68 | |
| | 250 | 2.398 | 0.023 | 96.32 | |
| | 500 | 2.699 | 0.014 | 97.76 | |

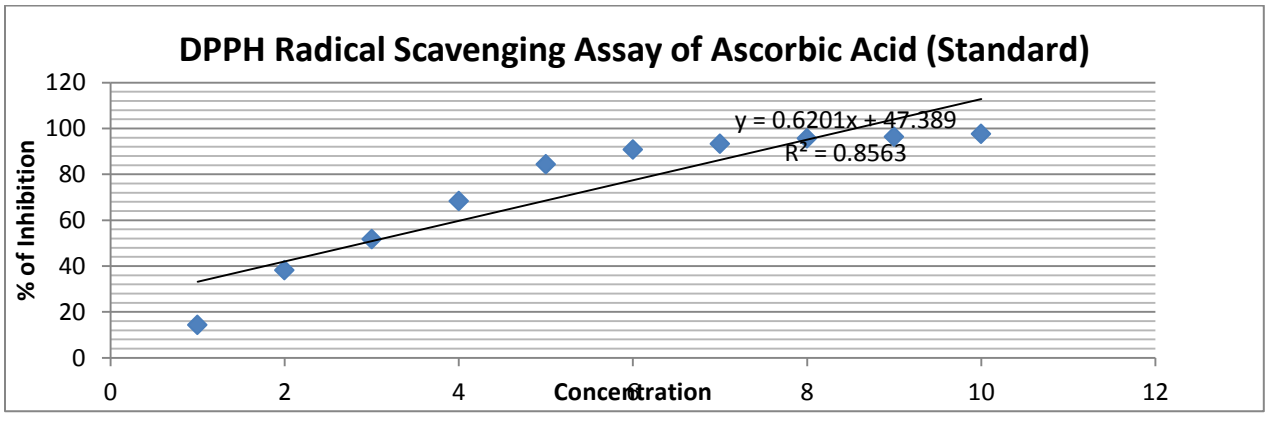



**Figure 6.2.1.: DPPH Radical Scavenging Assay of Ascorbic Acid (Standard)**

**Table 6.2.2.: DPPH Radical Scavenging Assay of *Spilanthes calva***

| Absorbance of Blank | Concentration µg/ml | Absorbance of the Sample | % of Inhibition | $IC_{50}$ (µg/ml) |
|---|---|---|---|---|
| 0.459 | 0.98 | 0.454 | 1.09 | 304.59 |
| | 1.95 | 0.449 | 2.17 | |
| | 3.91 | 0.443 | 3.49 | |
| | 7.81 | 0.436 | 5.01 | |
| | 15.63 | 0.425 | 7.41 | |
| | 31.25 | 0.406 | 11.55 | |
| | 62.5 | 0.398 | 13.3 | |
| | 125 | 0.356 | 22.44 | |
| | 250 | 0.245 | 46.62 | |
| | 500 | 0.103 | 75.56 | |

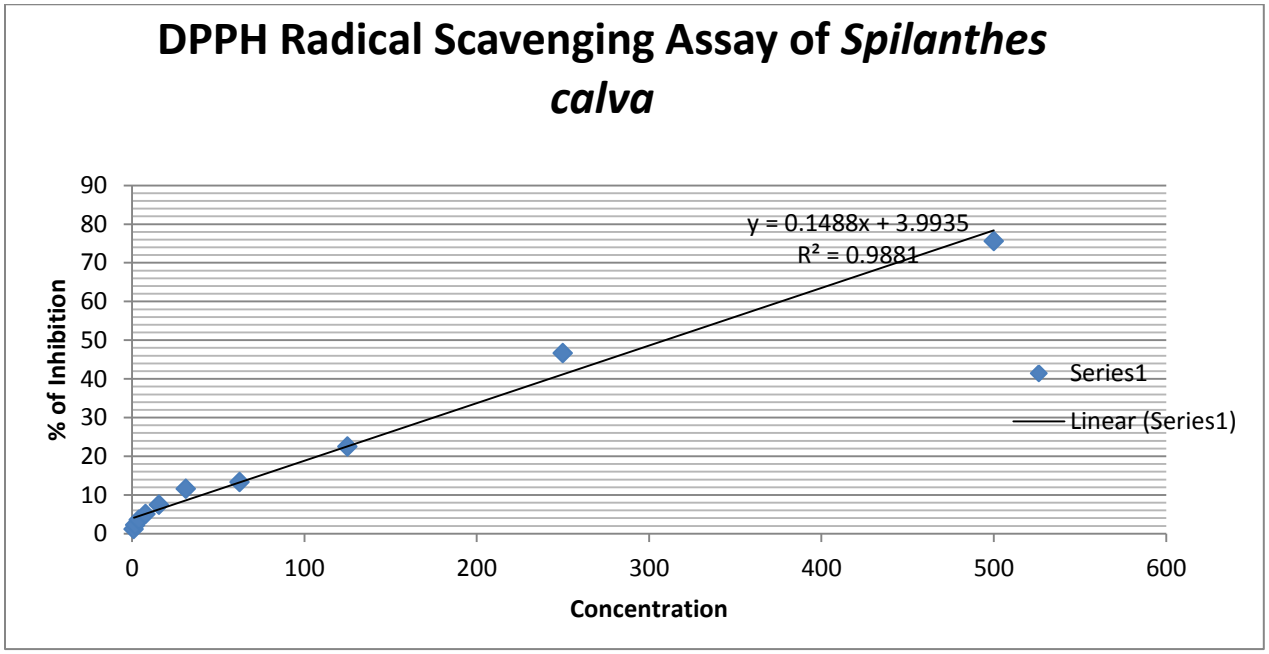



**Figure 6.2.2.: DPPH Radical Scavenging Assay of *Spilanthes calva***

**Table 6.2.3: DPPH Radical Scavenging Assay of *Solanum virginianum***

| Absorbance of Blank | Concentration µg/ml | Absorbance of the Sample | % of Inhibition | $IC_{50}$ (µg/ml) |
|---|---|---|---|---|
| 0.618 | 0.98 | 0.614 | 0.64 | 232.24 |
| | 1.95 | 0.595 | 3.72 | |
| | 3.91 | 0.548 | 11.33 | |
| | 7.81 | 0.523 | 15.37 | |
| | 15.63 | 0.507 | 17.96 | |
| | 31.25 | 0.468 | 24.27 | |
| | 62.5 | 0.461 | 25.40 | |
| | 125 | 0.404 | 34.63 | |
| | 250 | 0.233 | 62.29 | |
| | 500 | 0.075 | 87.86 | |

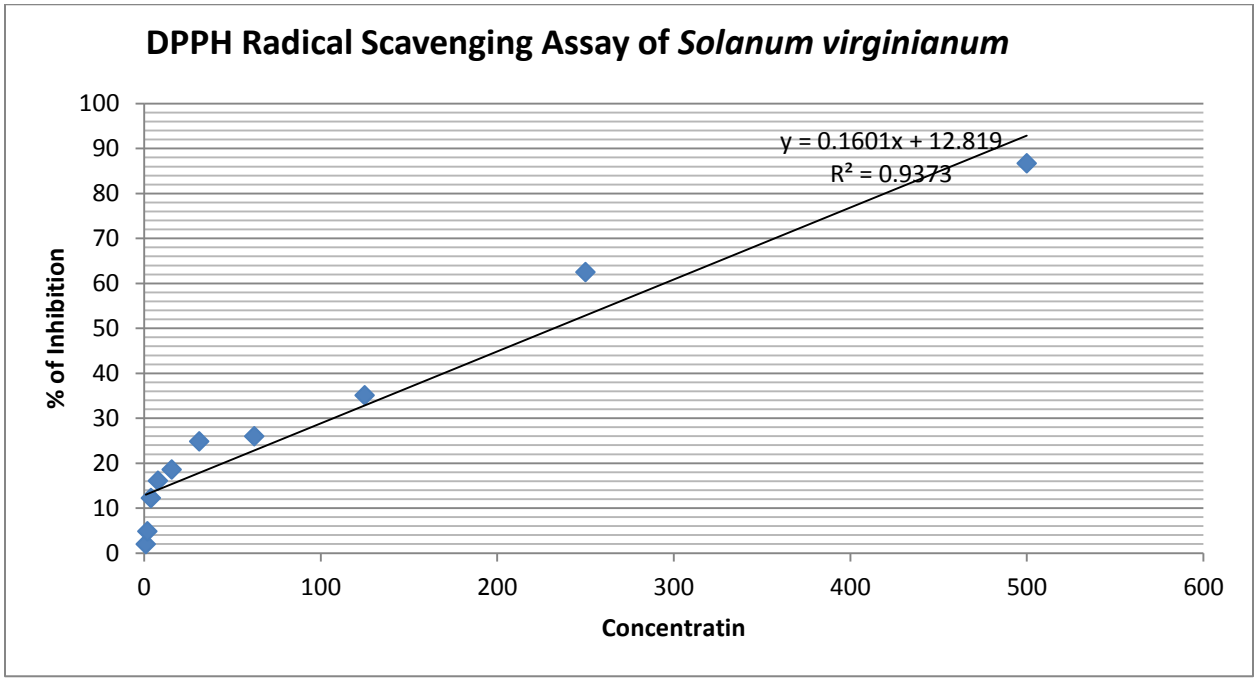



**Figure 6.2.3.: DPPH Radical Scavenging Assay of *Solanum virginianum***

**Table 6.2.4.: DPPH Radical Scavenging Assay of *Stevia rebaudiana***

| Absorbance of Blank | Concentration µg/ml | Absorbance of the Sample | % of Inhibition | $IC_{50}$ (µg/ml) |
|---|---|---|---|---|
| 0.612 | 0.98 | 0.465 | 24.01 | 111.16 |
| | 1.95 | 0.453 | 25.98 | |
| | 3.91 | 0.445 | 27.29 | |
| | 7.81 | 0.429 | 29.90 | |
| | 15.63 | 0.404 | 33.99 | |
| | 31.25 | 0.359 | 41.34 | |
| | 62.5 | 0.305 | 50.16 | |
| | 125 | 0.210 | 65.69 | |
| | 250 | 0.061 | 90.03 | |
| | 500 | 0.033 | 94.61 | |

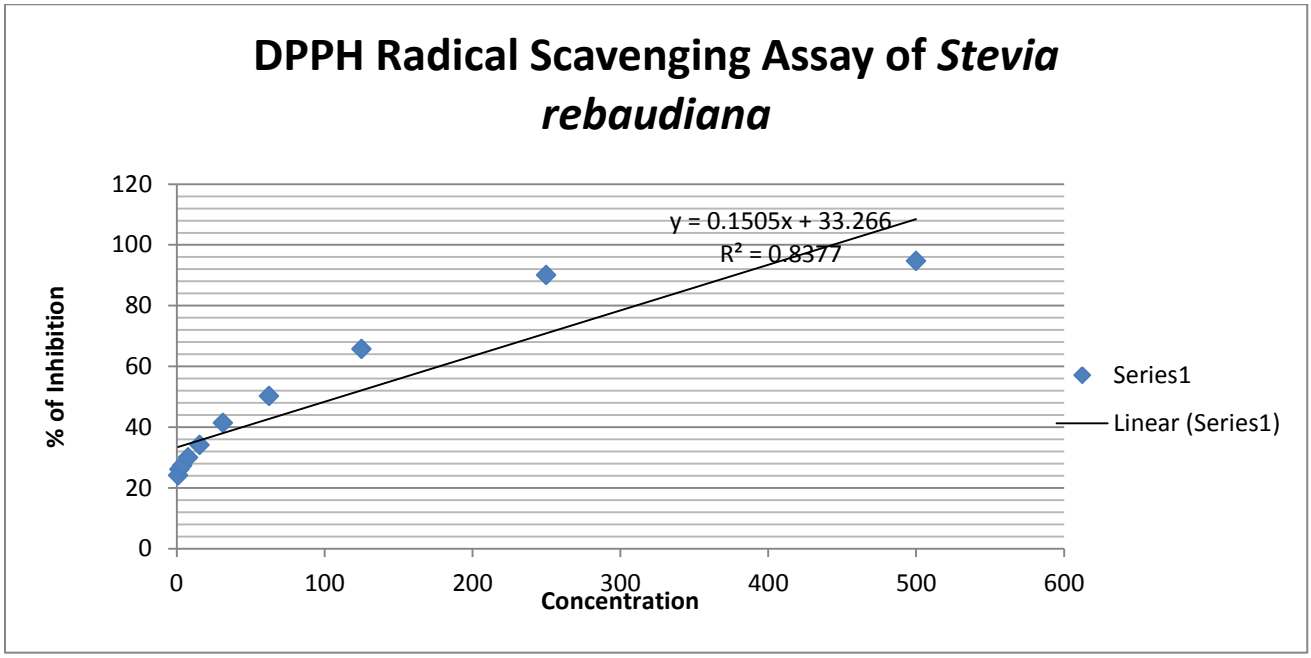



**Figure 6.2.4.: DPPH Radical Scavenging Assay of *Stevia rebaudina***

**Table 6.2.5.: DPPH Radical Scavenging Assay of *Ruellia tuberosa***

| Absorbance of Blank | Concentration µg/ml | Absorbance of the Sample | % of Inhibition | $IC_{50}$ (µg/ml) |
|---|---|---|---|---|
| 0.625 | 0.98 | 0.607 | 2.88 | 164.23 |
| | 1.95 | 0.592 | 5.28 | |
| | 3.91 | 0.436 | 30.24 | |
| | 7.81 | 0.410 | 34.4 | |
| | 15.63 | 0.391 | 37.44 | |
| | 31.25 | 0.350 | 44.0 | |
| | 62.5 | 0.314 | 49.76 | |
| | 125 | 0.250 | 60.1 | |
| | 250 | 0.165 | 73.6 | |
| | 500 | 0.123 | 80.32 | |

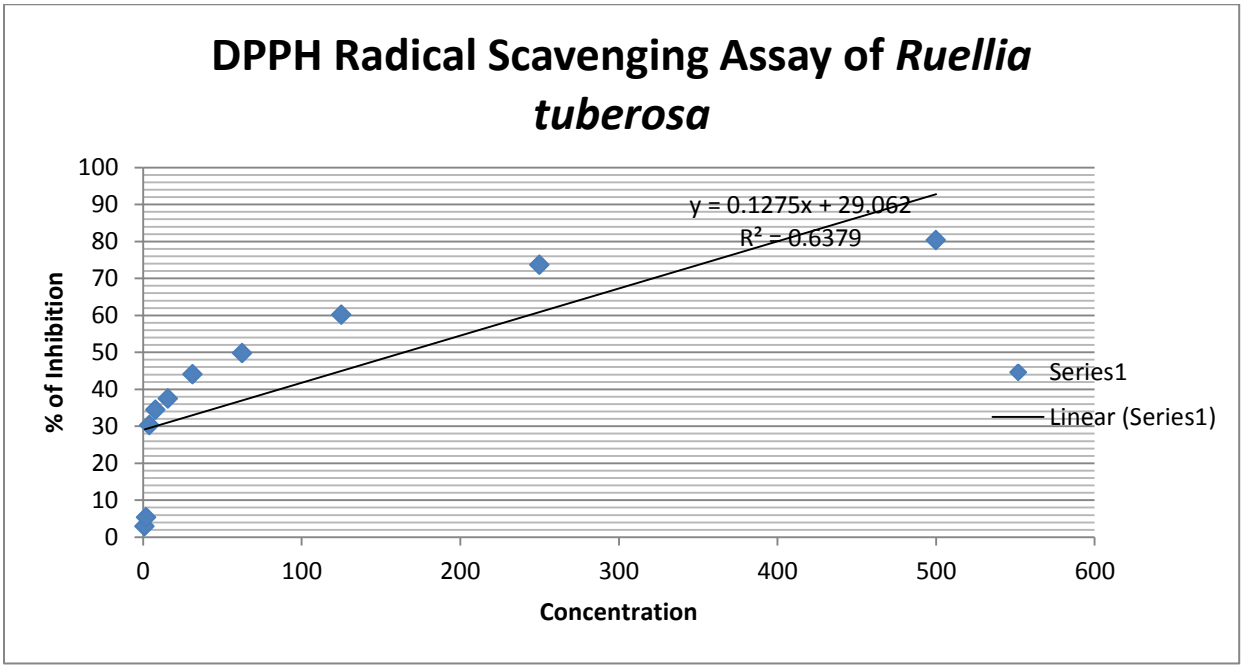



**Figure 6.2.5.: DPPH Radical Scavenging Assay of *Ruellia tuberosa***

**Table 6.2.6.: DPPH Radical Scavenging Assay of *Mikania cordata* (Cyclohexane fraction)**

| Absorbance of Blank | Concentration µg/ml | Absorbance of the Sample | % of Inhibition | $IC_{50}$ (µg/ml) |
|---|---|---|---|---|
| 0.580 | 0.98 | 0.541 | 6.72 | 193.84 |
| | 1.95 | 0.521 | 10.17 | |
| | 3.91 | 0.496 | 14.48 | |
| | 7.81 | 0.454 | 21.72 | |
| | 15.63 | 0.411 | 29.14 | |
| | 31.25 | 0.389 | 32.93 | |
| | 62.5 | 0.356 | 38.62 | |
| | 125 | 0.215 | 62.93 | |
| | 250 | 0.180 | 68.97 | |
| | 500 | 0.110 | 81.03 | |

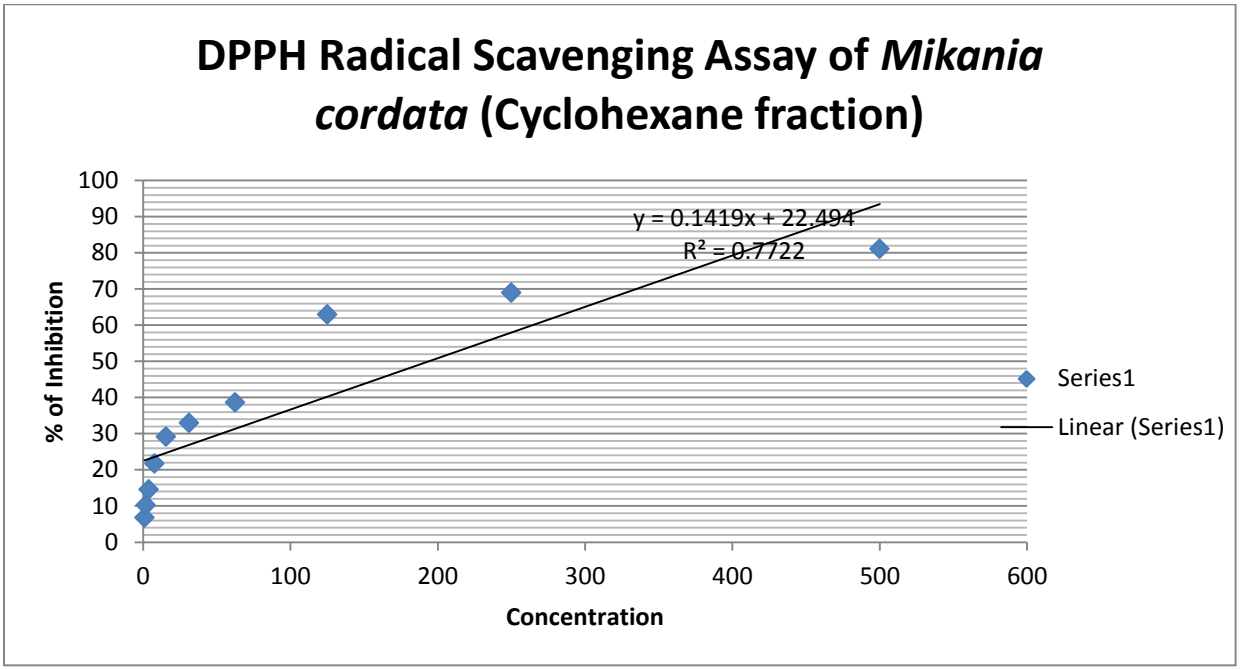



**Figure 6.2.6.: DPPH Radical Scavenging Assay of *Mikania cordata* (Cyclohexane fraction)**

**Table 6.2.7.: DPPH Radical Scavenging Assay of *Mikania cordata* (DCM fraction)**

| Absorbance of Blank | Concentration µg/ml | Absorbance of the Sample | % of Inhibition | $IC_{50}$ (µg/ml) |
|---|---|---|---|---|
| 0.481 | 0.98 | 0.454 | 5.61 | 181.58 |
| | 1.95 | 0.449 | 6.65 | |
| | 3.91 | 0.438 | 8.94 | |
| | 7.81 | 0.432 | 10.18 | |
| | 15.63 | 0.408 | 15.18 | |
| | 31.25 | 0.361 | 24.95 | |
| | 62.5 | 0.279 | 41.99 | |
| | 125 | 0.145 | 69.85 | |
| | 250 | 0.098 | 79.63 | |
| | 500 | 0.049 | 89.81 | |

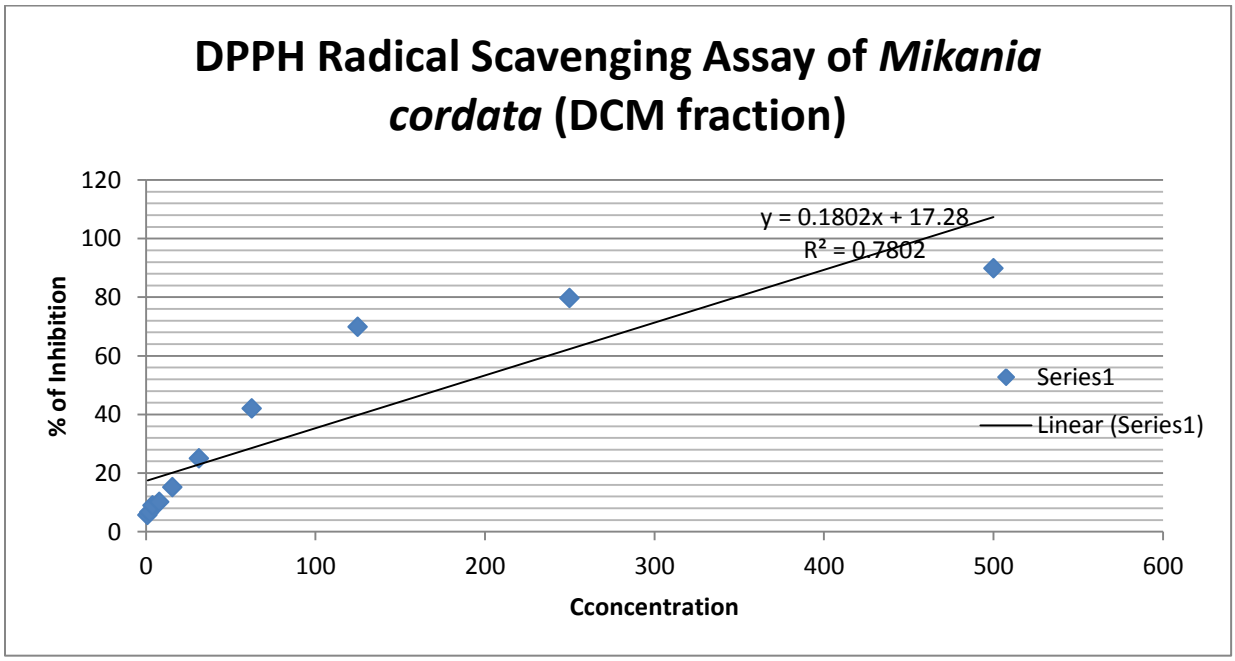



**Figure 6.2.7.: DPPH Radical Scavenging Assay of *Mikania cordata* (DCM fraction)**

**Table 6.2.8.: DPPH Radical Scavenging Assay of *Mikania cordata* (Methanolic fraction)**

| Absorbance of Blank | Concentration µg/ml | Absorbance of the Sample | % of Inhibition | $IC_{50}$ (µg/ml) |
|---|---|---|---|---|
| 0.612 | 0.98 | 0.471 | 23.03 | 117.74 |
| | 1.95 | 0.452 | 26.14 | |
| | 3.91 | 0.433 | 29.23 | |
| | 7.81 | 0.420 | 31.37 | |
| | 15.63 | 0.398 | 34.97 | |
| | 31.25 | 0.359 | 41.34 | |
| | 62.5 | 0.310 | 49.35 | |
| | 125 | 0.250 | 59.15 | |
| | 250 | 0.071 | 88.4 | |
| | 500 | 0.051 | 91.67 | |

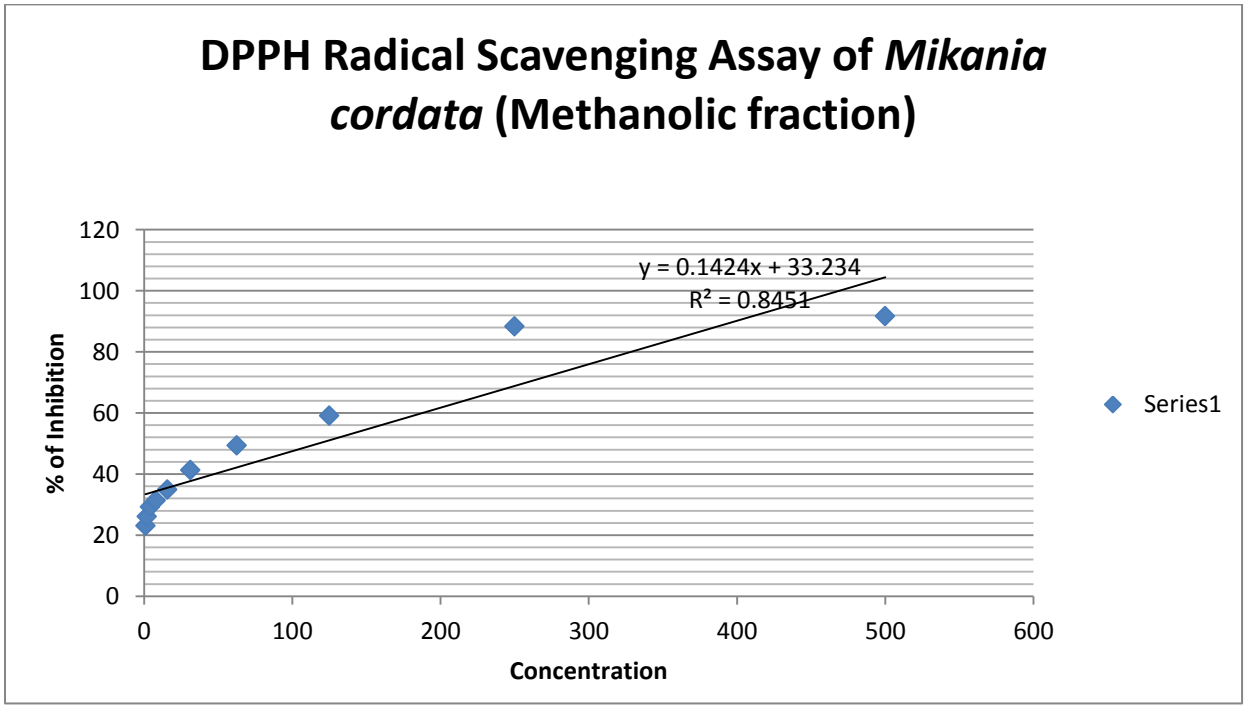



**Figure 6.2.8.: DPPH Radical Scavenging Assay of *Mikania cordata* (Methanolic fraction)**

### 6.2.9.: Comparison of $IC_{50}$ between Standard and Extracts

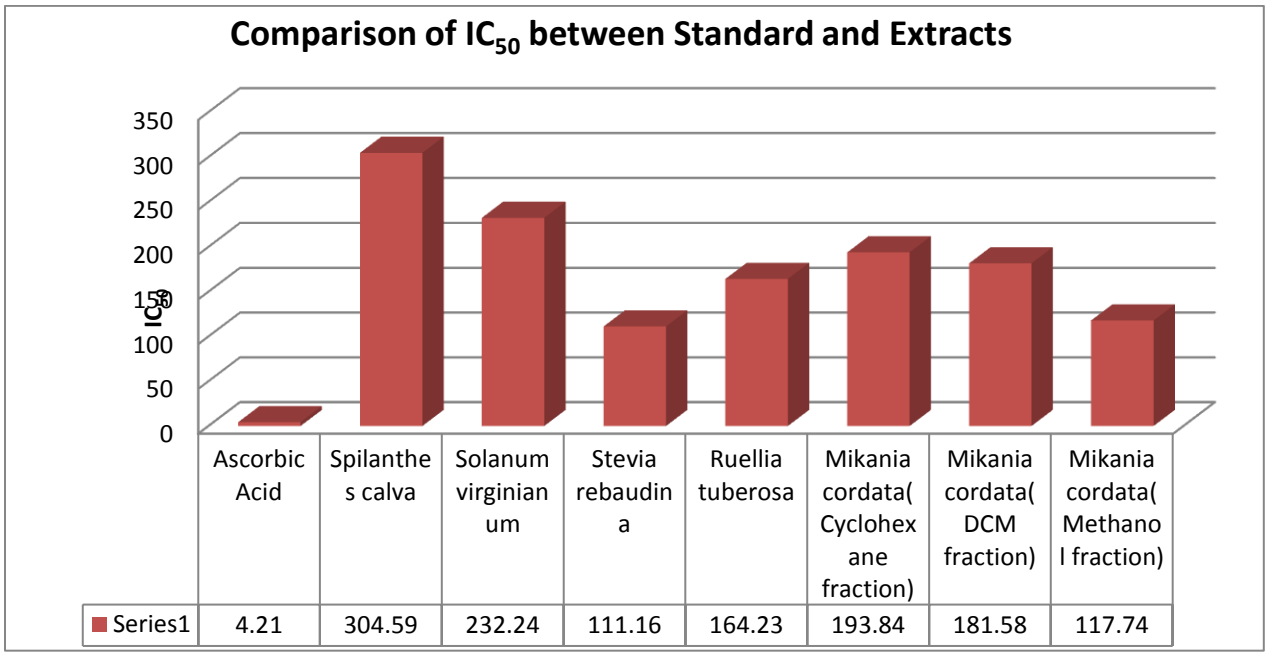



**Figure 6.2.9.: Comparison of $IC_{50}$ between Standard and Extract.**

**Discussion:**

The extract demonstrated an antioxidant activity by using DPPH where DPPH radical activity of methanolic extract of *Stevia rebaudiana* is good ($IC_{50}$ 111.16 µg/ml) after which is methanolic fraction of *Mikania cordata* ($IC_{50}$ 117.74 µg/ml), *Ruellia tuberosa* ($IC_{50}$ 164.23 µg/ml), DCM fraction of *Mikania cordata* ($IC_{50}$ 181.58 µg/ml).Cyclohexane fraction of *Mikania cordata* ($IC_{50}$ 193.84µg/ml) is better than *Solanum virginianum* ($IC_{50}$ 232.24µg/ml) and the least activity was shown by the *Spilanthes calva* ($IC_{50}$ 304.59 µg/ml).

Among all the different fractions of *Mikania cordata*, Methanolic fraction has highest radical scavenging activity ($IC_{50}$ 117.74 µg/ml).

### 6.3.: Nitric oxide Scavenging Assay

The Methanolic extract of *Spilanthes calva, Solanum virginianum, Stevia rebaudiana* and *Ruellia tuberosa* were subjected to Nitric oxide Scavenging Assay and ascorbic acid was used as reference standard in this experiment.

**Table 6.3.1: Nitric oxide scavenging assay of ascorbic acid (standard)**

| Absorbance of Blank | Concentration µg/ml | Absorbance of the Sample | % of Inhibition | $IC_{50}$ (µg/ml) |
|---|---|---|---|---|
| 0.030 | 0.98 | 0.034 | 13.33 | 12.16 |
| | 1.95 | 0.039 | 30.00 | |
| | 3.91 | 0.043 | 43.33 | |
| | 7.81 | 0.049 | 63.33 | |
| | 15.63 | 0.052 | 73.34 | |
| | 31.25 | 0.054 | 80.00 | |
| | 62.5 | 0.060 | 100 | |
| | 125 | 0.068 | 126.67 | |
| | 250 | 0.099 | 230.01 | |
| | 500 | 0.167 | 456.67 | |

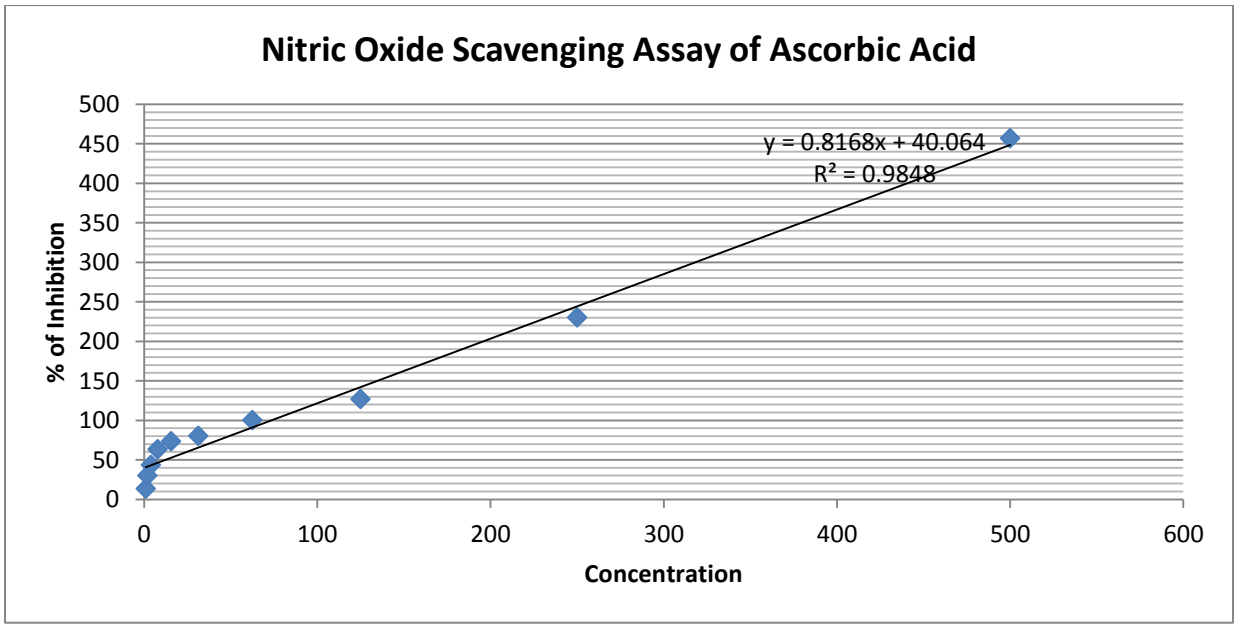



**Figure 6.3.1.: Nitric oxide scavenging assay of ascorbic acid (standard)**

**Table 6.3.2: Nitric oxide scavenging assay of *Spilanthes calva***

| Absorbance of Blank | Concentration µg/ml | Absorbance of the Sample | % of Inhibition | $IC_{50}$ (µg/ml) |
|---|---|---|---|---|
| 0.035 | 0.98 | 0.038 | 8.57 | 36.24 |
| | 1.95 | 0.041 | 17.14 | |
| | 3.91 | 0.045 | 28.57 | |
| | 7.81 | 0.049 | 40 | |
| | 15.63 | 0.053 | 51.42 | |
| | 31.25 | 0.057 | 62.86 | |
| | 62.5 | 0.061 | 74.29 | |
| | 125 | 0.076 | 117.14 | |
| | 250 | 0.081 | 131.43 | |
| | 500 | 0.132 | 277.14 | |

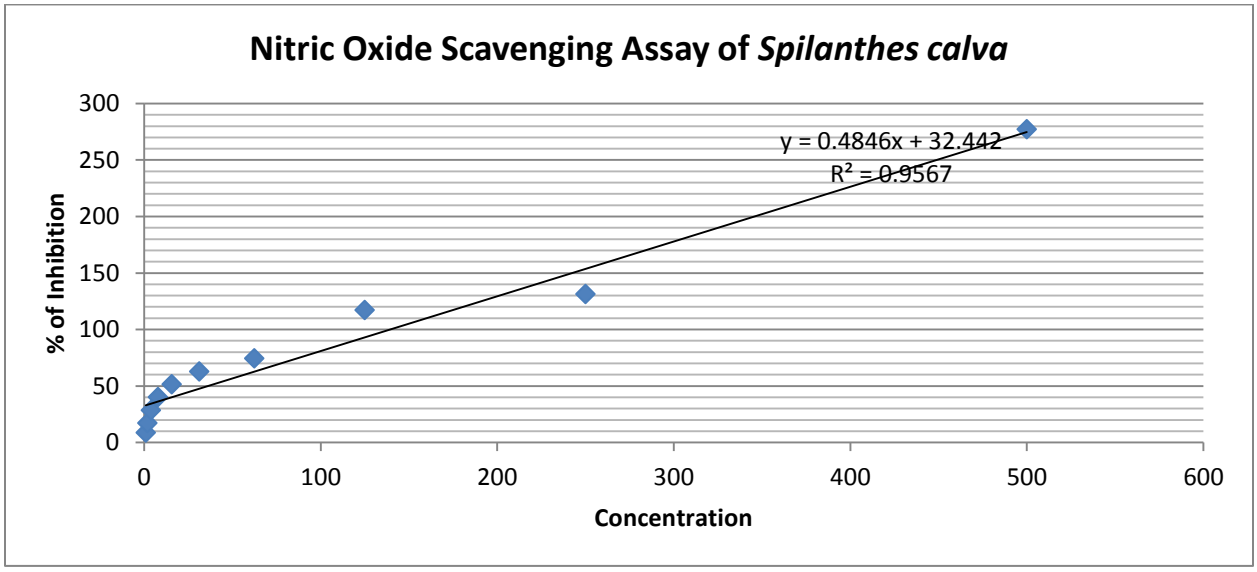



**Figure 6.3.2.: Nitric oxide scavenging assay of *Spilanthes calva***

**Table 6.3.3: Nitric oxide scavenging assay of *Solanum virginianum***

| Absorbance of Blank | Concentration µg/ml | Absorbance of the Sample | % of Inhibition | $IC_{50}$ (µg/ml) |
|---|---|---|---|---|
| 0.062 | 0.98 | 0.064 | 3.22 | 15.92 |
| | 1.95 | 0.070 | 12.9 | |
| | 3.91 | 0.114 | 83.87 | |
| | 7.81 | 0.061 | 1.61 | |
| | 15.63 | 0.079 | 27.41 | |
| | 31.25 | 0.085 | 37.09 | |
| | 62.5 | 0.128 | 106.45 | |
| | 125 | 0.270 | 335.55 | |
| | 250 | 0.524 | 745.16 | |
| | 500 | 0.751 | 1111.29 | |

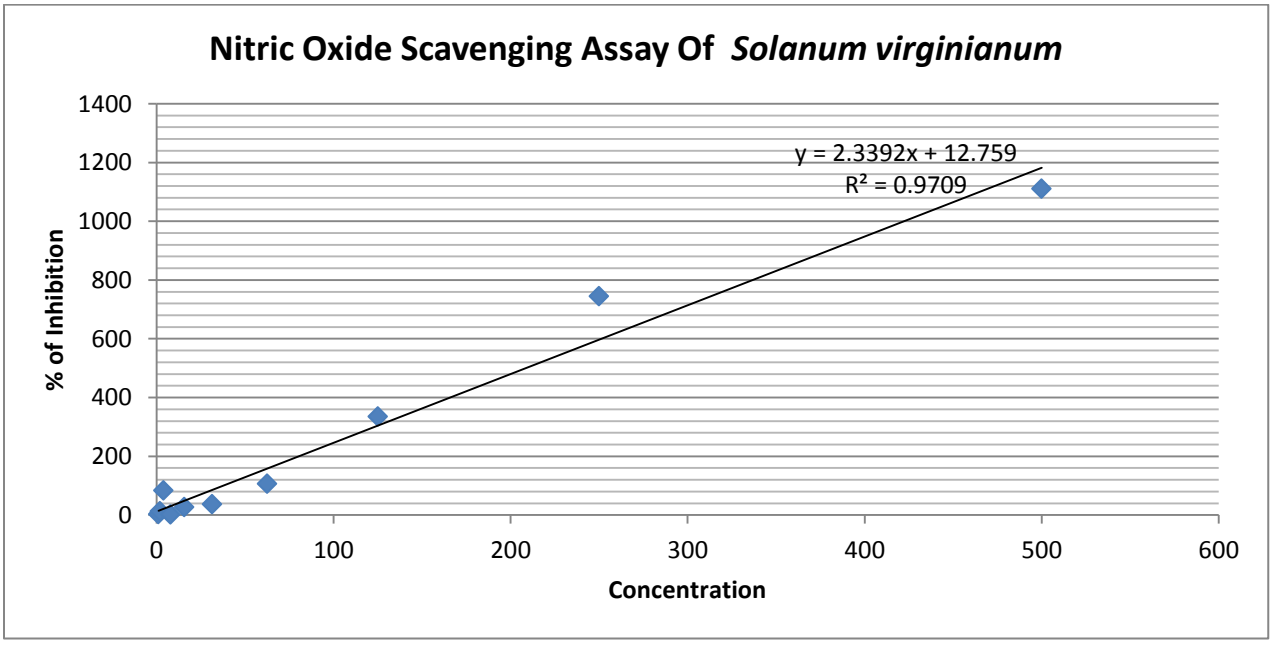



**Figure 6.3.3.: Nitric oxide scavenging assay of *Solanum virginianum***

**Table 6.3.4.: Nitric oxide scavenging assay of *Stevia Rebaudiana***

| Absorbance of Blank | Concentration µg/ml | Absorbance of the Sample | % of Inhibition | $IC_{50}$ (µg/ml) |
|---|---|---|---|---|
| 0.030 | 0.98 | 0.043 | 43.33 | 23.11 |
| | 1.95 | 0.049 | 63.33 | |
| | 3.91 | 0.054 | 80 | |
| | 7.81 | 0.061 | 103.32 | |
| | 15.63 | 0.078 | 160.00 | |
| | 31.25 | 0.085 | 183.32 | |
| | 62.5 | 0.128 | 100 | |
| | 125 | 0.270 | 326.67 | |
| | 250 | 0.289 | 863.33 | |
| | 500 | 0.301 | 903.33 | |

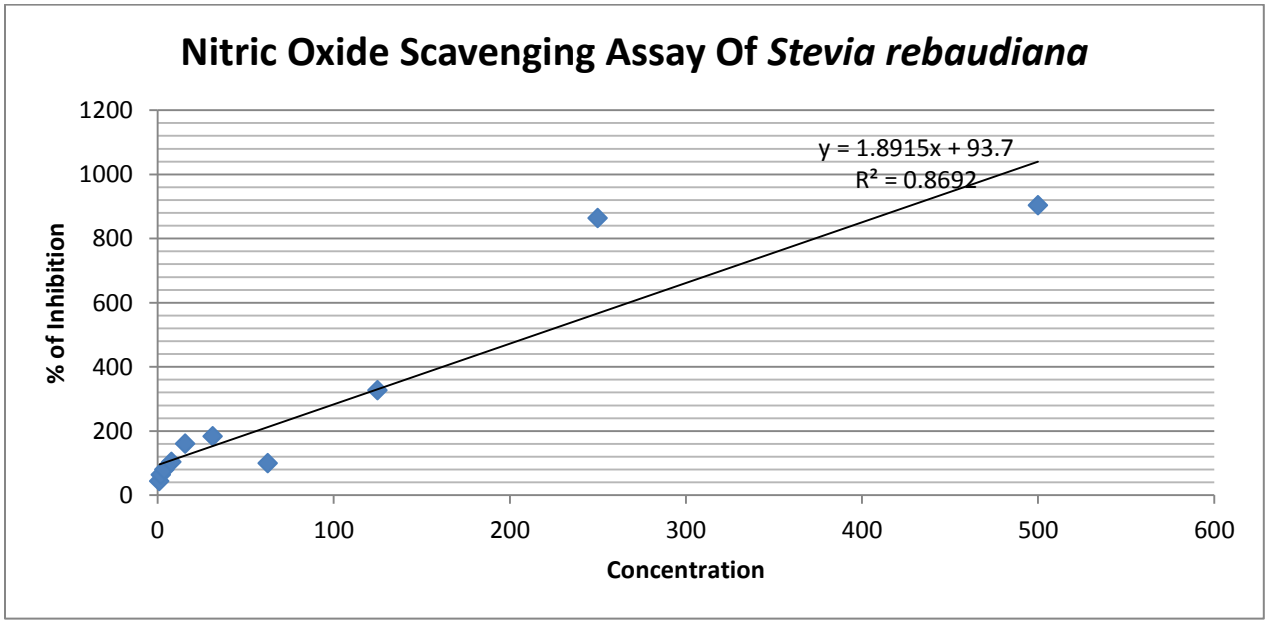



**Figure 6.3.4.: Nitric oxide scavenging assay of *Stevia Rebaudiana***

**Table 6.3.5.: Nitric oxide scavenging assay of *Ruellia tuberosa***

| Absorbance Abosorbance of Blank | Concentration µg/ml | Absorbance of the Sample | % of Inhibition | $IC_{50}$ (µg/ml) |
|---|---|---|---|---|
| 0.035 | 0.98 | 0.039 | 11.43 | 85.03 |
| | 1.95 | 0.040 | 14.28 | |
| | 3.91 | 0.041 | 17.14 | |
| | 7.81 | 0.042 | 20.00 | |
| | 15.63 | 0.044 | 25.71 | |
| | 31.25 | 0.044 | 25.71 | |
| | 62.5 | 0.046 | 31..43 | |
| | 125 | 0.051 | 45.71 | |
| | 250 | 0.074 | 111.43 | |
| | 500 | 0.130 | 271.43 | |

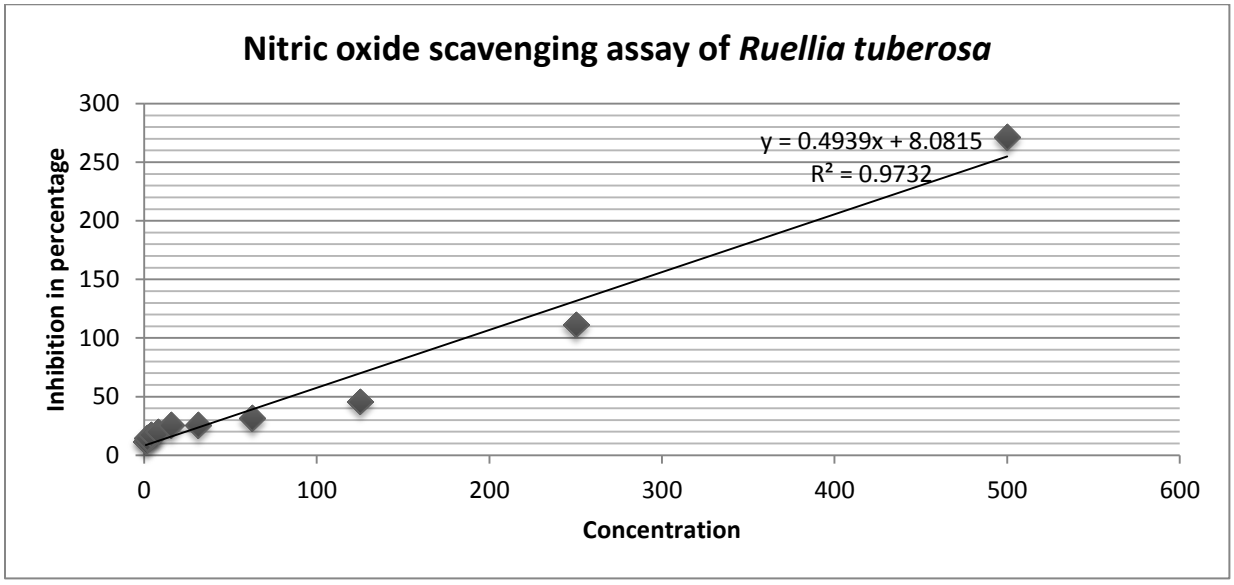



**Figure 6.3.5.: Nitric oxide scavenging assay of *Ruellia tuberosa***

**6.3.6.: Comparison of $IC_{50}$ values between standard and different extracts in Nitric oxide scavenging assay:**

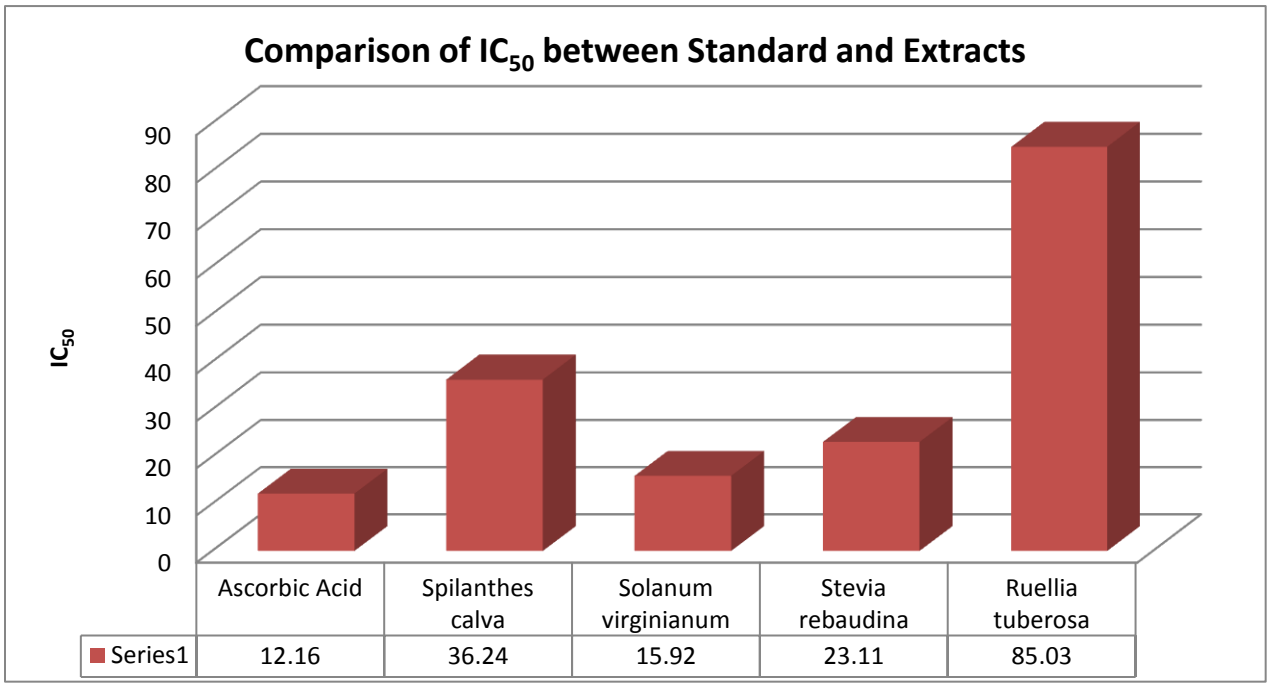



**Figure 6.3.6.: Comparison of $IC_{50}$ values between standard and different extracts in Nitric oxide scavenging assay**

**Discussion:**

The result of the tests is present in the following figure. The extract demonstrated an antioxidant activity in Nitric oxide scavenging assay where NO scavenging activity of methanolic extract of *Solanum virginianum* is good ($IC_{50}$ 15.92 µg/ml) after which is methanolic extract of *Stevia rebaudiana* ($IC_{50}$ 23.11 µg/ml). *Spilanthes calva* has moderate activity ($IC_{50}$ 36.24 µg/ml) and more than the methanolic extract of *Ruellia tuberosa* ($IC_{50}$ 85.03 µg/ml). *Ruellia tuberosa (*$IC_{50}$ *85.03 µg/ml*) showed least activity among the extracts.

**6.4 Reducing Power Assay**

The Methanolic extract of *Spilanthes calva, Solanum virginianum, Stevia rebaudiana* and *Ruellia tuberosa* were subjected to Reducing Power Assay and ascorbic acid was used as reference standard.

**Table 6.4.1 Reducing Power Assay of Ascorbic acid**

| SL. | Concentration µg/ml | Absorbance | | | Mean |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | |
| 1 | 0.98 | 0.096 | 0.098 | 0.097 | 0.097 |
| 2 | 1.95 | 0.105 | 0.105 | 0.102 | 0.104 |
| 3 | 3.91 | 0.141 | 0.145 | 0.147 | 0.144 |
| 4 | 7.81 | 0.223 | 0.221 | 0.222 | 0.221 |
| 5 | 15.63 | 0.284 | 0.281 | 0.285 | 0.283 |
| 6 | 31.25 | 0.438 | 0.439 | 0.440 | 0.439 |
| 7 | 62.5 | 0.801 | 0.804 | 0.806 | 0.802 |
| 8 | 125 | 0.964 | 0.965 | 0.966 | 0.965 |
| 9 | 250 | 1.592 | 1.595 | 1.593 | 1.593 |
| 10 | 500 | 2.96 | 2.94 | 2.93 | 2.94 |

**Table 6.4.2 Reducing Power Assay of *Spilanthes calva***

| SL. | Concentration µg/ml | Absorbance | | | Mean |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | |
| 1 | 0.98 | 0.006 | 0.007 | 0.009 | 0.007 |
| 2 | 1.95 | 0.013 | 0.012 | 0.015 | 0.013 |
| 3 | 3.91 | 0.027 | 0.022 | 0.024 | 0.025 |
| 4 | 7.81 | 0.030 | 0.029 | 0.027 | 0.029 |
| 5 | 15.63 | 0.038 | 0.040 | 0.037 | 0.038 |
| 6 | 31.25 | 0.054 | 0.053 | 0.055 | 0.054 |
| 7 | 62.5 | 0.077 | 0.076 | 0.079 | 0.077 |
| 8 | 125 | 0.119 | 0.121 | 0.123 | 0.121 |
| 9 | 250 | 0.156 | 0.159 | 0.158 | 0.158 |
| 10 | 500 | 0.294 | 0.296 | 0.297 | 0.296 |

**Table 6.4.3 Reducing Power Assay of *Solanum virginianum***

| SL. | Concentration µg/ml | Absorbance | | | Mean |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | |
| 1 | 0.98 | 0.065 | 0.061 | 0.063 | 0.063 |
| 2 | 1.95 | 0.081 | 0.083 | 0.084 | 0.083 |
| 3 | 3.91 | 0.104 | 0.101 | 0.103 | 0.106 |
| 4 | 7.81 | 0.120 | 0.121 | 0.126 | 0.122 |
| 5 | 15.63 | 0.143 | 0.142 | 0.145 | 0.143 |
| 6 | 31.25 | 0.156 | 0.157 | 0.159 | 0.157 |
| 7 | 62.5 | 0.194 | 0.191 | 0.196 | 0.194 |
| 8 | 125 | 0.281 | 0.285 | 0.283 | 0.283 |
| 9 | 250 | 0.451 | 0.452 | 0.455 | 0.453 |
| 10 | 500 | 0.649 | 0.647 | 0.642 | 0.646 |

**Table 6.4.4: Reducing Power Assay of *Stevia rebaudiana***

| SL. | Concentration µg/ml | Absorbance | | | Mean |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | |
| 1 | 0.98 | 0.041 | 0.043 | 0.045 | 0.043 |
| 2 | 1.95 | 0.062 | 0.064 | 0.065 | 0.063 |
| 3 | 3.91 | 0.074 | 0.076 | 0.073 | 0.064 |
| 4 | 7.81 | 0.084 | 0.085 | 0.087 | 0.085 |
| 5 | 15.63 | 0.093 | 0.096 | 0.099 | 0.096 |
| 6 | 31.25 | 0.112 | 0.115 | 0.114 | 0.114 |
| 7 | 62.5 | 0.176 | 0.172 | 0.171 | 0.173 |
| 8 | 125 | 0.231 | 0.234 | 0.238 | 0.234 |
| 9 | 250 | 0.391 | 0.395 | 0.401 | 0.396 |
| 10 | 500 | 0.571 | 0.574 | 0.576 | 0.574 |

**Table 6.4.5: Reducing Power Assay of *Ruellia tuberosa***

| SL. | Concentration µg/ml | Absorbance | | | Mean |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | |
| 1 | 0.98 | 0.031 | 0.035 | 0.032 | 0.033 |
| 2 | 1.95 | 0.042 | 0.046 | 0.049 | 0.046 |
| 3 | 3.91 | 0.053 | 0.057 | 0.061 | 0.057 |
| 4 | 7.81 | 0.072 | 0.075 | 0.073 | 0.073 |
| 5 | 15.63 | 0.096 | 0.092 | 0.102 | 0.097 |
| 6 | 31.25 | 0.123 | 0.124 | 0.128 | 0.125 |
| 7 | 62.5 | 0.175 | 0.171 | 0.174 | 0.173 |
| 8 | 125 | 0.215 | 0.214 | 0.218 | 0.216 |
| 9 | 250 | 0.341 | 0.343 | 0.342 | 0.342 |
| 10 | 500 | 0.492 | 0.495 | 0.496 | 0.494 |

**6.4.6 Comparison of Reducing Power Between Standard and Different Crude Extracts:**

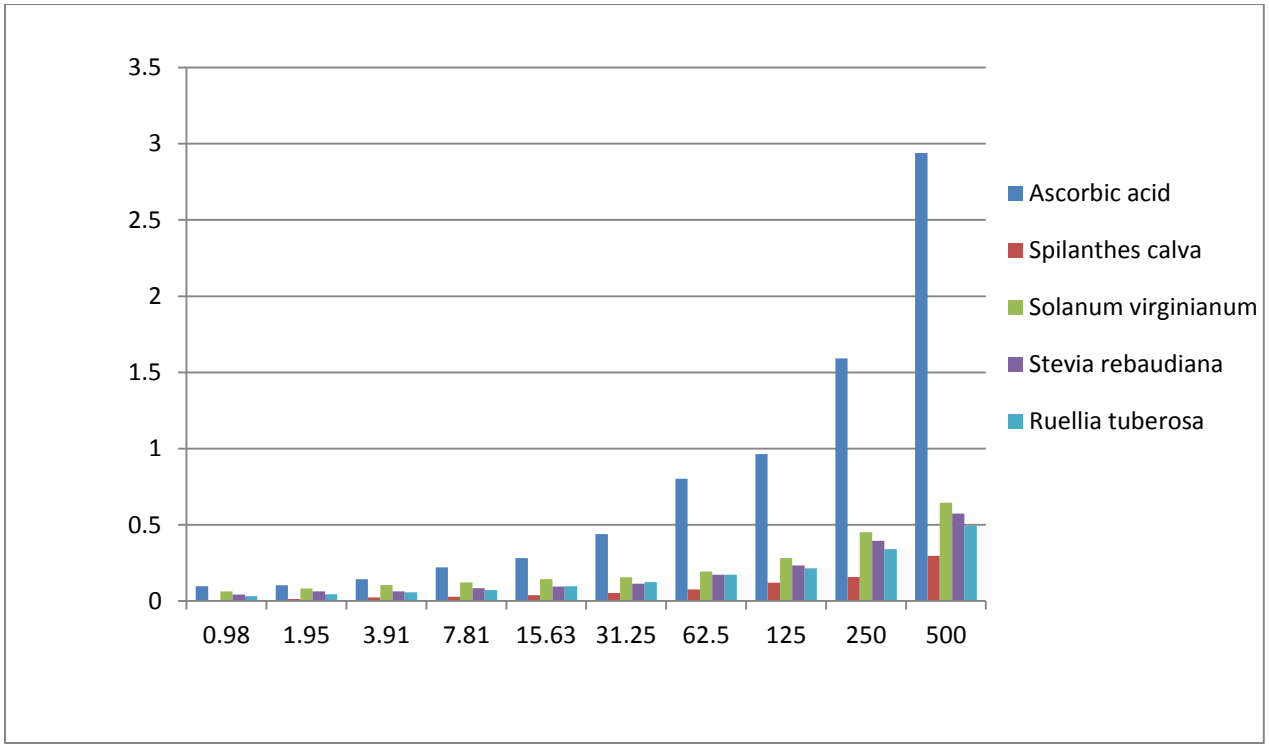



**Fig 6.4.6: Comparison of IC50 values between standard and different crude extract**

**Discussion:**

In reducing power assay higher absorbance of the reaction mixture indicates higher reductive potential. In the graph we can see that with the increase of concentration absorbance also increase. From the above graph we can say that among four different extract *Solanum virginianum* showed highest level of reducing power. *Stevia rebaudiana* and *Ruellia tuberosa* showed moderate. *Sp. Calva* showed least activity.

### 6.5 TLC of different fractions

#### 6.5.1 Solvent

| **Reagent** | **Amount** |
|---|---|
| Dichloromethane | 8 ml |
| Chloroform | 2 ml |


**Figure 6.5.1: Results of TLC before Charring**

**Figure 6.5.2: Results of TLC after Charring**

**6.5.2 Calculation of Rf value:**

**Rf value of Cyclohexane fraction:**

**Fraction 1 & 2 (F-12)**

1st spot: 0.29

2nd spot: 0.54

3rd spot: 0.67

**Fraction 3(F-3)**

1st spot: 0.33

2nd spot: 0.44

3rd spot: 0.56

4th spot:0.77

**Rf value of Cyclohexane : DCM (1:1) fraction:**

**Fraction 4 & 5( F-4,5)**

1st spot: 0.15

2nd spot: 0.33

3rd spot: 0.56

4th spot:0.73

**Fraction 6( F-6)**

1st spot: 0.12

2nd spot: 0.23

3rd spot: 0.31

**Rf value of DCM fraction**

**Fraction 7 ( F-7)**

1st spot: 0.1

2nd spot: 0.25

**Fraction 8( F-8)**

1st spot: 0.19

2nd spot: 0.33

3rd spot: 0.81

The compounds with less Rf value are more polar and the compounds with more Rf value are less polar. In case of dichloromethane and chloroform (8:2) solvent the naked eye view of the TLC showed no clear spot, under UV lamp it showed sky blue color spots in the cyclohexane fraction 1 & 2 (F-1,2) and also in the DCM fraction 7 (F-7); Orange color spots were found in the Cyclohexane fraction 3 (F-3) and also in the DCM fraction 8 (F-8). purple color and orange color spots were found in the Cyclohexane : DCM (1:1) Fraction i.e (F-4,5) and (F-6). Further study of these fractions may lead to the possible isolation of these compounds from the extracts.

# Conclusion

The study indicates that medicinal plants like *Spilanthes calva, Solanum virginianum, Stevia rebaudiana, Ruellia tuberosa* and *Mikania cordata* are good sources of antioxidant. Many substances consumed by man either through foods, drinks and inhalation, even effect of exogenous material (ultraviolet radiation) on the skin may be destructive to the health and thus, shortening the life span of man. Free radicals when generated in the body system of man causes damage to which eventually leads to death at shorter time. Continuous usage of the same vegetable oil, smoking, chronic alcoholism and exposure to pollution are the reason for which oxidative stress occurs in body. Report has shown that proper intake of antioxidant will help quench all these inevitably free radicals in the body thus, improving the health by lowering the risk of various diseases such as cancer. Synthetic antioxidants are recently reported to be dangerous to human health. Thus the search for effective, nontoxic natural compounds with antioxidative activity has been intensified in recent years. In addition to endogenous antioxidant defense systems, consumption of dietary and plant-derived antioxidants appears to be a suitable alternative. Dietary and other components of plants form a major source of antioxidants. Higher intake of foods with functional attributes including high level of antioxidants in functional foods is one strategy that is gaining importance. The RDA has been previewed therefore, the consumption of antioxidant rich diet will lead to lower health risks and people live longer and have fewer disabilities.

Newer approaches utilizing collaborative research and modern technology in combination with established traditional health principles will yield dividends in near future in improving health, especially among people who do not have access to the use of costlier western systems of medicine.

However, further studies are necessary to elucidate new compounds from these plants. This report may serve as a footstep to use these plants as a new source of medication**.**

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