Evaluation of Anticoagulation and Antioxidant Activity of Five Common Spices of Bangladesh

A Thesis

Submitted by

Fatema-Tuz-Zohura

Examination Roll: MS- 170705

Session 2016-2017

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Biotechnology and Genetic Engineering Discipline, Life Science School, Khulna University, Khulna

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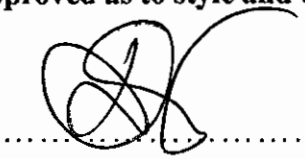

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Declaration

This thesis reports the original research work of the author, except as otherwise stated. The materials presented have not been submitted either in whole or in part for a degree from this or any other university. The findings of the research have not been/ will not be published anywhere without the concurrence of the concerned supervisor.

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DEDICATED TO
MY BELOVED FAMILY MEMBERS

Acknowledgement

At the very beginning, I offer my deepest gratitude to the almighty Allah for all his merciful blessings bestowed upon me. Allah, to whom all praises, goes for enabling me to complete my research work.

It is my pleasure to express my greatest and profound gratitude and heartfelt with best regards to my honorable supervisor, Dr. Kazi Md. Didarul Islam, Professor of Biotechnology and Genetic Engineering Discipline for his active guidance, keen interest, continuous inspiration and constructive criticism for which the work comes to light.

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Abstract

Pants and herbs have been used for the therapeutic purpose since time immemorial. Apart from boosting flavor, herbs and spices are also known for their preservative, antioxidant, antimicrobial and other medicinal properties. Spices are sources of wide variety of secondary metabolites which have been used for treatment of several human health disorders. Great pharmaceutical significance of bioactive metabolites with aspiring prospect has guided the present study to screen for anticoagulation activity, serine protease inhibitory assay and antioxidant activity of five common spices available in Bangladesh. *Z. officinile*, *A. sativum*, *C. verum*, *C. longa.*, *C sativum* were extracted separately with ethanol and water for bioactivity analysis. Anticoagulation activity was assessed by prothrombin time assay. *A. sativum* could inhibit the blood plasma to make a thrombin clot at 25.13±0.9781 min (at 1000µg/ml concentration). *C. verum* exhibited the highest serine protease inhibitory activity (78% inhibition of protease at 400 µg/ml concentration) among the five spices. Antioxidant activity was assessed by using DPPH free radical scavenging assay, reducing power assay and ferric reducing antioxidant power (FRAP) assay. *C. verum* extract demonstrated the highest free radical scavenging activity (IC50: 46.46 µg/ml) and FRAP value (375.28 µM Fe (II)/100µg dry weight of extract). Furthermore, total phenolic and flavonoid contents determined in the extracts of five spices where *C. verum* and *Z. officinale* demonstrated highest flavonoid (47.055±0.006 mg QE/g of extract) and phenolic content (153.33±0.012 mgGAE/g of extract) respectively. In conclusion among the five common spices of Bangladesh *A. sativum*, *C. verum* and *Z. officinale* were found to be potent anticoagulation and serine protease inhibitory activity which we postulate were exerted by phenolic and flavonoid molecules. The screens employed in this present study are preliminary and further study is required to ascertain whether the spices extracts could be utilized as source and template for the synthesis of potential drugs of anticoagulation and others.

Contents

Chapter One

INTRODUCTION

1.0 Introduction:

Natural sources are limitless inspiration for novel drug development because there are many promising drug candidates available [Newman *et al.*, 2012]. About more than 50% of FDA-approved drugs were natural sources derivatives [Kingston, 2011 & Chin *et al.*, 2006]. Among these sources "phytochemicals" (derived from plants) have been the single most productive source of leads for the development of drugs. Plants synthesize these chemicals for their particular needs, mainly in their defensive roles and they may become a base and natural blue print for the development of new drugs. Plants are sources of wide variety of secondary metabolites such as tannins, terpenoids, alkaloids, flavonoids, flavonoids, phenolics, glycosides, saponins and steroids etc. which has been used for treatment of several human health disorders. The biologically active compounds would provide selective ligands for disease-related targets and influence the disease-related pathways and eventually shift the biological network from disease status to the healthy status [Clardy *et al.*, 2004; Jiangyong *et al.*, 2013]. The past few years, however, have experienced a renewed interest in the use of phytochemicals and more importantly their role as a basis for drug development. Approximately one-half of all licensed drugs that were registered worldwide in the last 25 years period were natural products or their synthetic derivatives [Newman *et al.*, 2007].

Different parts of plants such as seed, fruit, root, bark, berry, bud or other vegetable substance are sometimes used in cooking or as a food substance for human being. Some of this substance distinguishes from herbs and parts of plant known as spices. In general, herbs are non-woody plants, and are leafy material in their fresh state [Roy *et al.*, 2004]. As long ago as 3500 BC the ancient Egyptians were using various spices for flavouring food, in cosmetics, and for embalming their dead. Spices and vegetables possess antioxidant activity that can be applied for preservation of lipids and reduce lipid peroxidation in biological systems. The antioxidant activity of these dietary spices suggests that in addition to imparting flavor to the food, they possess potential health benefits by inhibiting the lipid peroxidation. The antioxidant activity of spice extracts were retained even after boiling for 30 min at 100°C, indicating that the spice constituents were resistant to thermal denaturation [S Shobana *et al.* 2000]. These botanicals, also known as nature's pharmacy, have been used as traditional medicines in many parts of the world for generations [Lai *et al.*, 2004]. The medicinal values

of herbs and spices are strongly promoted in Canada. The uses of natural health products are given legal status [Volpe J., 1998]. Hippocrates said: "Let your food be your medicine, and your medicine be your food". Recent research has taken into consideration all aspects related to the use of traditional herbal remedies towards the development of modern medicines. Over the years, investigations on the curry spice, turmeric, have identified curcumin, an active anticancer agent, which is now in Phase clinical trials for colon cancer [Caragay *et al.*, 1992].

Herbs are usually grown in temperate or cooler climate, whereas spices are grown in the tropics. In general, herbs are green and have pleasant taste, whereas spices are brown, black or red in color with pungent smell. However, some plants can be both herb and spice. A herb may be harmless when used as food, but may have toxicity when used as medicine because of the relative higher dose administered, or because it can interact with other pharmaceutical medications [Argento *et al.*, 2000, Ernst *et al.*, 2003].

Spices are herbs, the leaves, flowers, or stems of plants used for flavoring or as a garnish. A spice is a seed, fruit, root, bark, or other plant substance primarily used for flavoring, coloring or preserving food [Thomas *et al.*, 2012]. The spice trade developed throughout the Indian subcontinent and Middle East by at earliest 2000 BCE with cinnamon and black pepper, and in East Asia with herbs and pepper [Steven E *et al.*, 2019]. The world's spices come from South East Asia, the spice trade helped drive the global economy starting in the Middle Ages. Spices were among the most demanded and expensive products available in Europe in the Middle Ages, [Duke, J.A., 2002]. Black pepper, cinnamon, cumin, nutmeg, ginger and cloves etc. were used as medieval medicine in Europe [Woodward, Penny 2003].

Ayurvedic Medicine brings the unique theories and traditions of Ayurveda alive so that they are accessible to the complementary health practitioner of today [S Pole *et al.*, 2006]. Ayurveda is reputed to have been practiced for over 3000 years, its history being divided into 4 periods [KN Udupa, 1975]. Some spices are use as ayurvedic medicine. The history of herbs and spices is as long as the history of mankind. People have used these plants for medicinal purposes since the earliest times, and the knowledge of herbs has been handed down from generation to generation for thousands of years [Brown, 1995]. About 80 % of the world population is dependent upon medicinal plants for primary healthcare, particularly in the developing economies where local communities are offered immediate access to safe and effective products so as to treat ill health through self-medication [Akerele, 1992].

Spices have substantial antioxidant activity, owing primarily to phenolic compounds, especially flavonoids, influence nutrition through many pathways, including affecting the absorption of other nutrients. One study found cumin and fresh ginger to be highest in antioxidant activity [Ninfali *et al.*, 2007]. Cardiovascular disease, such as deep vein thrombosis, strokes, heart attacks, and hypertension resulting from thrombosis and intravascular blood clots, are main causes of morbidity and mortality in world countries. Millions of people over the world are affecting by thromboembolic disorders, resulting from thrombosis, causing millions of death every years. Thrombosis is closely linked to activate platelet adhesion, aggregation, secretion functions, and activation of intrinsic and extrinsic pathways, causes blood coagulation and fibrin formation [Gong G., *et al.* 2011]. Blood coagulation pathway is classified two pathways one is intrinsic and another one is extrinsic pathway. Intrinsic pathway is renponse to tissue trauma which is initiated with exposure of tissue factor [Spanier T. B. *et al.*, 1998]. The coagulation factors are generally serine proteases (enzymes), which act by cleaving downstream proteins. The exceptions are tissue factor, FV, FVIII, FXIII. Tissue factor, FV and FVIII are glycoproteins, and Factor XIII is a transglutaminase [Pallister CJ & Watson MS, 2010]. The tissue factor and *contact activation* pathways both activate the "final common pathway" of factor X, thrombin and fibrin [Hoffbrand, A. V., 2002]. Both pathways consists of factor Xa and Va, those are bound together on phospholipid membrane by calcium ions. This complex converts prothrombin (factor II) to thrombin (factor IIa).

The extrinsic pathway is the mechanism by which coagulation is initiated in vivo in response to trauma. The intrinsic pathway is an alternative mechanism by which the coagulation system can be initiated. Thrombin generation occurs after the formation of the fibrin clot [Mann KG. *et al.*, 1998]. Thrombin is important for additional fibrin generation as well as for activation of factor XIII and the thrombin-activatable fibrinolysis inhibitor. Activated factor XIII (XIIIa) is atransglutaminase that stabilizes the clot by covalent cross-linking of fibrin [Davie EW 1995]. Coagulation defects may cause hemorrhage or thrombosis, and occasionally both, depending on the nature of the defect [Shapiro S.S., 2003]. Hemophilias (hemophilia A, hemophilia B, hemophilia C) thrombosis ischemia venous thrombosis etc. are coagulation factor disorders. Antithrombotic agents include anticoagulants, antiplatelets, and thrombolytics which reduce blood clots in the body by dissolving already fromed clot or inhibit clot formation [Webster C., 2001]. Anticoagulants are substances or agents which inhibit or prevent clot formation of blood in body [Roger N., 1978], by removing calcium

[Cheesbrough M., 2000]. Last few decays heparin, warfarin, rivaroxaban, dabigatran, enoxaparin, etc. and their derivatives have played a major role in the clinical setting. They are expensive medication and have life threatening side effects including development of thrombocytopenia, hemorrhagic effect, ineffectiveness in congenital or acquired antithrombin deficiencies and incapability to inhibit thrombin bound to fibrin have also been documented [Pereira M.S. *et al.*, 2002]. Spices can be the sources of anticoagulant agents due their biological activities.

Most of the enzymes generated during activation of coagulation are inhibited by the serine-protease inhibitor antithrombin, previously called antithrombin III. Antithrombin is, in itself, an inefficient serine-protease inhibitor, but heparin and the heparin-like molecules that are present on the surface of endothelial cells stimulate its activity [Albert LR, *et al.*, 1998]. Serine proteases have been shown to play a multifarious role in health and disease. Many pathological disorders are caused by deficiencies in the normally exquisite regulation of the activity of proteolytic enzymes, resulting in abnormal tissue destruction and/or the aberrant processing of other proteins and peptides. Their role in clot dissolution, the urokinase- and tissue-type plasminogen activators (uPA and tPA) play a critical role in a number of processes including extracellular matrix (ECM) remodeling [Dano K. *et al.*, 1985], angiogenesis [Fazioli F. & Blaisi F., 1994], wound healing [Pepper M.S., *et al.*, 1990], tumor invasion and metastasis [Andreasen P. A., *et al.* 1997]. The most widely studied group of serine proteases has been those involved in the coagulation cascade (thrombin, protein C, factor VII, IX, X and XII), complement system (C1r, C1s, C3 convertase, C5 convertase and factor D) and fibrinolysis (tPA, uPA and plasmin) [Mann K. G., et al., 1998 &Davie E. W., *et al.*, 1991]. Specific and selective protease inhibitors are potentially powerful tools for inactivating target proteases in the pathogenic process of human diseases such as emphysema, arthritis, pancreatitis, thrombosis, high blood pressure, muscular dystrophy, cancer and AIDS [Johnson and Pellecchia, 2006]. Serine protease inhibitors are isolated from Solanaceae, Fabaceae, Euphorbiaceae, Poaceae, and Cucurbitaceae families [Kim *et al.*, 2009]. These families plants has strong antioxidant, antimicrobial, and free radical scavenging activities [Manojlovic *et al.*, 2005 & Stef *et al.*, 2009].

A plant cell produces two types of metabolites: primary metabolites involved directly in growth and metabolism (carbohydrates, lipids and proteins); and secondary metabolites considered as end products of primary metabolism and not involved in metabolic activity (alkaloids, phenolics, sterols ,steroids, essential oils, lignins and tannins etc) [Irchhaiya, R. *et*

al., 2015] .In most references, it is stated that the secondary metabolites extracted from plants are subdivided in three major classes: terpenoids, alkaloids and phenolics. They contain numerous natural products with interesting pharmacology activities [Nicolaou, 2011]. Antioxidant substances are believed to play a potential role in interfering with the oxidation process by reacting with free radicals, chelating catalytic metals and scavenging oxygen in biological systems. Natural antioxidants are widely used because they are regarded as safer and causing fewer adverse reactions but the synthetic antioxidants which have been restricted by legislative rules because they are suspected to have some toxic effects and as possible carcinogens [Hirose, M., *et al.*, 1998].

Spices have been used for thousands of years to enhance the flavor, color and aroma of food. In addition to boosting flavor, spices are also known for their preservative, antioxidant, antimicrobial and other medicinal properties [Tajkarimi, M., 2011 & Sharifi-Rad, J., *et al.*, 2017]. The spice plant *Zingiber officinally* which known as ginger used as flavoring agent. Ginger helps alleviate nausea and vomiting resulting from chemotherapy or pregnancy is inconsistent [Giacosa A. *et al.*, 2015]. The rhizomes have been shown to be effective in the treatment of several medical conditions including stomach problems, nausea, vomiting, epilepsy, sore throat, cough, common cold, bruises, wounds, liver complaints, rheumatism, muscular pains, atherosclerosis, migraine headaches, high cholesterol, ulcers, and stomach discomfort [Shukla, Y. & M. Singh, 2007].

Allium sativum is one of the most common spices known as garlic. Garlic is used for many conditions related to the heart and blood system. These conditions include high blood pressure, low blood pressure, high cholesterol, inherited high cholesterol, coronary heart disease, heart attack, reduced blood flow due to narrowed arteries. Sulfur compounds, including allicin, appear to be the active components in the root bulb of the garlic plant [Ellen 2005]. Song *et al.*, 1960, isolated substance from garlic as anticoagulant. Studies show significant but modest lipid-lowering effects and antiplatelet activity. Garlic appears to have no effect on drug metabolism, but patients taking anticoagulants should be cautious [Ellen, 2005].

Cinnamomum verum is a member of Lauraceae family. Along with dietary fiber cinnamon is a good source of calcium, iron, vitamin K. It decreases plasma glucose [Costello RB., *et al.*, 2016]. Dietary cinnamon lowered total cholesterol, LDL-cholesterol and triglycerides [Khan A, *et al.*, 2003]. Cinnamon has a long history of use in traditional medicine as a digestive

system aide. Cinnamon has anti-inflammatory, antimicrobial, antioxidant, antitumor, cardiovascular, cholesterol-lowering, and immunomodulatory effects [Nazari Hossein, *et al.*, 2013]. Cinnamon plants for detection of its active ingredients and effect on blood clotting time [Bamosa *et al* 1997; & Van Wyk and Wink, 2004).

Curcuma longa is known as turmeric is a plant of Zingiberaceae family used as a coloring agent in cooking food. The bright yellow color of tumeric is due to curcumin. turmeric has been prescribed for many ailments in Indian natural medicines [Nadkarni, K.M. , 1976]. It is applied to fresh wounds and bruises and also acts as a counter-irritant in insect bites. Internally it is an anthelminthic. traditional Indian medicineuses turmeric powder for the treatment of biliary disorders,anorexia, coryza, cough, diabetic wounds, hepatic disorders, rheumatism and sinusitis [Ammon et al., 1992]. The antioxidant activity of curcumin was reported 77 as early as 1975 [Sharma, O. P. , 1976]. Curcumin shows anticoagulant activity by inhibiting col-lagen and adrenaline-induced platelet aggregation in vitroas well as in vivo in rat thoracic aorta [Srivastava, R. *et al.*, 1985].

Coriandrum sativum is an annual herb in the family Apiaceae. Coriander is used for digestion problems including upset stomach, loss of appetite, hernia, nausea, diarrhea, bowel spasms, and intestinal gas. It is also used to treat measles, hemorrhoids, toothaches, worms, and joint pain, as well as infections caused by bacteria and fungus. In foods, coriander is used as a culinary spice and to prevent food poisoning. The seeds of C. sativum are used as a traditional drug for the treatment of diabetes [Deepa, B & Anuradha, C V ,2011].

The objective of the study,

- Anticoagulant activity of the ethanolic and aqueous extracts common spices available in Bangladesh.
- Serine protease activity of the ethanolic and aqueous extracts common spices available in Bangladesh.
- Antioxidant activity of the ethanolic and aqueous extracts common spices of Bangladesh.

Chapter Two

Chapter 2

2 Literature Review

2.1.1 Literature Review of *Zingiber officinale*

General Information

Zingiber officinale is a flowering plant whose rhizome, ginger root or ginger, is widely used as a spice and a folk medicine. In Bangladesh it is commonly known as "Ada" .This plant is a member of the family Zingiberaceae.

2.1.1.1 Taxonomy of *Zingiber officinale*

Kingdom: Plantae

Subkingdom: Viridaeplantae

Infrakingdom: Streptophyta

Super Division: Tracheobionta

Division: Magnoliophyta

Subdivision: Spermatophytina

Infradivision: Angiospermae

Class: Liliopsida

Subclass: Zingiberidae

Superorder: Rosanae

Order: Zingiberales

Family: Zingiberaceae

Genus: Zingiber. L

Species: Zingiber officinale

2.1.1.2 Common Name of *Z. officinale*

Latin name: *Zingiber officinale*

Bengali name: Ada

English name: Ginger

Hindi name: Adrak

Tamil name: Inji

2.1.1.3 Geographical Distribution of *Z. officinale*:

Z. officinale is a popular medicinal plant originated from Island Southeast Asia. The rhizomes and the leaves were used to flavor food or eaten directly. It is found throughout the hot and humid part of India, China, Myanmar, Sri Lanka, Cambodia, Vietnam and Taiwan. It has also been found in the America, Australia and the Pacific Ocean islands. In 2016, global production of ginger was 3.3 million tonnes, led by India with 34% of the world total. Nigeria, China, and Indonesia also had substantial production [Beaujard P. 2011].

2.1.1.4 Description of the Plant:

Z. officinale is a perennial herb, which is vegetatively propagated with its rhizomes (underground stems). From the tuberous rhizome sections of sterile, reed-like stem grow up to 1m long, which are covered by the sheaths of the 20cm long, linear-lanceolate leaves. Some shorter shoots bear inflorescences with terminal yellow flowers that have a purple lip. This plant has profound medicinal use and is proved to have anticoagulants, adaptogenic, althelmintic, anti-inflmmatory [Terry R et al. 2011], antipyretic, analgesic, anti- allergic antibacterial, antidiabetic, antifilarial, antioxidant, nootropic, immunomodulatory, hypoglycemic and hepatoprotective activity [Suryawanshi et al. 2011].

Figure 2.1: Figure of *Z. officinale* flower and roots.

2.1.1.5 Medicinal and other uses

The roots of *Z. officinale* used as spices and flavoring agent in cooking food all over the world [USDA, NRCS. 2018] Young ginger rhizomes are juicy and fleshy with a mild taste. Powdered dry ginger root is typically used as a flavoring for recipes such as gingerbread, cookies, crackers and cakes, ginger ale, and ginger beer. It has a role in traditional ayurvedic medicine. preventive and ameliorative effects of ginger have been described in the treatment of catarrh, rheumatism, nervous diseases, gingivitis, toothache, asthma, stroke, constipation and diabetes [Chrubasik et al. 2005]. It is used in drinks both cold and hot and mostly used in tea in Indian Subcontinent. It helps in nausea, vomiting, dysmenorrhea, skin problem, gall stones, pain relief, though it was found superior over a place for postoperative nausea [Goa, et al. 2010]. The efficacy of garlic powders, especially in regard to cholesterol reduction, is controversial [H Amagase et al. 2003].

2.1.1.6 Biological and phytochemical compounds:

Ginger helps alleviate nausea and vomiting resulting from chemotherapy or pregnancy is inconsistent [Giacosa A. et al., 2015]. The rhizomes have been shown to be effective in the treatment of several medical conditions including stomach problems, nausea, vomiting, epilepsy, sore throat, cough, common cold, bruises, wounds, liver complaints, rheumatism, muscular pains, atherosclerosis, migraine headaches, high cholesterol, ulcers, and stomach discomfort [Shukla, Y. & M. Singh, 2007].

Ginger used for a wide array of unrelated ailments that include arthritis, rheumatism, sprains, muscular aches, pains, sore throats, cramps, constipation, indigestion, vomiting, hypertension, dementia, fever, infectious diseases and helminthiasis, rheumatism, nervous diseases, gingivitis, toothache, asthma, stroke, constipation and diabetes [Awang, 1992; Wang & Wang, 2005]. The pungency of fresh ginger is due primarily to the gingerols, which are a homologous series of phenols.It has been reported that treatment with a methanolic extract of dried rhizomes of ginger produced a significant reduction in fructose-induced elevation of lipid levels, bodyweight, hyperglycemia and hyperinsulinemia. Ghayur and Gilani 2005, reported that the crude extract of ginger induced a dose-dependent (0.3–3 mg/kg) fall in the arterial blood pressure. The anti-inflammatory properties of ginger have been known for centuries (Afzal et al., 2001; Grzanna *et al.*,2005). Serum protein binding of [6]-gingerol was 92.4% [Ding *et al.*, 1991]. High doses of ginger (500 mg/kg) significantly effective in lowering serum.Recent studirs showsthat [6]-gingerol is endowed with strong

anti-oxidant action both in vivo and in vitro, in addition to strong anti-inflammatory and anti-apoptotic actions [Kim *et al.*, 2007].

2.1.2 Literature Review of *Allium sativum*

General Information

Allium sativum (Liliaceae), known as garlic, is a strongly aromatic bulb crop believed to originate from Kazakhstan, Uzbekistan, and Western China [V. kuete2017]. Garlic grows in temperate and tropical regions all over the world. *A. sativum* is the most widely consumed bulb after onion. The bulb, composed of so-called cloves, is mainly used for flavoring meat, fish, sauces and salads, raw or cooked, or more recently in dehydrated form. Crushed raw garlic is strongly antibiotic, and it has a reputation for lowering blood pressure and cholesterol, and inhibiting thrombus formation [Le Bon, et al. 2000].

2.1.2.1 Taxonomy of *Allium sativum*

Kingdom: Plantae

Subkingdom: Viridiplantae

Infrakingdom: Streptophyta

Super Division: Embryophyta

Division: Tracheophyta

Subdivision: Spermatophytina

Class: Liliopsida

Subclass: Liliidae

Superorder: Liliianae

Order: Asparagales

Family: Amaryllidaceae

Genus: Allium L.

Species: Allium sativum L.

2.1.2.2 Common Name of *A. sativum*

Latin name: Allium sativum

Bengali name: Rosun

English name: Garlic

Hindi name: Lahasun

Tamil name: Punt

2.1.2.3 Geographical Distribution of *A. sativum*:

Garlic is believed to originate from Central Asia (Kazakhstan, Uzbekistan and western China). This was confirmed by phylogenetic analysis based on molecular and biochemical markers, also indicating a secondary diversity center in the Caucasus. Garlic spread to the Mediterranean in ancient times [Eoth,T. et al.1992]. At present Garlic is grown all over the world. Russian Federation, Ukraine and Spain are then the biggest producers of garlic in Europe [FAO 2008]. After onion, garlic is the second most widely used cultivated *Allium* [Fritsch, et al. 2002].

2.1.2.4 Description of the Plant:

A. sativum is a erect herb, usually grown as an annual from small bulbs (cloves), up to 150 cm tall; real stem very short, formed at the base of the plant in the form of a disk, with adventitious roots at base; bulb solitary, depressed globose to ovoid, up to 7 cm in diameter, whitish to purplish, composed of (1–)7–15(–40) sessile cloves, these ovoid to ellipsoid-oblong and borne in the axil of the 2, 3 or 4–5 last leaves, each clove consisting of a protective sheath, a single thickened storage leaf sheath and a small central bud; pseudostem formed by sheathing bases of successive leaves. It produces hermaphrodite flowers. It is pollinated by bees, butterflies, moths, and other insects [Meredith, et al. 2014].

Figure 2.2: The rhizome of *A. sativum* (garlic).

2.1.2.5 Medicinal Uses

Garlic showed lipid-lowering effects, antiplatelet activity and antiatherosclerotic activities. The cardiovascular effects of garlic are among the best investigated of all medicinal plants species [Keusgen 2002]. Louis Pasteur was the first to describe the antibacterial effect of onion and garlic juices. Garlic exhibits a broad antibiotic spectrum against both gram-positive and gram-negative bacteria [Friesen 2006]. Helicobacter pylori is a bacterium implicated in the etiology of stomach cancer and ulcers. The incidence of stomach cancer is lower in populations with high intake of Allium vegetables. Some antibiotic-resistant H. pylori strains are susceptible to garlic [Sivam 2001]. Garlic has anticoagulation, hypotensive, carminative, antiseptic, anthelmintic, diaphoretic and expectorant properties.

2.1.2.6: Biological and phytochemical compounds:

Garlic as a good remedy for intestinal disorders, flatulence, worms, respiratory infections, skin diseases, wounds, symptoms of aging and many other ailments. The use of garlic to treat wounds surfaced repeatedly through the middle ages into World War II, when garlic was used to treat the wounds of soldiers (Essman 1984). The bioactive compounds reduction of risk factors for cardiovascular diseases and cancer, a stimulation of immune function, enhanced foreign compound detoxification, radioprotection, restoration of physical strength, resistance to various stresses and potential antiaging effects. Its lowering the cholesterol level of human blood [Warshafsky, *et al.*, 1993].

The acidity of the stomach would be expected to prevent the conversion of allium to allicin [Freeman and Kodera 1995]. The main quality feature of garlic products is the distinct flavour of cloves, as a result of complex biochemical reactions [Randle & Lancaster, 2002]. The main compounds responsible for that flavour are mostly sulfur-containing, non-volatile amino acids [Block, *et al.*, 1993].

These sulfur compounds are also responsible for the renowned medicinal properties of garlic, such as anticancer, antidiabetic, anti-inflammatory, antimicrobial, antioxidant, cardioprotective and immunomodulatory activities [Alorainy, 2011].

2.1.3 Literature Review of *Cinnamomum verum*

General Information

Cinnamomum verum is a small evergreen tree belonging to the family Lauraceae. Cinnamon is native to Sri Lanka. Among other species, its inner bark is used to make cinnamon. Cinnamon is used mainly as an aromatic condiment and flavoring additive in a wide variety of cuisines, sweet and savoury dishes, breakfast cereals, snack foods, tea and traditional foods. Only a few Cinnamomum species are grown commercially for spice.

2.1.3.1 Taxonomy of *Cinnamomum verum*

Kingdom: Plantae

Subkingdom: Viridiplantae

Infrakingdom: Streptophyta

Super Division: Embryophyta

Division: Tracheophyta

Subdivision: Spermatophytina

Class: Magnoliopsida

Subclass: Zingiberidae

Superorder: Magnolianae

Order: Laurales

Family: Lauraceae

Genus: Cinnamomum

Species: *Cinnamomum verum*

2.1.3.2 Common Name of *C. verum*

Latin name: *Cinnamomum verum*

Bengali name: Darcini

English name: Cinnamon

Hindi name: Daalacheenee

2.1.3.3: Geographical distribution of *C. verum*

Cinnamomum (cinnamon) is a genus of the family Lauraceae, many of whose members are used as spices [Shan *et al.*, 2007]. *C. verum* is native to Sri Lanka and tropical Asia [Hamidpour *et al.*, 2015] and exotic to several African countries, such as Comoros, Ghana, Madagascar, Mauritius, Nigeria, Seychelles, Sierra Leone, Tanzania, and Uganda [Orwa *et al.*, 2009]. The barks are commonly used as spices and for the treatment of cardiovascular diseases and cancers [Kuete *et al.*, 2011; Nkanwen *et al.*, 2013]. Combined, Indonesia and China produced 75% of the world's cinnamon in 2016 when global production was 223,574 tonnes. Four countries accounted for 99% of the world total: Indonesia, China, Vietnam, and Sri Lanka.

2.1.3.4: Description of the plant

Cinnamomum verum is a small tree whose bark is commonly used as spice. Cinnamon is also traditionally used for many centuries for the treatment of diabetes and other conditions. Cinnamon trees are small evergreens with aromatic bark and leaves. The leaves of the tree are thick and oval or lanceolate in shape, growing on smooth gray branches. Cinnamon trees can reach heights of up to 20 m (66 ft) in the wild but are usually coppiced to smaller bushes under cultivation. Cinnamon trees have an economic lifespan of approximately 10 years.

Figure 2.3: The bark of *Cinnamomum verum*.

2.1.3.5 Medicinal uses:

Cinnamon (Cinnamomum zeylanicum), belonging to family Lauraceae, has been utilized as a potential therapeutic agent in various cultures for centuries. Historically, cinnamon bark is among the oldest known spices used against gastrointestinal complaints, chronic bronchitis, and inflammation of eyes in Ayurvedic medicine for over 6000 years. Cinnamon used as a supplement to treat problems with the digestive system, diabetes, loss of appetite, and other conditions. Dietary cinnamon lowered total cholesterol, LDL-cholesterol and triglycerides [Khan A., *et al.*, 2003].

2.1.3.6: Biological & phytochemical constitute:

Generally, food deterioration is caused by lipid rancidity. An antioxidant property of cinnamon inhibits lipid rancidity and used in food as a preservative for many centuries (Wu *et al.*, 1994). Cinnamon polyphenols possess a strong antioxidant potential, which is attributed due to the free radicals scavenging activity, modulatory effect on xenobiotic bio activation, and detoxification processes and inhibition of certain prooxidant enzymes, like lipoxygenases and nitric oxide synthase. Moreover, it induces the activity of various glutathione dependent antioxidant enzymes like glutathione reductase, glutathione S-transferase, and glutathione peroxidase. Cinnamaldehyde as an antidiabetic agent. It helps in the reduction of level of blood glucose in diabetic patients of noninsulin-dependent type.

Antiinflammatory activity has also been shown by cinnamon. Acute inflammation in mice was treated by ethanol extract of cinnamon [Kubo and Kinst-Hori, 1998]. Extracts and oil of cinnamon showed various antimicrobial activities against several fungi and bacteria. Influenza virus replication was inhibited by cinnamon aqueous extract [(Mancini et al., 1999].

Cinnamon bark showed an inhibitory effect against the gram +ve bacteria: Enterococcus faecalis, Micrococcus luteus, Bacillus cereus, and Staphylococcus aureus, and the gram −ve bacteria: Pseudomonas aeruginosa, Escherichia coli, Enterobacter cloacae, and Alcaligenes faecalis, and also against the fungi Rhizopus oligosporus and Aspergillus niger [Chao et al., 2000].

2.1.4 Literature Review of *Curcuma longa*

General Information

Curcuma longa is a flowering plant of the ginger family, Zingiberaceae whose root is widely used in cooking and herbal medicine. It appears to have originated from tropical Southeast Asia. The greatest diversity of Curcuma species by number alone is in India, at around 40 to 45 species. Turmeric has been used in Asia for thousands of years and is a major part of Ayurveda, Siddha medicine, traditional Chinese medicine, Unani [Chattopadhyay I, et al. 2004]. In Bangladesh it is commonly known as "Holud" .This plant is a member of the family Zingiberaceae.

2.1.4.2 Taxonomy of *Curcuma longa*

Kingdom: Plantae

Subkingdom: Viridaeplantae

Infrakingdom: Streptophyta

Super Division: Embryophyta

Division: Tracheophyta

Subdivision: Spermatophytina

Class: Magnoliopsida

Subclass: Zingiberidae

Superorder: Lilianae

Order: Zingiberales

Family: Zingiberaceae

Genus: Curcuma. L

Species: *Curcuma longa*

2.1.4.2 Common Name of *C. longa*

Latin name: *Curcuma longa*

Bengali name: Holud

English name: Turmeric

Hindi name: Haldee

Tamil name: Mancal, Manjalpadi

2.1.4.3 Geographical Distribution of *C. longa*:

Turmeric, originating from India, reached the coast of China in ad700 and reached East Africa 100 years later and West Africa 500 years later. Arab traders were instrumental in spreading the plant to the European continent in the thirteenth century [K.P. Prabhakaran Nair 2013]. They grow in warm, humid climates and thrive only in temperatures above 60°F (29.8°C). India is a leading producer and exporter of turmeric in the world. Andhra Pradesh, Tamil Nadu, Orissa, Karnataka, West Bengal, Gujarat, Meghalaya, Maharashtra, Assam are some of the important states cultivates turmeric, of which, Andhra Pradesh alone occupies 35.0% of area and 47.0% of production.

2.1.4.4 Description of the Plant:

Turmeric is a perennial herbaceous plant that reaches up to 1 m (3 ft 3 in) tall. Highly branched, yellow to orange, cylindrical, aromatic rhizomes are found. The leaves are alternate and arranged in two rows. They are divided into leaf sheath, petiole, and leaf blade [Grieve, M. 2017]. Turmeric root is actually a fleshy oblong tuber 2–3 in (5–10 cm) in length, and close to 1 in (2.54 cm) wide. The interior of the root is hard, firm, and either orange-brown or deeply rust-colored, with transverse resinous parallel rings. The root contains a bitter volatile oil, brown coloring matter, gum, starch, calcium chloride, woody fiber and a yellowish coloring material known as cumarin.

Figure 2.4: Figure of *C. longa* (turmeric) flower and root.

2.1.4.5 Medicinal uses:

Turmeric has several traditional uses, starting from the *Ayurveda*. The common use of turmeric is as an antiseptic. Johnson & Johnson, the well-known US drug company, makes turmeric bandages for the Indian market [MacGregor, 2006]. Turmeric pastes use to relief a pain. Powdered turmeric is taken with boiled milk to cure cough and related respiratory ailments [Sundaram, 2005]. This ancient treatment also combats dental problems and digestive disorders such as indigestion dyspepsia, acidity, indigestion, flatulence, and ulcers. It also alleviates adverse effects of hallucination due to consumption of hashish (cannabis) or other psychotropic drugs. It reduces heart diseases, serum cholesterol, triglyceride, risk of diabetes, coagulation of blood, arthritis. Turmeric also works as an anti-inflammatory and anti-bacterial, antioxidant agent, improve brain function, prevent cancer [Ireson, *et al.*, 2002].

2.1.4.6: Bioactive and phytochemical compound:

Turmeric (rhizome of Curcuma longa) is a medicinal herb of high repute all over the world particularly in South Asia, where it is also used as curry spice in foods [Gilani *et al.*, 2005; Goel *et al.*, 2008]. Turmeric has been traditionally used for its medicinal value in wound healing, inflammation, asthma, epilepsy, gall bladder stones, abdominal cramps, high cholesterol, congestion and AD [Duke, 2002].The anticancer properties of cancer is because of curcumin [Aggarwal *et al.*, 2003]. Curcumin is present in the largest quantity (75–80%) in curcuminoids and it is one of the well-studied biologically active molecules of the turmeric exhibiting antioxidant and anti-inflammatory activities [Eybl et al., 2006]. Turmeric used as a aid for gastrointestinal and respiratory problems such as chronic diarrhoea, flatulence, cough, cold, asthma and throat irritations (Kapoor, 1990; Bakhru, 1997).Apart from this, turmeric also contains proteins, carbohydrates, fats, minerals, fibres and vitamins (Bakhru, 1997). A study show that the established use of *C. longa* in cardiovascular disorders such as hypertension [Godfraind et al., 1986; Triggle, 1992].

2.1.5 Literature Review of *Coriandrum sativum*

General Information

C. sativum is an annual herb in the family Apiaceae. All parts of the plants are edible and widely used in cooking and herbal medicine. The dried seed used as spice. It appears to have originated from Iran [Batmanglij & Najmieh , 2018] medicine, Unani [Chattopadhyay I, et al. 2004]. The essential oil from coriander leaves and seeds contains mixed polyphenols and terpenes, including linalool ,as the major constituent accounting for the aroma and flavor of coriander [Zheljazkov, *et al.*, 2014].

2.1.4.2 Taxonomy of *C. sativum*

Kingdom: Plantae

Subkingdom: Plantae

Infrakingdom: Viridiplantae

Super Division: Embryophyta

Division: Tracheophyta

Subdivision: Spermatophytina

Class: Magnoliopsida

Superorder: Asteranae

Order: Apiales

Family: Apiaceae

Genus: Coriandrum L.

Species: Coriandrum sativum L

2.1.5.2 Common Name of *C. sativum*

Latin name: *Coriandrum sativum*

Bengali name: Dhoniya

English name: Corinder

Hindi name: Dhaniya

Tamil name: Kottamalli

2.1.5.3 Geographical Distribution of *C. sativum*:

Coriander has its origin in the Iran. Coriander is native to regions spanning from southern Europe and northern Africa to southwestern Asia. It is a soft plant growing to 50 cm (20 in)

tall. The leaves are variable in shape, broadly lobed at the base of the plant, and slender and feathery higher on the flowering stems. It cultivate all over Bangladesh. Whole plant is edible but fresh leaves and dried seeds are usually used in cooking and the stem has little or no use [Wangensteen *et al.*, 2004].

2.1.5.4 Description of the Plant:

All parts of the plant are edible, but the fresh leaves and the dried seeds are the parts most traditionally used in cooking, Coriander is used in cuisines throughout the world [Samuelsson & Marcus 2003]. It grows 25–60 cm (9–24 inches) in height, and has thin, spindle-shaped roots, an erect stalk, alternate leaves, and small, pinkish-white flowers. The plant flowers from June to July, and yields round fruits consisting of two pericarps. The plant is cultivated for its aromatic leaves and seeds [Maryam Eidi & Akram Eidi, 2011]. The seeds are globular, beaked, finely ribbed, yellow-brown, 5 mm in diameter, with five longitudinal ridges. They mature 3 months after planting. The seeds contain about 1% volatile oil.

Figure2.5: *C. sativum* (dhoniya) leaves, flower, seeds.

2.1.5.5 Medicinal uses:

The stem, leaves and fruits all have a pleasant aromatic odour. The entire plant when young is used in preparing chutneys and sauces, and the leaves are used for flavouring continental curries and soups. The fruits are extensively employed as a condiment in the preparations of curry powder, pickling spices, sausages and seasonings. They are also used for flavouring pastry, biscuits, buns and cakes, and in flavouring liquors, particularly gin. Coriander seeds are also known for their medicinal properties and are considered carminative, diuretic tonic, stomachic antibilious, refrigerant and aphrodisiac.

2.1.5.6: Bioactive and pharmacological compounds:

Coriander also has flavonoid contents including apigenin, rhamnetin, keampferol, and quercetin. Moreover, coriander seed contains dynamic phenolic components, which are vital in prevention of free radicals produced in the cellular system. The study of antioxidants found in spices is gaining worth and momentum because these antioxidants are easily absorbed in human system [Rajeshwari and Andallu, 2011]. Coriander has important place among savory plants and a potential source of phenolic compounds with pivotal biological actions [Rajeshwari and Andallu, 2011]. Due to the biological activities some phytochemicals are known to be nutraceuticals. The antioxidant activity of the extracts of coriander has also been reported by [Wangensteen *et al.* [2004]. The extracted essential oil from coriander not only used as a flavoring agent in the liquor, cocoa, and chocolate industries but it is also used in medicines for indigestion, rheumatism, joint pains [Wangensteen et al., 2004].

Many researchers have reported that the antidiabetic (Gallagher et al., 2003), antibacterial (Kubo et al., 2004; Matasyoh et al., 2009), anticancerus and antimutagenic (Chithra and Leelamma, 2000), antiaging, antiulcer, antitumor (Rajeshwari and Andallu, 2011) characters of its essential oil.

Chapter Three

Chapter 3

3.0 Materials and Methods

3.1 Plant materials

3.1.1 Collection of the sample

For present research work rhizomes of *Z. officinale, A. sativum, C. verum, C.longa, C.sativum* were collected from, borobajar Dakbangla, Khulna, Bangladesh on 12th July , 2018. These plant samples were identified by Biotechnology and Genetic Engineering, Discipline, Khulna University, Khulna, Bangladesh.

3.1.2 Preparation of Extract

Drying and Grinding

The rhizomes was carefully cleaned and separated from undesired materials. Rhizomes of the plants were separated and cut into small pieces. Sliced materials were dried under sunshade and air for several weeks. After completion of drying the plants materials were ground into powder form with a grinder. The powder of plants were weighted by an electric balance and stored in airtight plastic bags separately in a dark cool and dry place for further use.

Extraction with solvent

The powder of plant parts were taken into different clean, flat-bottomed glass jars and soaked into ethanol and water. They were then sealed and kept for a period of 7 days in a dark room. The mixtures were filtered coarsely using filter paper.

Evaporation and storage

Filtrates obtained from filtration were evaporated in rotary evaporator and then kept at room temperature under an electric fan until they were completely dried. The crude extracts were weighed and kept in refrigerator for further experiments.

3.2Anticoagulant Activity Test

3.2.1 Study population

Blood samples obtained from 10-15 students of Biotechnology and Genetic Engineering Discipline, Khulna University, Khulna, Bangladesh, were used to evaluate the anticoagulant effects of selected spices plant. Participants were 20-25 years old. They had been chosen for the study according to the following criteria: having normal prothrombin time, not suffering from any cardiovascular disease (hypertension, congestive heart failure, coagulation disorders such as,

Hemophilia A or B) or diabetes, not recently using nonsteroidal anti-inflammatory drugs, not obese or smokers and free from dyslipidemic disorders.

3.2. 2 Collection of blood samples

The blood samples were obtained from normal volunteers who were previously consented by using sterile syringes, withdrawn from vein of right arm of each individual and placed separately in EDTA tubes to prevent the scavenging process. Centrifugation (15 minutes at rate 3000 rpm) was carried out to separate the blood cells from plasma in order to obtain Pure Platelet Plasma (PPP) for prothrombin time test. The obtained plasma sample of each individual were pooled together in a plane container using automatic pipette and stored at 4°C until the use.

3.2.3 Preparation of stock solutions

30% DMSO solution preparation: To prepare 30% DMSO solution, 30ml of DMSO was taken to a beaker and added 70 ml of distilled water to it.

Sample preparation: To prepare the stock solutions of different ethanolic extracts, weighed 2 mg extracts and then dissolved in 2 ml of 30% DMSO to prepare 1000 µg/ml stock solution. And then serially diluted to adjust the concentrations of 500 µg/ml and 250 µg/ml respectively.

500 ml 25 mM CaCl2 solution: To prepare 500 ml 25 mM CaCl2 solution, 1.84 gm CaCl2 dissolved in 500 ml distilled water.

Standard solution: To prepare standard solution, commercial warfarin sodium (warin, 5mg) tablets of Incepta Pharmaceuticals Limited were dissolved in 1 ml of 30% (v/v) DMSO.

3.2.4 Determination of the anticoagulant activity

Prothrombin time (PT) refers to the time that requires for the liquid portion (plasma) of blood to

clot formation. PT assay is a reliable assay to predict the anticoagulant activity of samples. In this study, the action of the crude extracts in the extrinsic and common blood coagulation pathway is evaluated by the Prothrombin time (PT) assay. The technic is easily manageable and requires small amount of test materials. The principle of the test was based on re-enabling the blood to clot by adding calcium chloride. In the PT assay, 0.2 ml of the blood plasma was taken to a clean fussion test tube and then 0.1 ml of plant extract of different concentrations (1000, 500, 250 µg/ml) were added respectively. The tube was shaken briefly to mix them. After shaking, 0.3 ml of 25 mM CaCl2 solution was added to the test tube and vortexed the tube to prepare a homogenized reaction mixture. A second clean fussion test tube was taken and 0.2 ml plasma, O.1 ml of 30% DMSO solution instead of samples and 0.3 ml CaCl2 (25mM) was added which considered as negative control. Then 0.1 ml warfarin instead of samples, 0.2 ml plasma and 0.3 ml CaCl2 (25mM) was added to the third test tube which considered as positive control. The reaction tubes were then incubated at 37 °C in water bath. The tubes were shaken gently and tilted back and forth in every five seconds to allow the mixing of the reaction and the time taken to coagulate was recorded in seconds using a stopwatch. As soon as the clot formation begins the stopwatch is stopped. The assay was carried out in triplicates for each sample and the average values were noted.

1. The plant materials were collected and extracted with the solvent ethanol and water.
2. The blood samples were collected from normal 10-15 volunteers who were previously consented and placed separately in EDTA tubes.
3. The blood samples were centrifuged at rate of 3000 rpm for 15 minutes to get pure platelet plasma (PPP) and discarded the pellets.
4. The supernatants were collected and pooled them together using automatic pipette and stored at 4 °C until the experiment was conducted.
5. A clean fussion test tube was taken and 0.2 ml of plasma was added to the test tube.

6. Different concentrations (1000, 500, 250 µg/ml) of samples were prepared in 30% DMSO and 0.1 ml of plant extract of different concentrations (1000, 500, 250 µg/ml) were added respectively and shaken briefly to mix them properly.
7. After shaking, 0.3 ml of 25mM CaCl2 was added to the reaction tube and vortex to prepare homogenized reaction mixture.
8. A second clean fussion test tube was taken and 0.2 ml plasma, O.1 ml of 30% DMSO solution instead of samples and 0.3 ml CaCl2 (25mM) was added which considered as negative control. Then 0.1 ml warfarin instead of samples, 0.2 ml plasma and 0.3 ml CaCl2 (25mM) was added to the third test tube which considered as positive control.
9. The reaction tubes were then incubated at 37 °C in water bath. The tubes were shaken gently and tilted back and forth in every five seconds to allow the mixing of the reaction and the time taken to coagulate was recorded in seconds using a stopwatch.
10. The assay was carried out in triplicates for each sample and the average values were noted.

3.3 Serine Protease Inhibition Assay

Proteolytic enzymes play a crucial role in living systems. So far, polypeptides that have proteolytic activity reach about 140,000 chains [Rawlings *et al.*, 2008]. Many diseases such as infections, heart diseases, cystic fibrosis, cancers are related with protease or malfunction of protease. It is reported that there are 80 genetic diseases in human caused by mutation of protease genes, metastasis of tumor has also resulted from proteinase malfunction [Sabeh *et al.*, 2009]. The proteolytic enzymes are also actively participating in the process of inflammation in human beings. The high reaction of inflammation may cause tissues damage, necrosis [Armstrong, 2006, Segel *et al.*, 2010, Sharony *et al.*, 2010].

Protein degradation ability of protease is magnificently high, thus nature adapts many level of regulations: gene regulation, secretion, activation, or inhibition. Plants possess efficient mechanisms against pathogen and pathogenic proteases enzyme system. These mechanisms have included the secondary metabolism with polyphenol compounds and their derivatives.

Polyphenol is well-known as the antioxidant agent, and research has shown that polyphenol derivatives protect protein and genetic materials against free radical [Gharass, 2009]. Polyphenol, specifically the flavonoid may interact to protein/enzyme in different ways which reduce cell damage, tumor formation and metastasis [Ren *et al.*, 2003].

The high molecular polyphenol so call tannin can be unspecifically attached to protease resulting in precipitation and denaturation of protein [Chung *et al.*, 1998, Segel *et al.*, 2010]. Other compounds can mimic the substrate then act as a specific inhibitor, these inhibitors show high potential for pharmaceutical application with a lower side effects than the synthesis compounds.

3.3.1: The mechanism of serine proteases

The availability of relatively large amounts of serine proteases from a variety of sources has provided numerous research groups with a useful means to undertake detailed studies of their physiocochemical properties and catalytic specificity against a wide range of both endogenous protein/peptide and synthetic substrates. Many of the most valuable insights into structure, function and specificity have been gained through the use of site-directed mutagenesis studies (extensively reviewed in [20 Perona and Craik, 1995]).

Serine proteases are characterized chiefly by the presence of an active site serine residue [Ser 195 (chymotrypsin numbering)], the g hydroxyl group of which acts as a nucleophile during the hydrolytic process. Two other amino acid residues are directly involved in the catalytic mechanism, in addition to this serine residue. These are His 57, a general base/acid catalyst, and Asp 102, which is believed to orientate the catalytic histidine. In addition to this so-called catalytic triad, the enzyme possesses an oxyanion binding site. This is made from the backbone amide NH groups of Ser 195 and Gly 193. The mechanism is outlined in figure 1.

After noncovalent binding of the substrate to the enzyme active-site region, the OH group of Ser 195 attacks the carbonyl carbon of the scissile bond via general base catalysis by His 57. This action leads to formation of the tetrahedral intermediate. The structure of the tetrahedral intermediate is stabilized through the newly formed oxyanion component by forming hydrogen bonds to the backbone NH groups of Ser 195 and Gly 193 residues.

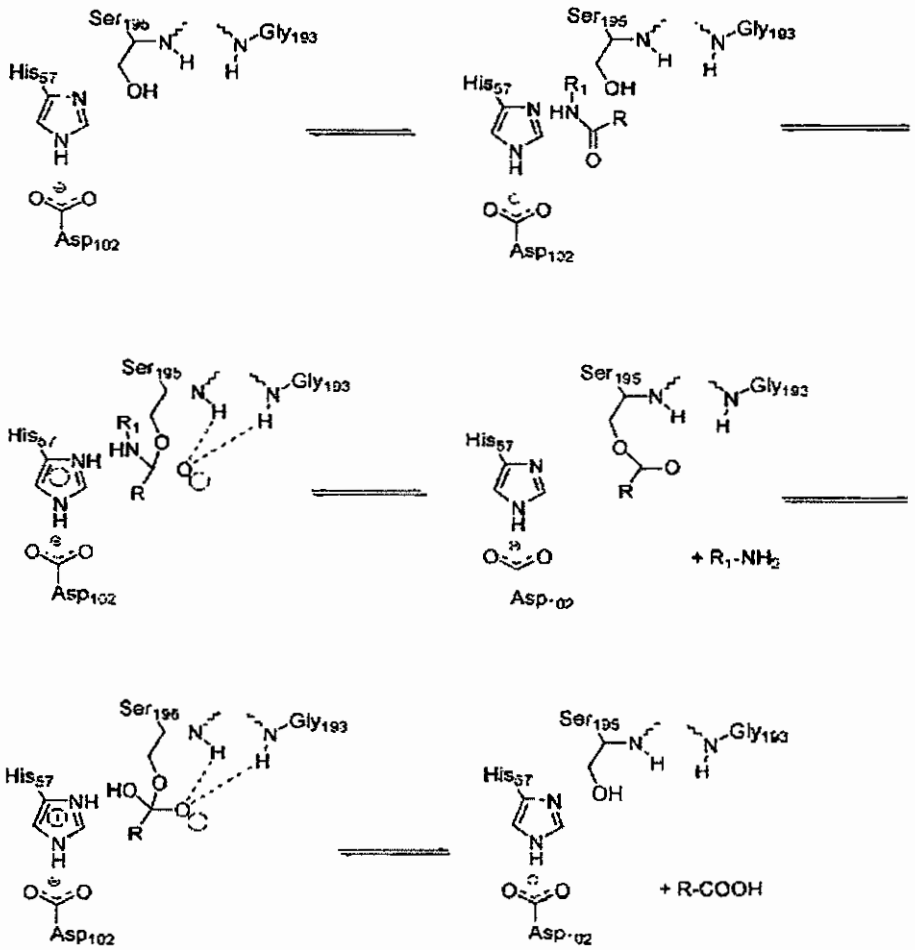


Figure 3.1. The mechanism of action of the serine proteases.

His 57, acting now as a general acid, transfers a proton to the amine of the tetrahedral intermediate; this subsequently breaks down, a product amine leaves and the covalent acyl enzyme intermediate is formed.

The acyl enzyme complex is attacked by a water molecule to generate a new tetrahedral intermediate. This intermediate collapses, assisted by general acid catalysis from His 57, resulting in the formation of product acid and the regenerated Ser 195 OH leaving group.

Whilst Barrett and Rawlings have revolutionized the nomenclature for the classification of all hydrolytic enzymes, describing species in so-called families and clans [Barrett and Rawlings, 1995]. For the purposes of this review we shall simply distinguish serine proteases on their differences in substrate specificity. The topology of the enzyme active site discriminates between peptides with defined amino acids sequences, particularly at the P1 specificity sub site [Schechter and Berger, 1968]. Serine proteases are generally classified according to their broad substrate specificity, and while there are notable exceptions (such as granzyme B,

which cleaves after Asp residues), there are three main categories: trypsin-like (cleaving after basic amino acids), chymotrypsin-like (cleaving after bulky hydrophobic residues) and elastaselike (cleaving after small, aliphatic residues). Almost all synthetic inhibitor development has been targeted against serine proteases with these broad substrate characteristics.

Protease inhibitor assay was performed against trypsin following the Kunitz method with some modifications [Kunitz, 1947].

Chemicals requirement

- 0.1M phosphate buffer pH 7.6
- 0.0025M HCl
- Trypsin (0.5 mg/mL prepared in 0.1 M phosphate buffer pH 7.6)
- 1% casein (prepared in 0.0025M HCl)
- 5 % trichloracetic acid

Methodology for Serine Protease Inhibition Assay

1. Standard (Quercetine) and Sample was dissolved in ethanol to make 400µg/ml concentration. Then each of them was serially diluted in 5 concentrations (400µg/ml, 200µg/ml, 100 µg/ml, 50 µg/ml, 25 µg/ml). From then 1 ml of Standard (Quercetine) or Sample was taken in 14 ml tube.

2. Then 1ml of Trypsin (previously heated at 37°c for 15 minutes before used) was added to the tube.

3. Then, 2 mL of 1% casein was added and the obtained mixture was allowed to stand for 30 min at 37°C.

4. Finally, 3 mL of 5% trichloroacetic acid (TCA) solution was added to stop the reaction.

5. After centrifugation of the reaction mixture (12,000 rpm, 15 min), the absorbance was measured at 280 nm.

6. % Inhibition was calculated by using following formula

$$\% \text{ Inhibition} = \frac{Absorbance\ of\ sample - Absorbance\ of\ blank}{Absorbance\ of\ blank} \times 100$$

3.4 Evaluation of Antioxidant Activity

Anti-oxidant

Oxidation is a type of chemical reaction that transfers electrons or hydrogen from a substance to an oxidizing agent. Oxidation reactions can produce free radicals that are highly active to react with other molecules. These free radicals can start chain reactions [Sies & Helmut, 1997]. These radicals are important part of life, which are produced during cellular metabolism and functional activities and these radicals have important roles in cell signaling, apoptosis, gene expression and ion transportation. Although oxidation reactions are crucial for life, they can also be damaging [Boonchoong *et al.*, 2004]. Excessive free radicals attack bases in nucleic acids, amino acid side chains in proteins and double bonds in unsaturated fatty acids, cause oxidative stress, which can damage DNA, RNA, proteins and lipids resulting in an increased risk for cardiovascular disease, cancer, autism and other diseases. Antioxidant is a molecule that inhibits the oxidation of other molecules. Cells are normally able to defend themselves against free radicals damage through the use of intracellular enzymes to keep the homeostasis of free radicals at a low level (Figure 3.1). However, during times of environmental stress and cell dysfunction, free radicals levels can increase dramatically, causes significant cellular damage in the body. In order to prevent or reduce the free radicals-induced oxidative damage, the human body and other organisms have developed an antioxidant defense system that includes enzymatic, metal-chelating and free radical-scavenging activities to neutralize these radicals after they have formed. In addition, intake of dietary antioxidants may help to maintain an adequate antioxidant status in the body [Giles *et al.*, 2002].

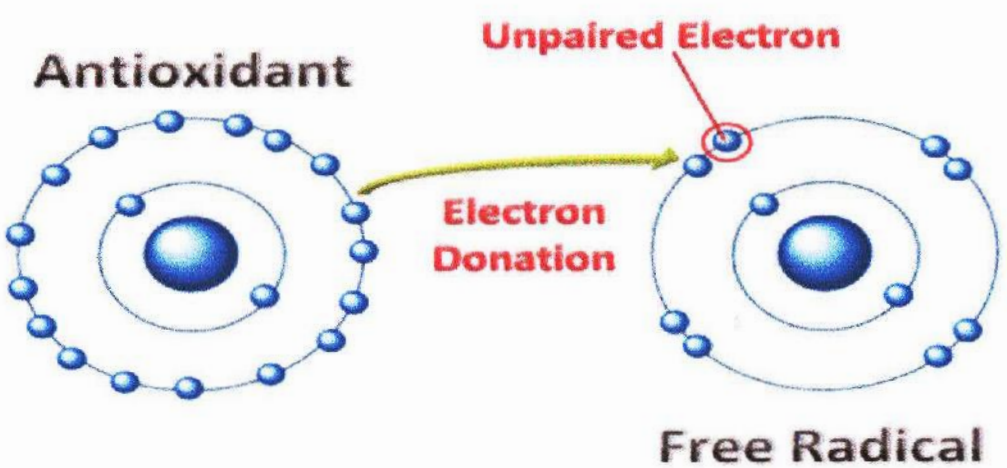
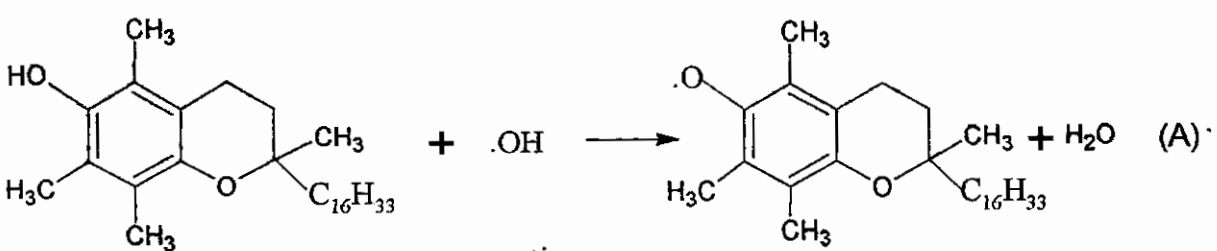


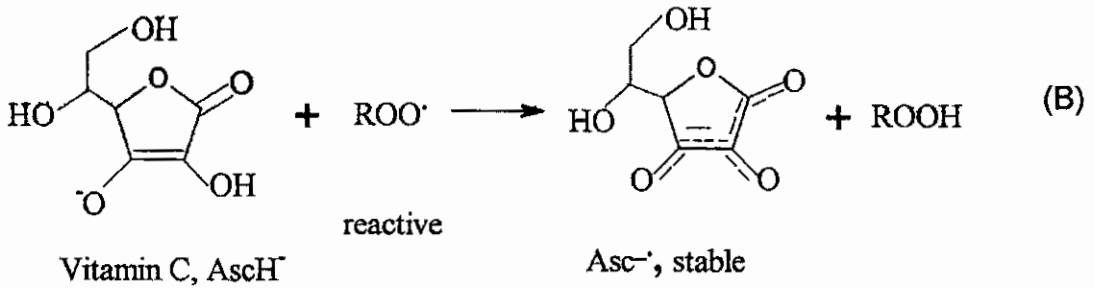
Figure 3.2: Mechanism and action of antioxidants.

Antioxidants can neutralize free radicals by accepting or donating electrons to eliminate the unpaired condition of the radical. The antioxidant molecules may directly react with the reactive radicals and destroy them, while they may become new free radicals which are less active, longer-lived and less dangerous than those radicals they have neutralized. They may be neutralized by other antioxidants or other mechanisms to terminate their radical status. Antioxidants are classified into two broad divisions, depending on whether they are soluble in water (hydrophilic) or in lipids (hydrophobic). In general, water-soluble antioxidants react with oxidants in the cell cytosol and the blood plasma, while lipid-soluble antioxidants protect cell membranes from lipid peroxidation. Many antioxidants have aromatic ring structures and are able to delocalize the unpaired electron. For example, Vitamin C ($AscH^-$) in the aqueous phase and vitamin E (TOH) in the lipid phase will directly react with or neutralize hydroxyl, alkoxyl ($LO\cdot$) and lipid peroxyl ($ROO\cdot$) radicals and form H_2O, alcohol and lipid hydroperoxides, (Figure 3.2) respectively. Vitamin E itself becomes a phenyl radical ($TO\cdot$) and vitamin C turns to a very stable radical ($Asc^{-\bullet}$), due to its delocalized structure. Furthermore, vitamin C can also neutralize the radical form of other antioxidants such as glutathione radical and vitamin E radical and regenerate these antioxidants. Vitamin C itself is readily regenerated from $Asc^{-\bullet}$ with NADH or NADPH-dependent reeducates [Hossain *et al.*, 1985]. Many antioxidants may directly react with free radical intermediates induced by reactive oxygen species and terminate the chain reaction, thereby stopping the free radical-induced damage [DeFeudis *et al.*, 2003].



Vitamin E, TOH

TO., less reactive



$$AscH^{-} + TO^{\cdot} \longrightarrow TOH + Asc^{-\cdot} \quad (C)$$

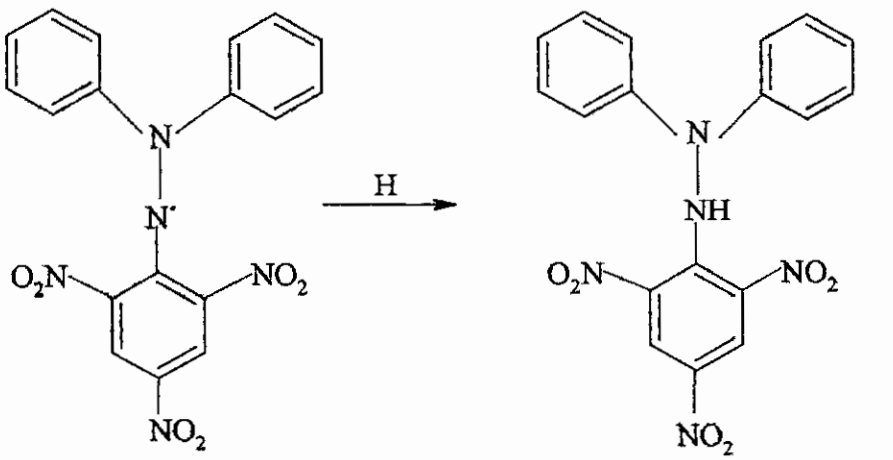
Figure 3.4: Direct reactions of vitamin E (TOH) with hydroxyl radical (A) and vitamin C ($AscH^-$) with lipid peroxyl radical (ROO·) (B), and regeneration of vitamin E from vitamin C (C).

Small molecules such as vitamin C, vitamin E, uric acid and glutathione play important roles as cellular antioxidants. Synthetic antioxidants such as tert-butylhydroxyl-toluene, tert-butylhydroxyanisole and tert-butylhydroquinone have been widely used in the food industry to retard lipid oxidation. However, such synthetic antioxidants are not preferred for pharmacologic use due to toxicological concerns. Thus, more and more interests have focused on identifying plant extracts to use as dietary antioxidant supplements [Rababah *et al.*, 2004]. Many research models have been established in chemical or biological systems for the studies of antioxidant activity as well as identification of new antioxidants especially from natural substances. Generally, the antioxidant ability was measured and presented as 1,1-diphenyl-2-picryl-hydrazyl free radical (DPPH) scavenging activity, total antioxidant activity, total antioxidant potentials, ferric chloride reducing power, trolox equivalent antioxidant capacity, total radical absorption potentials and oxygen radical absorption capacity. These methods are based on either a single-electron transfer reaction or a hydrogen atom transfer reaction from an antioxidant or oxidant to a free radical. The change of optical absorbance of either antioxidant or oxidant is measured for the antioxidant capability.

3.4.1 DPPH Free Radical Scavenging Assay

3.4.1.1 Principle

A simple, rapid and inexpensive method to measure antioxidant capacity of sample involves the use of free radical, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) [Sharma & Bhat, 2009]. It is a dark-colored crystalline powder composed of stable free-radical molecules and a trap ("scavenger") for other radicals. The DPPH method can be used for solid or liquid samples and is not specific to any particular antioxidant component, but applies to the overall antioxidant capacity of the sample. Antioxidants reduce 2, 2-diphenyl-1-picrylhydrazyl (purple color) to 2, 2-diphenyl-1-picrylhydrazine (colorless compound). The odd electron in the DPPH free radical gives a strong absorption maximum at 517 nm reduces from 9660 to 1640 when the odd electron of DPPH radical becomes paired with hydrogen from free radical scavenging antioxidant to form the reduced DPPH-H. The resulting decolorization is stoichiometric with respect to number of electrons captured (Figure 3.3) [Cuvelier *et al.*, 1992].



2,2-diphenyl-1-picrylhydrazyl 2,2 diphenyl-1-picrylhydrazine

Figure 3.4: Reaction between DPPH and antioxidant.

3.4.1.2 Chemical Requirements

The chemicals were ethanol, distilled water, 0.004% DPPH ethanolic solution, Quercetin (as positive control).

3.4.1.3 Equipments

The equipments used for this experiment were test tubes, beakers, digital balance, vortex machine, refrigerator, marker, Micropipette (20µl, 200µl and 1000µl), pipettes (5ml, 10ml), spoon, test tube stand, aluminum foil and UV spectrophotometer.

3.4.1.4 Methodology of DPPH free radical scavenging assay:

1. At first 9 test tubes were taken to make aliquots of 9 concentration (1.57, 3.13, 6.25, 12.50, 25, 50, 100, 200 and 400 µg/ml) by serial dilution technique for plant extract and 9 test tubes were taken to make aliquots of 9 concentration (1.57, 3.13, 6.25, 12.50, 25, 50, 100,200 and 400 µg/ml) by serial dilution technique for Quercetin which was taken as positive control.
2. Sample extract and quercetin were weighed and dissolved in ethanol to make homogeneously stock solution with the highest concentration i.e. 400 µg/ml, with the help of vortex and sonicator.
3. DPPH was also weighed and dissolved in ethanol to make 0.004% (w/v) solution.
4. After making the desired concentrations, 2ml of 0.004% DPPH solution was applied on each test tube containing 1ml of solution by pipette. The final volume of the solution was 3ml. This was done for both the quercetin and sample extract.
5. Then the test tubes were kept for 30 minutes in dark to complete the reaction.
6. DPPH was also applied on the blank test tubes at the same time where only ethanol was taken as blank.
7. After 30 minutes, absorbance of each test tube was determined by UV sepectrophotometer at 517 nm.
8. Percentage of inhibition was calculated as-

$$\% \text{ Inhibition} = \frac{Absorbance\ of\ blank - Absorbance\ of\ samle}{Absorbance\ of\ sample} \times 100$$

9. IC_{50} value is the concentration of sample required to scavenge 50% of DPPH free radical and was calculated from the plot of % inhibition against the log concentration of sample extract.

3.4.2 FRAP (Ferric ion Reducing Antioxidant Power) Assay

Ferric ion reducing antioxidant power (FRAP, also Ferric reducing ability of plasma) is a simple test to determine the total antioxidant power of sample. The FRAP assay was first performed by Iris Benzie and J. J. Strain of the Human Nutrition Research Group at the University of Ulster, Coleraine. It is often used to measure the antioxidant capacity of plant extract, foods, beverages and nutritional supplements containing polyphenols [Benzie et *al.*,1996].

3.4.2.1 Principle

The FRAP assay (ferric ion reducing antioxidant power) evaluates total antioxidant power and is chosen to assess the presumable effects of medicinal plants [Szollosi *et al.*, 2002]. FRAP assay depends upon the ferric tripyridyltriazine (Fe(III)-TPTZ) complex to the ferrous tripyridyltriazine (Fe(II)-TPTZ) by the action of electron donating antioxidants at low pH. Fe(II)-TPTZ has an intensive blue color and can be monitored at 593 nm. The FRAP assay was determined according to the method of Benzie and Strain (1996) [Benzie *et al.*, 1996].

3.4.2.2 Chemicals required

The chemicals were acetate buffer (300 mM; pH 3.6), TPTZ (2, 4, 6-tripyridyl-s-triazine), ferric chloride ($FeCl_3.\ 6H_2O$).

3.4.2.3 Equipments

Test tubes, beakers, digital balance, vortex machine, water bath, micropipette (20µl, 200µl and 1000µl), pipettes (5ml), UV spectrophotometer, marker and aluminum foil.

3.4.2.4 Acetate buffer (300 mM; pH 3.6) preparation

3.1gm sodium acetate trihydrate was weighed and added into 16 ml of glacial acetic acid and make the volume to 1L with distilled water.

3.4.2.5 TPTZ (2, 4, 6-tripyridyl-s- triazine) preparation

0.031gm TPTZ dissolve in 10ml of 40mM HCl and kept at 50°C in water bath for 20minutes.

3.4.2.6 Ferric Chloride $FeCl_3.6H_2O$ (20 mM) preparation

0.054gm $FeCl_3.6H_2O$ dissolves in 10ml distilled water.

3.2.2.7 FRAP reagent preparation

The working FRAP reagent was prepared by mixing 25ml acetate buffer, 2.5ml TPTZ solution and 2.5ml $FeCl_3.6H_2O$ solution, then warmed at 37°C before using.

3.4.2.8 Protocol for FRAP assay

1. Sample extract and Quercetin were weighed and dissolved in distilled water to make homogeneously stock solution with the highest concentration i.e. 400 µg/ml, with the help of vortex and sonicator.
2. Five test tubes were taken to make aliquots of 5 concentrations (25, 50, 100,200 and 400 µg/ml) by serial dilution technique for plant extract and 5 test tubes were taken to make

aliquots of 5 concentrations (25, 50, 100,200 and 400 µg/ml) by serial dilution technique for Quercetin which was taken as positive control.

3. Another five test tubes were taken with 0.2ml extract and quercetin solution.
4. Then 3ml FRAP reagent was applied on each test tube.
5. The mixture was kept at 37°C in water bath for 30minutes.
6. After cooling, the absorbance was measured at 593nm.

3.4.3 Reducing Power Assay

3.4.3.1 Principle

Reducing power assay measures the electron-donating capacity of an antioxidant. In this assay, the yellow color of the test solution changes to various shades of green color depending on the reducing power of each compound. Reducing power assay method is based on the principle that substances, which have reduction potential, react with potassium ferricyanide (Fe3+) to form potassium ferrocyanide (Fe2+), which then react with ferric chloride to form ferric ferrous complex. Therefore, measuring the formation of Perl's Prussian blue at 700 nm can monitor the Fe2+ concentration. Potassium ferricyanide + Ferric chloride Antioxidant Potassium ferrocyanide + Ferrous chloride.

The reducing power was determined according to the method of Oyaizu (1986) with some modification [Oyaizu 1986].

3.4.3.2Chemicals required

The chemicals for the experiment were phosphate buffer (0.2M, pH 6.6), potassium ferricyanide (1% w/v), trichloro acetic acid (10%), ferric chloride (0.1%) and ascorbic acid (1%) as positive control.

3.4.3.3 Equipments

Test tubes, beakers, digital balance, vortex machine, centrifuge machine, water bath, measuring cylinder, micropipette (20µl, 200µl and 1000µl), pipettes (5ml, 10ml), UV spectrophotometer, marker and aluminum foil.

3.4.3.4 Phosphate Buffer preparation

Dibasic sodium phosphate (37.50ml of 0.2M) is mixed with monobasic sodium phosphate (62.5ml of 0.2M) and diluted to 100ml of distilled water.

3.4.3.5 Protocol for Reducing Power

1. Sample extract and ascorbic acid were weighed and dissolved in distilled water to make homogeneously stock solution with the highest concentration i.e. 400 µg/ml, with the help of vortex.

2. Four test tubes were taken to make aliquots of 4 concentrations (50, 100,200 and 400 µg/ml) by serial dilution technique for plant extract and 4 test tubes were taken to make aliquots of 4 concentrations (50, 100,200 and 400 µg/ml) by serial dilution technique for ascorbic acid which was taken as positive control.

3. After making the desired concentrations, 2.5ml of phosphate buffer and 2.5ml of potassium ferricyanide was applied on each test tube. The solution was mixed by vortex.

4. The mixture was kept at 50°C in water bath for 20minutes.

5. After cooling, 2.5ml of 10% trichloro-acetic acid was added and centrifuged at 1400rpm for 10minutes.

6. Then 2.5ml of the upper layer of solution was take in each test tube and mixed 2.5ml of distilled water.

7. Finally, freshly prepared 0.1% ferric chloride solution was added and mix by vortex.

8. The absorbance was measured at 700nm.

A blank tube was prepared in similar manner excluding test sample or standard. Increased absorbance of the reaction mixture indicates increase in reducing power.

The percent increase in reducing power was calculated using the following equation: Increase in reducing power (%) = $A\ test - A\ blank A\ blank \times 100$ [Where A test is absorbance of test solution; A blank is absorbance of blank].

3.4.4 Total Phenolic Content Assay

Phenolic compounds are among the most widely occurring secondary metabolites in the plant kingdom, acting mainly as phytoalexins, attractants for pollinators and plant pigmentation, antioxidants and protective agents against UV light [Gottlieb *et al.*, 2000]. Studies have indicated that the phenolic compounds are a major source of natural antioxidants because their structure allows them to donate hydrogen atoms or electrons for free radical scavenging.

Some of the commonly used methods to determine the content of phenolics are approximately 100 years old, initially were developed by Folin and Colleagues at Harvard Medicinal School to study the metabolism of proteins in humans. Then Folin and Denis (1915) reported a method which applied to the determination of phenolics in sample. Due to the limitation of this method Folin and Ciocalteu (1927) was reported a modified method, mainly the modification was occurred on reagent, which referred to as the Folin-Ciocalteu reagent, was used to determine the content of phenolics from a wide range of sources. The Folin–Ciocalteu reagent (FCR) is a mixture of phosphomolybdate and phosphotungstate, clear bright yellow solution. Total phenolic content was estimated by the Folin-Ciocalteu method described by Singleton and Rossi [Singleton & Rossi, 1965].

3.4.4.1 Principle

Polyphenols in plant extracts react with redox reagents (Folin-Ciocalteu reagent) to form blue complex chromogens that can be detected by visible-light spectrophotometry. The color development is due to the transfer of electrons at basic pH to reduce the phosphomolybdic/phosphotungstic acid complexes to form chromogens [Katzung *et al.*, 2009].

3.4.4.2 Chemicals requirement

Sodium Carbonate (7% w/v), Folin-Ciocalteu (FC) reagent (diluted 10-fold with distilled water), Gallic acid (standard).

3.4.4.3 Equipments

Test tubes, beakers, digital balance, vortex machine, micropipette (20µl, 200µl and 1000µl), pipettes (5ml, 10ml), UV spectrophotometer, marker and aluminum foil.

3.4.4.4 Methodology for Total Phenolic Content

1. Gallic acid was used as standard. 250µg/ml stock standard solution of gallic acid was prepared by dissolving 0.0025 gm in 10 ml distilled water and then diluted to five concentration (50, 100, 150, 200 and 250 µg/ml).
2. The extract was prepared at concentration of 100µg/ml.
3. 9 ml distilled water and 1 ml FC reagent was added to each diluted gallic acid and extract solution.
4. The mixture was allowed to stand at room temperature for 5 minutes.
5. Then, 10 ml of 7% sodium carbonate was added to the mixture and mixed gently.
6. 25 ml adjust with distilled water.
7. After standing at room temperature for 60 minutes, the absorbance was measured at 750nm using spectrophotometer.

The standard calibration curve of gallic acid was plotted.

3.4.5 Total Flavonoid Content

Flavonoids are bioactive polyphenols which play a vital role in photosynthesising cells [Cushnie *et al.*, 2005]. They are secondary metabolites characterised by flavan nucleus and C6-C8-C6 carbon-skeleton (Figure 3.4) [Heim et al. 2002; Peterson et al. 1998; Tsuchiya et al. 2010]. The basic structural feature of flavonoid is 2-phenyl-benzo-γ-pyrane nucleus consisting of two benzene rings (A and B) linked through a heterocyclic pyran ring (C) as shown in following figure [Cushnie et al. 2005]

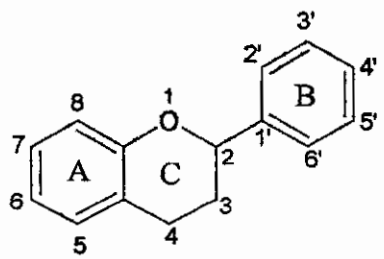


Figure 3.5: Basic structure of flavonoids.

Flavonoids differ in their arrangement of hydroxyl, methoxy and glycosidic side groups and in the conjunction between A and B rings [Heim *et al.*, 2002]. A variation in C ring provides division of subclasses. According to their molecular structure, they are divided into eight classes (Figure 3.5) [Tsuchiya *et al.*, 2010]

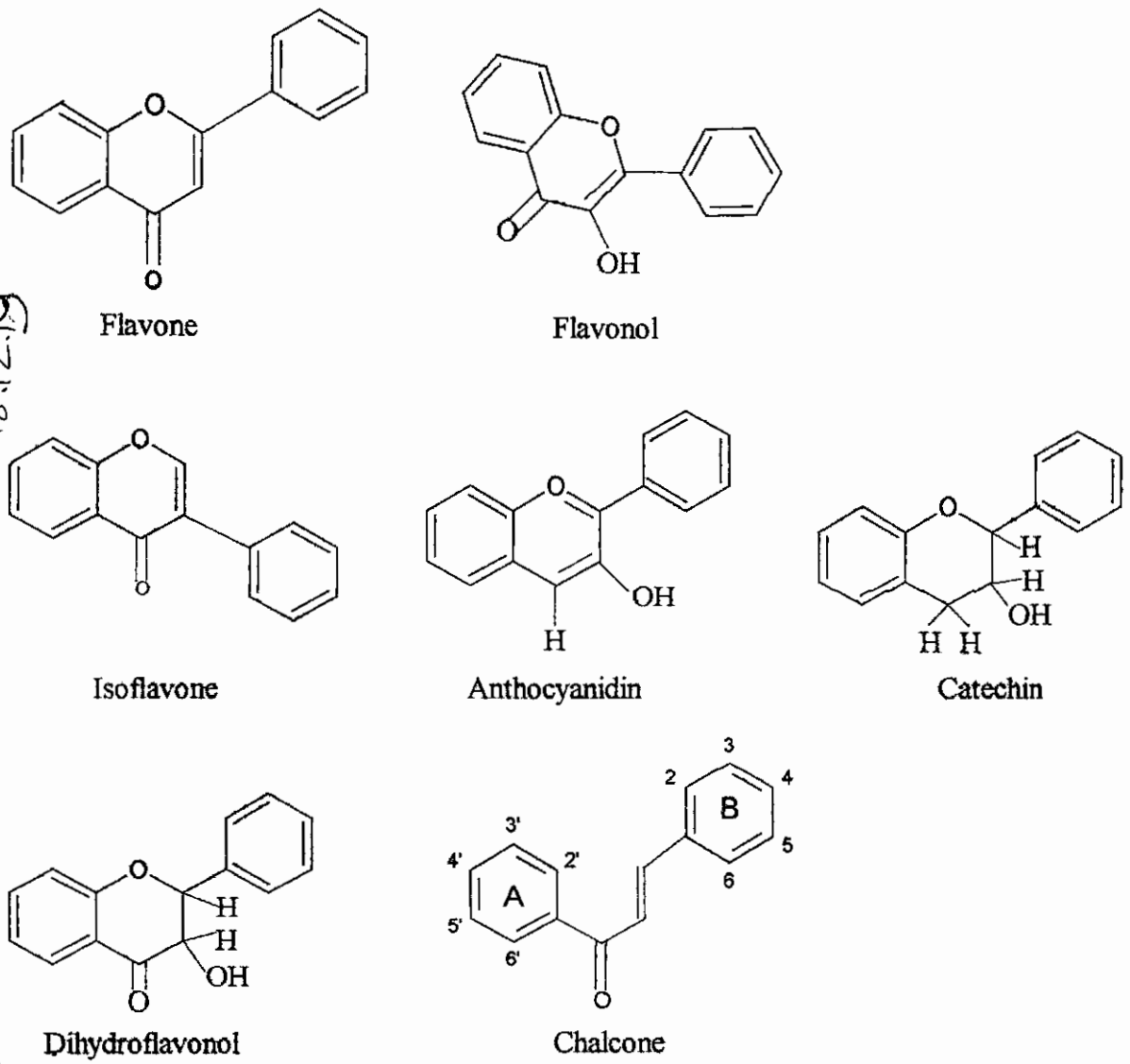


Figure 3.6: Chemical structure of different types of flavonoids [Cook *et al.*, 1996].

In plants, flavonoids are often present as O-glycosides or C-glycosides. The O-glycosides possess sugar substituent bound to –OH of aglycone, usually at position 3 or 7, whereas, C-glycosides possess sugar groups bound to Carbon of aglycone usually 6-C or 8-C [Buhler & Miranda, 2000].

Flavonoids are phenolic substances isolated from a wide range of plants, with over 8000 individual compounds known. They act in plants as photoreceptors, visual attractors, feeding repellants and for light screening [Pietta, 2000]. The flavonoids have aroused considerable interest recently because of their potential beneficial effects on human health. Many studies have suggested that flavonoids exhibit biological activities, including antibacterial, antiallergenic, antiviral, anti-inflammatory [Cushnie *et al.*, 2005, Cook *et al.*, 1996, Murray,

1998], cytotoxic, anti tumour, treatment of neurodegenerative diseases and vasodilating actions [Murray, 1998, Williams *et al.*, 2004, Tsuchiya *et al.*, 2010, Chebil *et al.*, 2006]. In addition flavonoids are known to inhibit lipid-peroxidation, platelet aggregation, capillary permeability and fragility, cyclo-oxygenase and lipoxygenase enzyme activities. They exert these effects as antioxidants, free radical scavengers, chelators of divalent cation [Cook *et al.*, 1996, Chebil *et al.*, 2006, Middleton *et al.*, 2000]. These are also reported to inhibit variety of enzymes like hydrolases, hyalouronidase, alkaline phosphatise, arylsulphatase, cAMP phosphodiesterase, lipase, α-glucosidase, kinase [Narayana *et al.*, 2001]. However, most interest has been devoted to the antioxidant activity of flavonoids, which is due to their ability to reduce free radical formation and to scavenge free radicals. The position of hydroxyl groups and other features in the chemical structure of flavonoids are important for their antioxidant and free radical scavenging activities. Flavonoids are powerful antioxidants against free radicals and are described as free-radical scavengers [Pal *et al.*, 2009]. This activity is attributed to their hydrogen-donating ability. Indeed, the phenolic groups of flavonoids serve as a source of a readily available "H" atoms such that the subsequent radicals produced can be delocalized over the flavonoid structure [Tripoli *et al.*, 2007]. Free radical scavenging capacity is primarily attributed to high reactivities of hydroxyl substituents that participate in the reaction [Heim *et al.*, 2002] as shown in fig. 3.6:

Figure 3.7: Scavenging capacity of free radical (R.)

Flavonoids inhibit lipid peroxidation in vitro at an early stage by acting as scavengers of superoxide anion and hydroxyl radicals. They terminate chain radical reaction by donating hydrogen atom to a peroxy radical as in fig (V), thus, forming flavonoids radical, which, further reacts with free radicals thus terminating propagating chain [Cook *et al.*, 1996, Ferreira *et al.*,2010].

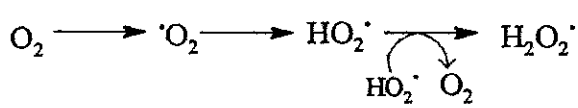


Figure 3.8: Formation of peroxy radical

$$\cdot O_2^- + \cdot O_2^- \xrightarrow[SOD]{2H+} H_2O_2 + O_2$$

Figure 3.9: Mechanism catalysed by SOD

According to kinetic studies of aroxyl radical formation and decomposition reactions, the antioxidant capacity of a flavonoid is linked to its three structural groups as shown in fig.

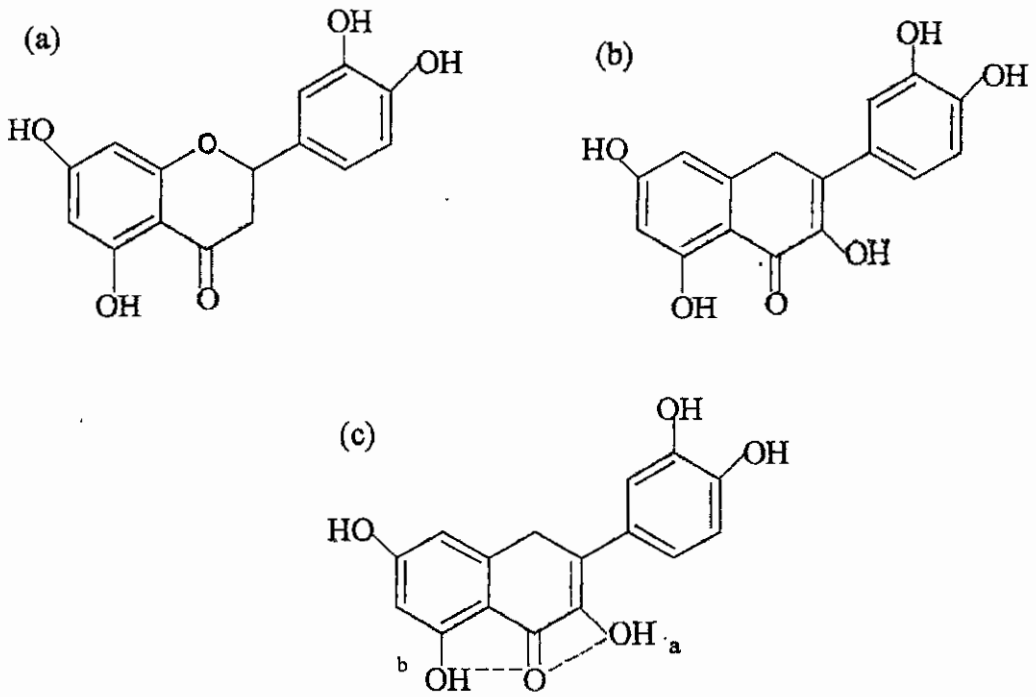


Figure 3.10: Structural groups responsible for antioxidant activity [Tripoli *et al.*, 2007].

1. The ortho-dihydroxy (catechol) structure in the B-ring, which confers greater stability to aroxyl radicals, possibly through hydrogen bonding, and which participates in electron dislocation.

2. The 2,3-double bond, in conjugation with a 4-oxo function, responsible for electron dislocation from the B-ring.

3. The presence of both 3-(a)-and 5-(b)-hydroxyl groups (Fig.VII(c)).

3',4'-catechol structure in B-ring strongly enhances lipid peroxide inhibition and this arrangement is an important characteristic of most potent scavengers of peroxyl, superoxide and peroxynitrite radicals [Heim *et al.*, 2002] and its absence decreases antioxidant activity. The absence of the hydroxyl group at position 3 in flavanones and flavones decreases their

antioxidant ability [Tripoli *et al.*, 2007]. The content of flavonoids in the examined plant extracts was determined using spectrophotometric method. Total flavonoids were determined following the procedure by Dewanto, Wu, Adom, and Liu, (2002) [Dewanto *et al.*, 2002].

3.4.5.1 Principle

Flavonoids in the sample extracts react with sodium nitrite, followed by the development of coloured flavonoid-aluminum complex formation using aluminum chloride in alkaline condition which can be monitored spectrophotometrically at maximum wavelength of 510 nm [Tsuchiya *et al.*, 2010].

3.4.5.2 Chemicals requirement

5% Sodium nitrite (5% NaNO2): 5gm in 100ml distilled water.

10% Aluminum chloride (10% AlCl3): 10gm in 100ml distilled water.

1 M Sodium hydroxide (1 M NaOH): 4gm NaOH in 100ml distilled water.

3.4.5.3 Equipments

Test tubes, beakers, volumetric flask, digital balance, vortex machine, micropipette (200µl and 1000µl), pipettes (5ml, 10ml), UV spectrophotometer, marker and aluminum foil.

3.4.5.4 Methodology for Total Flavonoid Content

1. Quercetin was used as standard and were weighed and dissolved in distilled water to make homogeneously stock solution with the highest concentration i.e. 400 µg/ml, with the help of vortex and sonicator.
2. Five test tubes were taken to make aliquots of 5 concentrations (25, 50, 100,200 and 400 µg/ml) by serial dilution technique for Quercetin.
3. The extract was prepared at concentration of 100µg/ml.
4. 5 ml distilled water and 0.3 ml of 5% $NaNO_2$ was added to each diluted Quercetin and 1ml of extract solution.
5. After 5 minutes 0.6 ml of 10% $AlCl_3$ was added.
6. After 5 minutes 2 ml of 1M NaOH.
7. Then the mixture was allowed to stand at room temperature for 5 minutes.
8. After standing at room temperature, the absorbance was measured at 510 nm using spectrophotometer.

The standard calibration curve of Quercetin was plotted.

Chapter Four

Chapter 4

Result and Discussion

4.0 Result

4.1 Determination of anticoagulant activity

Coagulation of blood is a complex procedure where numerous numbers of factors in the plasma and tissues are involved. The intrinsic and extrinsic pathways play a vital role in this coagulation process [Shanmugam M. & Mody K.H., 2000]. Anticoagulants, commonly referred to as blood thinners, are chemical substances that prevent or reduce coagulation of blood, prolonging the clotting time. Anticoagulants are closely related to antiplatelet drugs and thrombolytic drugs by manipulating the various pathways of blood coagulation. Common anticoagulants include warfarin and heparin [Ron Winslow & Avery Johnson, 2007]. Different studies shows that the effects of warfarin use in patients with end stage renal disease and atrial fibrillation, there was no increased risk of stroke incidence with warfarin use, but there was a significantly increased risk of all-cause bleeding, compared to alternate treatments (aspirin, dabigatran, rivaroxaban) or no warfarin use [Tan *et al.*, 2016]. For this reason, researchers have been trying to trace new anticoagulant from natural sources [Edemeka D. B. U. & Ogwu A.S, 2000; Khouya T. *et al.*, 2015]. There are several assays such as aPTT, PT, TT involving in the anticoagulation study using normal citrated plasma in an attempt to check the anticoagulant effect of different plants and spices. . A number of studies have been conducted byvarious researchers to find out the herbs and natural food sources and their supplements having antithrombotic(anticoagulant and antiplatelet) effect and there is evidence that consuming such food leads to prevention of coronary events and stroke [K.J. Joshipura *et al.*, 1999; S. Liu, 2000 & W.D. Ratnasooriya *et al.*,2008]. In this study, the extrinsic prothrombin time (PT) test was conducted in normal plasma to evaluate the anticoagulant effect of selected five spices, *Z. officinally*, *A. sativum*, *C. vurem*, *C. longa*, *C. sativum*. The test sample shows different results in prothrombin time at different concentrations. The prothrombin times were determined through plot of mean prothrombin time against the concentrations of extracts. Warfarin sodium considered as a standard in this study.

4.1.1 Warfarin sodium as standard

Warfarin sodium (tablets) and warfarin sodium (for injection), are the common anticoagulant that act by inhibiting vitamin K dependent coagulation factors. The chemical name of warfarin sodium is 3-(α-acetonylbenzyl)-4-hydroxycoumarin sodium salt, which is a racemic mixture of the R- and S-enantiomers. Warfarin, sold under the brand name Coumadin among others. Crystalline warfarin sodium is an isopropanol clathrate. Its empirical formula is $C_{19}H_{15}NaO_4$, and molecular weight is 330.315 g/mol. Its structural formula is represented by the following:

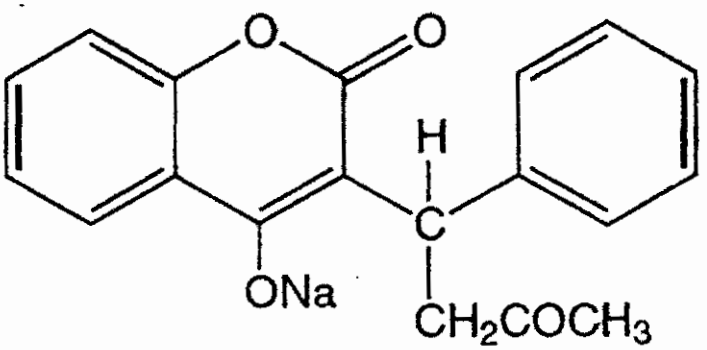


Figure 4.1.1: The structural formula of warfarin sodium.

Crystalline warfarin sodium occurs as a white, odorless, powder that is discolored by light. It is very soluble in water, freely soluble in alcohol, and very slightly soluble in chloroform and ether. The prothrombin time of standard (warfarin sodium) evaluated from prothrombin time (PT) assay. The concentrations (100, 500, 250,125, 62.5 µg/ml) exhibited different prothrombin time.

Table 4.1.1: The effect of standard (Warfarin) against different concentrations on prothombin time of plasma in a prothombin time test.

Concentration (µg/ml)	Mean prothrombin time (minutes)	Standard deviation
1000	47.45333	1.494468
500	42.02333	1.686308
250	36.61333	1.634513
125	21.51333	2.390112
62.5	14.62	1.393879
Negative Control	1.41	0.189

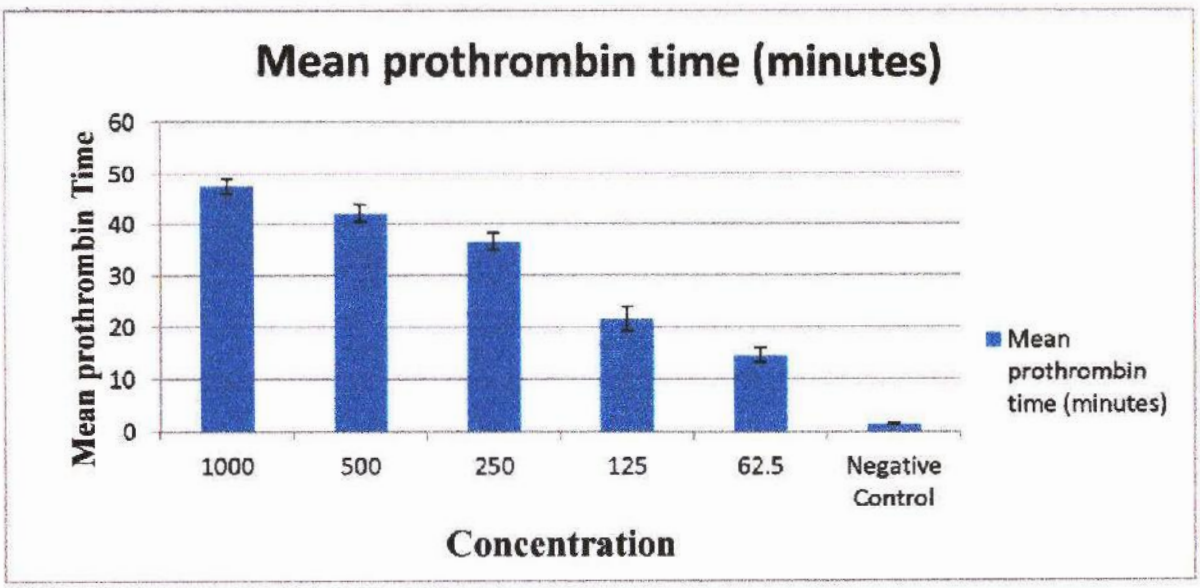


Figure 4.1.2: Plot of different concentrations of standard (Warfarin) versus mean prothrombin time; NC-Negative control.

4.1.2 *Z. officinale* ethanolic extract

The prothrombin time of ethanolic extract of Z. *officinale* evaluated from prothrombin time (PT) assay. The extract was tested in different concentrations which exhibited different clotting times. The results are presented in table (Table 4.1.2). The prothrombin time of each

concentration was obtained through a plot of mean prothrombin time against different concentrations of extract (Figure 4.1.3).

Table 4.1.2: The effect of ethanolic extract of *Z. officinale* at different concentrations on prothombin time of plasma in a prothombin time test.

Concentration (µg/ml)	Mean prothrombin time (minutes)	Standard deviation
1000	21.98333	0.441852
500	19.01667	0.603352
250	15.79	0.469361
125	12.34667	0.894725
62.5	9.95	0.377492
NC	1.41	0.189

❖ The coagulation time was recorded as mean time of coagulation± standard deviation. Significant ($P < 0.05$) difference between the samples and the control present.

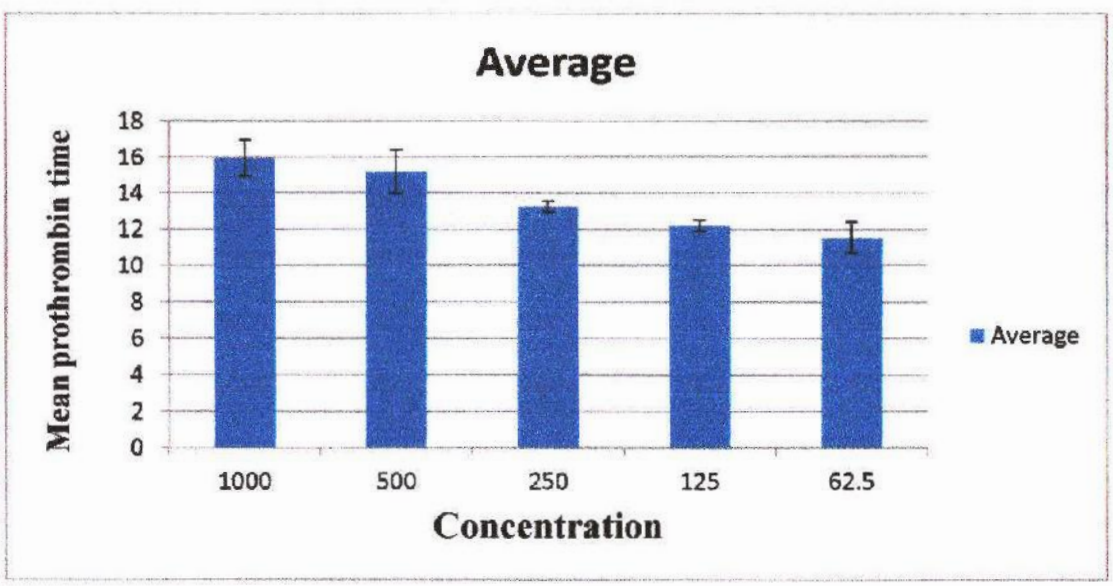


Figure 4.1.3: Plot of concentration of ethanolic extract of *Z. officinale* versus mean prothrombin time.

4.1.3 *Z. officinale* aqueous extract

The prothrombin time of aqueous extract of Z. officinale evaluated from prothrombin time (PT) assay. The extract was tested in different concentrations which exhibited different clotting times. The results are presented in table (Table 4.1.3). The prothrombin time of each concentration was obtained through a plot of mean prothrombin time against different concentrations of extract (Figure 4.1.4).

Table 4.1.3: The effect of *Z. officinale*, (Aqueous) at different concentrations on prothombin time of plasma in a prothombin time test.

Concentration (µg/ml)	Mean prothrombin time (minutes)	Standard deviation
1000	15.93667	1.020653
500	15.18333	1.223942
250	13.23667	0.289885
125	12.21667	0.300721
62.5	11.55333	0.871799
Negative Control	1.41	0.189

- The coagulation time was recorded as mean time of coagulation± standard deviation. Significant ($P < 0.05$) difference between the samples and the control present.

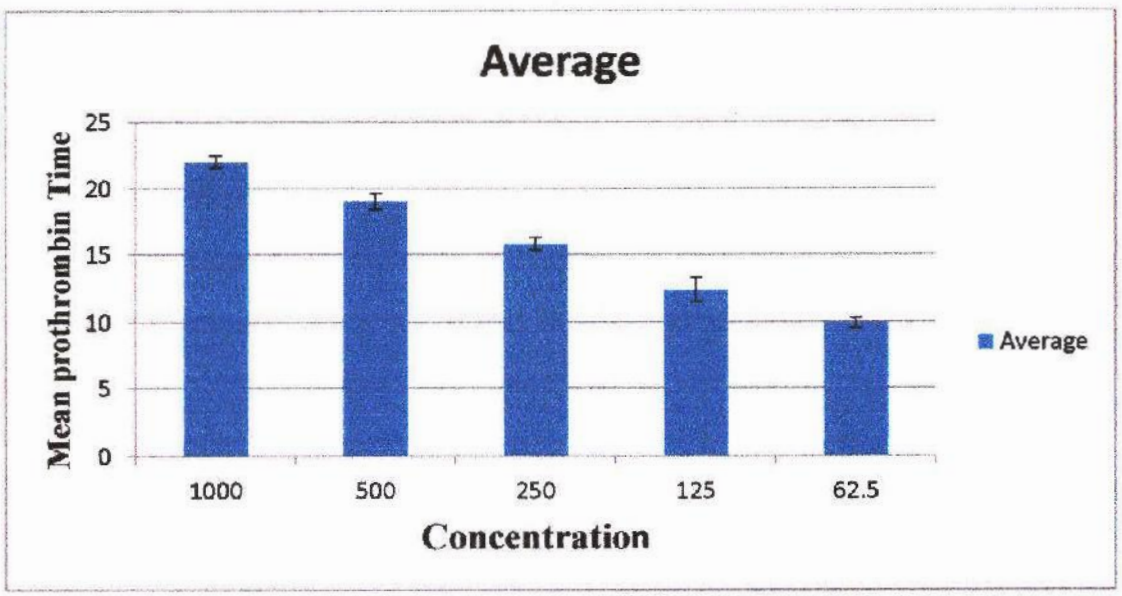


Figure 4.1.4: Plot of concentration of aqueous extract of *Z. officinale* versus mean prothrombin time.

4.1.4 *A. sativum* ethanolic extract

The prothrombin time of ethanolic extract of *A. sativum* evaluated from prothrombin time (PT) assay. The extract was tested in different concentrations which exhibited different clotting times. The results are presented in table (Table 4.1.4). The prothrombin time of each concentration was obtained through a plot of mean prothrombin time against different concentrations of extract (Figure 4.1.5).

Table 4.1.4: The effect of, *A. sativum* (ethanolic) at different concentrations on prothombin time of plasma in a prothombin time test.

Concentration (µg/ml)	Mean prothrombin time (minutes)	Standard deviation
1000	25.13	0.97811
500	22.35333	0.941771
250	21.31333	0.761074
125	19.09667	0.610765
62.5	14.53667	0.599694
NC	1.41	0.189

❖ The coagulation time was recorded as mean time of coagulation± standard deviation. Significant ($P < 0.05$) difference between the samples and the control present.

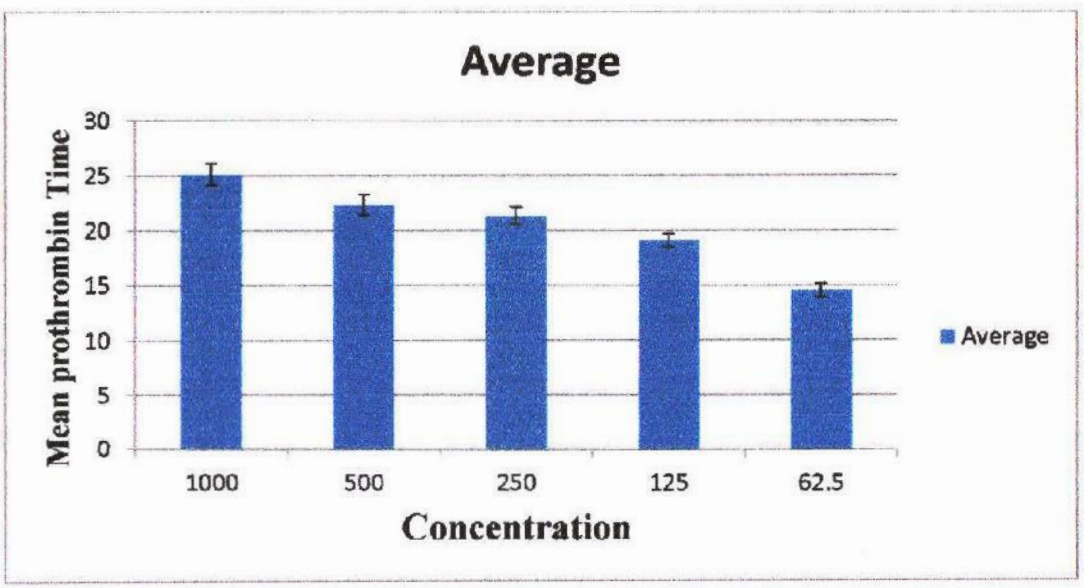


Figure 4.1.5: Plot of concentration of ethanolic extract of *A. sativum* versus mean prothrombin time.

4.1.5 *A. sativum* aqueous extract

The prothrombin time of aqueous extract of *A. sativum* evaluated from prothrombin time (PT) assay. The extract was tested in different concentrations which exhibited different clotting times. The results are presented in table (Table 4.1.5). The prothrombin time of each concentration was obtained through a plot of mean prothrombin time against different concentrations of extract (Figure 4.1.6).

Table 4.1.5: The effect of *A. sativum* (aqueous) at different concentrations on prothombin time of plasma in a prothombin time test.

Concentration (µg/ml)	Mean prothrombin time (minutes)	Standard deviation
1000	21.97333	0.801582
500	18.57667	0.547205
250	17.85	0.560268
125	16	0.614898
62.5	12.66333	0.356417

❖ The coagulation time was recorded as mean time of coagulation± standard deviation. Significant ($P < 0.05$) difference between the samples and the control present.

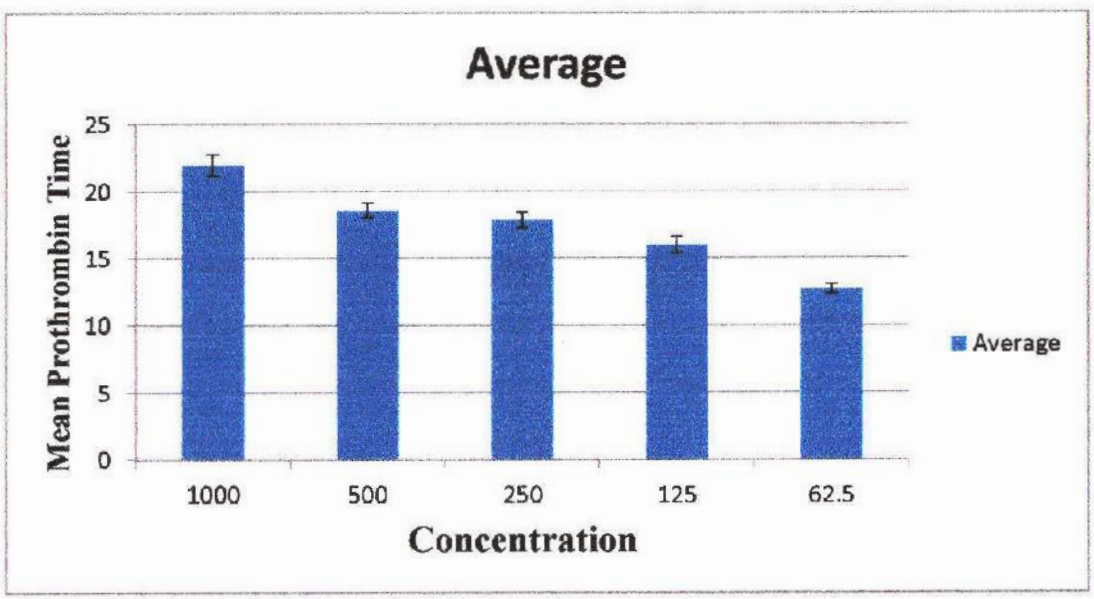


Figure 4.1.6: Plot of concentration of aqueous extract of *A. sativum* versus mean prothrombin time.

4.1.6 *C. verum* ethanolic extract

The prothrombin time of ethanolic extract of *C. verum* evaluated from prothrombin time (PT) assay. The extract was tested in different concentrations which exhibited different clotting times. The results are presented in table (Table 4.1.6). The prothrombin time of each concentration was obtained through a plot of mean prothrombin time against different concentrations of extract (Figure 4.1.7).

Table 4.1.6: The effect of C. verum (ethanolic) at different concentrations on prothombin time of plasma in a prothombin time test.

Concentration	Mean prothrombin time (minutes)	Standard deviation
1000	14.39	0.577
500	13.15	0.533
250	11.69	0.308
125	10.37	0.858
62.5	8.61	0.641
NC	1.41	0.189

❖ The coagulation time was recorded as mean time of coagulation± standard deviation. Significant ($P < 0.05$) difference between the samples and the control present; NC-Negative control.

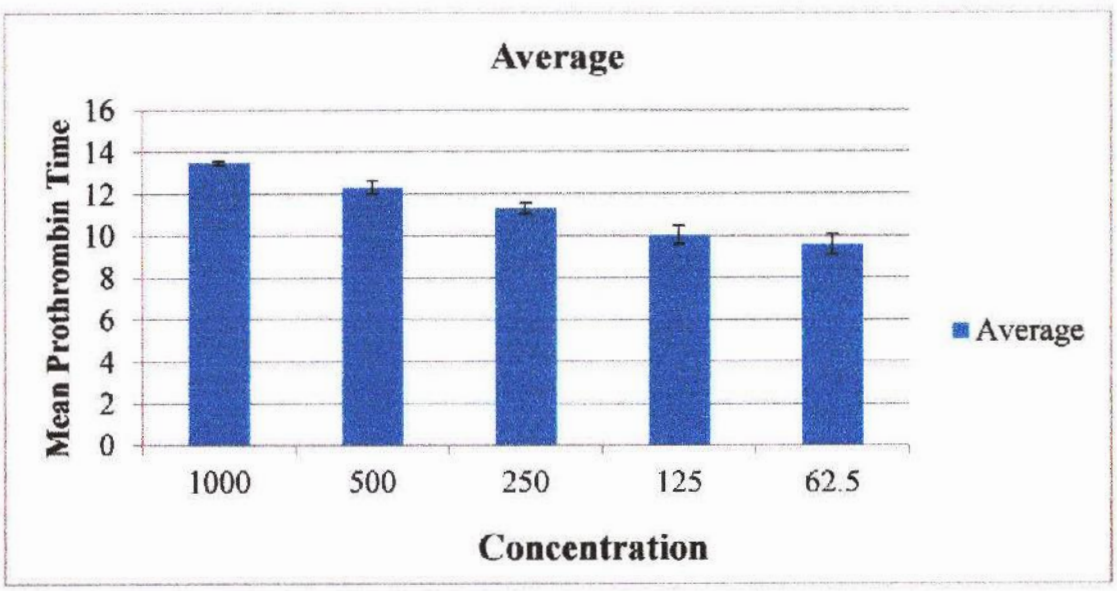


Figure 4.1.7: Plot of concentration of ethanolic extract of *C. verum* versus mean prothrombin time.

4.1.7 *C. verum* aqueous extract

The prothrombin time of aqueous extract of *C. verum* evaluated from prothrombin time (PT) assay. The extract was tested in different concentrations which exhibited different clotting times. The results are presented in table (Table 4.1.7). The prothrombin time of each concentration was obtained through a plot of mean prothrombin time against different concentrations of extract (Figure 4.1.8).

Table 4.1.7: The effect of C. verum (aqueous) at different concentrations on prothombin time of plasma in a prothombin time test.

Concentration	Mean prothrombin time (minutes)	Standard deviation
1000	13.49	0.088
500	12.35	0.3051
250	11.33	0.2523
125	10.05	0.4387
62.5	9.62	0.4844
NC	1.41	0.189

- The coagulation time was recorded as mean time of coagulation± standard deviation. Significant ($P < 0.05$) difference between the samples and the control present; NC-Negative control.

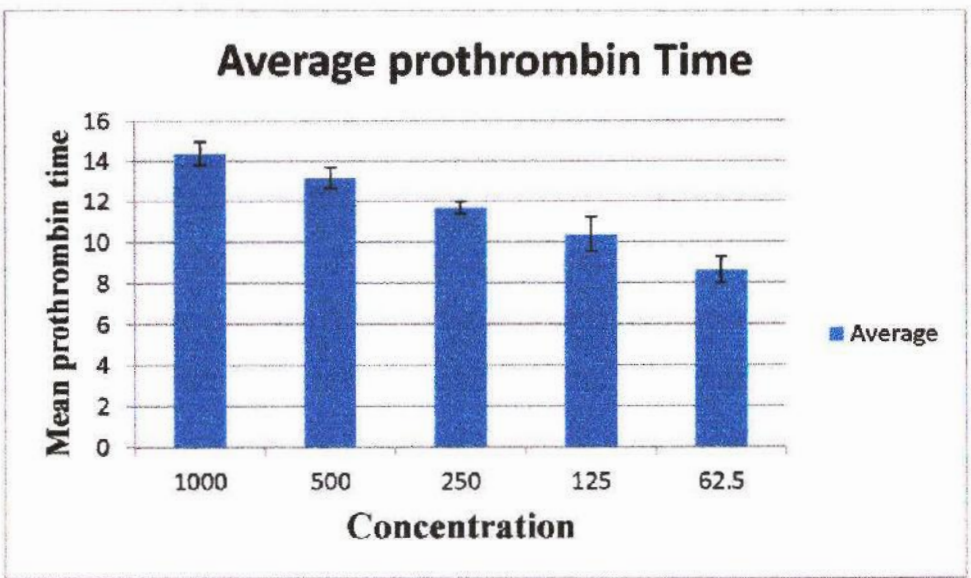


Figure 4.1.8: Plot of concentration of aqueous extract of *C. verum* versus mean prothrombin time.

4.1.8 *C. longa* ethanolic extract

The prothrombin time of ethanolic extract of *C. longa* evaluated from prothrombin time (PT) assay. The extract was tested in different concentrations which exhibited different clotting times. The results are presented in table (Table 4.1.8). The prothrombin time of each concentration was obtained through a plot of mean prothrombin time against different concentrations of extract (Figure 4.1.9).

Table 4.1.8: The effect of *C. longa* (ethanol) at different concentrations on prothombin time of plasma in a prothombin time test.

Concentration (µg/ml)	Mean prothrombin time (minutes)	Standard deviation
1000	16.33	0.293
500	15.24	0.826
250	14.45	0.860
125	11.82	0.532
62.5	9.56	0.453
NC	1.41	0.189

- The coagulation time was recorded as mean time of coagulation± standard deviation. Significant ($P < 0.05$) difference between the samples and the control present; NC-Negative control.

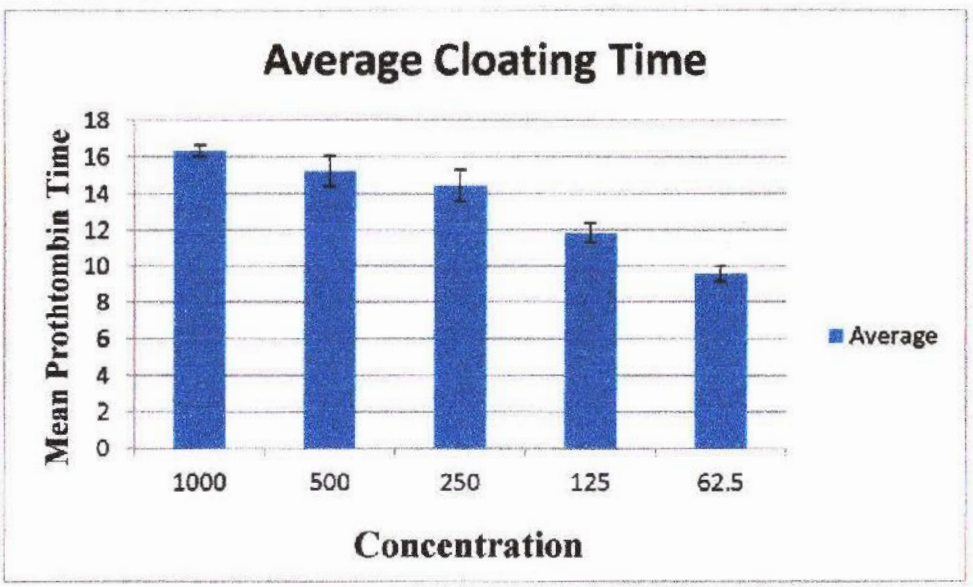


Figure 4.1.9: Plot of concentration of ethanolic extract of *C. longa* versus mean prothrombin time.

4.1.9 *C. longa* aqueous extract

The prothrombin time of aqueous extract of *C. longa* evaluated from prothrombin time (PT) assay. The extract was tested in different concentrations which exhibited different clotting times. The results are presented in table (Table 4.1.9). The prothrombin time of each concentration was obtained through a plot of mean prothrombin time against different concentrations of extract (Figure 4.1.10).

Table 4.1.9: The effect of *C. longa* (ethanol) at different concentrations on prothombin time of plasma in a prothombin time test.

Concentration (µg/ml)	Mean prothrombin time (minutes)	Standard deviation
1000	13.67	1.491073
500	10.97667	0.746749
250	11.56	1.780646
125	11.71333	2.069042
62.5	8.623333	0.536781

- The coagulation time was recorded as mean time of coagulation± standard deviation. Significant ($P < 0.05$) difference between the samples and the control present; NC-Negative control.

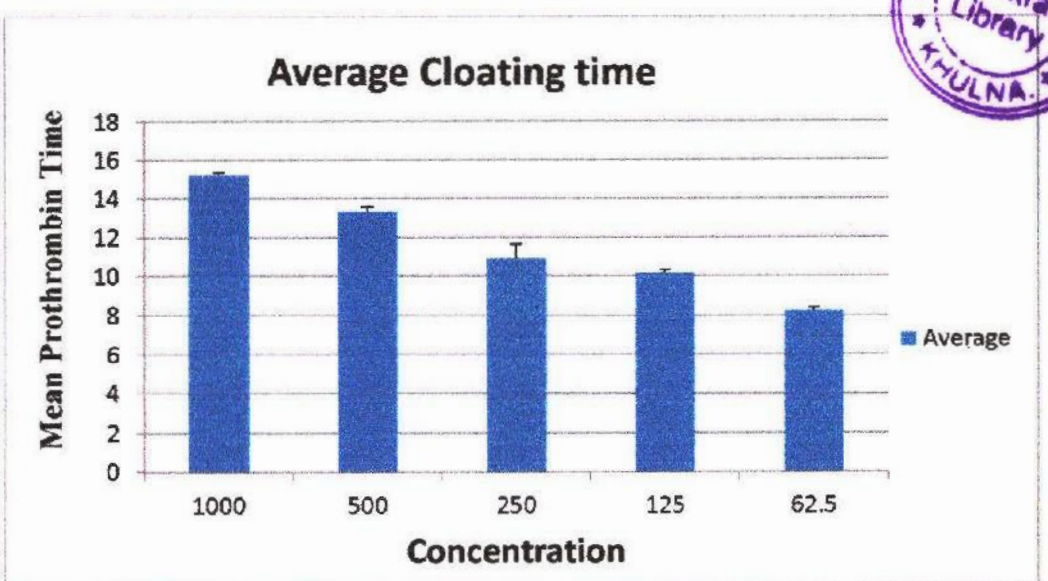


Figure 4.1.10: Plot of concentration of aqueous extract of *C. longa* versus mean prothrombin time.

4.1.10 *C. sativum* ethanolic extract

The prothrombin time of ethanolic extract of *c. sativum* evaluated from prothrombin time (PT) assay. The extract was tested in different concentrations which exhibited different clotting times. The results are presented in table (Table 4.1.10). The prothrombin time of each concentration was obtained through a plot of mean prothrombin time against different concentrations of extract (Figure 4.1.11).

Table 4.1.10: The effect of *C. sativum* (ethanol) at different concentrations on prothombin time of plasma in a prothombin time test.

Concentration (µg/ml)	Mean prothrombin time (minutes)	Standard deviation
1000	16.58	0.634114
500	15.32333	1.265003
250	14.06	0.639296
125	13.52	0.849235
62.5	12.93333	0.929157

- The coagulation time was recorded as mean time of coagulation± standard deviation. Significant ($P < 0.05$) difference between the samples and the control present; NC-Negative control.

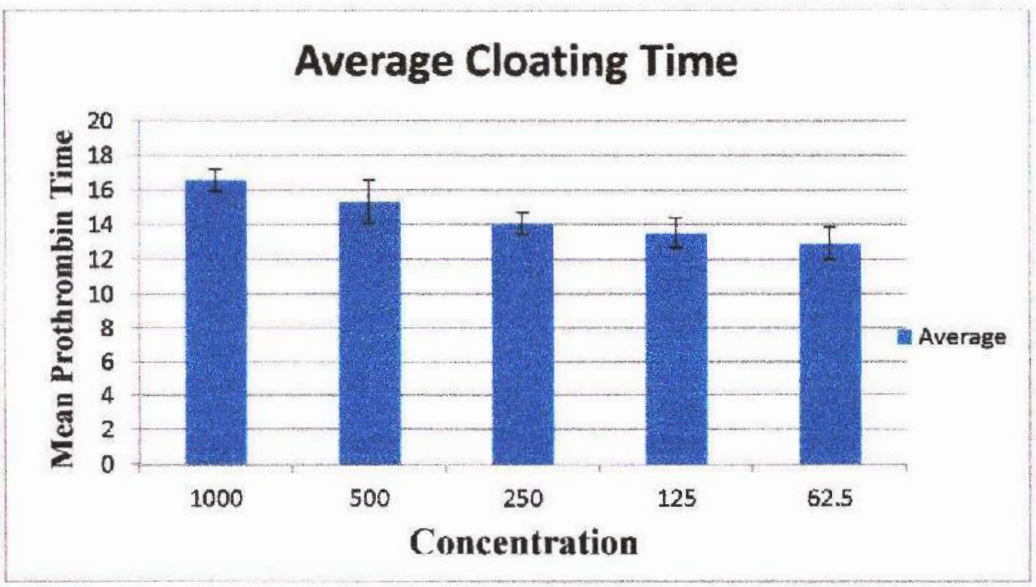


Figure 4.1.11: Plot of concentration of ethanolic extract of *C. sativum* versus mean prothrombin time.

4.1.11 *C. sativum* aqueous extract

The prothrombin time of aqueous extract of *c. sativum* evaluated from prothrombin time (PT) assay. The extract was tested in different concentrations which exhibited different clotting times. The results are presented in table (Table 4.1.11). The prothrombin time of each concentration was obtained through a plot of mean prothrombin time against different concentrations of extract (Figure 4.1.12).

Table 4.1.11: The effect of *C. sativum* (aqueous) at different concentrations on prothombin time of plasma in a prothombin time test.

Concentration (µg/ml)	Mean prothrombin time (minutes)	Standard deviation
1000	14.91	0.761774
500	11.51	0.960156
250	10.72667	0.499233
125	9.99	0.432782
62.5	8.693333	0.480139

- The coagulation time was recorded as mean time of coagulation± standard deviation. Significant ($P < 0.05$) difference between the samples and the control present.

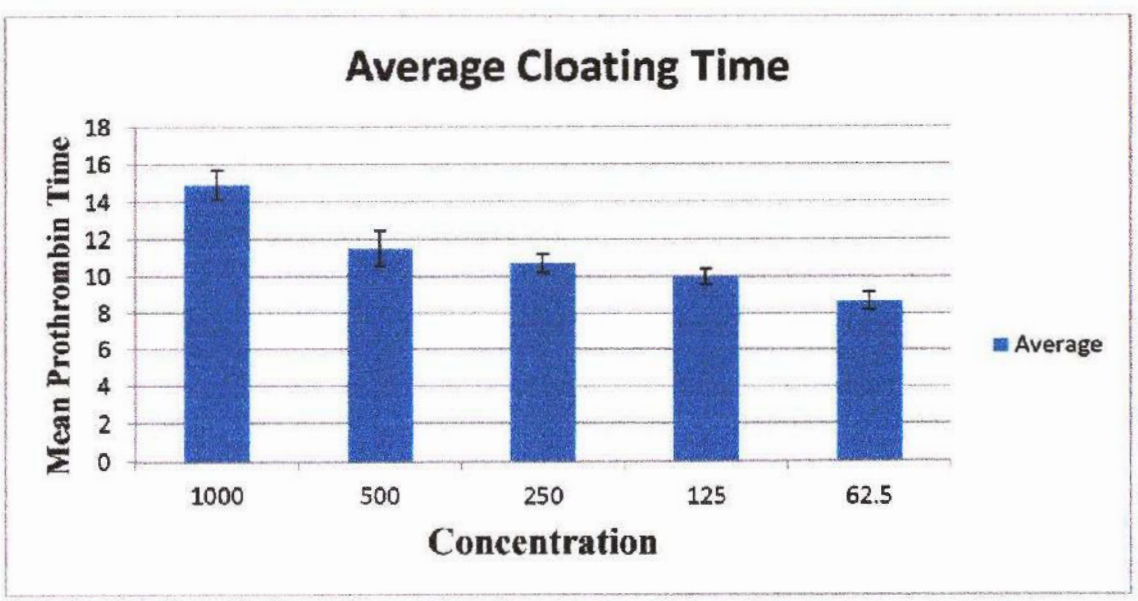


Figure 4.1.12: Plot of concentration of aqueous extract of *C. sativum* versus mean prothrombin time.

❖ **The Prothrombin Time Assay result of all spices in a graph.**

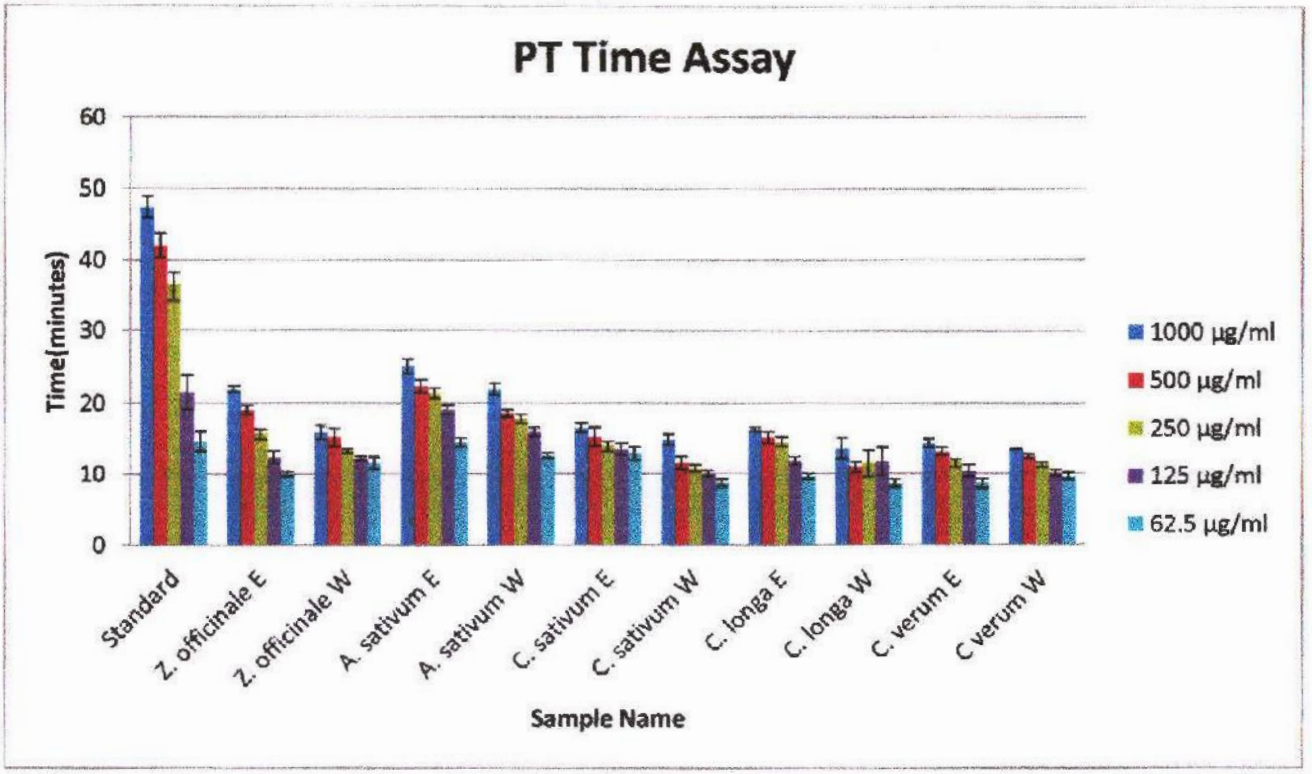


Figure 4.1.13: The overview of PT time Assay with standard and spices sample.

4.1.11 Discussion on Anticoagulation

Blood coagulation is an essential part of homeostasis in which platelets, a non-nucleated cell derived from megakaryocyte plays a major role to prevent excessive bleeding (Rang H. P. *et al.*, 2003]. Platelets change shape and adhere or aggregate to themselves resulting in plug formation which is then strengthened by fibrin containing clot to stop bleeding and for repairing of damaged vessel to begin. Disorders of coagulation are known to cause haemorrhage or thrombosis [Rapaport S. I., 1993]. Fibrin is the most important component of clots that form in veins but platelets are the major component of clots that form in arteries where they can cause heart attacks and strokes by blocking the flow of blood in the heart and brain, respectively (Hirsh J., 2008). Herbs ans spice plants contain a number of bioactive compounds like arabinose, those are able to prevent clots formation in veins and arteries (Premanathan M. et al., 1999). The prothrombin time was found to be increased with increasing the concentration of extracts. The prothrombin time was obtained through a plot of mean prothrombin time versus different concentrations of extracts. In the PT assay, 30%

DMSO was considered as negative control which exhibited a mean clotting time of 1.41 minutes. The aqueous extract of *Z. officinale* was tested at different concentrations (1000, 500 and 250 µg/ml) which exhibited a mean clotting time of 15.93, 15.18 and 13.23 minutes respectively where as the standard, warfarin sodium showed a mean clotting time of 47.45, 42.02 and 36.61 minutes respectively at 1000, 500 and 250 µg/ml concentrations. The ethanolic *Z. officinale* extract of was tested at different concentrations (1000, 500 and 250 µg/ml) which exhibited a mean clotting time of 21.98, 19.01 and 15.79 minutes respectively where as the standard, warfarin sodium showed a mean clotting time of 47.45, 42.02 and 36.61 minutes respectively at 1000, 500 and 250 µg/ml concentrations. The ethanolic extract of A. sativum was tested at different concentrations (1000, 500 and 250 µg/ml) which exhibited a mean clotting time of 25.13, 22.35 and 21.31 minutes respectively where as the standard, warfarin sodium showed a mean clotting time of 47.45, 42.02 and 36.61 minutes respectively at 1000, 500 and 250 µg/ml concentrations. The ethanolic extract of *C. verum* was tested at different concentrations (1000, 500 and 250 µg/ml) which exhibited a mean clotting time of 14.39, 13.15 and 11.69 minutes respectively where as the standard, warfarin sodium showed a mean clotting time of 47.45, 42.02 and 36.61 minutes respectively at 1000, 500 and 250 µg/ml concentrations. The ethanolic extract of *C. longa* was tested at different concentrations (1000, 500 and 250 µg/ml) which exhibited a mean clotting time of 16.33, 15.24 and 14.45 minutes respectively where as the standard, warfarin sodium showed a mean clotting time of 47.45, 42.02 and 36.61 minutes respectively at 1000, 500 and 250 µg/ml concentrations. The ethanolic extract of *C. sativum* was tested at different concentrations (1000, 500 and 250 µg/ml) which exhibited a mean clotting time of 16.58, 15.32 and 14.06 minutes respectively where as the standard, warfarin sodium showed a mean clotting time of 47.45, 42.02 and 36.61 minutes respectively at 1000, 500 and 250 µg/ml concentrations.

Warfarin sodium which was used as the positive control also exhibited higher anticoagulant activity than the ethanolic plant extracts. This is logical because the warfarin sodium is a pure compound but the extract is a crude and mixture of many compounds. The mechanism by which the extract showed anticoagulant activity is not understood but chelating agents, warfarin and vitamin K antagonists are known to interfere with blood clotting processes. The warfarin can inhibit clotting factor IXa, XIa and thrombin. The action of warfarin on factor Xa accounts for its potency as an anticoagulant [Melvin J. S., 1977]. The synthesis of clotting factors II, VII, IX and X in the liver depends on adequate amounts of vitamin K. The anticoagulant drugs resists the synthesis of non-functional forms of coagulant proteins and thereby prevents blood clot formation [Schneeweiss S. *et al.*, 2012].

The highest anticoagulation potential was observed in *A. sativum* (ethanol) > *Z. officinale* (ethanol) > *C. longa* (ethanol) > *C. sativum* (ethanol) > *C. verum* (ethanol).

In aqueous extracts *A. sativum* > *Z. officinale* > *C. longa* > C. sativum > *C. verum*.

4.2 Serine Protease Inhibition Assay

Serine protease inhibition assay was performed according to Kunitz, 1947 with some modifications. In this method, sample donate electron in trypsine casine reaction. Sample inhibited trypsine to react with casine. Trichloracetic acid was used to stop the reaction. And finally the reaction mixture was centrifuge at 12,000 rpm, 15 min and the supernatant was measured at 280 nm. In this method Quercetine was used as standard.

In this study, Serine protease inhibition activity of ethanolic and aqueous extracts of spices (*Z. officinale*, *A. sativum*, *C. vurem*, *C. longa*, *C. sativum*) were examined and analyze their graphical representation.

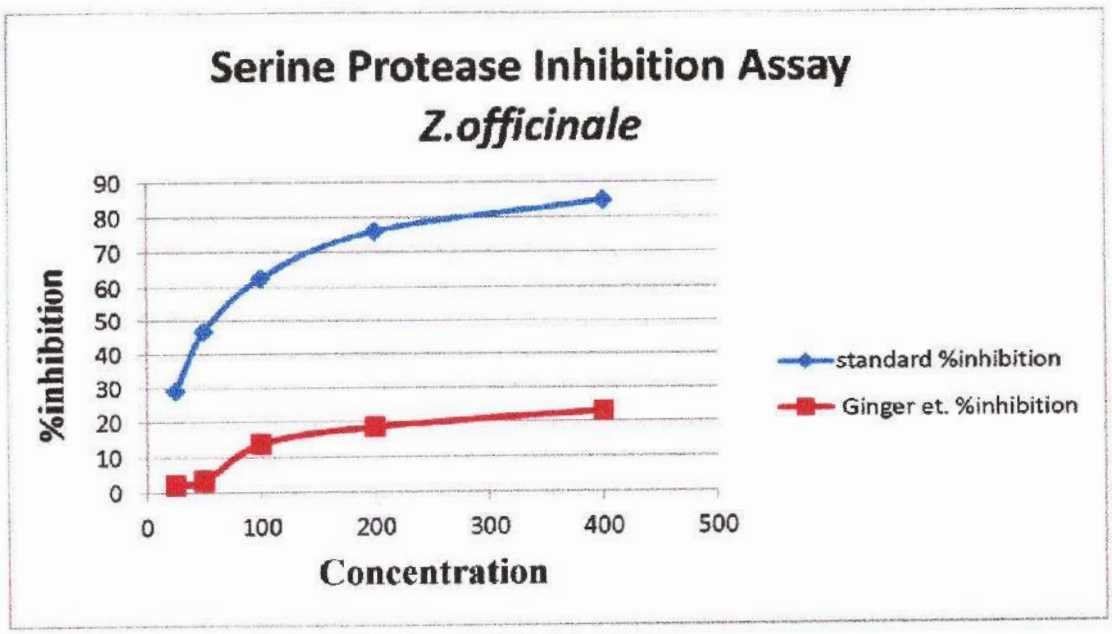


Figure 4.2.1: Serine protease inhibition assay of *Z.officinale* ethanolic extract (Absorbance vs concenitration).

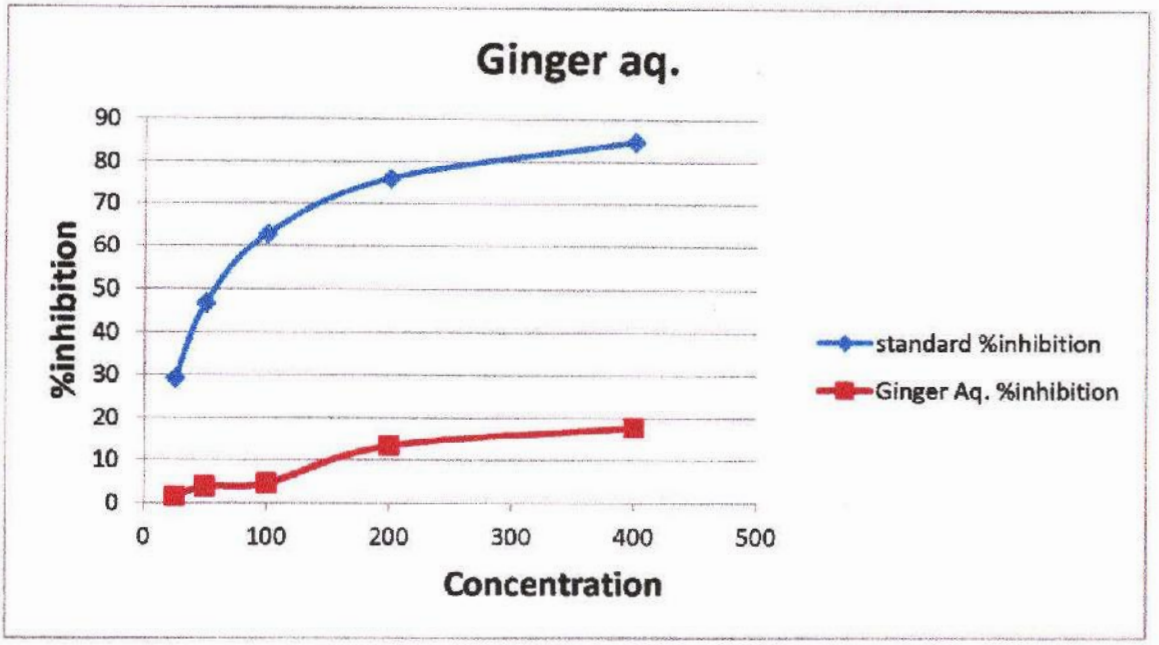


Figure 4.2.3: Serine protease inhibition assay of *Z. officinale* (ginger) aqueous extract (Absorbance vs concenitration).

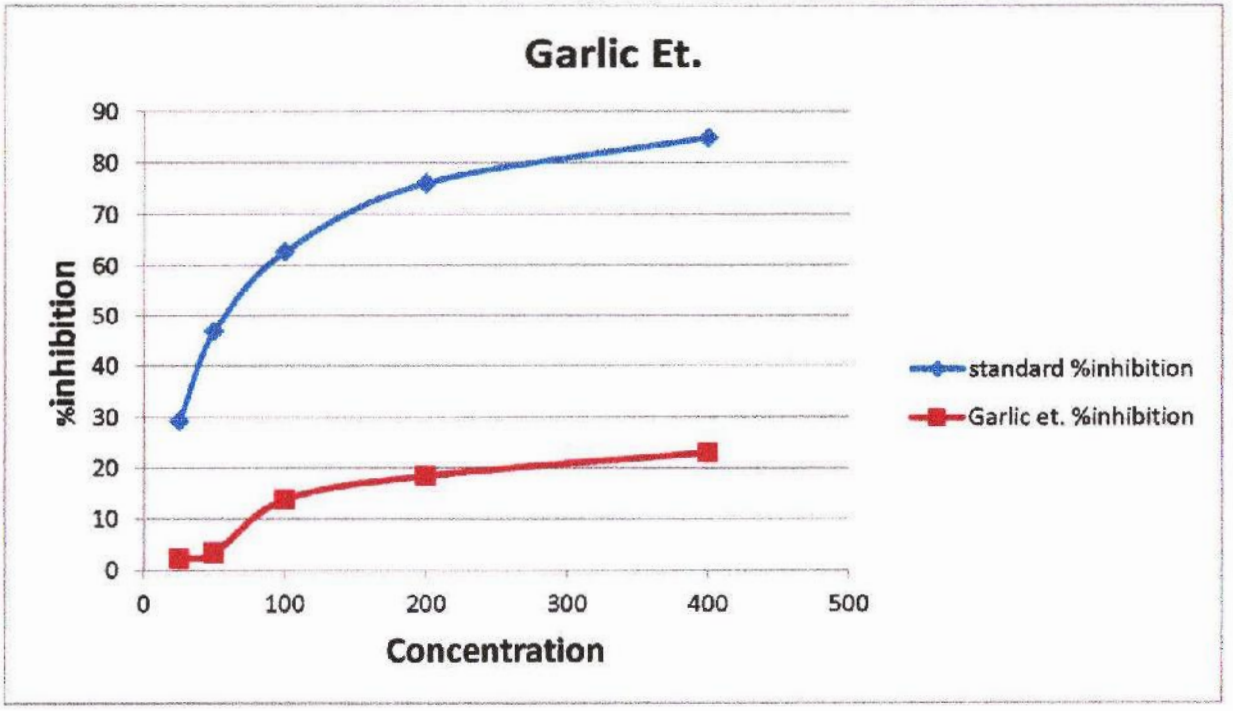


Figure 4.2.4: Serine protease inhibition assay of *Allium sativum*(garlic) ethanolic extract (Absorbance vs concenitration).

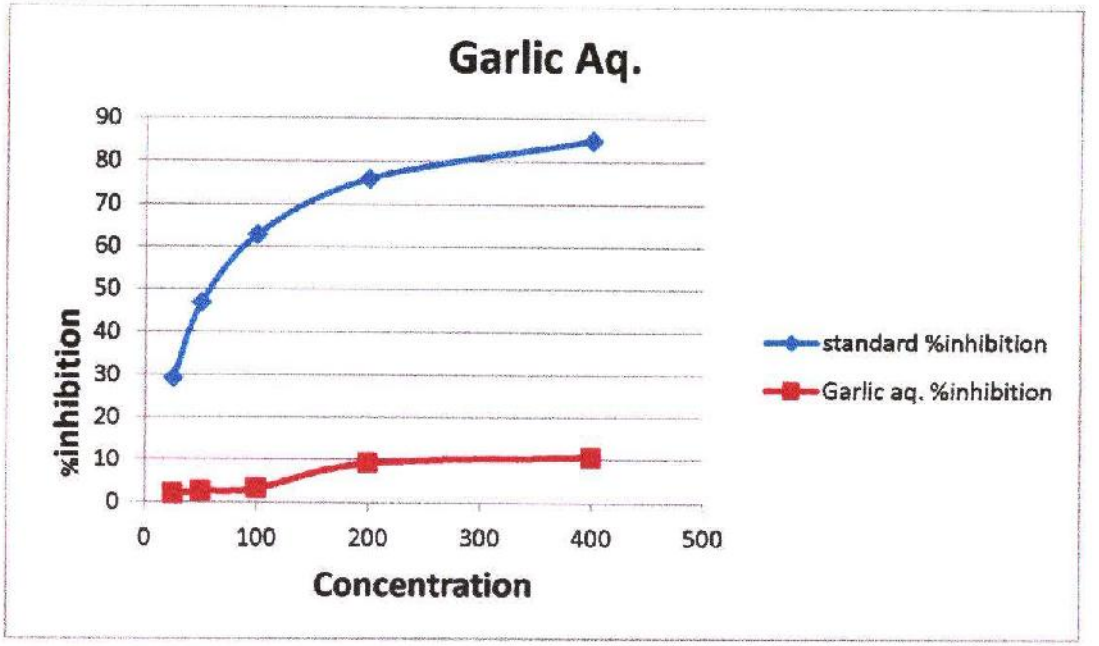


Figure 4.2.5: Serine protease inhibition assay of *Allium sativum*(garlic) aqueous extract (Absorbance vs concenitration).

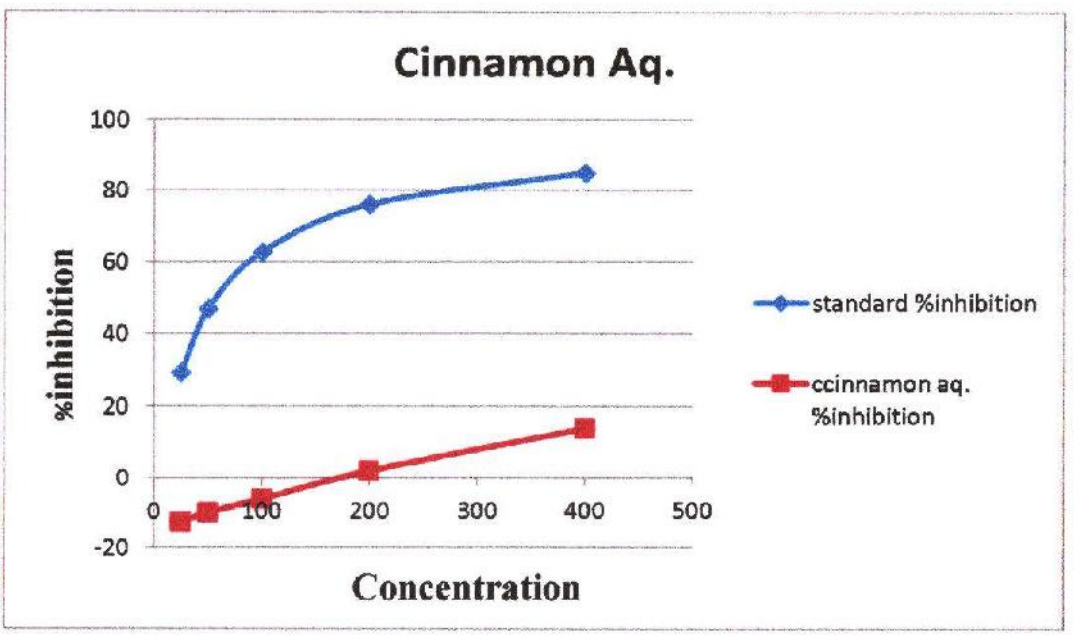


Figure 4.2.6: Serine protease inhibition assay of *C. verum* (cinnamon) aqueous extract (Absorbance vs concenitration).

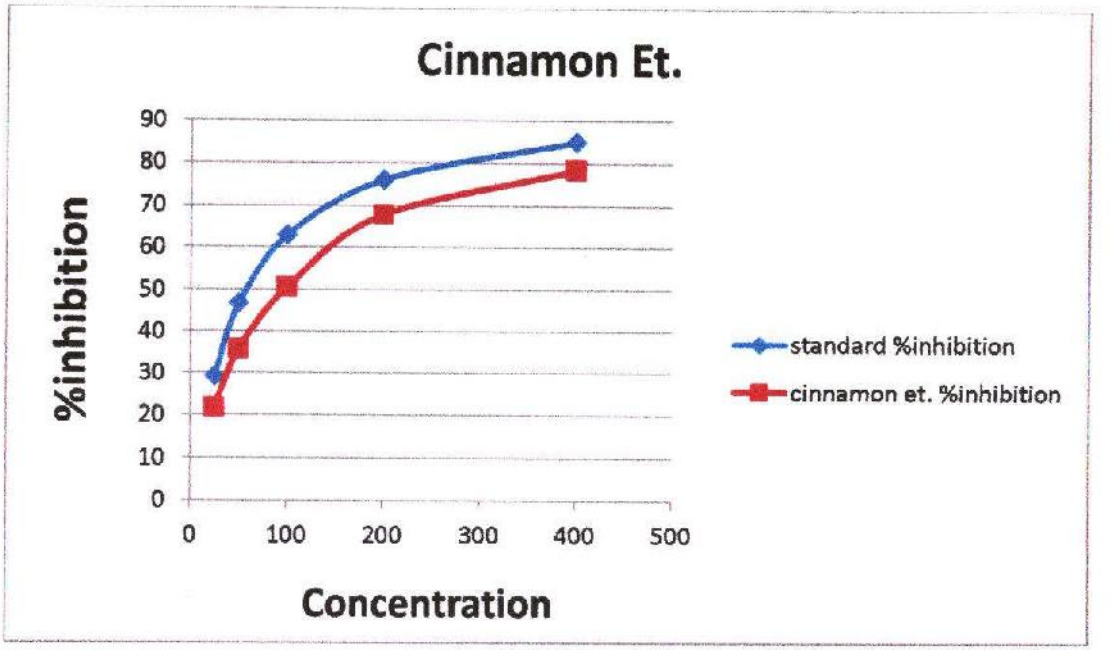


Figure 4.2.7: Serine protease inhibition assay of C. verum (cinnamon) aqueous extract (Absorbance vs concenitration).

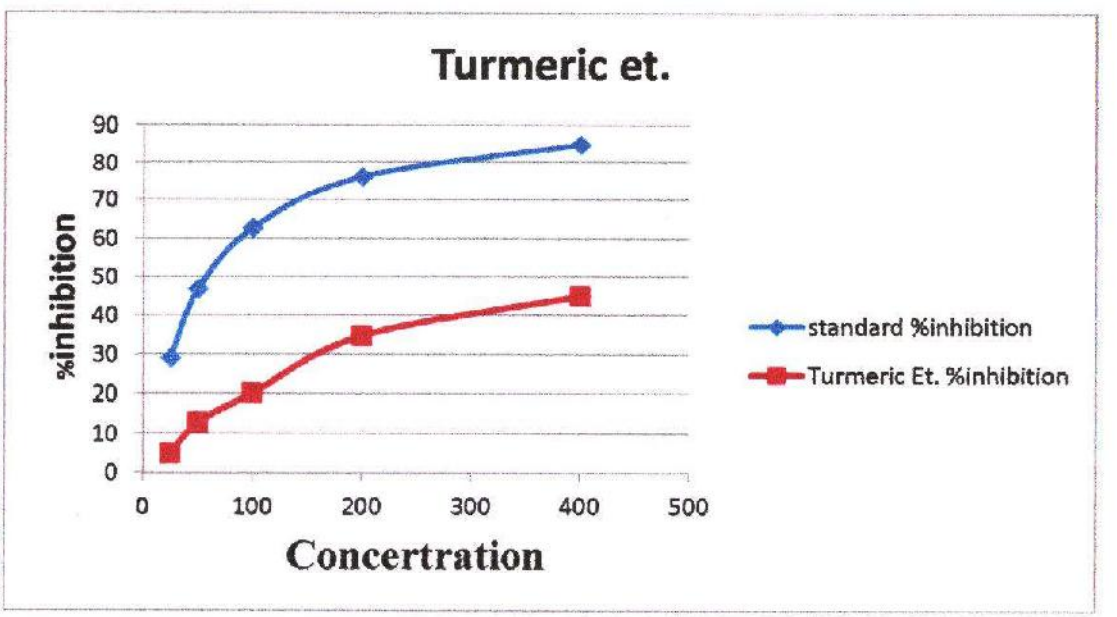


Figure 4.2.8: Serine protease inhibition assay of *C. longa* (turmeric) ethanolic extract (Absorbance vs concenitration).

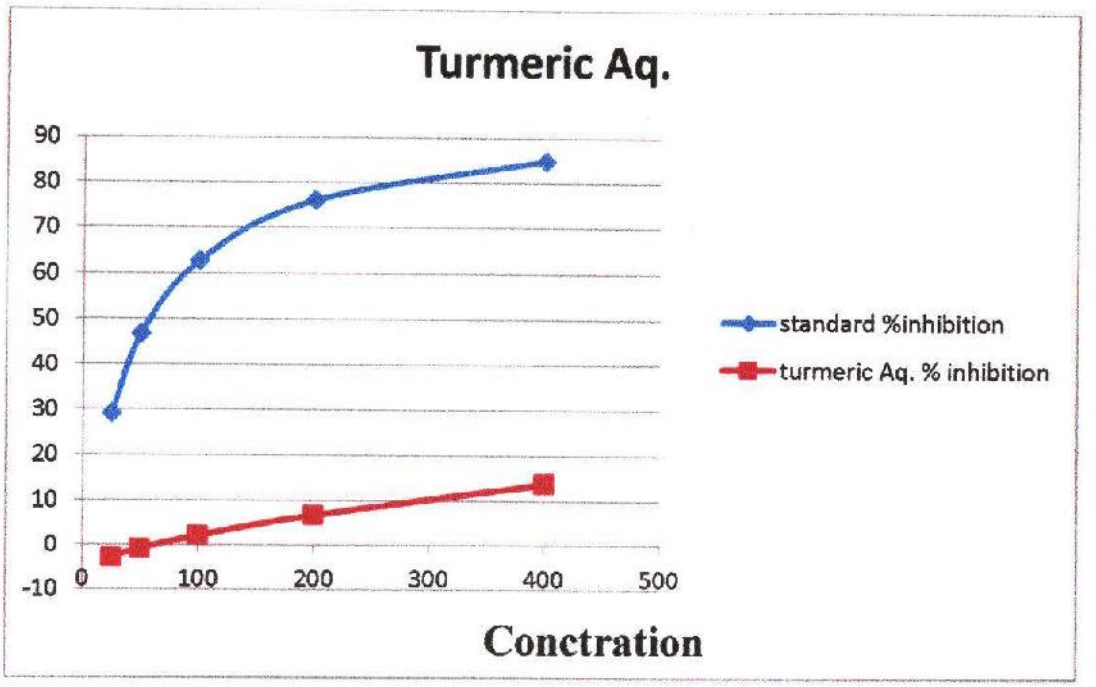


Figure 4.2.9: Serine protease inhibition assay of *C. longa* (turmeric) aqueous extract (Absorbance vs concenitration).

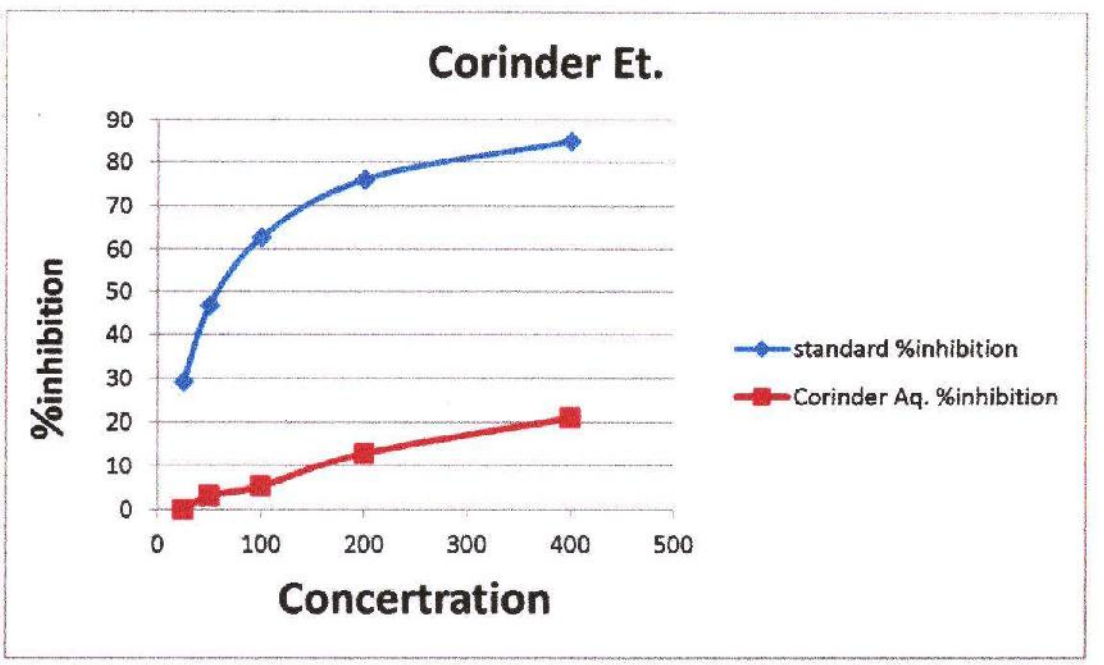


Figure 4.2.10: Serine protease inhibition assay of *C. sativum* (corinder) ethanolic extract (Absorbance vs concenitration).

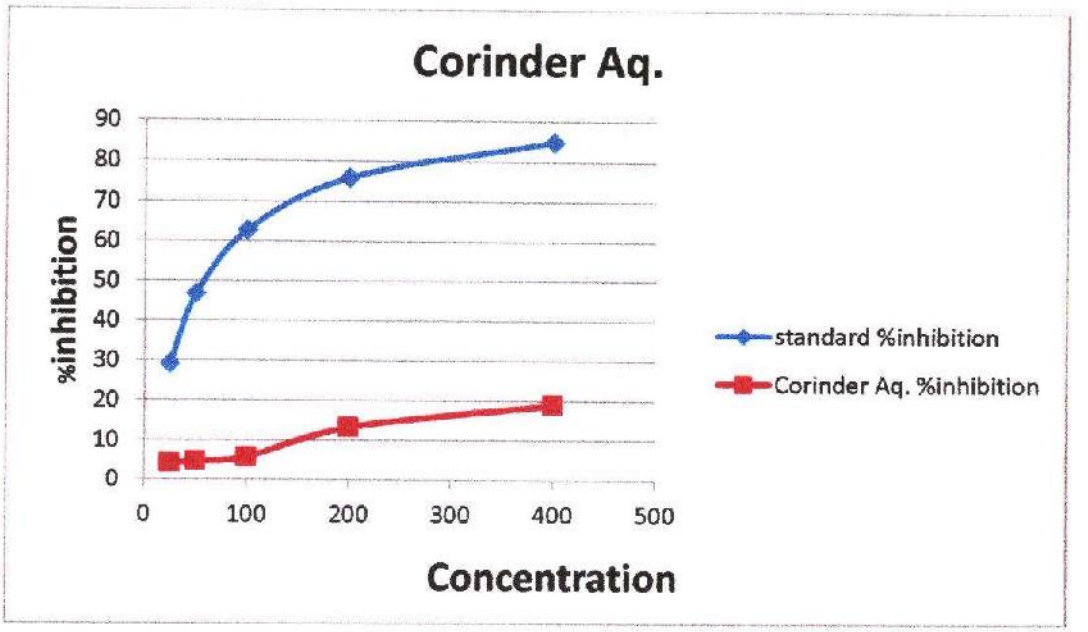


Figure 4.2.11: Serine protease inhibition assay of C. sativum (corinder) aqueous extract (Absorbance vs concenitration).

4.2.1: Discussion

Serine protease inhibition activity was determined by the method of Kunitz, 1947 with some modifications. According to literature we found that spice family has potential protease inhibition activity. For this reason we select common spices of our country for our experimental sample. Ethanolic extract of all the five samples exhibited, ginger 23%, garlic 27% ,cinnamon 78%, turmeric 45%, coriander 21% - tripsine inhibition at 400 µg/ml and standard Quercetine exhibited at highest 400 µg/ml concentration is 85.01 % inhibition. This ethanolic extract of spices has moderate protease inhibition activity but the cinnamon has high protease inhibition activity. This is due to the presence of phenolic acid, flavonoid etc. This result support our DPPH free radical scavenging assay, FRAP assay, phenolic content, flavonoid content. Water extract of spices exhibited moderately low tripsine inhibition at 400µg/ml concentration. This is due to the presence of less phenolic and flavonoid content.

4.3 Antioxidant Related Assay

4.3.1 DPPH Free Radical Scavenging Assay

The DPPH antioxidant assay is a rapid, direct and reliable method for the antioxidant screening of plant extracts. A number of methods are available for the determination of free radical scavenging activity but the assay employing the stable 2, 2-diphenyl-1-picryl-hydrazyl radical (DPPH) has received the maximum attention owing to its ease of use and its convenience. This test is based on the ability of DPPH, a stable nitrogen-centered free radical, to decolorize in the presence of antioxidants. The DPPH radical contains an odd electron, which is responsible for the absorbance at 515-517 nm and also for a visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound, the color of DPPH changes from violet to yellow, which can be quantitatively measured from the changes in absorbance. The purple color of DPPH fades due to conversion of it to 2, 2-diphenyl-1-picryl hydrazine resulting in decrease in absorbance. The DPPH radical scavenging activities of sample increased gradually as the concentration increased. Decrease in absorbance of DPPH solution depends on intrinsic antioxidant activity of samples as well as on speed of reaction between DPPH and antioxidant. Quercetin was chosen as the reference antioxidant for this test.

In this study, antioxidant activity of ethanolic and aqueous extracts *Zingiber officinale, Allium sativum, Cinnamomum verum, Curcuma longa, Coriandrum sativum* were examined and analyze their graphical representation.

By using the equation $y = mx + c$ (where 'c' is intercept and 'm' is slope); IC50 value of extract was found.

Table 4.3.1: DPPH Scavenging assay of Quercetin

Concentration	% inhibition	IC50 (µg/ml)
400	0.117	17.105
200	0.202	
100	0.227	
50	0.235	
25	0.252	
12.5	0.389	
6.25	0.447	

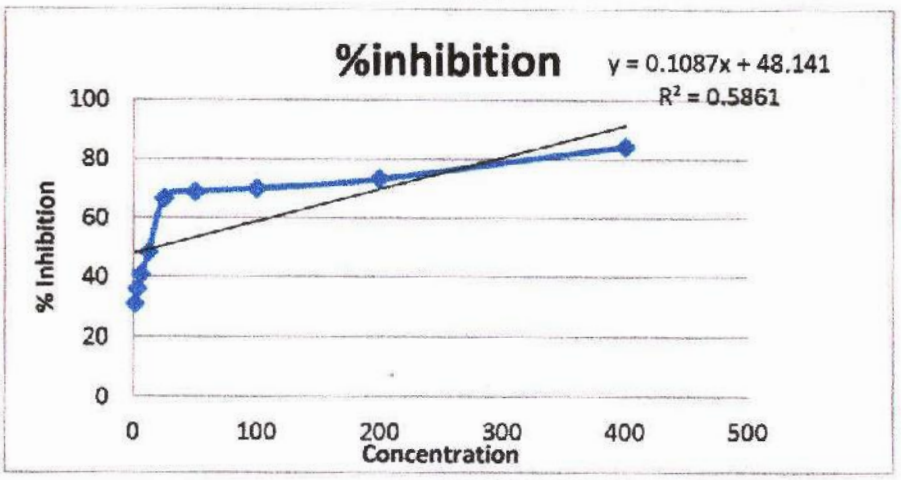


Figure 4.3.1: DPPH Scavenging assay of Quercetin (% inhibition vs Concentration)

Table 4.3.2: DPPH Scavenging assay of *Zingiber officinale* (Ada) ethanolic extract

Concentration (µg/ml)	% inhibition	IC_{50} value (µg/ml)
400	76.82	158.89
200	67.98	
100	57.75	
50	45.36	
25	37.95	
12.5	26.29	
6.25	23.62	

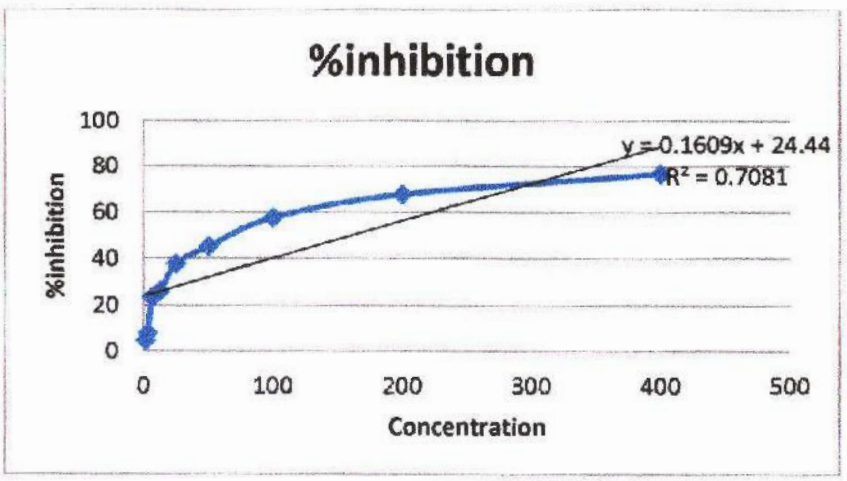


Figure 4.3.2: DPPH Scavenging assay of *Zingiber officinale* (Ada) ethanolic extract (% inhibition vs Concentration)

Table 4.3.3: DPPH Scavenging assay of *Zingiber officinale* (Ada) aqueous extract

Concentration (µg/ml)	% inhibition	IC_{50} value (µg/ml)
400	64.55	237.91
200	50.78	
100	43.46	
50	31.98	
25	26.22	
12.5	17.51	
6.25	15.33	

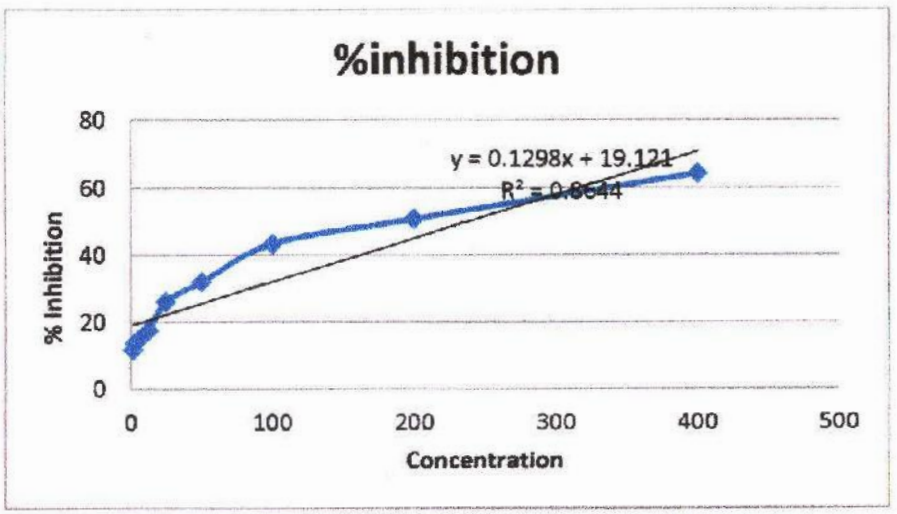


Figure 4.3.3: DPPH Scavenging assay of *Zingiber officinale* (Ada) aqueous extract (% inhibition vs Concentration).

Table 4.3.4: DPPH Scavenging assay of *Allium sativum* (Rosun) ethanolic extract

Concentration (µg/ml)	% inhibition	IC_{50} value (µg/ml)
400	.261	185.68
200	.430	
100	.48	
50	.595	
25	.664	
12.5	.704	
6.25	.720	

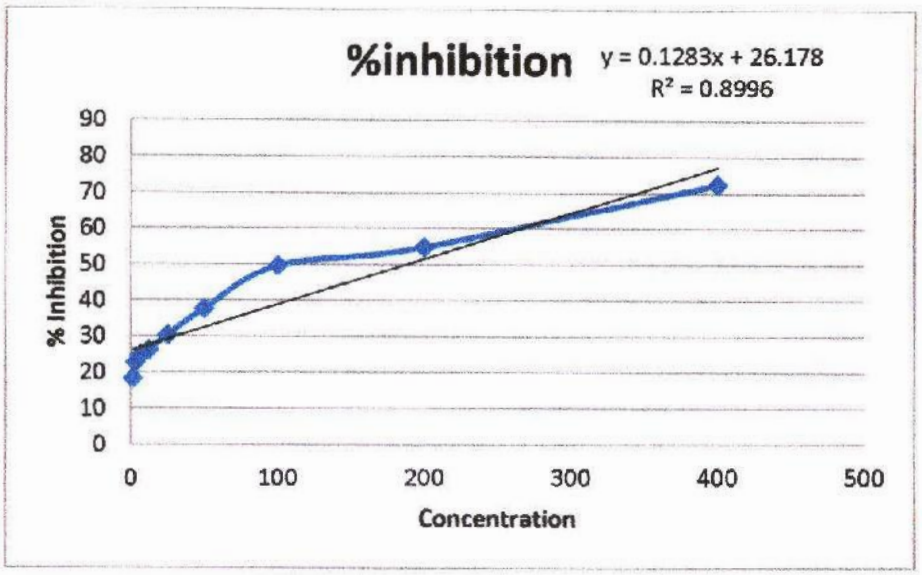


Figure 4.3.4: DPPH Scavenging assay of *Allium sativum* (Rosun) ethanolic extract (%inhibition vs concentration).

Table 4.3.5: DPPH Scavenging assay of *Allium sativum* (Rosun) aqueous extract

Concentration (µg/ml)	%inhibition	IC_{50} value (µg/ml)
400	.318	203.34
200	.460	
100	.481	
50	.518	
25	.624	
12.5	.721	
6.25	.738	

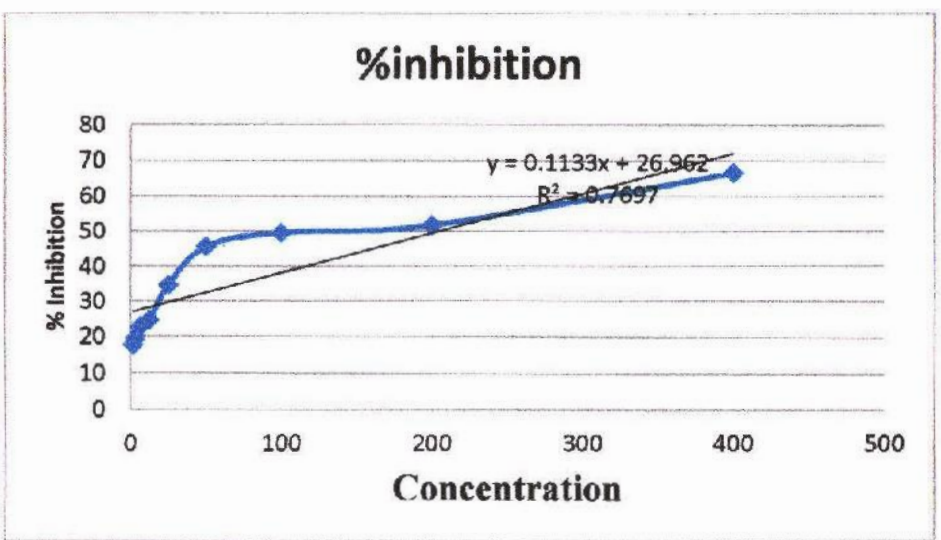


Figure 4.3.5: DPPH Scavenging assay of *Allium sativum* (Rosun) aqueous extract (%inhibition vs concentration).

Table 4.3.6: DPPH Scavenging assay of *Cinnamomum verum* (Darcini) ethanolic extract

Concentration (µg/ml)	%inhibition	IC_{50} value (µg/ml)
400	.151	46.462
200	.155	
100	.160	
50	.165	
25	.278	
12.5	.393	
6.25	.477	

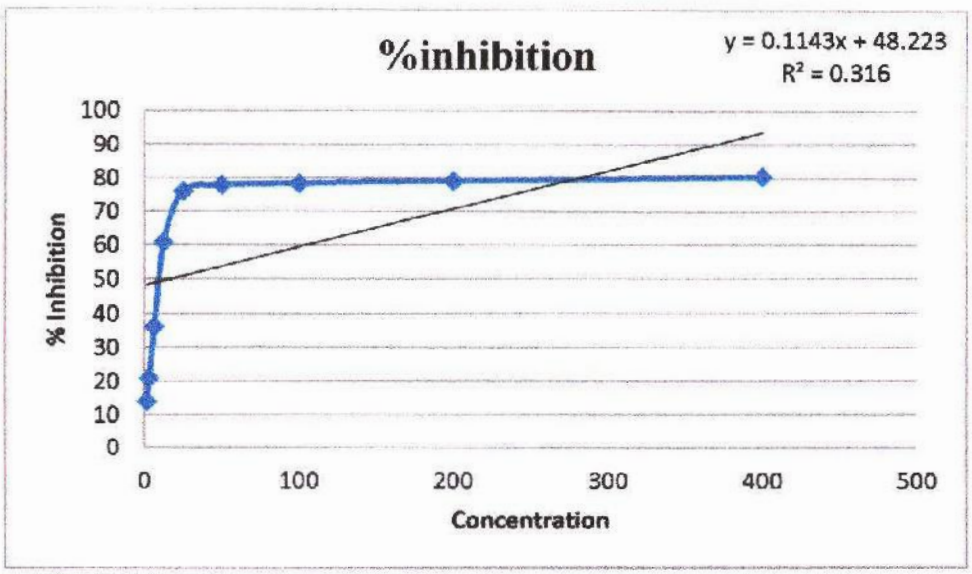


Figure 4.3.6: DPPH Scavenging assay of *Cinnamomum verum* (Darcini) ethanolic extract (%inhibition vs concentration).

Table 4.3.7: DPPH Scavenging assay of *Cinnamomum verum* (Darcini) aqueous extract

Concentration (µg/ml)	%inhibition	IC_{50} value (µg/ml)
400	.184	155.050
200	.206	
100	.247	
50	.415	
25	.518	
12.5	.560	
6.25	.654	

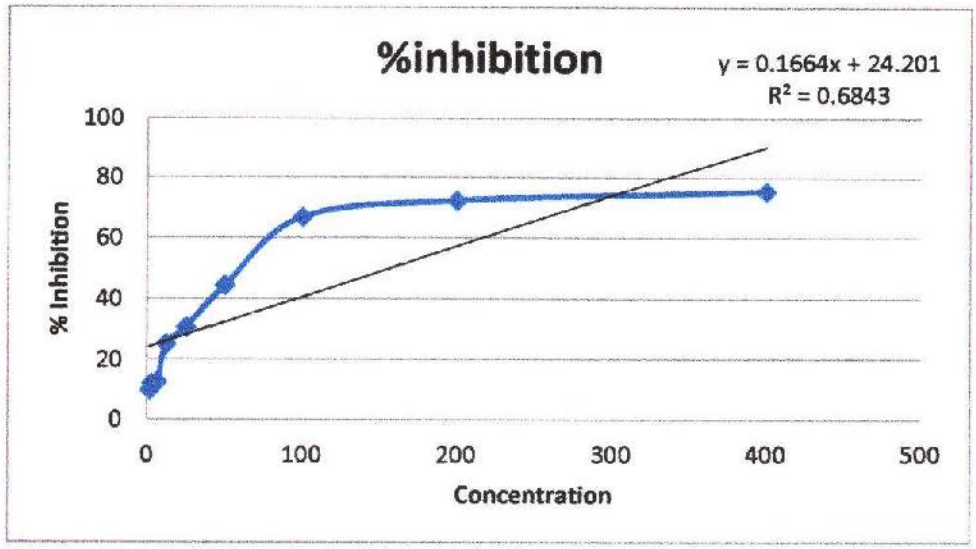


Figure 4.3.7: DPPH Scavenging assay of Cinnamomum verum (Darcini) aqueous extract (%inhibition vs concentration).

Table 4.3.8: DPPH Scavenging assay of *Curcuma longa* (Holud) ethanolic extract

Concentration (µg/ml)	%inhibition	IC_{50} value (µg/ml)
400	.357	228.33
200	.454	
100	.482	
50	.508	
25	.633	
12.5	.720	
6.25	.797	

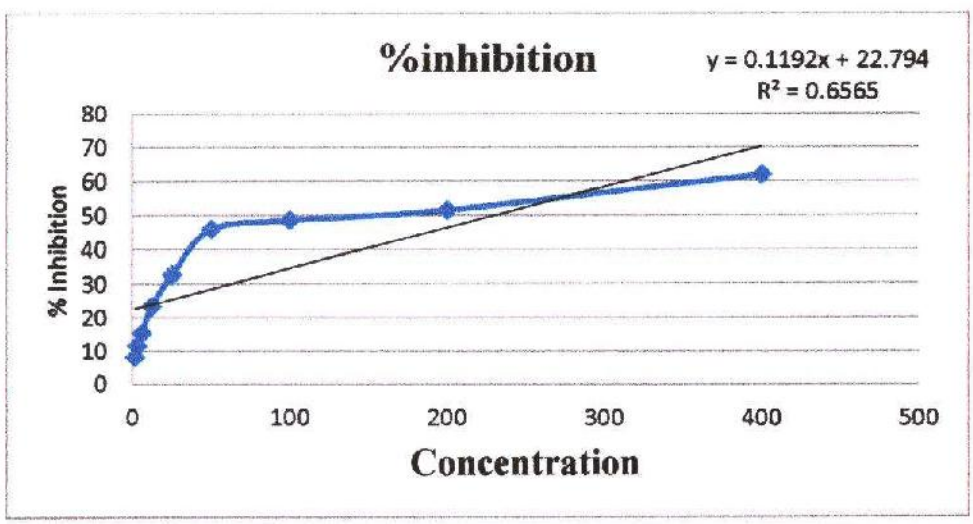


Figure 4.3.8: DPPH Scavenging assay of *Curcuma longa* (Holud) ethanolic extract (%inhibition vs concentration).

Table 4.3.9: DPPH Scavenging assay of *Curcuma longa* (Holud) aqueous extract

Concentration (µg/ml)	%inhibition	IC_{50} value (µg/ml)
400	.411	290.145
200	.504	
100	.622	
50	.717	
25	.815	
12.5	.844	
6.25	.873	

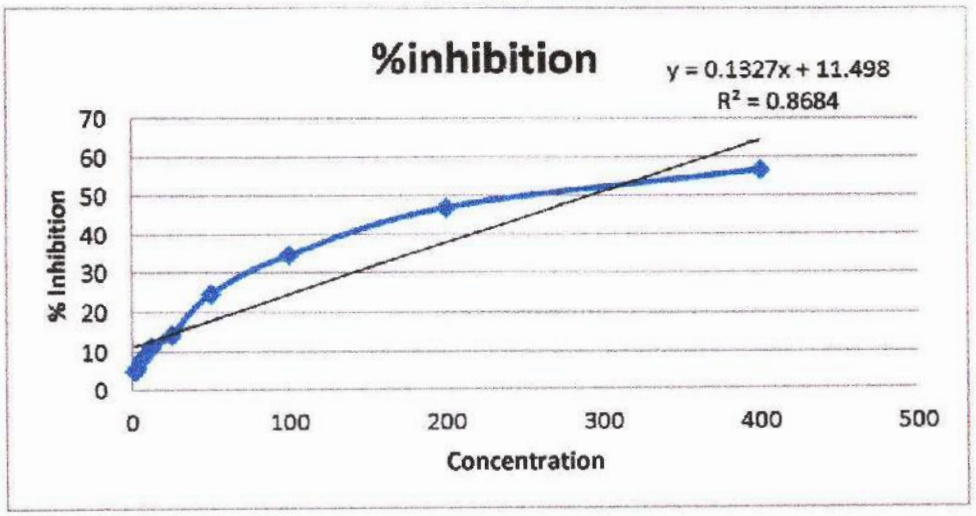


Figure 4.3.9: DPPH Scavenging assay of *Curcuma longa* (Holud) aqueous extract (%inhibition vs concentration).

Table 4.3.10: DPPH Scavenging assay of *Coriandrum sativum* (Dhoniya) ethanolic extract

Concentration (µg/ml)	%inhibition	IC_{50} value (µg/ml)
400	.540	384.356
200	.587	
100	.616	
50	.697	
25	.830	
12.5	.854	
6.25	.882	

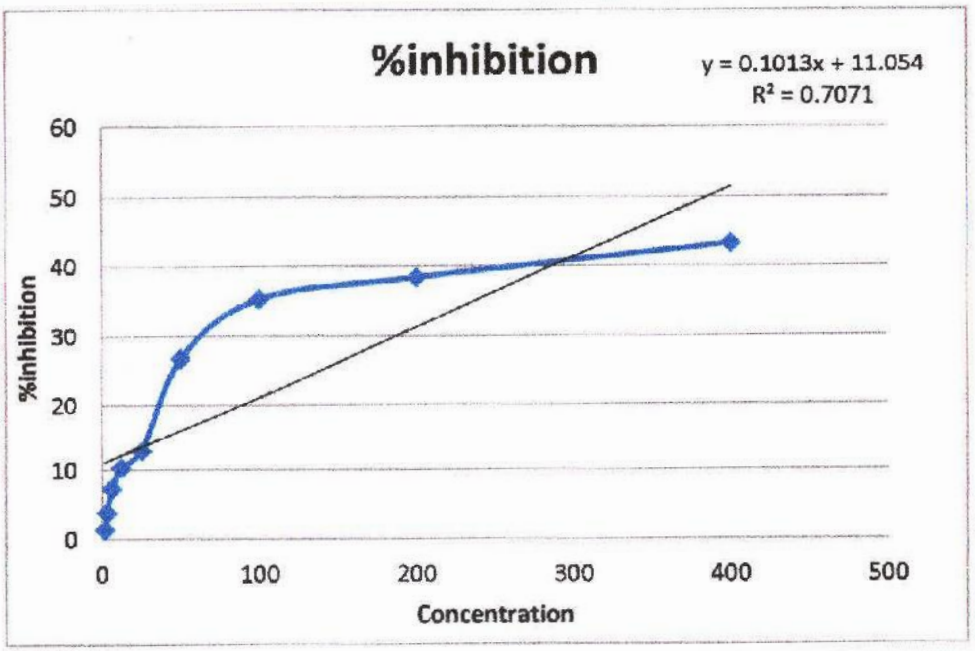


Figure 4.3.10: DPPH Scavenging assay of *Coriandrum sativum* (Dhoniya) ethanolic extract (%inhibition vs concentration).

Table 4.3.11: DPPH Scavenging assay of *Coriandrum sativum* (Dhoniya) aqueous extract

Concentration (µg/ml)	%inhibition	IC_{50} value (µg/ml)
400	.411	290.145
200	.504	
100	.622	
50	.717	
25	.815	
12.5	.844	
6.25	.873	

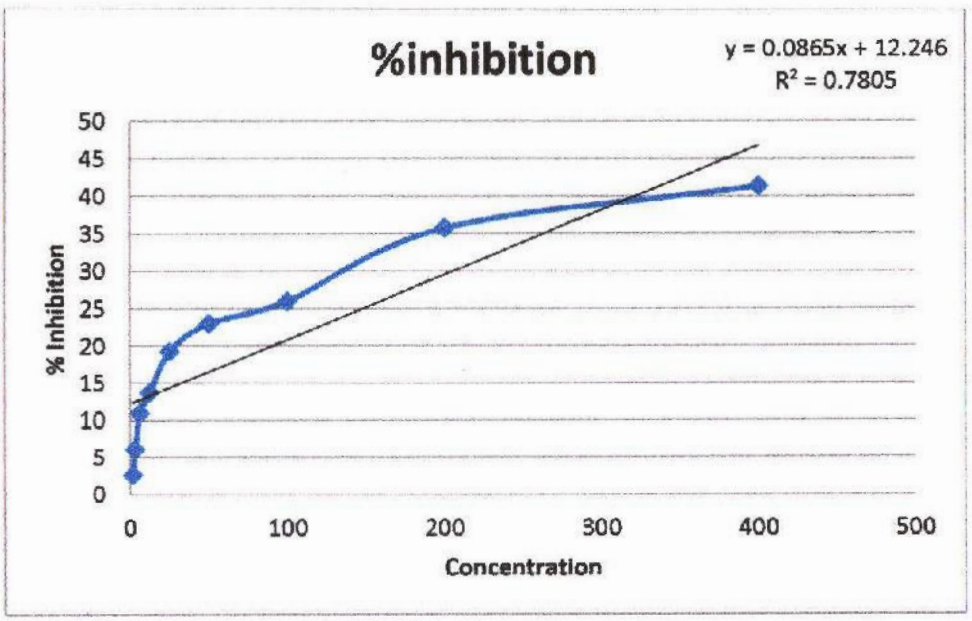


Figure 4.3.11: DPPH Scavenging assay of *Coriandrum sativum* (Dhoniya) aqueous extract (%inhibition vs concentration).

4.4 Reducing power assay

Reducing power of the extracts is determined by direct electron donation in the reduction of ferri cyanide $[Fe(CN)_6]_3$- to ferro cyanide $[Fe(CN)_6]_4$-. The product is visualized by addition of free Fe_{3+} ions after the reduction reaction, by forming the intense prussian blue color complex, $(Fe_{3+})_4[Fe_{2+}(CN-)_6]_3$ and quantified by absorbance measurement at 700 nm. The reducing power of the extracts increases with the increase in amount of sample. Increased absorbance of the reaction mixture indicates increased reducing power.

Table 4.4.1: Reducing power assay of Standard Ascorbic Acid

Concentration (µg/ml)	Absorbance ± SD	% Increase in reducing power
Blank	0.1483±0.0028	
25	0.3893 ± 0.0025	162.471
50	0.5473 ± 0.0020	268.988
100	0.7676 ± 0.0025	417.528
200	1.3756 ± 0.0020	827.415
400	2.3153 ± 0.00351	1460.898

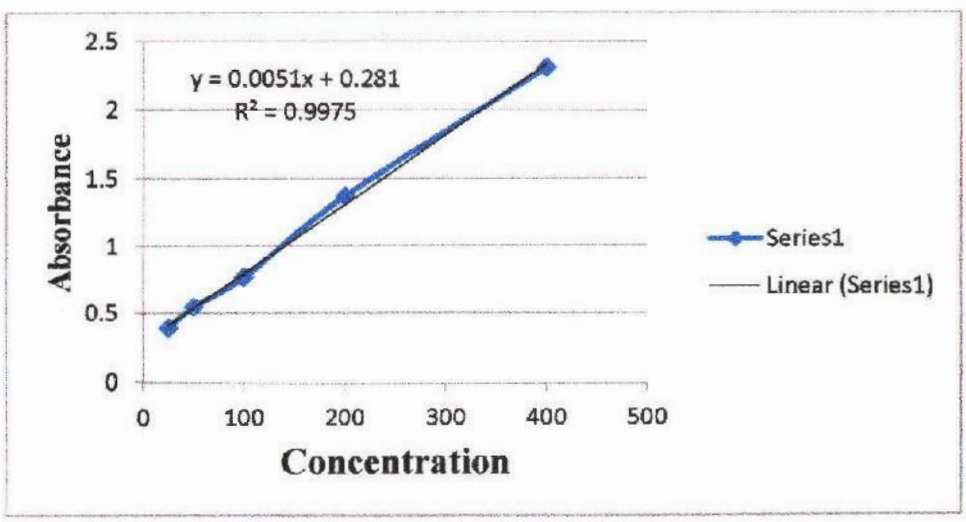


Figure 4.4.1: Reducing Ability of Ascorbic Acid at different concentration.

4.4.1: Ethanolic extract of *Z. officinile*

In the case of *Z. officinale* extract, table 4.4.2 shows that the ethanolic extract exhibited the ranges from 85.37 to 785.731 whereas standard ascorbic acid exhibited the ranges from 162.47 to 1460.89. Figure 4.4.2 shows the result of ethanolic extract of *Z. officinile.*

Table 4.4.2: Reducing power assay of ethanolic extract of *Z. officinile* .

Concentration (µg/ml)	Absorbance ± SD	% Increase in reducing power
Blank	0.1167	
25	0.207	85.373
50	0.3003	168.95
100	0.376	256.11
200	0.6533	486.86
400	0.9533	753.73

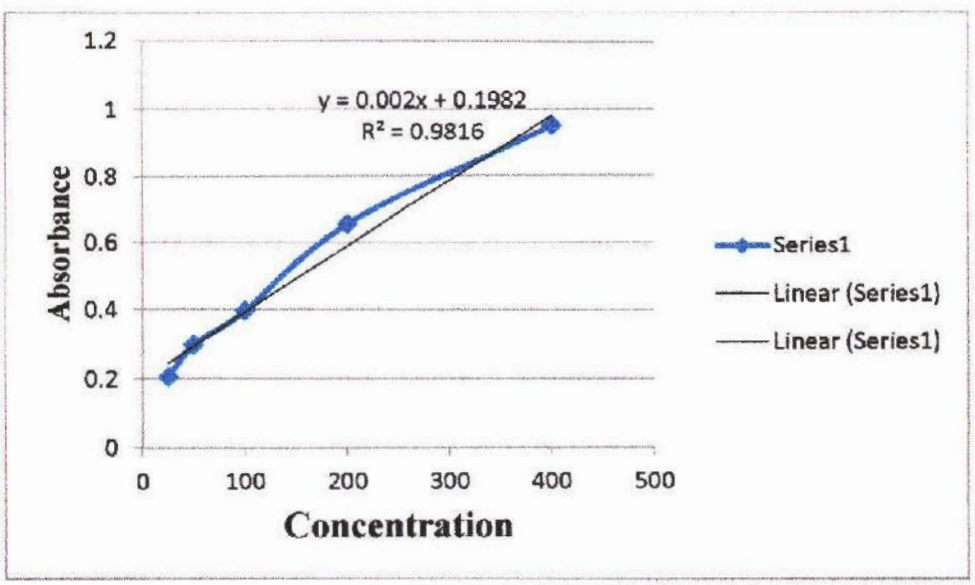


Figure 4.4.2: Reducing Ability of *Z. officinile* ethanolic extract , (concentration vs absorbance).

4.4.2: Aqueous extract of *Z. officinile*

In the case of *Z. officinale* extract, table 4.4.3 shows that the aqueous extract exhibited the ranges from 37.51 to 252.83 whereas standard ascorbic acid exhibited the ranges from 162.47 to 1460.89. Figure 4.4.3 shows the result of aqueous extract of *Z. officinile.*

Table 4.4.3: Reducing power assay of Aqueous extract of *Z. officinile* .

Concentration (µg/ml)	Absorbance ± SD	% Increase in reducing power
Blank	0.111	
25	0.154	37.91
50	0.167	49.55
100	0.180	61.19
200	0.207	85.97
400	0.394	252.83

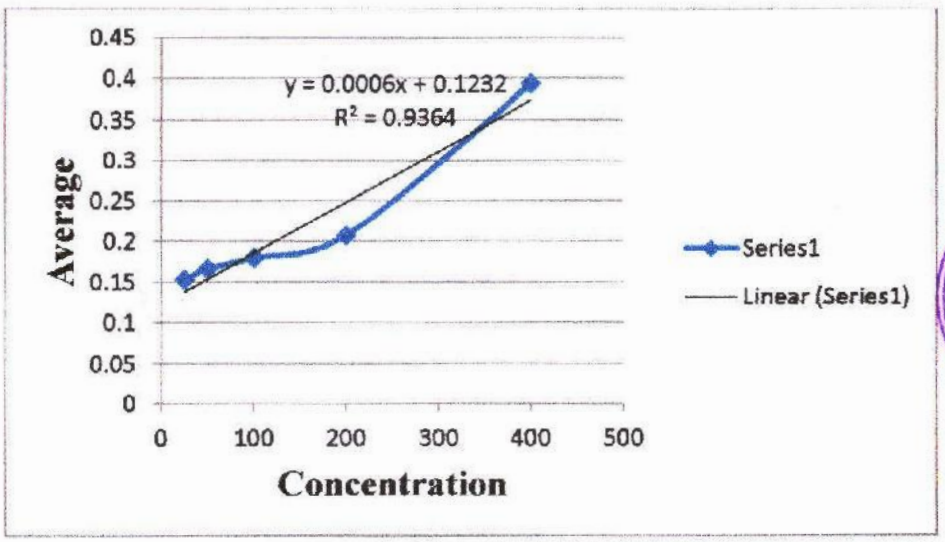


Figure 4.4.3: Reducing Ability of *Z. officinile* aqueous extract,(concentration vs absorbance).

4.4.3: Ethanolic extract of *A.sativum*

In the case of *A. sativum* extract, table 4.4.4 shows that the ethanolic extract exhibited the ranges from 110.029 to 745.7227 whereas standard ascorbic acid exhibited the ranges from 162.47 to 1460.89. Figure 4.4.4 shows the result of ethanolic extract of *A. sativum.*

Table 4.4.4: Reducing power assay of ethanolic extract of *A. sativum* .

Concentration (µg/ml)	Absorbance ± SD	% Increase in reducing power
Blank	0.113	
25	0.237	110.029
50	0.326	189.085
100	0.449	297.64
200	0.671	494.395
400	0.955	745.722

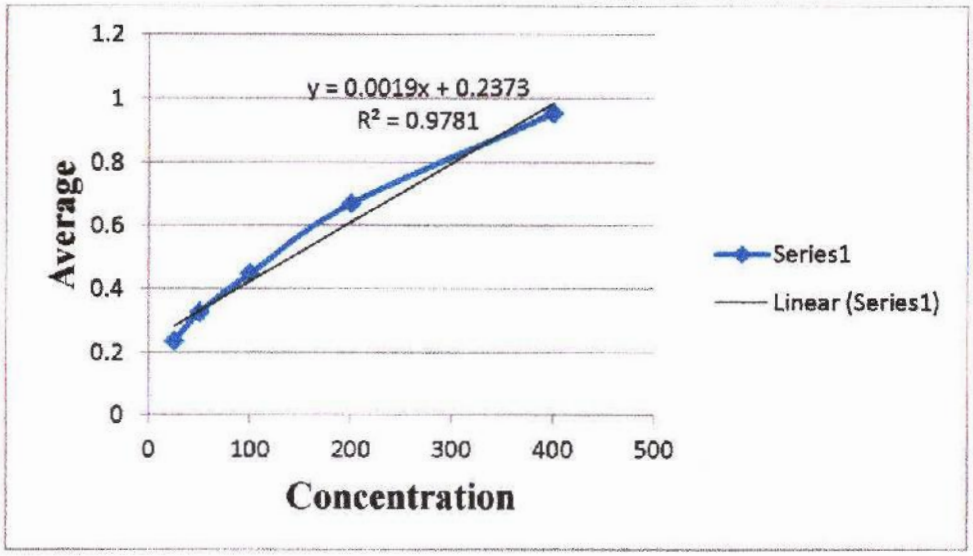


Figure 4.5.3: Reducing Ability of *A. sativum* ethanolic extract,(concentration vs absorbance).

4.4.4: Aqueous extract of *A.sativum*

In the case of *A. sativum* extract, table 4.4.5 shows that the aqueous extract exhibited the ranges from 9.73 to 218.73 whereas standard ascorbic acid exhibited the ranges from 162.47 to 1460.89. Figure 4.4.5 shows the result of aqueous extract of *A. sativum.*

Table 4.5.5: Reducing power assay of aqueous extract of *A. sativum.*

Concentration (µg/ml)	Absorbance ± SD	% Increase in reducing power
Blank	0.113	
25	0.124	9.73
50	0.133	17.69
100	0.163	44.24
200	0.260	130.38
400	0.360	218.87

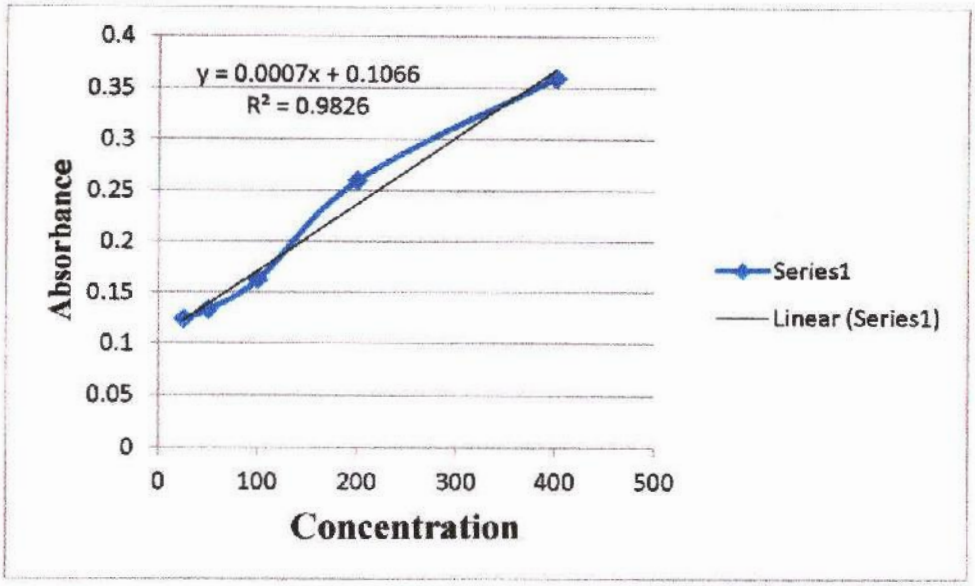


Figure 4.4.5: Reducing Ability of *A. sativum* aqueous extract,(concentration vs absorbance).

4.4.5: Ethanolic extract of *C. verum*

In the case of *C.verum* extract, table 4.4.6 shows that the ethanolic extract exhibited the ranges from 74.32 to 793.13 whereas standard ascorbic acid exhibited the ranges from 162.47 to 1460.89. Figure 4.4.6 shows the result of ethanolic extract of *C. verum.*

Table 4.4.6: Reducing power assay of ethanolic extract of *C. verum.*

Concentration (µg/ml)	Absorbance ± SD	% Increase in reducing power
Blank	0.111	
25	0.194	74.32
50	0.322	188.65
100	0.41	267.16
200	0.653	484.77
400	0.997	793.13

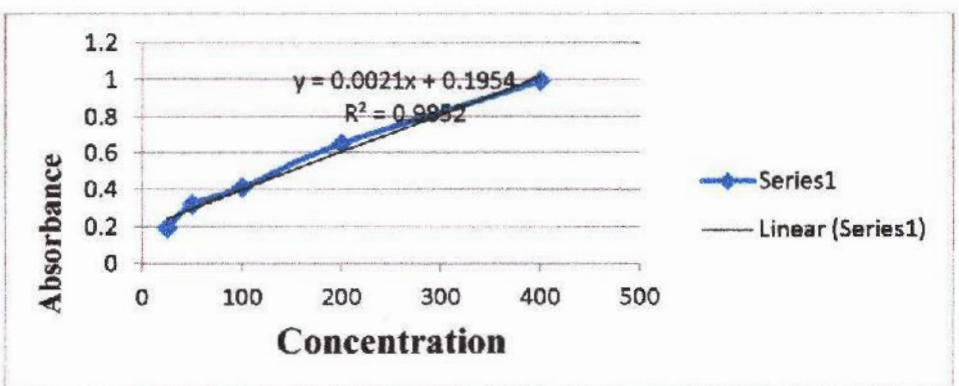


Figure 4.4.6: Reducing Ability of *C.verum* ethanolic extract,(concentration vs absorbance).

4.4.6: Aqueous extract of *C. verum*

In the case of *C. verum* extract, table 4.4.7 shows that the aqueous extract exhibited the ranges from 34.32 to 388.806 whereas standard ascorbic acid exhibited the ranges from 162.47 to 1460.89. Figure 4.4.7 shows the result of aqueous extract of *C. verum.*

Table 4.4.7: Reducing power assay of aqueous extract of *C. verum.*

Concentration (µg/ml)	Absorbance ± SD	% Increase in reducing power
Blank	0.111	
25	0.15	34.32
50	0.172	54.02
100	0.219	96.41
200	0.313	180.29
400	0.490	388.80

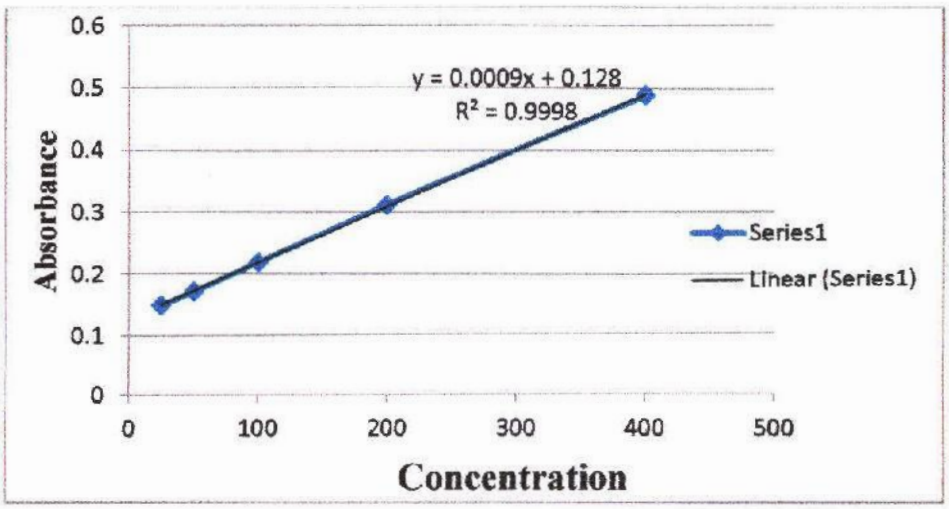


Figure 4.4.7: Reducing Ability of *C.verum* aqueous extract,(concentration vs absorbance).

4.4.7: Ethanolic extract of *C. longa*

In the case of *C. longa* extract, table 4.4.8 shows that the ethanolic extract exhibited the ranges from 96.46 to 574.63 whereas standard ascorbic acid exhibited the ranges from 162.47 to 1460.89. Figure 4.4.8 shows the result of ethanolic extract of *C. longa.*

Table 4.4.8: Reducing power assay of ethanolic extract of *C. longa.*

Concentration (µg/ml)	Absorbance ± SD	% Increase in reducing power
25	0.222	96.46
50	0.264	134.21
100	0.355	234.15
200	0.489	333.03
400	0.762	574.63

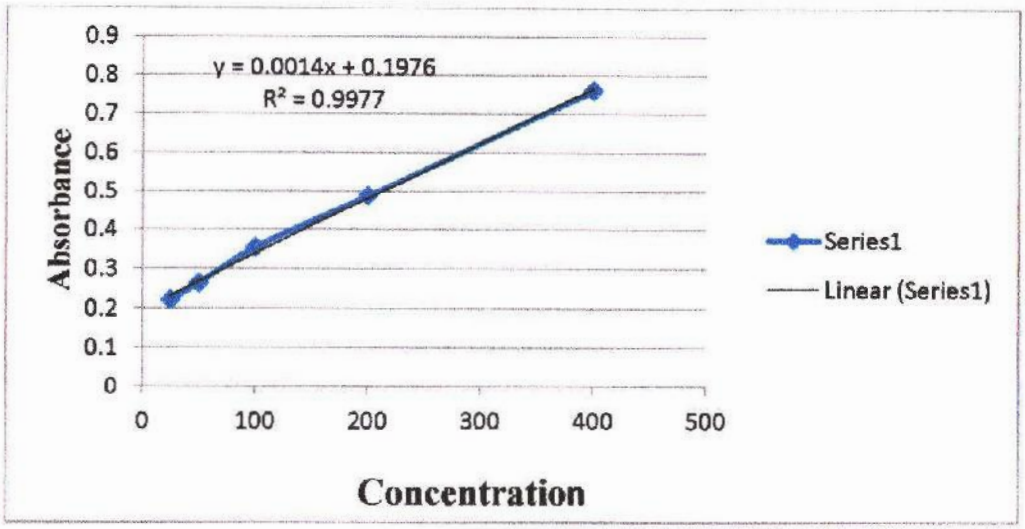


Figure 4.4.8: Reducing Ability of *C. longa* ethanolic extract,(concentration vs absorbance).

4.4.8: Aqueous extract of *C. longa*

In the case of *C. longa* extract, table 4.4.9 shows that the aqueous extract exhibited the ranges from 45.93 to 153.77 whereas standard ascorbic acid exhibited the ranges from 162.47 to 1460.89. Figure 4.4.9 shows the result of aqueous extract of *C. longa.*

Table 4.5.9: Reducing power assay of aqueous extract of *C. longa.*

Concentration (µg/ml)	Absorbance ± SD	% Increase in reducing power
25	0.222	
50	0.264	
100	0.355	234.15
200	0.489	333.03
400	0.762	574.63

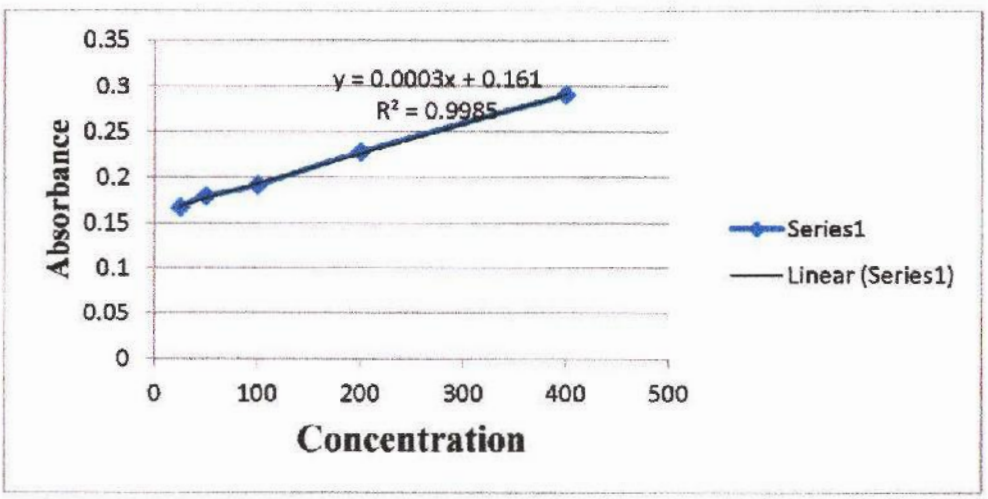


Figure 4.4.9: Reducing Ability of *C. longa* aqueous extract,(concentration vs absorbance).

4.4.9: Ethanolic extract of *C. sativum*

In the case of *C. sativum* extract, table 4.4.10 shows that the ethanolic extract exhibited the ranges from 25.77 to 423.29 whereas standard ascorbic acid exhibited the ranges from 162.47 to 1460.89. Figure 4.4.10 shows the result of ethanolic extract of *C. sativum.*

Table 4.4.10: Reducing power assay of ethanolic extract of *C. sativum.*

Concentration (µg/ml)	Absorbance ± SD	% Increase in reducing power
25	0.135	25.77
50	0.244	127.63
100	0.398	271.11
200	0.460	328.88
400	0.561	423.29

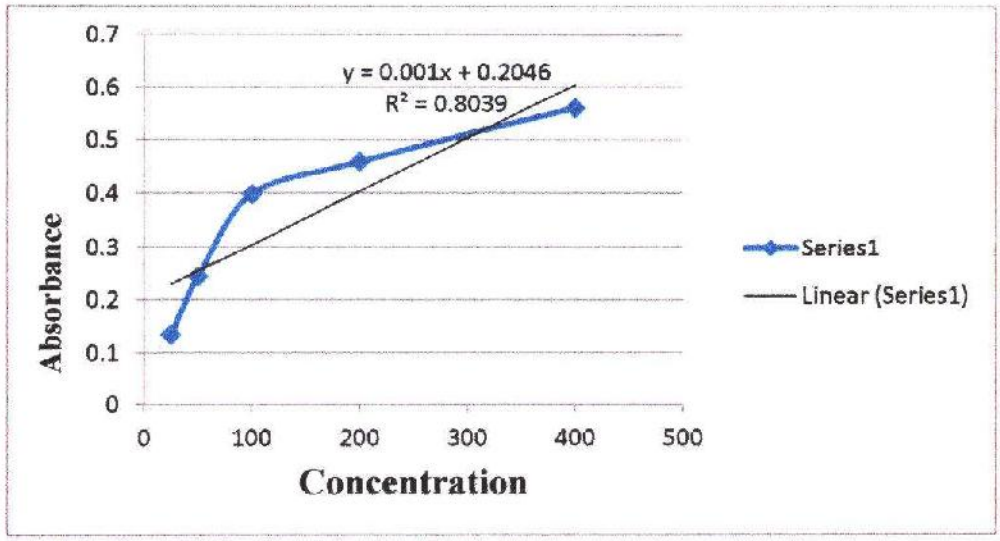


Figure4.4.10: Reducing Ability of *C.sativum* ethanolic extract,(concentration vs absorbance).

4.4.10: Aqueous extract of *C. sativum*

In the case of *C. sativum* extract, table 4.5.11 shows that the aqueous extract exhibited the ranges from 9.063 to 99.093 whereas standard ascorbic acid exhibited the ranges from 162.47 to 1460.89. Figure 4.5.11 shows the result of aqueous extract of *C. sativum.*

Table 4.4.11: Reducing power assay of aqueous extract of *C. sativum.*

Concentration (µg/ml)	Absorbance ± SD	% Increase in reducing power
25	0.135	25.77
50	0.244	127.63
100	0.398	271.11
200	0.460	328.88
400	0.561	423.29

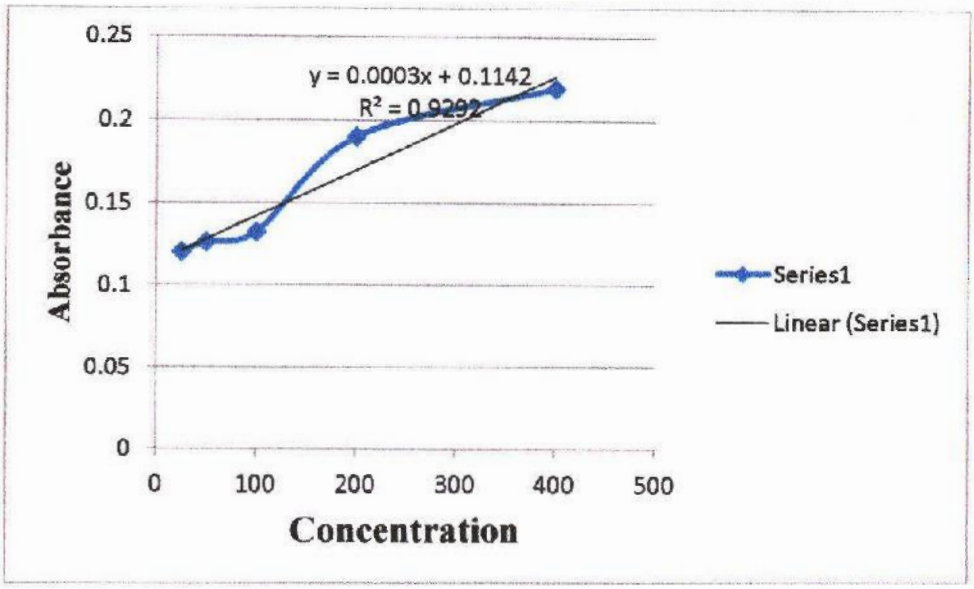


Figure4.4.11: Reducing Ability of *C. sativum* aqueous extract,(concentration vs absorbance).

4.5 FRAP (ferric reducing antioxidant power) Assay

The total antioxidant potential of sample was determined using FRAP assay as a measure of antioxidant power. FRAP assay measures the change in absorbance at 593 nm owing to the formation of a blue colored FeII-tripyridyltriazine compound from colorless oxidized FeIII form by the action of electron donating antioxidants. In this research work, the total antioxidant potential of was determined by using FRAP reagent with standard $FeSO_4.7H_2O$. Standard curve was prepared using different concentrations (400-25) µg/ml. In the FRAP assay the antioxidant efficiency of the antioxidant under the test was calculated with reference to the reaction signal given by an Fe^{2+} solution of known concentration, this representing a one-electron exchange reaction. The results were expressed in µM Fe (II)/100g dried plant material using the equation $y = 0.004x + 0.129$, coefficient $R^2 = 0.991$, where y is absorbance at 593 nm and x is the total antioxidant potential of different extracts. Absorbance of $FeSo_4.7H_2O$ in different concentration is given in table below.

Table 4.5.1: Absorbance of standard FeSO4. 7H2O at different concentration

Concentration (µg/ml)	Avg. of Absorbance (593 nm)	Absorbance ± SD
400	1.86	1.8673 ± 0.0025
200	1.067	1.067 ± 0.011
100	.566	0.5697 ± 0.0040
50	.353	0.353 ± 0.0072
25	.210	0.2107 ± 0.0035
Blank	0.0955	

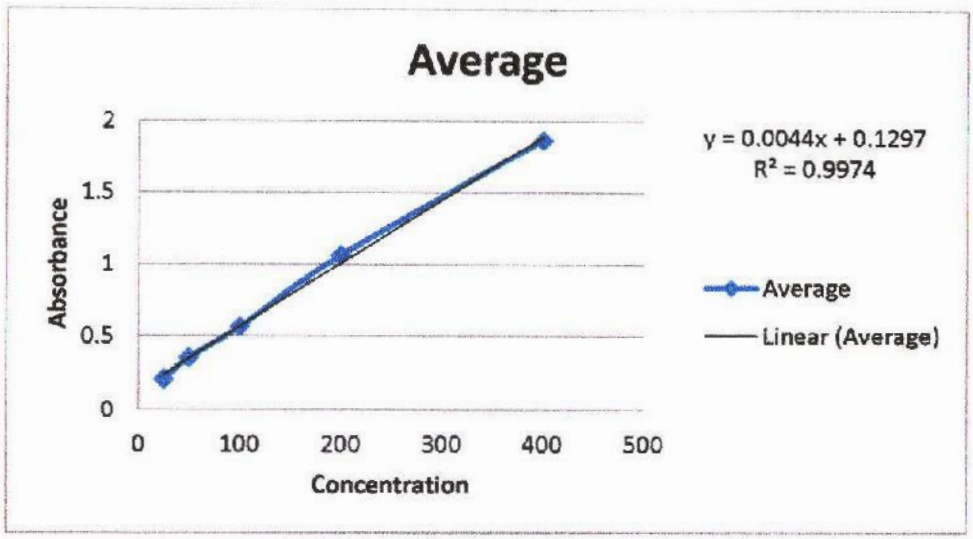


Figure 4.5.1: Calibration curve of standard $FeSo_4.7H_2O$ for determination of FRAP assay.

Table 4.5.2: FRAP value of Spices extracts (ethanolic and aqueous)

Sample	R1	R2	R3	Average	SD	FRAP
Ascorbic acid	2.557	2.651	2.782	2.66333	0.11300	575.825
Z. officinile Aq.	1.217	1.255	1.232	1.23466	0.01914	251.128
Z. officinile Et.	1.479	1.478	1.519	1.492	0.02338	309.613
A. sativum Aq.	1.173	1.154	1.306	1.211	0.08281	245.75
A. sativum Et.	1.233	1.246	1.237	1.23866	0.00665	252.037
C. sativum Aq.	1.18	1.181	1.33	1.23033	0.08631	250.143
C. sativum Et.	1.205	1.237	1.342	1.26133	0.07166	257.189
C. longa Aq.	1.223	1.221	1.225	1.223	0.002	248.477
C. longa Et.	1.347	1.35	1.348	1.34833	0.00152	276.962
C. verum Aq.	1.79	1.62	1.814	1.74133	0.10576	366.280
C. verum Et.	1.79	1.75	1.81	1.78333	0.03055	375.825

4.6 Total Phenolic Content

The amount of total phenol was determined with the Folin-Ciocalteu assay using Gallic acid as a standard phenolic compound. This method is a fast and simple method and can be useful in characterizing and standardizing botanical samples. Folin-Ciocalteu method is based on oxidation of phenolics by a molybdotungstate in Folin-Ciocalteu reagent to yield a colored product with max 745 – 750 nm [Javanmardi *et al* .2003].

A linear calibration curve of Gallic acid, in the range of 25–250µg/ml with coefficient of determination (R2) value of 0.968 was obtained. Absorbance of Gallic acid in different concentration is obtained and from which calibration curve plotted in figure 4.13. Using this calibration curve, the total amount of phenolic content of plant extract was determined using the equation y = 0.002x – 0.036, where y is the absorbance at 750 nm and x is total phenolic content in the different plant extracts.

Table 4.6.1 : Absorbance of standard gallic acid at different concentration

Concentration (µg/ml)	Avg. of Absorbance (750 nm)	Absorbance ± SD
250	0.481333	0.481333 ± 0.0065
200	0.436667	0.436667 ± 0.0073
150	0.365	0.365 ± 0.0060
100	0.256333	0.256333 ± 0.0011
50	0.139	0.139 ± 0.009539
25	0.052467	0.052467 ± 0.00023

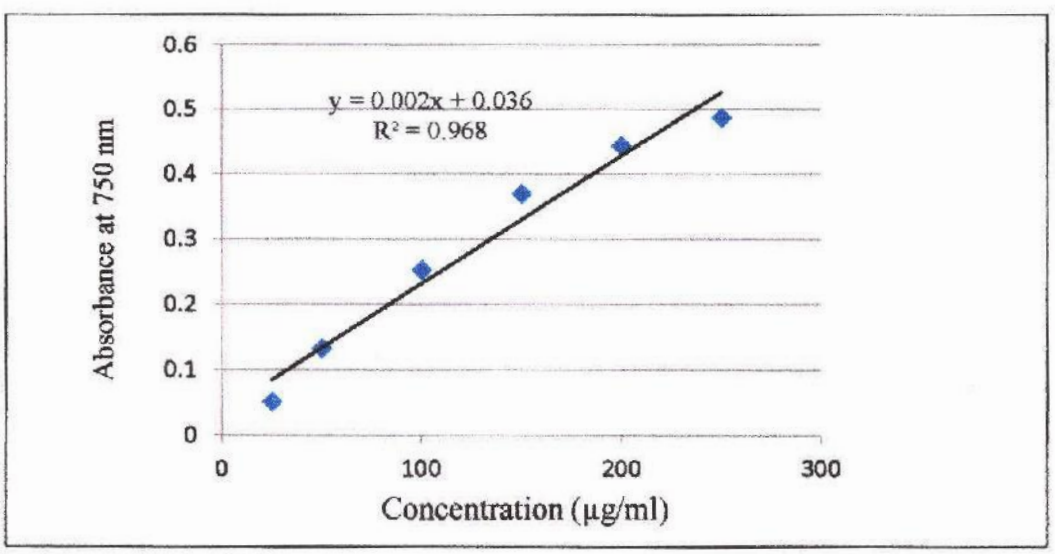


Figure 4.6: Calibration curve of standard Gallic acid for determination of total phenolic contents.

Table 4.7.2: Total phenolic content

Extract	Conc. (µg/ml)	Total Phenolic Content (mgGAE/g of extract)
Z. officinale (Ada) [Ethanol]	100	153.33 ****
Z. officinale (Ada) [Aqueous]	100	100.33 ****
A.sativum (Rosun) [Ethanol]	100	86.66 ****
A.sativum (Rosun) [Aqueous]	'100	65.67 ****
C. verum (Darcini) [Ethanol]	100	76.17 ****
C. *verum* (Darcini) [Aqueous]	100	63.50 ****
C. longa (Holud) [Ethanol]	100	48.83 ****
C. longa (Holud) [Aqueous]	100	44.50 ****
C. sativum (Dhoniya) [Ethanol]	100	21.166 ****
C. sativum (Dhoniya) [Aqueous]	100	17.18 ****

******= P value is <0.0001, The Statistical analysis done with one way ANOVA**

4.8 Total Flavonoid Content

A linear calibration curve of Quercetin, in the range of (400-25) µg/ml with coefficient of determination (R2) value of 0.984was obtained. Absorbace of Quercetin in different concentration was obtained from which calibration curve plotted in figure 4.14. Using this calibration curve, the total amount of flavonoid content of plant extract was determined using the equation y = 0.001x + 0.023, where y is the absorbance at 510 nm and x is total flavonoid content in the different plant extracts.

Table 4.8.1 : Absorbance of standard quercetin at different concentration

Concentration (µg/ml)	Avg. of Absorbance (510 nm)	Absorbance ± SD
Blank	0.076	
400	0.718	0.718 ± 0.001
200	0.369	0.369 ± 0.013
100	0.258	0.258 ± 0.002
50	0.093	0.093 ± 0.003
25	0.04	0.04 ± 0.006

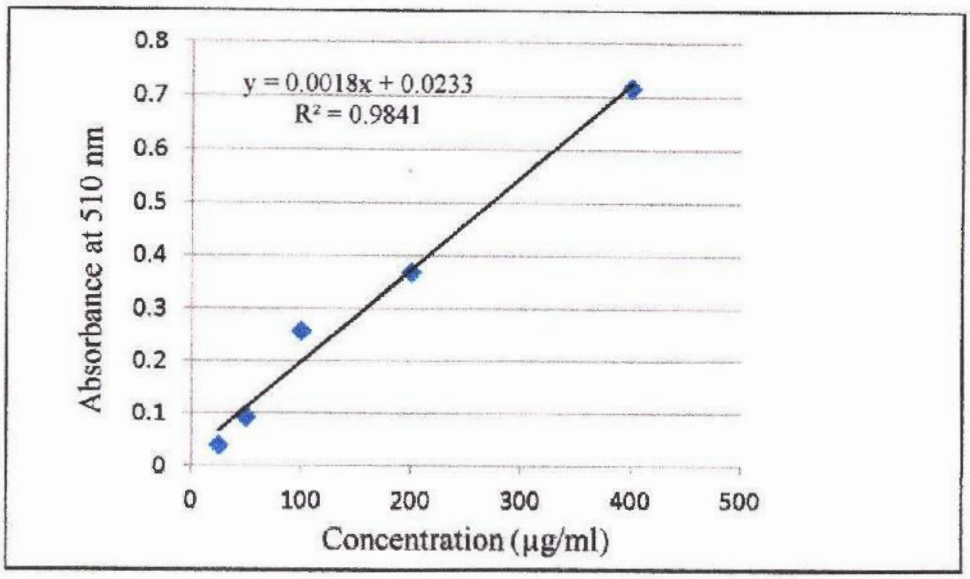


Figure 4.8: Calibration curve of standard Quercetin for determination of total flavonoid contents.

Table 4.8.2: Total Flavonoid content of Different Spices (with statistical analysis)

Extract	Conc. (µg/ml)	Total Flavonoid Content (mg QE/g of extract)
Z. officinale (Ada) [Ethanol]	100	45.01 ****
Z. officinale (Ada) [Aqueous]	100	35.94 ****
A.sativum (Rosun) [Ethanolic]	100	41.5 ****
A.sativum (Rosun) [Aqueous]	100	36.87 ****
C. verum (Darcini) [Ethanol]	100	47.05 ****
C. verum (Darcini) [Aqueous]	100	39.64 ****
C. longa (Holud) [Ethanol]	100	32.61 ****
C. longa (Holud) [Aqueous]	100	17.79 ****
C. sativum (Dhoniya) [Ethanol]	100	18.35 ****
C. sativum (Dhoniya) [Aqueous]	100	14.64****

******= P value is <0.0001**

The Statistical analysis done by one way ANOVA (Graph Pad Prizom)

Statistical Analysis

Level of Significance of Antioxidant test

The statistical analysis done with two way Anova (graph pad prizom)

Sample	DPPH assay (µg/ml)	FRAP assay (µM Fe(II)/g of dried plant material)	Reducing power assay (µg/ml)
Z. officinale (Ada) [Ethanol]	158.899 ****	309.613 ****	153.608 ****
Z. officinale (Ada) [Aqueous]	237.916 ****	251.1288 ****	599.888 ****
A.sativum (Rosun) [Ethanol]	185.684 ****	252.0379 ****	163.109 ****
A.sativum (Rosun) [Aqueous]	203.343 ****	245.75 ****	617.557 ****
C. verum (Darcini) [Ethanol]	46.462 ****	365.825 ****	147.509 ****
C. *verum* (Darcini) [Aqueous]	155.050 ****	366.280 ****	409.348 ****
C. longa (Holud) [Ethanol]	228.334 ****	276.962 ****	217.423 ****
C. longa (Holud) [Aqueous]	290.145 ****	248.477 ****	1040.531 ***
C. sativum (Dhoniya) [Ethanol]	384.356 ****	257.189 ****	353.679 ****
C. sativum (Dhoniya) [Aqueous]	436.333 ****	250.143 ****	1403.583 ***

******= P value is <0.0001**

4.9: Discussion on Antioxidant activity related assay

Antioxidants, inhibitors of lipid peroxidation, are important not only for food protection but also for the defense of living cells against oxidative damage.

The present study was an attempt to investigate antioxidant activities of *Z.officinale, A. sativum C. verum, C. longa, C. sativum* ethanolic and aqueous extracts. *C. verum* ethanolic extract showed maximum inhibitory activity (IC50~46.462.µg/ml) against the DPPH free radical among all extracts, whereas *Z. officinale, A. sativum, C. longa, C. sativum* extracts did lower exhibit scavenging activity with the IC50 value of 158.68 µg/ml and 185.684 µg/ml, 228.33µg/ml 384.35µg/ml, respectively. In parallel, the IC50 value 17.1046 µg/ml of Qurcetin, was also estimated. The antioxidant values of *C. verum* extract ranged from 46.46 ± 0.004% and 155.05 ± 0.015% DPPH radical inhibition Moreover, there is an agreement with previous study which showed that at concentration 50 µg/ml, 50% ethanolic extract inhibited 82.8% DPPH free radical [El-Ghorab, 2010], whereas current study exhibited 79 % inhibition at same concentration of *C. verum* ethanolic extract.

It is an issue whether the antioxidant capacity of these extracts shown in this scavenging assay is specific to DPPH radical or not. To gain insight into the heterogenous antioxidant capability of the extracts, ferric reducing capability was determined. For this purpose, reducing power assay using potassium hexacyanoferrate and ferric reducing antioxidant power (FRAP) assay using tripyridyltriazine (TPTZ) were conducted followed by DPPH scavenging assay.

Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity. Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants [Jung et al. 2008].This assay has proven that the effectiveness of this five spices extracts both ethanolic and aqueous. Literature reports are evident that the reducing power of bioactive compounds is associated with antioxidant activity. The presence of reductants (i.e. antioxidants) in extracts causes the reduction of the Fe3+/ferricyanide complex to the ferrous form. Therefore, the Fe2+ can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. In the concentration range investigated, all the extracts demonstrated reducing power that increased linearly with concentration. In case of ethanolic extract of *C. verum*, the maximum absorbance is found (0.9973 ± 0.0173) at 400 µg/ml concentration; whereas (2.315 ± 0.0035) is found for standard

ascorbic acid. In case of ethanolic extract of *Z. officinile* the absorbance is found (0.953± 0.0771) at 400 µg/ml concentration; whereas (2.315 ± 0.0035) is found for standard ascorbic acid. The ethanolic extract showed more reducing power activity in compare with the aqueous extract of those spices. *C.verum* possesses moderate reducing potential in compared to the standard Ascorbic acid. Table 4.5.7 and figure 4.5.7 showed the reducing ability of *C. verum*. All these five spices in case of ethanolic extract the reducing ability like in this way *C. verum* > *Z. officinale* > *A. sativum* > *C. longa* > *C. sativum*. In case of aqueous extract the ability of reducing is *C. verum* >*Z. officinale* >*A. sativum* >*C. longa* >*C. sativum*.

In the present investigation, ethanolic extract of *C. verum* possess highest reducing potentiality and highest scavenging activity in DPPH assay in compared to all extracts. The reducing power of the extract might be due to their hydrogen donating ability. Possibly, ethanolic extract of *C.verum* contain high amounts of reductive, which could react with radicals to stabilize and terminate radical chain reactions.

FRAP assay measure the reducing potential of an antioxidant reacting with a ferric tripyridyltriazine [Fe3+-TPTZ] complex and producing a colored ferrous tripyridyltriazine [Fe2+-TPTZ] [Guo *et al.*, 2003]. Generally, the reducing properties are associated with the presence of compounds which exert their action by breaking the free radical chain by donating a hydrogen atom. FRAP assay treats the antioxidants in the sample as a reductant in a redox-linked colorimetric reaction [Ercan *et al.*, 2011].

The reducing capacity of an extracts may serve as a significant indicator of its potential antioxidant activity. FRAP assay showed variability between these extracts. In this research using FRAP assay from the quantitative estimation of total antioxidant potential of *C. verum* ethanolic and aqueous extract exhibited 375.82 ±0.030551 and 366.280 ±0.10576 µM Fe (II)/ 100g of dried plant material respectively. Ethanolic extract of *C. verum* showed significant antioxidant power, whereas ascorbic acid which has potential antioxidant potential showed 575.825 ±0.11300. Investigating the results of the present study, *C. verum* ethanolic extract showed the slightest antioxidant capacity which is closest to ascorbic acid.
In this research work, previous assays also showed that the ethanolic extract had a greater antioxidant potentiality than the aqueous extract.

Phenolics or polyphenols are secondary plant metabolites that are ubiquitously present in plants and plant products. Many of the phenolic compounds have been shown to contain high levels of antioxidant activities [Razali *et al.*, 2008]. The antioxidant properties of phenolic

compounds originate from their properties of proton loss, chelate formation and dismutation of radicals. In fact, in some studies, theoretical methods have been proposed to estimate the antioxidant activities of phenolic substances. Phenols are compounds that have the ability to destroy radicals because they contain hydroxyl groups [Das, N. and T. Pereira, 1990]. Antioxidant activity of flavonoids depends on the structure and substitution pattern of hydroxyl groups [Sharififar, *et al.*, 2009]. They are gaining interest due to their wide variants and number of members. Flavonoids are powerful antioxidants against free radicals and are described as free-radical scavengers [Pal, R.S., *et al.*, 2009]. The presence of an ortho-dihydroxyl structure in phenolics is largely responsible for their excellent anti-radical activity. 3-Hydroxyl was also essential to generate a high radical-scavenging activity. An increasing number of hydroxyls on flavones with a 3',4'-dihydroxyl basic structure, the presence of a third hydroxyl group at C-5', a phloroglucinol structure, glycosylation and methylation of the hydroxyls, and some other hydroxyls, for example 5-, and 7-hydroxyl in ring A, decreased the radical-scavenging activities of flavonoids and other phenolics [Zheng, C.-D., *et al.*, 2010]. These important plant components give up hydrogen atoms from their hydroxyl groups to radicals and form stable phenoxyl radicals; hence, they play an important role in antioxidant activity [HATANO, T., *et al.*, 1989]. Tannin compounds are polyphenolic compounds that are very complex and widely distributed in almost all plant species [Okuda, T. 2005]. These complex polyphenolic compounds are soluble in water and organic solvents [Haslam 1996].

The quantitative estimation of total phenolic content of ethanolic extract of *Z. officnale* exhibited 153.33 ± 0.006 mg GAE/g of extract. And other four ethanolic extracts of *A.sativum* ,*C. verum, C. longa, C.sativum* showed lower amount of phenol containing 86.66 ± 0.0064 mg GAE/g , 76.166 ± 0.0040 mg GAE/g ,48.83± 0.00251 mg GAE/g, 21.16± 0.0170 mg GAE/g of extract respectively.

Phenolic compounds of plants fall into several categories; flavonoids are the most dominant class of phenolic compounds among these categories which have potent antioxidant activities [Nunes, X.P., *et al.*, 2012]. Compering the spices it was found that *C. verum* has the highest total flavonoid content and *Z. officinale* near to *C. verum*.

Chapter 5

5. Conclusion

Spices are potentially of great commercial and food value in our daily life. They not only enhance the flavor, aroma, taste, color but also the have a good nutrition quality and pharmacological activity. From the ancient period spices used as traditional medicine. Commonly used five spices in Bangladeshi cooking were targeted to make samples; antioxidant and anticoagulation activities were found at different levels by giving a comparative evaluation. All the extracts showed varying degrees of anticoagulation as well as antioxidant activity on the test methods. In this present study, an investigative extraction was conducted for different plant of spices family viz. *Z. officinale* , *A. sativum, C. verum, C. longa* and *C. sativum,* using the polar solvent ethanol and water to obtain an extract containing varying polar compounds which possess anticoagulant and antioxidant activities. In this study, the aqueous extracts of plants exhibited low to significant levels of anticoagulant and antioxidant activities in prothrombin time (PT) assay, protease (serine) inhibitory assay, DPPH, ferric ion reducing power assay, phenol and flavonoid assay. The ethanolic sample exhibited high significant level of anticoagulation and antioxidant assays. The investigation result showed that *A. sativum* exhibited the most significant anticoagulation activities among the extracts. *C. verum* exhibited the most significant serine protease inhibitory activity and antioxidant activities also. The extracts of *C. longa, C. sativaum* showed comparatively low significant value than other extract of *Z. officinally, A.sativum, C. verum. A. sativum* and *Z. officinale* showed antioxidant activity near to *C.verum.* It is suggested that *A. sativum* can be the replacement of anticoagulation drugs. It can be the home remedy, safe, without side effects and cheaper than anticoagulant drug (warfarin). *C. verum* can be used as a protease inhibitor more specifically serine protease inhibitor. Furthermore, the distinctive results of this study provide baseline information for the potential and constructive use of these plants. It also generates our anticipation that detailed investigation may guide to the isolation of interesting pharmaceuticals of plant origin by the identification of compounds responsible for respective activity. Since the assays of current study were conducted in vitro, investigation for pharmacological activity may lead to the evaluation of the spices through in vivo.

Chapter Six

Chapter 6

6 References

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