PHYTOCHEMICAL INVESTIGATION OF *SYZYGIUM CUMINI* SEEDS AND ITS IMPACT IN AMELIORATING LIFE STYLE ASSOCIATED DISEASES

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Certificate

*This is to certify that the work embodied in the thesis entitled "**Phytochemical investigation of Syzygium cumini seeds in ameliorating lifestyle associated diseases**" has been carried out by **Ms. Syama. H. P** under my supervision at Agroprocessing and Technology Division, National Institute for Interdisciplinary Science and Technology, CSIR, Thiruvananthapuram, in partial fulfilment of the requirements for the award of the Degree of Doctor of Philosophy in Biotechnology, under the Faculty of Science, Cochin University of Science and Technology, and the same has not been submitted elsewhere for any other degree.*

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Declaration

*I hereby declare that the work embodied in the thesis entitled "***Phytochemical investigation of Syzygium cumini seeds in ameliorating lifestyle associated diseases***" is the result of the investigations carried out by me, at the Agroprocessing and Technology Division, National Institute for Interdisciplinary Science and Technology (formerly Regional Research Laboratory), CSIR, Thiruvananthapuram, under the supervision of Dr. P Jayamurthy and the same has not been submitted elsewhere for any other degree.*

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Dedicated to all my teachers & family members

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Preface

Lifestyle diseases are characterized as those diseases which occur with the way people live their life. The risk factors for these diseases are mainly tobacco use, unhealthy diet and physical inactivity. Diseases that impact on our lifestyle are mainly heart disease, type II diabetes and their associated problems like inflammation. Currently, synthetic drugs are used for the treatment of these diseases that are known to have adverse effects such as kidney complications, stomach upset, diarrhoea, dizziness, skin rashes, etc. There is a continuous drive globally to investigate more effective agents as alternatives with comparatively lesser side effects for the management of these diseases. Natural compounds from traditional plants are the attractive alternatives for synthetic drugs, which can efficiently counter the side effects, high cost and poor availability.

Epidemiological studies showed that there exist a positive correlation between consumption of fruits and vegetables and delayed onset and progression of life style associated diseases. Thus plant based health care is gaining more importance. Traditional plants that are known to have therapeutic potential are lacking scientific validations. Modern techniques are now available for the scientific investigation and identification of active principles from medicinal plants.

The phytochemical content of active fraction is subject to large variations due to variety, age, maturity of the plants used, season, geo-agro-climatic conditions, agronomical practices, post-harvest handling, storage, processing, etc. The active principle thus can vary tremendously and that, in turn, would affect the biopotency. It is in this context that chemical profiling of the plants is important to produce products with consistent quality.

Syzygium cumini is the plant selected for the present study. It is a tropical plant of Myrtacea family, found predominantly in India, Pakistan, Sri Lanka and Malaysia. Traditionally the plant products have been used in the treatment of diabetes and various other ailments. Among the several anatomical parts and whole plant studied by various researchers, seed is found to be the most important as evidenced by the volume of data generated by standardized analytical methods. However, the major literature gap found is the lack of knowledge about the detailed mechanism of action of *Syzygium cumini* seed in diseases like diabetes, cardiovascular diseases and inflammation. Therefore, the present study focuses on the mechanism of action of *Syzygium cumini* seeds and its fractions in ameliorating diseases such as diabetes, cardiovascular diseases and inflammation.

Chapter 1 gives a general introduction about lifestyle associated diseases including inflammation, cardiovascular diseases and diabetes. It also describes about *Syzygium cumini* and the phytochemical importance of its different parts.

Chapter 2 deals with the extraction procedures and the antioxidant potential of three different geographical variants (Trivandrum-TVM, Trichy-TCH, Malampuzha-MPA) of *Syzygium cumini* seeds and comparision of phenolic profiling in order to know the effect of geographical variation in phenolic composition. Total phenolic and flavonoid content of *Syzygium cumini* seeds were analyzed. Antioxidant activities in terms of DPPH, ABTS, nitric oxide and superoxide radical scavenging assays were performed. The most active fractions were subjected to HPLC profiling to identify the phenolic composition. TVM variant of *Syzygium cumini* seed showed potent antioxidant activity among all the variants. There existed a linear correlation between phenolic content and antioxidant activity. HPLC profiling of different fractions of TVM variant revealed the presence of phenolic compounds with high concentrations of ellagic acid and gallic acid. The presence of phenolics in

different fractions of TVM variant were confirmed by LC-MS/MS data. The differences in phenolic concentration due to geographical changes might be the reason for higher antioxidant potential showed by 70% methanol fraction of TVM variant. The study revealed that 70% methanolic fraction of *Syzygium cumini* can act as a novel source of natural antioxidant. An attempt was made to isolate and identify the compounds present in 70% methanol fraction of TVM variant. A compound named β-sitosterolglucoside was isolated and confirmed by NMR and HRMS data.

Chapter 3 explores the cardioprotective effect of *Syzygium cumini* seed fractions in modulating angiotensin converting enzyme (ACE), HMG-CoA reductase, LDL oxidation and tertiary butyl hydrogen peroxide (TBHP) induced oxidative stress in H9c2 cardiac cell lines. *Syzygium cumini* effectively attenuated the cellular oxidative stress in H9c2 cardiomyoblasts. These fractions possess inhibitory potential against ACE, HMG-CoA reductase and LDL oxidation. Molecular docking studies of the predominant polyphenols with ACE and HMG-CoA proteins revealed the binding interactions of these compounds, thus confirming their modulation of activity. The present study demonstrated the cardioprotective efficacy of *Syzygium cumini* seed fractions which can be attributed to the presence of phenolic acids and flavonoids.

Chapter 4 deals with the antidiabetic potential of fractions of *Syzygium cumini* seeds and its mechanism of action. Potential α-glucosidase and α-amylase inhibition was demonstrated by 70% methanol fraction. The highest antiglycation potential was exhibited by ethyl acetate fraction. The methanol fraction showed potent DPP-IV inhibition. Major phenolics identified were docked with target enzymes of diabetes investigated in the present study and analyzed. A four-fold increase in 2-NBDG uptake in L6 skeletal muscle cells following the pretreatment of 70% methanol fraction further confirmed the antidiabetic

potential of *Syzygium cumini* seeds. Western blot analysis of key signaling molecules involved in IRS-1 pathway and the gene expression analysis of GLUT 4, IRS-1, PI3K, PDK-1 confirmed the molecular mechanism behind the antidiabetic activity. These results suggest that *Syzygium cumini* seed fractions, rich in flavonoids and phenolics, have potential in the management of diabetes.

Chapter 5 deals with the anti-inflammatory activity of *Syzygium cumini* seeds and to reveal the underlying mechanism of action which would form an additional proof to the traditional knowledge of this plant. The anti-inflammatory potential was studied in ethyl acetate, methanol and 70% methanol fractions of *Syzygium cumini*. The anti-inflammatory effects of *Syzygium cumini* were studied by using lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. *Syzygium cumini* seeds effectively reduced the production of nitric oxide (NO) and pro-inflammatory cytokines. Results showed that *Syzygium cumini* inhibited LPS-activated lipoxygenases and reduced the levels of iNOS and COX-2 mRNA expression in murine macrophages. These effects were mediated by impaired translocation of nuclear factor (NF)-κB and inhibition of the phosphorylation of JNK, ERK and p38 in LPS-stimulated RAW 264.7 cells. This study gives scientific evidence that *Syzygium cumini* seeds inhibits the LPS-induced expression of inflammatory mediators via suppression of JNK, ERK and p38 mediated NF-κB pathway as well as down regulation of iNOS and COX-2, indicating that *Syzygium cumini* seeds have a potential application against inflammation.

Chapter 6 summarizes the work with main summary and conclusions.

CHAPTER 1

Introduction

1.1. INTRODUCTION

Lifestyle diseases are diseases that are linked with the way people live their life. They include arthritis, atherosclerosis, asthma, cancer, liver cirrhosis, type 2 diabetes, cardiovascular disease, stroke, obesity, depression, etc. These lifestyle diseases are preventable and can be controlled with changes in diet, lifestyle, and environment. The lifestyle diseases are insidious, as they take years to develop, and once encountered, do not lend them to cure easily (Sochaliya *et al*., 2012). There are controllable risk factors and uncontrollable risk factors that can lead to lifestyle diseases. Controllable risk factors include unhealthy diet, lack of physical exercise, use of alcohol and smoking habits. Uncontrollable risk factors include age, heredity, gender, ethnicity, etc.

A report, jointly prepared by the World Health Organization and the World Economic Forum, says that due to unhealthy lifestyles and faulty diet, an accumulated loss of $236.6 billion occurred in India during 2015. The income loss to Indians because of chronic diseases like heart disease, stroke, cancer, diabetes and respiratory infections, was $8.7 billion in 2005, is projected to rise to $54 billion in coming years.

According to the report, 80% of deaths due to non-communicable diseases occurred in low and middle-income countries like India. In India, the burden of infectious diseases, poor maternal and prenatal conditions and nutritional deficiencies are crippled by an ever increasing rate. The loss of productive life-years annually to chronic diseases in countries like Brazil, Russia, China, and India is expected to grow by 65% by 2030 (WHO 2008).

Factors such as lack of exercise, sunlight exposure, poor nutrition, excessive intake of alcohol, tobacco, exposure to industrial pollutants and environmental toxins, poor quality of sleep, etc. might play a confounding role in the onset of associated lifestyle diseases.

1.1.1. Cardiovascular diseases

Cardiovascular disease (CVD) is characterized by multiple factors (Rahman *et al*., 2006) and is said to be the leading cause of death worldwide. These include heart disease (i.e., myocardial infarction and angina), stroke, hypertension, ischemia, hardening of the arteries, and other circulatory system diseases such as arteriosclerosis, aortic aneurysm, etc. As per WHO reports, 17.5 million people died from CVD in 2012 that represents 31% of all global deaths. Out of 82% death rate due to noncommunicable diseases, 37% were caused by CVDs (WHO fact sheet, 2016). Tobacco smoking, high blood pressure, and household air pollution are the three leading risk factors for global disease burden including CVD (Lim *et al*., 2013). The number of global annual death due to cardiovascular diseases is expected to grow to more than 23.6 million by 2030 (Fig 1.1).

1.1.1.1. Types of Cardiovascular Diseases

Cardiovascular disease is a class of disease that involves the disease of the circulatory system including the heart and blood vessels. Different types of cardiovascular diseases are:

<u>a) Coronary heart disease</u>

Coronary heart diseases is the most commonly occurring heart disease that involves the disease of the blood vessels supplying the heart muscle. This happens when the arteries supplying blood to the heart get narrow or harden due to the build up of plaque. High blood pressure, cholesterol, physical inactivity, use of tobacco, unhealthy diet, age, diabetes, genetic disposition all these can lead to coronary heart disease. Other risk factors include mental depression, poverty, low educational status, inflammation and blood clotting disorders.

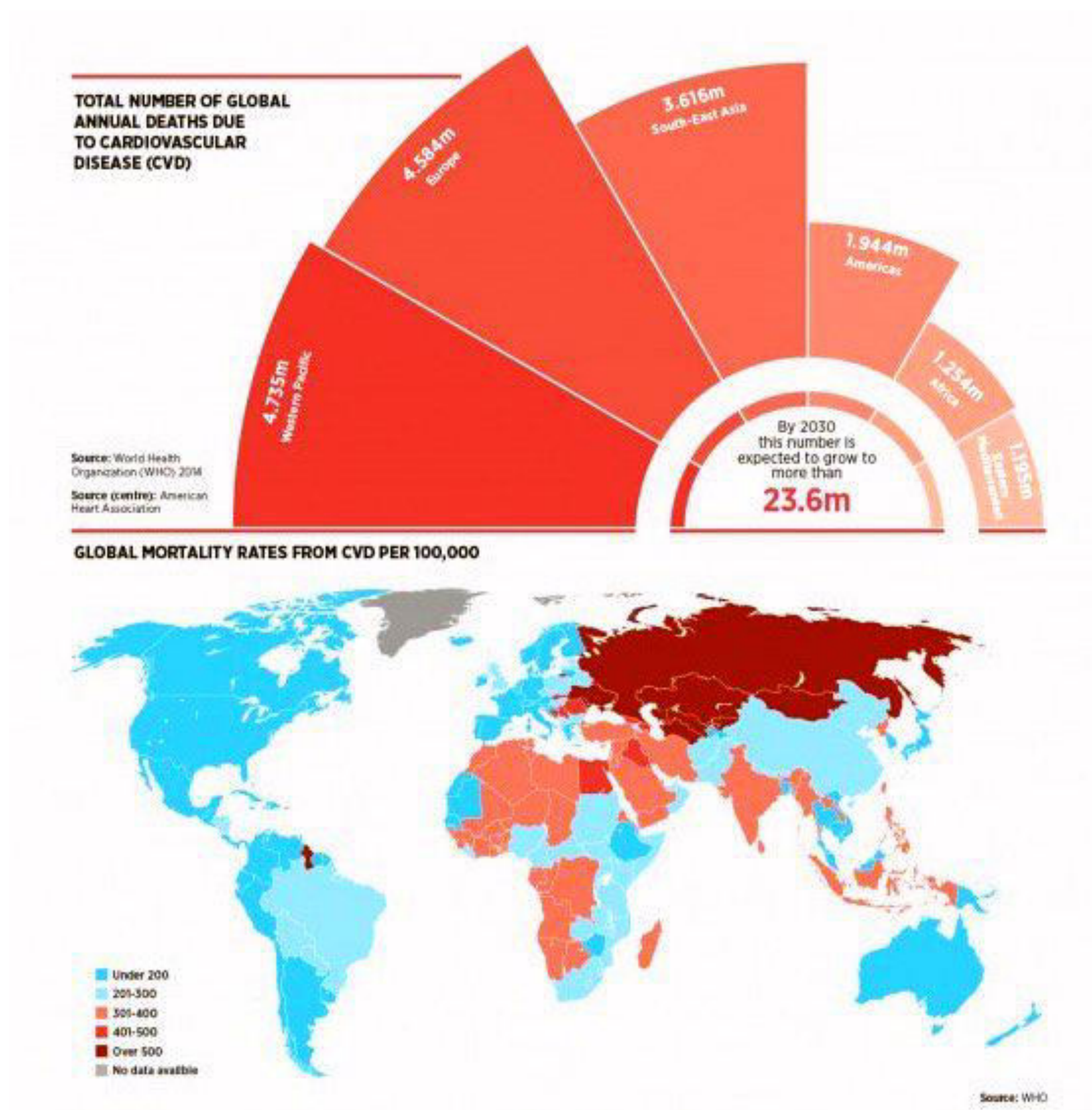


Fig 1.1: Global cardiovascular disease outlook (https://www.raconteur.net)

b) Stroke

Strokes occur due to disruption of the blood supply to the brain. This may happen from either blockage (ischemic stroke) or rupture of a blood vessel (hemorrhagic stroke). Due to this, the brain is deprived of oxygen which results in damage to the brain. This can lead to problems with speech or vision, weakness or paralysis. Risk factors include a

heart rhythm disorder, high blood pressure, atrial fibrillation, high blood cholesterol, use of tobacco, unhealthy diet, physical inactivity, diabetes, and advancing age.

c) Hypertensive heart disease

This disease occurs due to high blood pressure that may overburden the heart and blood vessels. Certain specific diseases or infections can lead to high blood pressure which includes the tumor in the adrenal glands, damage or disease of the kidneys or their blood vessels. This can lead to aneurysm, a bulge or weakness in the ball of blood vessels due to high blood pressure.

d) Inflammatory heart disease

This occurs when the pericardium of the heart gets affected due to inflammation, fluid accumulation and stiffness. This condition also occurs when the heart valve gets damaged due to rheumatic fever, connective tissue disorders or certain medications or radiation treatment for cancer.

e) Other cardiovascular diseases

Other cardiovascular diseases include tumors of the heart, disorders of the heart muscle (cardiomyopathy), disorders of the lining of the heart, heart valve diseases (Mackay *et al*., 2004).

1.1.1.2. Drugs used to treat cardiovascular diseases

The most common drugs used for the treatment of cardiovascular diseases are discussed below:

1.1.1.2.1. Anticoagulants

Anticoagulants help to prevent the formation of the clot in the blood vessels. It is often prescribed to prevent first or recurrent stroke. Some of the common anticoagulants are rivaroxaban, dabigatran, apixaban, heparin, warfarin, etc.

1.1.1.2.2. Antiplatelet agents

These drugs help to prevent clotting in patients who had a heart attack, ischemic strokes, unstable angina and other forms of cardiovascular diseases. Thus it retards blood clot formation by preventing blood platelet from sticking together. Commonly prescribed antiplatelet agents are aspirin, clopidogrel, ticagrelor, prasugrel, etc.

1.1.1.2.3. Angiotensin converting enzyme inhibitors (ACE inhibitors)

These drugs are used in cardiovascular conditions like high blood pressure and heart failure. It lowers the level of vasoconstrictor angiotensin II and makes the blood flow easier. Commonly prescribed ACE inhibitors are captopril, enalapril, fosinopril, lisinopril, quinapril, trandolapril, etc.

1.1.1.2.4. Angiotensin II receptor blockers (ARB)

Angiotensin II receptor blockers are used to treat high blood pressure and heart failure. Angiotensin II receptor blockers prevent this chemical from having any effects on heart and blood vessels. Commonly used angiotensin II receptor blockers are candesartan, eprosartan, irbesartan, losartan, valsartan, etc.

1.1.1.2.5. Angiotensin receptor neprilysin inhibitors (ARNIs)

These are a new drug combination of a neprilysin inhibitor and an ARB. Neprilysin breaks down natural substances in the body that open narrowed arteries. By inhibiting the effect of neprilysin, it enhances the opening of the artery and increases blood flow, reduces sodium (salt) retention, and decrease strain on the heart. Sacubitril/valsartan is commonly used as angiotensin receptor neprilysin inhibitor.

1.1.1.2.6. Beta blockers

The major function of beta blockers is to lower blood pressure. It is used in treating chest pain (angina) and cardiac arrhythmias (abnormal heart rhythms). It helps to

prevent future heart attacks in patients who have had a heart attack. Thus beta blockers decreases the heart rate and cardiac output, lowers blood pressure and thus make the heart beat more slowly. Commonly prescribed beta blockers are acebutolol, atenolol, betaxolol, bisoprolol, propranolol, nadolol, sotalol, etc.

1.1.1.2.7. Combined alpha and beta-blockers

Combined alpha and beta-blockers are prescribed for patients having high blood pressure who were at risk for heart failure. It is used as an IV drip for those patients experiencing a hypertensive crisis. Commonly used combined alpha and beta-blockers are carvedilol, labetalol hydrochloride. One of the possible side effects of combined alpha and beta-blockers is the chance of drop in blood pressure when patients stand up.

1.1.1.2.8. Calcium channel blockers

Calcium channel blockers can decrease the heart's pumping strength and relax blood vessels. It is used to cure chest pain (angina) caused by reduced blood supply to the heart muscle, high blood pressure, and some arrhythmias (abnormal heart rhythms). The commonly used calcium channel blockers are amlodipine, diltiazem, felodipine, nimodipine, verapamil., nisoldipine.

1.1.1.2.9. Cholesterol-lowering medications

Usually, statins are used to lower bad cholesterol levels (LDL). Commonly used cholesterol-lowering drugs are statins, nicotinic acids, cholesterols absorption inhibitors.

1.1.1.2.10. Digitalis preparations

Digitalis preparations help to improve the force of heart's contractions, which can be beneficial in heart failure and for arrhythmias. This is used when the patient is not responding to ACE inhibitors and diuretics. Commonly prescribed digitalis is lanoxin.

1.1.1.2.11. Diuretics

Diuretics helps to remove excess of fluids in lungs and other parts of the body. Thus it helps to lower blood pressure, reduce edema from excess buildup of fluid in the body. The commonly used diuretics are amiloride, bumetanide, chlorothiazide, furosemide, indapamide, spironolactone.

1.1.1.2.12. Vasodilators

Vasodilators help to relax blood vessels and increase the supply of blood and oxygen to the heart while reducing its workload. They come in pills to be swallowed, chewable tablets and as cream for topical application. It helps to ease angina. Commonly prescribed vasodilators are isosorbide dinitrate, nesiritide, hydralazine, nitrates, minoxidil, etc.

1.1.1.3. Side effects of Cardioprotective drugs

Each type of cardioprotective drug has their adverse effect. A study conducted on side effects of cardioprotective drugs showed that majority of side effects are due to pharmacological causes and the remaining were because of immunologic character. Women were suffering more than men from side effects of cardioprotective drugs. Most of the side effects are caused by coronary vasodilating drugs (31.53%), calcium antagonists (18%), cardiac glycosides (12.6%), etc. (Koleva *et al*.,1988). Anticoagulants have the side effects of bleeding and necrosis of the skin. Use of angiotensin converting enzyme can cause cough, hyperkalemia, low blood pressure, headache, weakness, etc. The major side effects due to cardioprotective drugs are depicted in Table 1.1.

Table 1.1. Drugs used to treat cardiovascular diseases and their side effects *(Kaiser et al., 2013)*

Drug class	Adverse effects
Thiazide and loop diuretics	• Hypokalemia, hyponatremia, hypomagnesemia • Volume-depletion and orthostatic hypotension • Renal impairment, hyperuricemia, gout, lipid alterations, hyperglycemia, insulin resistance • NSAIDs reduce thiazide potency • Erectile dysfunction and possibly impotence • Reduction of lithium excretion and precipitate lithium toxicity • Potential to increase fatigue and lethargy • Pro-diabetogenic potential in combination with BBs • Increase of urinary frequency, leg cramps • Decrease of renal blood flow, creatinine clearance, GFR
Potassium-sparing diuretics	• Hyperkalemia, hypotension
BBs	• Sinus bradycardia, fatigue, AV-nodal heart block bronchospasm, aggravation of acute heart failure • Intermittent claudication, confusion, hyperglycemia • Diabetes mellitus • Drowsiness, lethargy, sleep disturbance, visual hallucinations, depression, blurring of vision, nightmares • Pulmonary side-effects (increased airway resistance in asthmatics) • Peripheral vascular side-effects (cold extremities, Raynaud's phenomenon) • Erectile dysfunction
ACEIs	• Cough, hyperkalemia • Angioneurotic edema • Rash, altered taste sensation, renal impairment
ARBs	• Hyperkalemia, renal impairment
CCBs (non-dihydropyridines)	• Rash, sinus bradycardia, heart block, heart failure, constipation (verapamil), gingival hyperplasia • Ankle edema, headache and postural hypotension
CCBs (dihydropyridines)	• Peripheral edema, heart failure, tachycardia • Aggravation of angina pectoris (short-acting agents)
Direct vasodilators	• Tachycardia, fluid retention • Angina pectoris
Alpha1-adrenergic antagonists	• Hypotension
Alpha-beta adrenergic blockers (vasodilator-beta adrenergic blockers)	• Hypotension, heart block, sinus bradycardia, bronchospasm
Central-acting substances	• Sedation, constipation, dry mouth
Direct renin inhibitors	• Mild diarrhea

1.1.2. Diabetes

Diabetes mellitus (DM), is one of the lifestyle diseases characterized by polyuria. This is a chronic syndrome of impaired carbohydrate, protein, and fat metabolism. The abnormalities in carbohydrate, fat and protein metabolism will lead to chronic hyperglycemia and results in insufficient secretion of insulin or target-tissue insulin resistance. Complications of diabetes mellitus include both macrovascular (cardiovascular) and microvascular (retinopathy, nephropathy, or neuropathy) diseases (Alberti *et al.*, 1998). Though the etiology of this disease is not well defined, viral infection, autoimmune disease, and environmental factors have been implicated (Paik *et*

al., 1982). Polyuria, polydipsia, weight loss, sometimes with polyphagia, and blurred vision are some of the symptoms of marked hyperglycemia. Patients with diabetes are more susceptible to atherosclerotic cardiovascular, peripheral arterial, and cerebrovascular disease (American Diabetes Association, 2010).

1.1.2.1. Classification of diabetes

1.1.2.1.1. Type-I diabetes

Type 1 diabetes occurs due to the auto immune destruction of beta cell that leads to absolute insulin deficiency. It is also known as insulin-dependent diabetes or juvenile-onset diabetes. This type of diabetes usually accounts for 10-20% of total reported cases of diabetes (American Diabetes Association, 2004). An individual with a Type 1 diabetes may be metabolically normal before the disease is clinically manifest, but the process of beta–cell destruction can be detected (Alberti *et al*., 1998).

Symptoms of Type I diabetes includes- abnormal thirst and dry mouth; frequent urination; tiredeness; hunger; weight loss; blurred vision

1.1.2.1.2. Type 2 Diabetes

Type 2 diabetes is also known as non-insulin dependent diabetes mellitus. It is the most common form of diabetes which is characterized by disorders of insulin action, a defect in glucose uptake in muscle, a disruption of the secretory function of adipocytes, dysfunction of the pancreatic beta cells, and an impaired insulin action in the liver. Genetic and environmental factors are the predominant causes of type II diabetes. The comparison of Type I and Type II diabetes mellitus has been tabulated in Table 1.2

Symptoms of Type II diabetes includes - excessive thirst; blurred vision; frequent urination; weight loss

Table 1.2: Comparison of Type I and Type II diabetes mellitus

Features	Type 1	Type II
Clinical	Onset <20 years Normal weight Decreased blood insulin Anti-islet cell antibodies	Onset >30years Obesity Normal or increased blood insulin No anti-islet cell antibodies
Genetics	Ketoacidosis common Human leukocyte antigen (HLA)-D linked	Ketoacidosis rare No HLA association
Pathogenesis	Autoimmunity, immunopathologic mechanisms Severe insulin deficiency	Insulin resisitance Relative insulin deficiency
Nutritional status at the time of onset	Frequently under nourished	Obesity usually present
Prevalence	10-20% of diagnosed diabetes	80-90% of diagnosed diabetics
Genetic predisposition	Moderate	Very strong
Defect or deficiency	β cells are destroyed, eliminating the production of insulin	Inability of β cells to produce insulin

1.1.2.2. Diabetes Prevalence

Diabetes and its complications are major causes of death in most countries. Type II diabetes is the most prevalent form of diabetes and has increased alongside cultural and societal changes. According to International Diabetic Federation (IDF), in 2015, 215.2 million men and 199.5 million women had been affected with diabetes (Fig 1.2). This has been expected to increase by 328.4 million men and 313.3 million women by 2040.

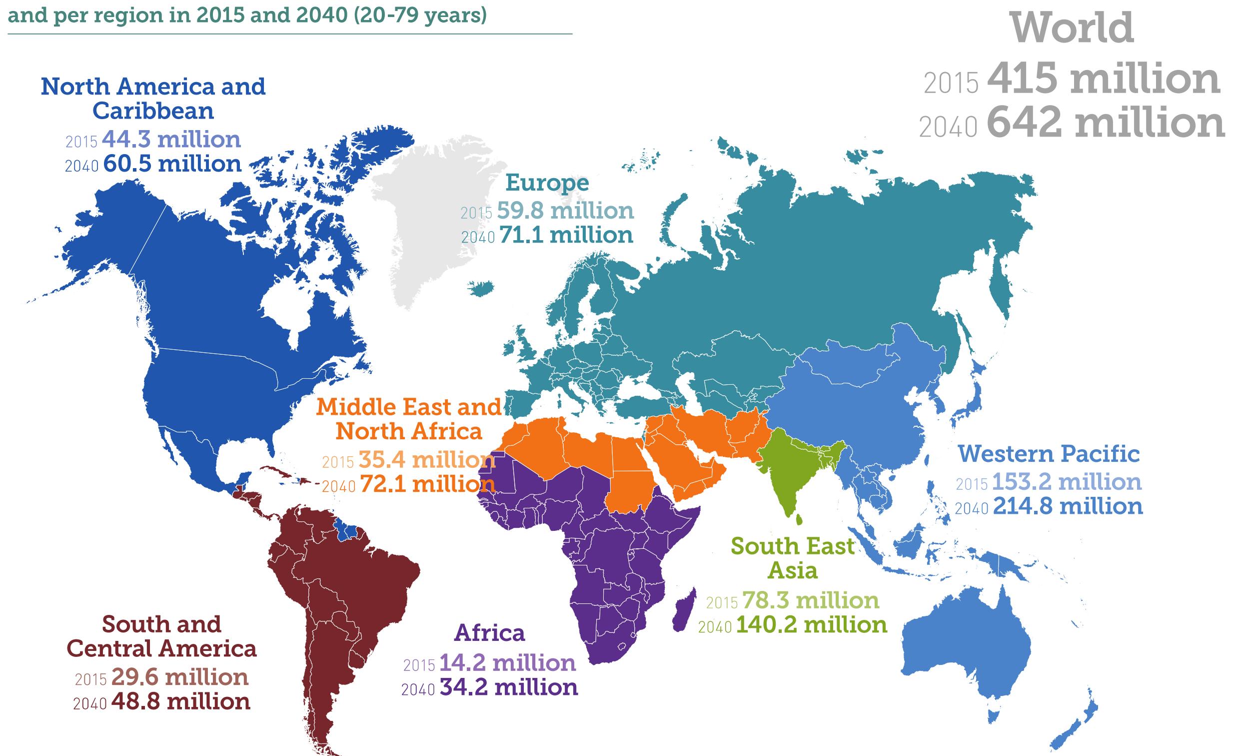


Fig 1.2: Global diabetic prevalence (International Diabetic Federation Atlas, 2015)

Among the top 10 countries with the number of people with diabetes, India comes in second place with 69.2 million suffering from diabetes (IDF 2015) and is expected to increase to 123.5 million in 2040. Kerala is known as diabetes capital of India, as prevalence of diabetes is as high as 14.6% (Fig 1.3), which is double the national average (Unnikrishnan et., 2016).

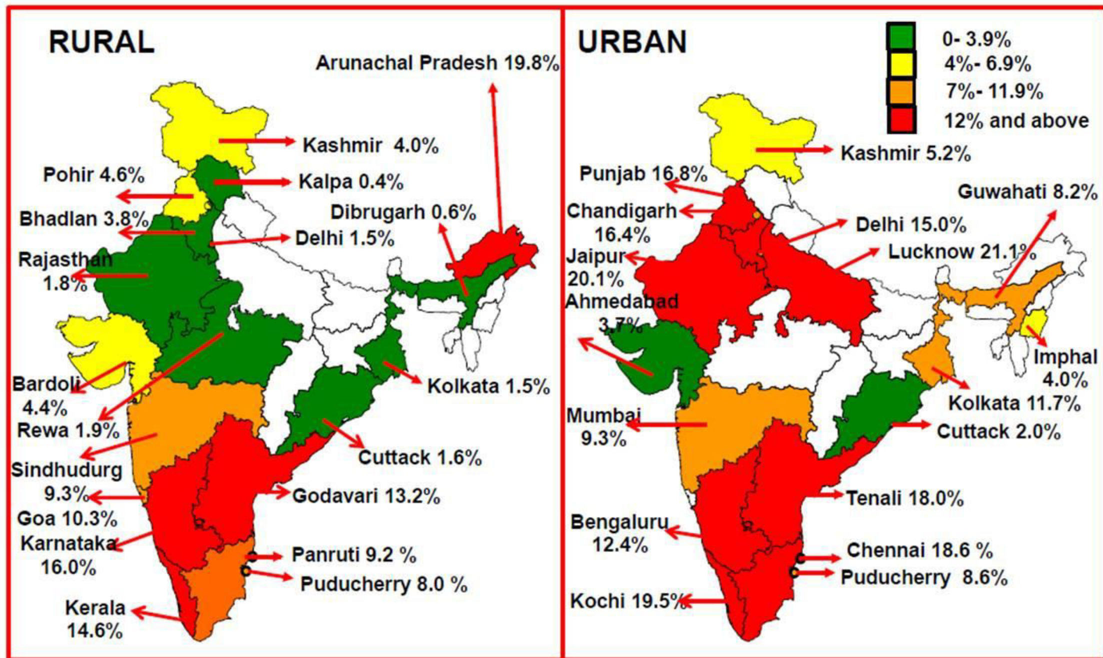


Fig 1.3: Diabetic prevalence in India (Unnikrishnan et al., 2016)

1.1.2.3. Drugs used to treat type - II diabetes and their associated side effects

Several drugs are used for the treatment of type II diabetes, and they differ in the way they function in the body to reduce blood glucose. Site of action of these drugs which are currently used for the treatment of type II diabetes is shown in Fig 1.4.

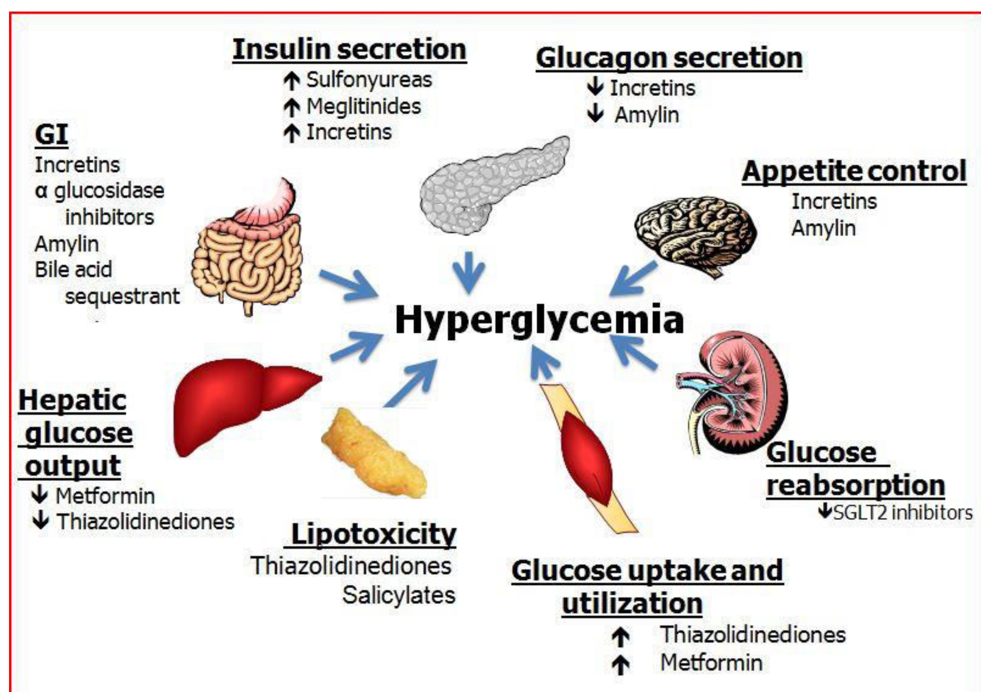


Fig. 1.4: Site of action of current pharmacological therapies for the treatment of type II diabetes (Evans et al., 2016)

Similar to all synthetic drugs, diabetic medications also been reported for their side effects. Some diabetic drugs include common side effects such as nausea or an upset in stomach. Continuous use of some other drugs can lead to hypoglycemia, back pain, etc. Common adverse side effects observed due to the use of some antidiabetic drugs are shown in Table 1.3.

Table 1.3: Drugs used for the treatment of Type II diabetes and its side effects

Type of drug	Brand/Generic Name	Side effects
Sulfonylureas	Glimepiride, Glipizide, Glyburide	Skin rash, irritability, upset stomach
Biguanides	Glucophage, Metformin, Glucophage XR	Bloating, gas, diarrhoea, loss of appetite
Thiazolidinediones	Pioglitazone	Low blood glucose
α-glucosidase inhibitors	Acarbose	Upset stomach, abdominal pain, diarrhoea, gas
Meglitinides	Prandin, Starlix, Nateglinide	Back pain, joint pain, cough, a stuffy nose, and diarrhoea. Cause constipation and feelings of numbness, dizziness
Dipeptidyl peptidase 4 inhibitors	Januvia, Onglyza	Head ache, skin rash, gastrointestinal disturbances

1.1.3. Inflammation

Inflammation is a set of biological response of body tissues against harmful stimuli such as pathogens, irritants, or damaged cells. Acute inflammation is characterized by the increased movement of plasma and innate immune system cells, such as neutrophils and macrophages, from the blood into the injured tissues which eventually leads to pain, heat, redness, swelling and loss of function. It is a protective attempt by the organism to remove the injurious stimuli and to initiate the healing

process for the tissues (Pan *et al*., 2010). Excess levels of nitric oxide (NO), prostaglandin E2 (PGE2) and cytokines (tumor necrosis factor-α [TNF-α], interleukin-1ß [IL-1ß] and IL-6) are secreted by activated inflammatory cells during the process of inflammation (Yoon *et al*., 2009). These substances induce not only cell and tissue damage but also activation of macrophages in diseased conditions such as rheumatoid arthritis and chronic hepatitis. Regulation of the inflammatory response by reducing the level of nitric oxide and cytokines is a fundamental element in the pathogenesis of chronic inflammation-related disorders like hay fever, periodontitis, atherosclerosis and rheumatoid arthritis (Dang *et al*., 2008).

Inflammation and its markers can act as a link between diabetes and cardiovascular diseases (Fig 1.5). Thus by reducing inflammation, the chances for prevailing diabetes and cardiovascular diseases can be minimised.

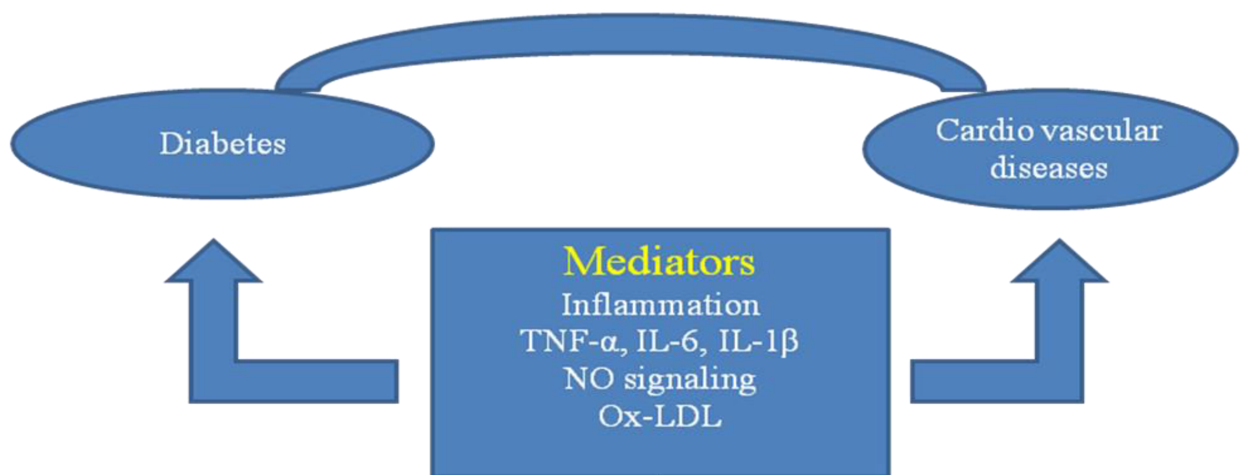


Fig. 1.5: Link between diabetes and cardiovascular diseases

1.1.3.1. Types of inflammation

Inflammation is mainly acute inflammation or chronic inflammation. Acute inflammation starts rapidly and is induced by tissue damage due to trauma, noxious compounds, or microbial invasion. Chronic inflammation means long term inflammation

which last for several months and years. The major differences between acute and chronic inflammation are depicted in Table 1.4.

Table 1.4: Differences between acute and chronic inflamamtion
(Ref: https://virtualmedic.files.wordpress.com/2012/01/differences-between-acute-and-chronic-inflammation.pdf)

Features	Acute inflammation	Chronic inflammation
Onset	Rapid onset	Insidious/delayed onset
Duration of Course	Short (Days)	Long (Weeks to Months)
Specificity	Non-specific	Specific as it involves Acquired Immunity
Cardinal Signs	Pain, Heat, Redness, Swelling, Loss of Function	Absent in any of cardinal signs
Causative Agents	Physical and Chemical damages; Pathogen invasion; Tissue necrosis; Immune response	Presistent infection; Presence of foreign bodies; Autoimmunity
Fundamental Cells	Neutrophils; Macrophages	Lymphocytes; Macrophages; Fibroblasts
Fluid Exudation and Edema	Present	Absent
Fibrosis	Absent	Present
Angiogenesis	Absent	Present
Systemic Manifestation	High grade fever Other 5 cardinal signs	Low grade fever; Loss of weight; Loss of appetite
Peripheral Blood Changes	Neutrophil Leukocytosis (bacterial infection); Lymphocytosis (viral infection)	Often absent; Increase in the level of Antibodies
Primary Mediators	Vasoactive amines; Serotonin; Histamine; Eicosanoids; Prostaglandins; Thromboxane	Interferon Gamma; TNF alpha; Growth Factor; ROS; Hydrolyzing enzymes

1.1.3.2. Drugs used to treat inflammation

Non-steroidal anti-inflammatory drugs (NSAIDs) are a drug class that are not related to steroids. This helps to reduce inflammation and provide analgesic (pain killing) and antipyretic action. These drugs help to relieve pain in non-inflammatory conditions such as a migraine, period pain and postoperative pain, sprains and strains, flu, and to reduce fever. Some commonly used NSAIDs include - ibuprofen, naproxen, diclofenac, celecoxib, mefenamic acid, etoricoxib, indometacin, high-dose aspirin, etc. Mode of action of some of the NSADs are shown in Fig 1.6.

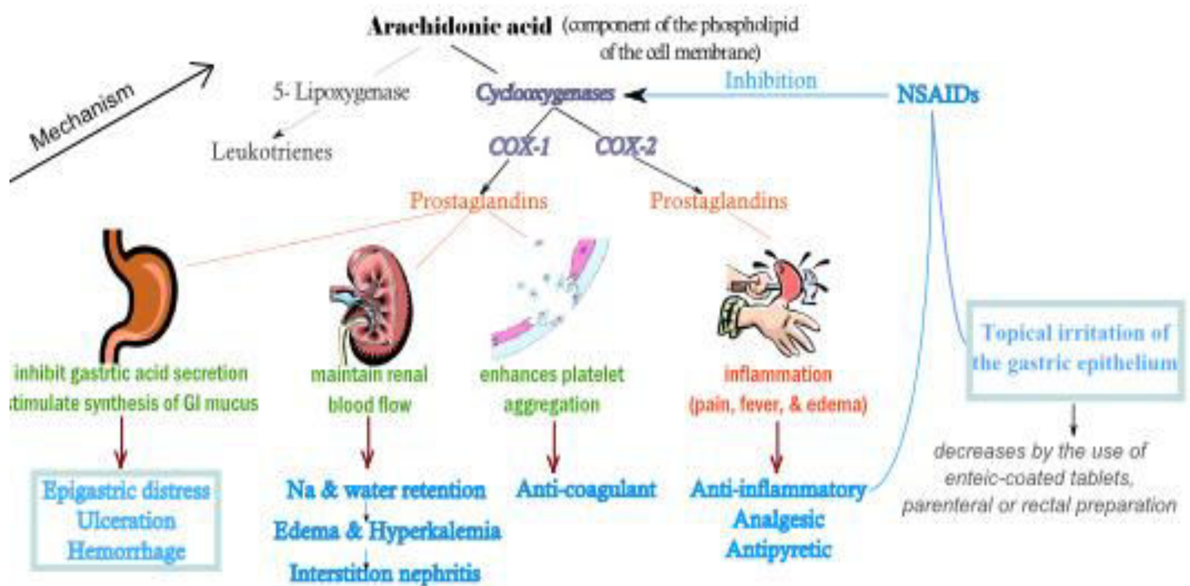


***Fig.1.6: Mechanism of action of non-steroidal anti-inflamamtory drugs** (https://pharmacotherapy.wordpress.com)*

1.1.3.3. Adverse effects of NSAIDs

Long-term use of NSAIDs can cause unwanted adverse effects such as indigestion, stomach upset (including nausea or feeling sick), ulcers & bleeding in the stomach and other parts of the gastrointestinal tract (gut). Use of NSAIDs can also cause diarrhoea, headache, dizziness, salt and fluid retention, high blood pressure, etc. Other less common side effects include ulcers of the esophagus, heart failure, hyperkalemia

(high levels of potassium in the blood), reduced kidney function, bronchospasm (difficulty breathing), skin irritations, etc.

Many synthetic drugs are used for the treatment of various lifestyle associated diseases, and almost all drugs are associated with detrimental side effects. Therefore it is a clinical requisite to identify more effective agents with lesser side effects. Medicinal plants have lesser side effects and have more favourable pharmacological profile with better therapeutic potential. Moreover, natural products are cost effective alternatives and are biocompatible for the treatment and prevention of various diseases.

1.1.4. Natural products and lifestyle associated diseases

From the beginning of human civilization, herbs have been used for the treatment of different diseases. Herbs are valued for both their culinary and medicinal properties. According to WHO, Traditional Medicine (TM) refers to health practices, approaches, knowledge, and belief involving plant, animal and mineral based medicine, spiritual therapies, manual techniques and exercises applied singularly or in combination to treat, diagnose and prevent illness or well-being plant-based healthcare (Zhang *et al*., 2004). One of the oldest records related to the plant-based medicine is the Papyrus Eber written nearly 1500 BC and contains information of more than 500 natural ingredients (Ghalioungui, 1969). Indian traditional medicine "Ayurveda" is a storehouse of knowledge on traditional healthcare. Besides Ayurveda, Homeopathy, Siddha, and Unani are several other complementary and alternative systems of medicine like which are also practiced and developed with the course of time in India, where plants and plant-based formulations are employed for health care and disease treatments. Variations in geographical landscaping and biodiversities in the Indian subcontinent have helped to develop the use of a variety of plant species and other natural resources for health care and contributed to TM. The inherited cumulative knowledge and experience in

Ayurveda, as well as other TM, may be of interest because of new leads to modern approach for diseases treatment and management. There is a consensus that TM generated important knowledge about the therapeutically potent plant(s), plant part, preparation methods, dose, etc. but, lack scientific evidences. The only remedies to answer the unanswered questions are to do further research based on evidences to address safety, efficacy, and quality.

Several drugs derived from plant products can act as antimicrobial agent (e.g. Resveratol, bereberine) (Cowan *et al*., 1999; Lewis *et al*., 2006., Daglia, 2012), analgesic (e.g. epibatidine, cannabinoid) (McCarberg *et al*., 2007), antihypertensive (e.g. tetramethylpyrazine, tetrandrine) (Kwan, 1994), and anti-inflammatory drugs (e.g. Bromelain, reserpine etc.) (Maurer, 2001). Moreover, plant derived antitumor agents (e.g. vincristine, vinorelbine, camptothecin) (Pezzuto *et al*., 1997; Lee, 1999) as well as cardioprotective agents (e.g. digitalis, acetyldigox in, digoxin, etc.) (Charles *et al*., 1953) are also being used currently. Table 1.5 shows some important medicinal plants that are currently used as drugs.

Table 1.5: Major biological activities of some medicinal plants used in ayurveda *(Patwardhan et al., 2004)*

Sl. no.	Sanskrit name	Botanical name	Main activity
1	Amalaki	*Phyllanthus emblica*	Rasayana
2	Ashwagandha	*Withania somnifera*	Immunomodulatory
3	Bhallataka	*Semecarpus anacardium*	Antiarthritc
4	Bilva	*Aegle mermelos*	Antidiarrhoeal
5	Chandan	*Santalum album*	Antiviral
6	Chitraka	*Plumbago zeylanica*	Antitumour
7	Dadima	*Punica granatum*	Antidiarrhoeal
8	Eranda	*Ricinus communis*	Hepatoprotective
10	Guduchi	*Tinospora cordifolia*	Immunomodulatory

11	Haridra	*Curcuma longa*	Antimicrobial
12	Haritaki	*Terminalia chebula*	Hypolipidemic
13	Manjishtha	*Rubia cordifolia*	Antioxidant
14	Maricha	*Piper nigrum*	Bioenhancer
15	Nimba/Neem	*Azadirachta indica*	Antidiabetic
16	Pippali	*Piper longum*	Bioenhancer
17	Sariva	*Hemeidesmus indicus*	Antiulcer
18	Shunthi	*Zingiber officinale*	Antiemetic
19	Vacha	*Acorus calamus*	Psychotropic
20	Vidanga	*Embelia ribes*	Antifertility
21	Yashtimadhu	*Glycyrrhiza glabra*	Antiulcer

The global market analysis for botanical and plant-derived drugs showed that the demand for natural products was $23.2 billion in 2013 and $24.4 billion in 2014 (Fig 1.7). By the year 2015, total market is expected to reach $25.6 billion and nearly $35.4 billion in 2020, with a compound annual growth rate (CAGR) of 6.6% (Lawson 2013).

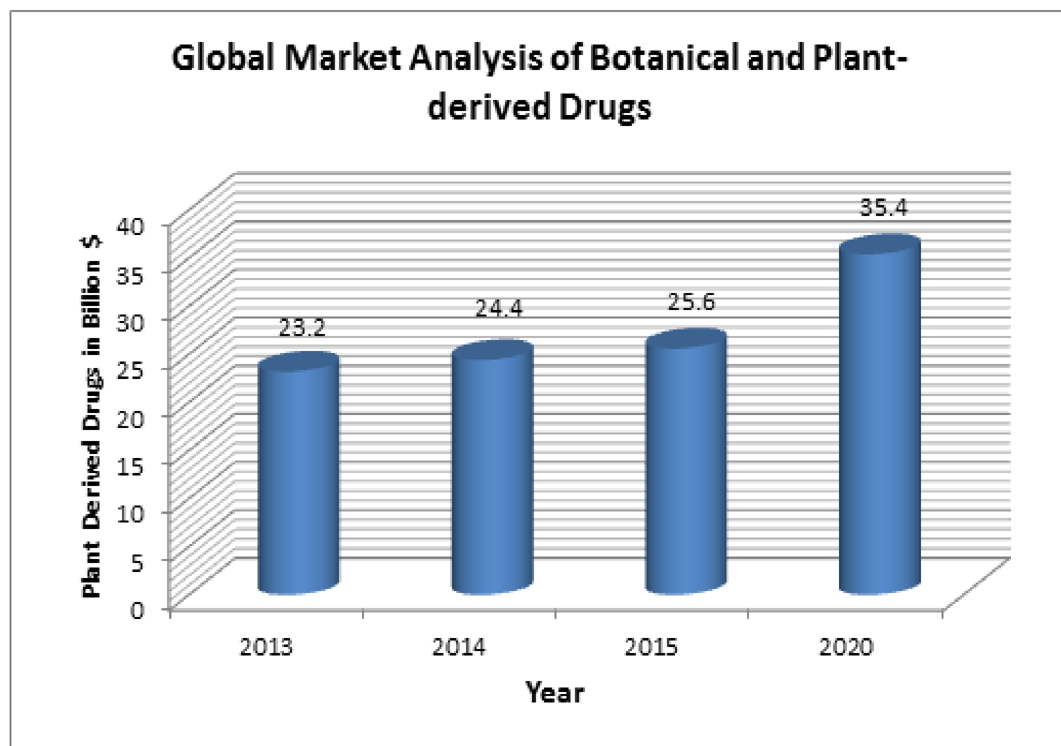


Fig.1.7: Statistics showing the demand for global market analysis of botanical and plant derived drugs

1.1.4.1. Plant-based antidiabetic agents

Some recent studies endorsed the antidiabetic potential of some medicinal plants which were used in traditional medicine. Heart wood of *Pterocarpus marsupium* Rox. (Tandon *et al*., 2016; Patil *et al*., 2011), *Cassia auriculata* flowers, *Osbeckia octandra* leaves, *Syzygium cumini* bark, *Phyllanthus emblica* fruits and *Scoparia dulcis* (Perera *et al*., 2015) etc.have been used to treat diabetes mellitus. The antihyperglycemic effect of these plants can inhibit the intestinal absorption of glucose, improve the antioxidant status and lipid metabolism, increase insulin output and thus restore the function of pancreatic tissues. (Bailey *et al*., 1989).

The presence of glycosides, alkaloids, terpenoids, flavonoids, cartenoids, etc., present in plants mainly attributes to their antidiabetic effects. Though there are more than 400 plant species having antidiabetic activity available in literature, however, inquiring for noval antidiabetic drugs from medicinal plants is still interesting because they contain substances which demonstrate alternative and safe effects on diabetes mellitus. Antidiabetic activities of few medicinal plants is depicted in Table 1.6.

Table 1.6: Antidiabetic activity of some medicinal plants and their parts used

Plant	Parts	Reference
Achyranthes aspera	Whole plant	Akhtar *et al*., 1991
Aegle marmelos	Seed	Kesari *et al*., 2006
Allium cepa	Bulb	Kumari *et al*., 1995
Azadirachta indica	Leaves	Mostofa *et al*., 2007
Cinnamomum zeylanicum	bark	Verspohl *et al*., 2005
Coriandrum sativum	Seeds	Gray *et al*., 1999
Cuminum nigrum	Seeds	Ahmad *et al*., 2000
Curcuma longa	Rhizome	Kuroda *et al*., 2005

Daucus carota	seeds	Vasudevan *et al*., 2006
Mangifera indica	stem, bark, leaves	Bhowmik *et al*., 2009
Mentha piperita	leaves	Büyükbalci *et al*., 2008
Momordica charantia	fruits	Joseph *et al*., 2013
Murraya koenigii	leaves	Kesari *et al*., 2005
Ocimum sanctum	leaves	Chattopadhyay*et al*., 1993
Panax ginseng	fruit	CHIOU *et al*., 2008
Pterocarpus marsupium	fruit	Anila *et al*., 2000
Syzygium cumini	seed	Saravanan *et al*., 2006
Syzygium cumini	bark	Kumar *et al*., 2013
Terminalia chebula	fruits	Kumar *et al*., 2006
Triticum vulgare	Whole plant	Makheswari *et al*., 2012
Vinca rosea	Whole plant	Ahmed *et al*., 2010
Withania somnifera	root	Udayakumar *et al*., 2009
Zingiber officinale	rhizome	Akhani *et al*., 2004

1.1.4.2. Plant based cardioprotective agents

Currently, phytochemicals are widely evaluated for the treatment and management of cardiovascular diseases. Compounds like arjunolic acid, many flavonoids, phenolics, and anthocyanins demonstrated significant cardioprotectiev effects. Cardioprotective action of some medicinal plants and their parts used are depicted in Table 1.7.

Table 1.7: Cardioprotective activity of some medicinal plants and their parts used

Plant	Parts	Reference
Aerva lanata	leaves	Goyal *et al*., 2011
Allium cepa	leaves	Park *et al*., 2009
Allium sativum	bulb	Saravanan *et al*., 2004
Aloe barbadensis	leaves	Jain *et al*., 2010
Asparagus racemosus	roots	Visavadiya*et al*., 2009
Azadirachta indica	leaves	Peer *et al*., 2008
Bacopa monnieri	Leaves, root, stem	Kamesh *et al*., 2012
Cassia fistula	Bark, flowers	Nirmala *et al*., 2008
Curcuma longa	Rhizome	El-Sayed *et al*., 2011
Emblica officinalis	Fruit	Bhattacharya *et al*., 2002
Glycyrrhiza glabra	Roots, rhizome	Ojha *et al*., 2013
Hemidesmus indicus	Root	Khandelwal *et al*., 2010
Syzygium cumini	Seeds	Mastan *et al*., 2009

1.1.4.3. Plant based anti-inflamamtory agents

Pharmacologically active medicinal plants with effective anti-inflammatory properties has been widely explored currently. Compounds like curcumin, colchicine, resveratrol, capsaicin, epigallocatechin-3-gallate (EGCG), quercetin, etc. were reported to have anti-inflammatory potential (Fürst *et al*., 2014). Some medicinal plants reported to have anti-inflamamtory effect are shown in Table 1.8.

Table 1.8: Anti-inflamamtory activity of some medicinal plants and their parts used

Plant	Parts	Reference
Oscimum sanctum	Leaves	Singh *et al*., 1996
Zingiber officinale	rhizome	Thomson *et al*., 2002
Azadirachta indica	Leaves	Tidjani *et al*., 1989
Glycyrrhiza glabra	Leaves	Siracusa*et al*., 2011
Curcuma longa	rhizome	Araujo *et al*., 2001
Capsicum annum	fruit	Hernández-Ortega *et al*., 2012
Boswellia serrata	gum	Singh *et al*., 1986
Aloe barbadensis	leaves	Vázquez *et al*., 1996
Nigella sativa	seed	Chehl *et al*., 2009

1.1.5. *Syzygium cumini*

1.1.5.1. Botanical description

Syzygium cumini is a fast-growing tropical and sub-tropical tree preferring moist, riverine habitats. It may reach up to 30 m tall in India. The bark is rough, cracked, flaking and discolored on the lower part of the trunk, becoming smooth and light-grey. Evergreen leaves have a turpentine smell with oval or elliptic shape and tapering to a point at the apex. The leaves are pink in color at the younger stage and become leathery and glossy. On the top, it is dark-green but lighter beneath. When mature, a conspicuous, yellowish midrib appears. Flowers are fragrant with a funnel-shaped calyx and 4-5 united petals. Fruit appear in clusters of just a few or 10-40, with 1.25-5 cm long and round or oblong, often curved in shape. Fruit then turns from green to light-magenta, then dark-purple or nearly black. The texture of skin is thin, smooth and glossy. The pulp of *Syzygium cumini* is purple or white, very juicy. The pulp is enclosed by a 4 cm long

single, oblong, green or brown seed. In some fruits, 2-5 seeds are present within a leathery coat, and some are seedless. The fruit is usually astringent, sometimes unpalatable, and the flavor varies from acid to moderately sweet. *Syzygium cumini* propagates easily from fresh seed. The seed cotyledons consist of single layered epidermis. Mesophyll is composed of isodiametric thin-walled, parenchymatous cells fully packed with simple starch grains, oval, rounded measuring 7-28 μ in diameter a few schizogenous cavities are also found (*Syzygium cumini* begins bearing fruit when 8-10 years old. *Syzygium cumini* is a tropical species preferring mean annual temperatures around 25-27°C (Morton, 1987). *Syzygium cumini* grows best in wet regions with annual rainfall, more than 1000 mm and up to 4000 mm. *Syzygium cumini* is thus native to India, Sri Lanka, and Myanmar but also reported to be grown in Australia, Philippines, Pemba Kenya, Florida West Indies, East and West Africa, etc. All parts of *Syzygium cumini* are shown in Fig 1.8.

.1.1.5.2. Taxonomy

Common names: Duhat, jambolan, plum, jamelonguier, kavika, mesegerak, black plum; Indian blackberry; jambolan; jambolan; jamun; Java plum; Malabar plum; Portuguese plum

1.1.5.3. Botanical names**:** *Syzygium cumini* (L.) skeels, *Calyptranthes jambolana* Willd, *Eugenia cumini* (L.) Druce, *Eugenia jambolana* Lam., *Myrtus cumini* L., *Syzygium jambolana* Lam., *Syzygium jambolanum* DC (ITIS Report, 2017).

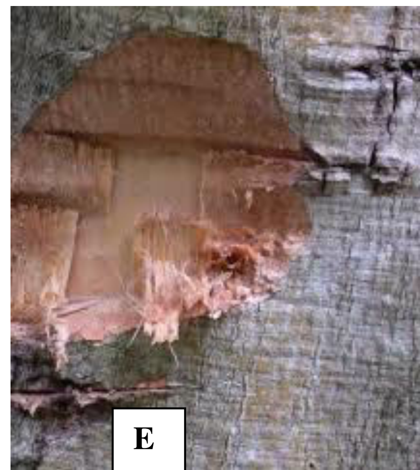
Kingdom	:	*Plantae*
Subkingdom	:	*Viridiplantae* - Vascular plants
Infrakingdom	:	Streptophyta
Superdivision	:	*Embryophyta*
Division	:	*Tracheophyta*

Subdivision	:	*Spermatophytina* - Seed plants
Class	:	Magnoliopsida - Dicotyledons
Superorder	:	*Rosanae*
Order	:	*Myrtales*
Family	:	*Myrtaceae* - Myrtle family
Genus	;	*Syzygium*
Species	:	*Syzygium cumini* (L.) Skeels

1.1.5.4. Pharmacological and biological activities of *Syzygium cumni*

1.1.5.4.1. Antioxidant activity

In vitro and *in vivo* antioxidant activities of different parts of *Syzygium cumini* namely its fruit skin, pulp, kernel, seed coat and leaves were reported. *Syzygium cumini* fruit skin is reported to have significant antioxidant activity due to the presence of phenolics, tannins, anthocyanins and vitamins. The antioxidant properties of anthocyanin rich fruit skin were reported by Banerjee *et al*., and its radical scavenging capacity was found to be proportional to phenolic compounds present in the fruit skin (Banerjee *et al*., 2005).The DPPH scavenging activity of hot aqueous infusion prepared from fruit peels of *Syzygium cumini* dried for 7 days showed IC_{50} value 168 µg/mL. (Banerjee *et al*., 2005). *Syzygium cumini* peel extract inhibited the iron-induced lipid peroxidation in rat brain, liver, liver mitochondria, testes and human erythrocyte ghost cells. Trolox equivalent antioxidant capacity (TEAC) values of *Syzygium cumini* fruit at pH 5.0–9.0 is (15 µmol Trolox/g fruit (Luximon-Ramma *et al*., 2003). In another study, TEAC and ORAC values of *Syzygium cumini* fruit were 9.7 µmol Trolox/g fruit, pH 7.0 and 16.4 µmol Trolox/g fruit, pH 7.4 respectively (Faria *et al*., 2011). The fruit pulp is also reported to have antioxidant activity evidenced by the results of TEAC and ferric



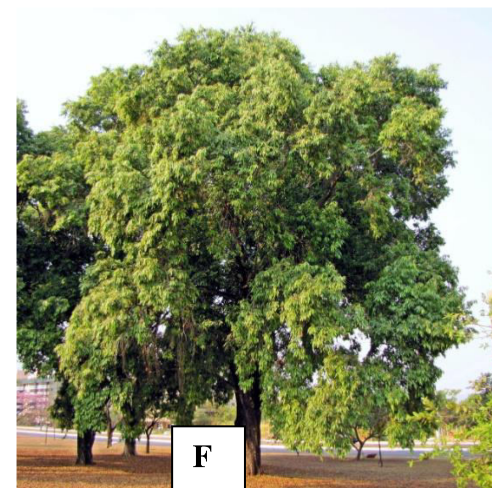


Fig. 1.8: Different parts of Syzygium cumini A) Flower B) Fruit C) Seed D) Leaf E) Bark F) Syzygium cumini plant
(http://tropical.theferns.info)

reducing antioxidant potential (FRAP) (Luximon-Ramma *et al*., 2003). The high antioxidant activity of the extract at extremely low concentrations makes *Syzygium cumini* a potential source of antioxidants (Veigas *et al*., 2007). When compared to standards, such as catechin and trolox, *Syzygium cumini* seed kernel of the jamun fruits showed significant activity against the superoxide anion and hydroxyl radical (Benherlal *et al*., 2007) and aqueous extract of *Syzygium cumini* seed kernel is reported to ameliorate antioxidant status (Prince *et al*., 1998). *Syzygium cumini* seed kernel extracts has been reported to enhance plasma antioxidant capacity by increasing levels of enzymatic antioxidants such as superoxide dismutase and catalase (Mandal *et al*., 2008). *Syzygium cumini* seed kernel extracts have been shown to protect pancreatic beta cells from alloxan-induced oxidative stress in animal models and reversed hyperglycemia by regenerating beta cells and enhanced other endogenous antioxidant levels in plasma (Ravi *et al*., 2004). Leaf extracts of *Syzygium cumini* have been demonstrated for their radioprotective activity in the γ radiation-induced micronuclei formation assay in cultured human peripheral blood lymphocytes (Jagetia *et al*., 2004). However, a comprehensive antioxidant activity evaluation of different sequential fractions of *Syzygium cumini* seeds has not been reported.

1.1.5.4.2. Cardioprotective activity

Methanolic extract of *Syzygium cumini* seed showed cardioprotective action against glucose-induced oxidative stress (Atale *et al*., 2013) and also isoproterenol-induced myocardial infarction in rats (Mastan *et al*., 2009). It showed maximum anti glycoxidative potential and therefore proposed to play a therapeutic role for diabetic complications associated with heart (Atale *et al*., 2013). The presence of flavonoids in *Syzygium cumini* seed extracts reduced intracellular oxidative stress and depletion of cellular antioxidants (Devkar *et al*., 2012). Aqueous extract of *Syzygium cumini* seed was

reported to play a crucial role in modulating PPAR, reduce dyslipidemia, oxidative stress, and TNF-α in type 2 diabetic rats and hence ameliorate insulin resistance and β-cell dysfunction (Sharma *et al*., 2012). The hydroalcoholic extract of *Syzygium cumini* leaves has been reported to exhibit potential to inhibit arterial tone and extracellular calcium influx in Wistar rats and hence to show antihypertensive effect (Ribeiro *et al*., 2014).

1.1.5.4.3. Antidiabetic activity

Different parts of the *Syzygium cumini* especially fruits, seeds and stem bark possess promising activity against diabetes mellitus, and it has been confirmed by several experimental and clinical studies. *Syzygium cumini* bark was reported to have anti-diabetic activity substantiated with positive oral glucose tolerance test in a mouse model (Villasenor *et al*., 2006) and Streptozotocin (STZ) induced diabetic rat model (Saravanan *et al*., 2008). Studies conducted in type 2 diabetic human volunteers showed that administration of whole *Syzygium cumini* fruit extract decreased serum glucose level significantly in a dose-dependent manner (Stanely *et al*., 1997). Several studies reported the antidiabetic potential of the anatomical parts of the fruit. Water and alcohol extracts of edible portion of fruit (pulp) were reported to have antihyperglycemic activity (Pepato *et al*., 2005; Sharma *et al*., 2006). Leaves were also reported for their hypoglycemic effect (Damasceno *et al*., 2002). *Syzygium cumini* seed aqueous extract has been studied for the capacity to ameliorate glucose metabolizing enzymes in alloxan induced diabetic rats (Stanely *et al*., 1997). In STZ induced diabetic rats too, the methanol extract of kernel ameliorated glucose metabolism evidenced by the recovery in the activities of hexokinase, glucose-6-phosphatase, and glucose-6-phosphate dehydrogenase activity in liver besides normalizing fasting blood glucose, liver and skeletal muscle glycogen level and plasma insulin level (Mallick *et al*., 2006). *Syzygium cumini* seed kernel extracts

showed significant α-glucosidase activity (Shinde *et al*., 2008). Ethyl acetate (200 mg/Kg) extract, methanol extract (400 mg/Kg) and a compound isolated from methanol extract, mycaminose (40 mg/Kg), had been reported for their anti-diabetic effects against STZ-induced diabetic rats (Kumar *et al*., 2013). In the early 1960s to 1970s, various reports came regarding the antidiabetic activity of different parts of *Syzygium cumini* in diabetic animals (Sepha *et al*., 1956; Sigogneau-Jagodzinski *et al*., 1967; Chirvannia *et al*., 1974; Vikrant *et al*., 2001). Based on this data, authors suggested that *in vivo* hypoglycemic effect might be due to the fraction containing gummy fiber. Seed coat does not show antidiabetic activity (Ravi *et al*., 2004). Antidiabetic activity has been reported using alcoholic extracts of seeds (Teixeira *et al*., 2004; Sridhar *et al*., 2005; Singh *et al*., 2007;). Flavonoid-rich extracts prepared from *Syzygium cumini* seed alcohol extract was found to have comparatively better hypoglycemic activity than that of the kernel crude methanol extract (Sharma *et al*., 2008). Administration of lyophilized *Syzygium cumini* plant powder has been reported to have antihyperglycemic effect in streptozotocin (STZ) induced diabetic rat (Grover *et al*., 2000). Apart from antihyperglycemic effect, the whole plant is known to reduce renal hypertrophy and urinary albumin level in STZ induced diabetic rat models (Grover *et al*., 2001). It has been reported that feeding rats with 400 mg plant extract/day for 15 days prevented hyperglycemia induced by high fructose diet (Vikrant *et al*., 2001). Hypoglycemic effect of plant extract has also been shown in STZ induced diabetic rats (Grover *et al*., 2002). Inorganic contents such as Zn, Cr, V, K and Na in *Syzygium cumini* seed has been reported to exhibit normoglycemia and better glucose tolerance in STZ induced diabetic rats (Ravi *et al*., 2004). Scientific investigations on *Syzygium cumini* containing herbo mineral formulations viz. “hyponidd,” “Dianex,” “Diakyur,” have been reported to exhibit anti-hyperglycemic activity in a double blind placebo controlled studies (Babu and Prince, 2004; Joshi *et al*.,

2007; Mutalik *et al*., 2005). Evaluation of *Syzygium cumini* containing anti-diabetic poly herbal formulation in alloxan induced diabetic rats also showed significant hypoglycemic activity, positive glucose tolerance activity, and reduced lipid peroxidation in various organs compared to that of the diabetic control animals (Joshi *et al*., 2007). Another study that evaluated four classical antidiabetic formulations, all of them contained *Syzygium cumini* as one of many ingredients and reported that all formulations exhibited significant hypoglycemic activity (Rafeeuddin *et al*., 2004). The absence of antidiabetic activity was also reported. Oleveira *et al*., evaluated crude ethanol extract, water and butanol fractions of *Syzygium cumini* leaves and reported no significant reduction in glycemia compared to that of control animals (Oliveira *et al*., 2005). Absence of antihyperglycemic activity of *Syzygium cumini* leaf has also been reported by several authors (Pepato *et al*., 2001; Teixeira *et al*., 2006).

1.1.5.4.4. Anti-inflammatory activity

Syzygium cumini leaf and bark are reported to have anti-inflammatory activity (Slowing *et al*., 1994). The ethanolic extract of *Syzygium cumini* bark showed anti-inflammatory activity in animal models, and up to a dose of 10.125 g/kg, the extract was found non-toxic (Muruganandan *et al*., 2001). Chloroform extract of *Syzygium cumini* seeds was reported to inhibit carrageenan, kaolin and other mediator-induced edema in experimental animals. The extract also inhibited potency to inhibit inflammation, migration of leucocytes, granuloma formation (Chaudhuri *et al*., 1990). Another recent study also reported the anti-inflammatory activity of *Syzygium cumini* seed alcohol extract on carrageenan-induced rat paw edema (Kumar *et al*., 2008). *Syzygium cumini* leaves have been reported for their anti-inflammatory activities in animal models (Brito *et al*., 2007; Lima *et al*., 2007).

1.1.5.4.5. Other biological activities

Syzygium cumini pulp is shown to have gastro protective and anti-ulcerative activity (Ramirez and Roa Jr, 2003; Chaturvedi *et al*., 2007). According to Ayurveda, the bark is used for sore throat, bronchitis, asthma, thirst, dysentery and to cure ulcers (Kirtikar *et al*., 1975) and also reported to have excellent wound healing property (Nadkarni *et al*., 1954). Anti-human immuno deficiency virus (HIV) type-1 protease inhibitor activity was observed in the *Syzygium cumini* bark extract (Kusumoto *et al*., 1995). The seed extract have been used to treat cold, cough, fever and skin problems such as rashes and the mouth, throat, intestines and genitourinary tract ulcers (infected by *Candida albicans*) by the villagers of Tamil Nadu (Chandrasekaran *et al*., 2004). Central nerve system stimulation activity by the alcohol extract of *Syzygium cumini* seed was also reported (Kumar *et al*., 2007). In Unani medicine, the ash of *Syzygium cumini* leaves is used to strengthen the gums and teeth. *Syzygium cumini* is also reported for antibacterial and anti-HIV activity (Ravi *et al*., 2004). The other biological activities include neuropsychopharmacological (Chakraborty *et al*., 1986), anti-microbial (Chandrasekaran *et al*., 2004), anti-bacterial (Nascimento *et al*., 2000), anti-HIV (Kusumoto *et al*., 1995), antileishmanial and antifungal (Braga *et al*., 2007), nitric oxide scavenging (Jagetia *et al*., 2004), antidiarrheal (Mukherjee *et al*., 1998), anorexigenic (Krikorian-Manoukian *et al*., 1967) gastroprotective and anti-ulcerogenic (Ramirez *et al*., 2003), and radioprotective (Jagetia *et al*., 2003) activities. Other therapeutically significant properties of *Syzygium cumini* plant parts have also been reported. *Syzygium cumini* plant extract also reported having a hypotensive and diuretic effect (Cirqueira et al., 2005). The whole plant is said to have antileishmanial and antifungal activity (Braga *et al*., 2007). Methanol and water extracts of *Syzygium cumini* seed were found to inhibit the growth of several species of bacteria and fungus (Chandrasekaran et al., 2004;

Deshpande *et al*., 2005). *Syzygium cumini* leaf was reported to have better inhibitory activity against many multidrug resistant bacterial species (Oliveira *et al*., 2007). A tincture prepared from the bark of *Syzygium cumini* plant was reported to have strong fungicidal activity (Dutta *et al*., 2000). Shafi *et al*. reported the antibacterial activity of *Syzygium cumini* essential oil (Shafi *et al*., 2002).

1.1.6. Chemical constituents reported in *Syzygium cumini* seed

Syzygium cumini seeds are reported to have compounds belonging to the class of flavonoids, alkaloid, tannin, lipid, coumarine, glucoside, alkane, benzenoids, etc. A compiled data on the literature review of all the compounds present in *Syzygium cumini* seeds is depicted in Table 1.9.

Table 1.9: Chemical constituents reported in Syzygium cumini seed

Compound	Class	Reference
Jamboline	Glucoside	Ayyanar *et al*., 2012
Rutin	Flavonoid	Arun *et al*., 2011
Mycaminose		Srivastava *et al*., 2013
Medioresinol-4"-O-β-glucoside	Lignan glycoside	Martin *et al*., 1998
Pinoresinol-O-β-glucoside	Lignan glycoside	Martin *et al*., 1998
Jambosine	Alkaloid	Ayyanar *et al*., 2012
Cuminoside		Farswan *et al*., 2009
3,6-hexahydroxy Diphenoylglucose	Tannin	Bhatia and Bajaj, 1975
4,6-hexahydroxydiphenoylglucose	Tannin	Bhatia and Bajaj, 1975
1-galloylglucose	Tannin	Bhatia and Bajaj, 1975

3- galloylglucose	Tannin	Bhatia and Bajaj, 1975
ß-sitoterol	Triterpenoid	Sagrawat *et al*., 2006
Quercetin	Flavonoid	Sagrawat *et al*., 2006
Taxifolin	Flavonoid	Bhatia and Bajaj, 1975
4-(2-2-dimethyl-6-6-methylenecyclohexyl)butanol	sesquiterpenoids	Kumar *et al*., 2009
Decahydro-8a-ethyl-1,4a,6-tetramethyl naphthalene		Kumar *et al*., 2009
Eicosane	Acyclic Alkane	Kumar *et al*., 2009
Heptacosane	Acyclic Alkane	Kumar *et al*., 2009
1-chlorooctadecane	Acyclic Haloalkane	Kumar *et al*., 2009
Octacosane	Acyclic Alkane	Kumar *et al*., 2009
Tetratetracontane	Acyclic Alkane	Kumar *et al*., 2009
Octadecane	Acyclic Alkane	Kumar *et al*., 2009
Diphenic acid	benzenoid	Bhatia and Bajaj, 1975
Corilagin	Tannin	Bhatia and Bajaj, 1975
3-3'-4-tri-O-methyl ellagic acid	Coumarin	Bhatia and Bajaj, 1975
3-3'-di-O-methyl ellagic acid	Coumarin	Bhatia and Bajaj, 1975
gallic acid	Benzenoid	De Lima *et al*., 1998
Coniferyl alcohol	Phenylpropanoid	Martin *et al*., 1998
Pinoresinol-O-ß- glucoside	Lignan	Martin *et al*., 1998
Syringaresinol-O-ß-glucoside	Lignan	Martin *et al*., 1998
Lectin	Protein	Tabora *et al*., 1980
Elaeostearic acid	Lipid	Das *et al*., 1995

Lauric acid	Lipid	Daulatabad *et al*., 1988
Oleic acid	Lipid	Daulatabad *et al*., 1988
Linoleic acid	Lipid	Daulatabad *et al*., 1988
Malvalic acid	Lipid	Daulatabad *et al*., 1988
Myristic acid	Lipid	Daulatabad *et al*., 1988
Palmitic acid	Lipid	Daulatabad *et al*., 1988
Stearic acid	Lipid	Daulatabad *et al*., 1988
Sterculic acid	Lipid	Daulatabad *et al*., 1988
Vernolic acid	Lipid	Daulatabad *et al*., 1988
Caffeic acid	Phenol	Daulatabad *et al*., 1988
Ferulic acid	Phenol	Daulatabad *et al*., 1988
Resorcinol dimethyl ether	Ether (Aromatic aroma and Flavour)	Daulatabad *et al*., 1988
ß- pinene	Monoterpene	Williamson., 2002
α-terpinene	Monoterpene	Williamson., 2002
Terpinolene	Monoterpene	Williamson., 2002
Borbeneol	Terpene	Williamson., 2002
ß-phellandrene	Monoterpene	Williamson., 2002
α -terpineol	Monoterpene	Williamson., 2002
Eugenol	Monoterpene	Williamson., 2002
Furfural,5-(hydroxy-methyl)	Oxygen heterocycle	Martin *et al*., 1998
Medioresinol-4''-o-betabglucoside	Lignan	Martin *et al*., 1998

1.2. Aims and objectives of the study

Literature studies showed a positive correlation between consumption of fruits and vegetables and delayed onset and progression of lifestyle diseases. Recent statistics regarding increased demand for plant-based healthcare products is also gaining importance. However, literature survey showed that many plants that have medicinal properties are not scientifically validated. This increases the demand for scientific investigation on traditionally used plants to confirm known therapeutic properties and identification of active principles/fractions. The phytochemical contents present in different geographical variants vary due to variety, age, maturity of the plants used, season, geo-agro-climatic conditions, agronomical practices, post-harvest handling, storage, processing, etc. The phytochemical constituents present in different fractions vary, and that would affect the biopotency. *Syzygium cumini* was the plant selected for the present study. *Syzygium cumini* is a tropical plant of *Myrtaceae* family, endemic to India, Pakistan, Sri Lanka and Malaysia. Different parts of *Syzygium cumini* has been used in various traditional medicines for the treatment of diabetes, and various other diseases. Medicinal properties of *Syzygium cumini* seeds are reported by different authors but, most of the studies are limited to single solvent fractions. Though several researchers studied the anatomical parts and whole plant of *Syzygium cumini*, the seed is found to be the most important part as it is clear cut by the volume of data generated by standardized analytical methods. However, the literature study showed a major literature gap in the lack of knowledge about the mechanism of action on antidiabetic, cardioprotective and anti-inflammatory activities of different sequential fractions of *Syzygium cumini* seeds. This study is the first to compare the antioxidant activity of different variants of *Syzygium cumini* seeds. Based on the literature gap as well as extending the possibility of

Syzygium cumini seed for prophylactic and therapeutic applications, the objectives of present study are summarized as:

1. To check the antioxidant potential and chemical characterisation of *Syzygium cumini* seed fractions through HPLC and LC-MS/MS.
2. To elucidate the cardioprotective efficacy of *Syzygium cumini* seed fractions in H9c2 cardiac myoblast cells.
3. To find out the molecular mechanism behind the anti-diabetic potential of *Syzygium cumini* seed fractions.
4. To determine the anti-inflammatory mechanism of different fraction of *Syzygium cumini* seed.

The proposed work has significant relevance in the present scenario on the under-utilized product as phytochemicals with health benefits. The scientific knowledge generated on health benefits of *Syzygium cumini* seeds would help to use this seed for value-added nutraceutical formulations.

1.2.1. Societal impact of the study

Kerala, situated in the region of Western Ghats is rich in traditional and indigenous knowledge. The region of Western Ghats is rich in all biodiversity, richness, and endemism of different species. More than 2000 medicinal plants with excellent therapeutic potential are available in this region. Nowadays, the possibilities of balanced conservation of biodiversity and indigenous knowledge remain unexplored. Though Kerala is a state of high literacy and improved healthcare facilities, the prevalence of lifestyle diseases is very high. Hence it is high time to develop cost effective strategies for the management of lifestyle associated diseases to reduce the incidence and prevalence of this epidemic. The prevalence of lifestyle diseases especially diabetes and cardiovascular diseases can be reduced to a certain extent by utilizing the biodiversity

and indigenous knowledge. The plant selected for present study is available in Western Ghats. The study explores the beneficial role of *Syzygium cumini* seeds which remains under-utilized, that can be made useful to the mankind if it is expected to uncover more scientific information for its use in lifestyle associated diseases. This study helps to scientifically validate the traditional knowledge for universal acceptance and wider use.

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CHAPTER 2

Antioxidant activity and chemical characterization of Syzygium cumini seeds

2.1. INTRODUCTION

The effect of free radicals in biology is producing a medical revolution that ensures a new age of health and disease management. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are the predominant by-products of cellular redox processes. These free radicals possess both toxic and beneficial effects. ROS and RNS exert beneficial effects on cellular responses and immune function at low or moderate level. They generate oxidative stress at high concentrations, which is a deleterious process that can damage cell structure and function (Oyagbemi *et al*., 2009). Oxidative stress is an important factor in the progression of chronic degenerative diseases including coronary heart disease, cancer, and arthritis (Zhang *et al*., 201). The human body can counteract this oxidative stress with the support of exogenous antioxidants and by producing various endogenous antioxidants. For the past few decades, the secondary metabolites from plants have been well known for their antioxidant potential (Pandey *et al*., 2009).

Plant phenolics are the most fascinating antioxidants which include predominantly phenolic acids, flavonoids, and tannins. Phenolic acids can be either derivatives of benzoic acid such as gallic acid, or derivatives of cinnamic acid such as coumaric, caffeic and ferulic acid. The most abundant phenolic acid in many fruits and vegetables is caffeic acid (Manach *et al*., 2004). Researchers and food manufacturers have become more engrossed in polyphenols due to their potent antioxidant properties, their richness in the diet, and their ability to prevent various oxidative stress associated diseases (Nanda *et al*., 2014). Antioxidants are defined as compounds that can delay, inhibit, or prevent the oxidation of oxidizable materials by scavenging free radicals and diminishing oxidative stress. The inverse relationship between the dietary intake of fruits and vegetables and the chance of oxidative stress associated diseases has been partially

accredited to phenolics (Grosso *et al*., 2016). The phenolic compounds in plants are reported to have antidiabetic, anticancer, anti-inflammatory, antimutagenic, antimicrobial and other activities. Moreover, polyphenols can modulate the activity of a wide range of enzyme and cell receptors. In this way, in addition to having antioxidant properties, polyphenols have several other specific biological actions in preventing and or treating diseases.

Medicinal plants are an important source of antioxidants. Phenolic compounds from natural products are gaining importance because of their relatively safe and wide acceptance by consumers. The increasing interest in the search for natural replacements for synthetic antioxidants has led to the antioxidant evaluation of some plant species. Antioxidants have the ability to counteract the damaging effects of free radicals inside our body. If free radicals formed are left unchallenged, they would eventually lead to the etiology of a wide range of diseases.

Syzygium cumini (L.) Skeels, belonging to the family Myrtaceae, is one of the best-known species and is often distributed in Asia (East India, Malaysia, and China). *Syzygium cumini* is widely used in traditional systems of medicines in India, like Ayurveda, Unani, and Siddha. Different parts of *Syzygium cumini* are reported to have several medicinal properties like antidiabetic (Bose *et al*., 1956), antimicrobial (Chandrasekaran *et al*., 2004), anti-inflammatory (Chauduri *et al*., 1990) and free radical scavenging potential (Banerjee *et al*.,2006; Silva *et al*., 2006). The seeds have been reported to possess compounds like jambosine, gallic acid, ellagic acid, corilagin, 3,6-hexahydroxy diphenoylglucose, 1-galloylglucose, 3-galloylglucose, quercetin, β-sitoterol and 4,6 hexahydroxydiphenoylglucose (Rastogi *et al*., 1990; Sagrawat *et al*., 2006).

The phytochemical content of active fraction is subject to large variations due to variety, age, maturity of the plants used, season, geo-agro-climatic conditions, agronomical

practices, post-harvest handling, storage, processing, etc. The active principle thus can vary tremendously and that, in turn, would affect the biopotency. It is in this context that chemical profiling of the plants is important to produce products with consistent quality. Therefore, in the present study, we compared the antioxidant activity and profiling of *Syzygium cumini* seeds collected from three different geographical locations of southern part of India.

2.2. MATERIALS AND METHODS

2.2.1. Chemicals

Gallic acid, chlorogenic acid, caffeic acid, syringic acid, coumaric acid, ferulic acid, ellagic acid, cinnamic acid, catechol, myricetin, quercetin, kaempferol, apigenin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), sodium carbonate, aluminium chloride, potassium acetate and all other chemicals and biochemicals unless otherwise noted were from Sigma (St. Louis, MO, USA). All the positive controls used were of HPLC grade. Folin Ciocalteau reagent, HPLC grade methanol and acetic acid were supplied by Merck, Germany; All other chemicals used were of standard analytical grade.

2.2.2. Plant Material

The fully mature *Syzygium cumini* fruits were collected from Trivandrum - TVM (8° 29' N, 76° 59’ E), Trichy - TCH (10° 48’ N, 78° 41’ E) and from Malampuzha - MPA (10.7° N, 76.6° E). The samples were authenticated by Dr. E. S. Santhosh Kumar, Technical Officer, Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Palode, Trivandrum, Kerala and voucher specimens (Collection No: SC-APNP-CSIR-100, 101 and 102) were deposited in the herbarium of JNTBGRI, Palode,

Trivandrum, Kerala, INDIA. Samples were collected during the month of April and stored at -80°C until processed.

2.2.3. Preparation of plant fractions

Syzygium cumini seeds were separated from fruits and washed well using distilled water. The seeds thus obtained were dried in the oven at 40°C, seed coats were removed, and seeds were coarsely powdered using a motor and pestle. Dried powder of *Syzygium cumini* (2 Kg) was extracted sequentially with hexane (HE), ethyl acetate (EA), methanol (ME), 70% methanol (70% ME) and water (WE) at room temperature (27 ± 1°C). The extraction process was repeated till each solvent became colourless. These fractions were filtered through Whatman No. 1 filter paper. The fractions were evaporated in rotavapor and stored at 4°C, protected from light for further analysis.

2.2.4. Experimental design

The work flow of this chapter is illustrated in the schematic representation in Fig 2.1.

2.2.5. Yield of extracts

The extract (1 mL) was pipetted out to a pre-weighed Petri dish and kept in the oven for 4 h at 100°C. The weight of the Petri dish was then measured. The petri dish was kept in the oven till the weight become constant. The difference in weight of the petri dish gave the yield of extract in 1 mL.

2.2.6. Antioxidant assays

2.2.6.1. DPPH scavenging activity

The DPPH scavenging activity of different extracts was evaluated according to the method of Brand Williams (Brand-Williams *et al*., 1995). 1 mL of 0.1 mM DPPH solution in methanol was mixed with 1 mL of extract solution of varying concentrations. Corresponding blank sample were prepared and gallic acid was used as reference standard. Mixture of 1 mL methanol and 1 mL DPPH solution was used as control. The

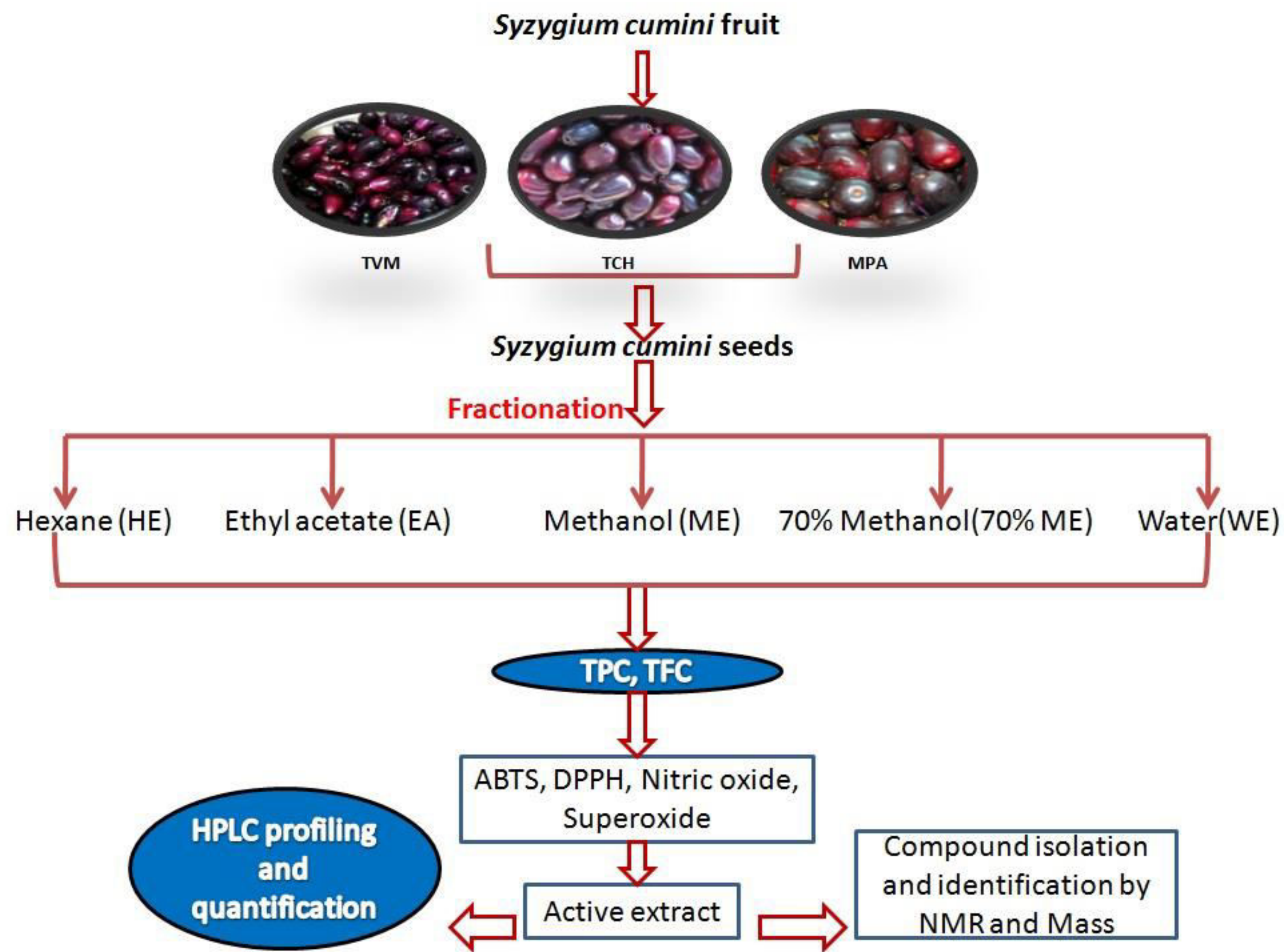


Fig. 2.1: Schematic representation of experimental design

TVM-Trivandrum, TCH-Trichy, MPA-Malampuzha, TPC-Total phenolic content, TFC-Total flavonoid content

mixture was shaken well and incubated for 30 min in dark. The reaction was carried out in triplicate and the decrease in absorbance was measured at 517 nm after incubation using a multiplate reader (Synergy 4 Biotek.USA). The scavenging activity was expressed as IC_{50} (µg/mL).

The % inhibition was calculated using the formula

$$\text{DPPH radical scavenging activity (\%)} = \left(\frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}}\right) \times 100$$

2.2.6.2. Nitirc oxide (NO) scavenging activity

Nitric oxide scavenging activity was estimated according to the method of Marcocci *et al*., (1994) with slight modification. The reaction mixture contained 1 mL of 10 mM SNP, phosphate buffered saline (pH 7.4) and various concentration of extracts in a final volume of 1.1 mL. After incubation for 150 min at room temperature, pipetted out 100 µL into wells plate and 100 µL of Griess reagent was added. The mixture was incubated for 10 min at 25°C. The pink chromophore generated was measured spectrophotometrically at 540 nm against a blank sample. All tests were performed in triplicates. Ascorbic acid was used as the standard. The percentage inhibition of nitric oxide radical generation was calculated using the following formula:

$$\text{NO radical scavenging activity (\%)} = \left(\frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}}\right) \times 100$$

2.2.6.3. Superoxide radical scavenging activity

Superoxide radical scavenging activity of different extracts was measured by the reduction of NBT according to a previously reported method (Fontana *et al.*, 2001). The non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS/NADH) system generates superoxide radicals, which reduce nitro blue tetrazolium (NBT) to a purple formazan. The 1 mL reaction mixture contained phosphate buffer (20 mM, pH

7.4), NADH (73 µM), NBT (50 µM), PMS (15 µM) and various concentrations of sample solution. After incubation for 5 min at room temperature, the absorbance at 562 nm was measured against an appropriate blank to determine the quantity of formazan generated. Quercetin was used as the standard and the percentage radical scavenging capacity was determined using the formula:

$$\text{Superoxide radical scavenging activity (\%)} = \left(\frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}}\right) \times 100$$

2.2.6.4. ABTS scavenging activity

ABTS scavenging potential of extracts were analyzed by the method of Arnao *et al.*, (2001) with some modifications. The working solution was prepared by mixing the stock solutions - 7 mM ABTS and 2.45 mM potassium persulfate solution in equal quantities, and allowing them to react for 14 h at room temperature in the dark. The solution was then diluted with ethanol to make the absorbance in the range 0.70 ± 0.01 units at 734 nm. Different concentrations of extracts were allowed to react with 1 mL of the ABTS solution for 7 min, and the absorbance was taken at 734 nm. The ABTS scavenging capacity of the extract was compared with that of ascorbic acid, and percentage inhibition was calculated as

$$\text{ABTS radical scavenging activity (\%)} = \left(\frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}}\right) \times 100$$

2.2.7. Determination of Total phenolic content (TPC)

The TPC was determined by Folin Ciocalteau method (Singleton *et al.*, 1965) with slight modifications. Briefly, different concentrations of fractions (20-100 µL) were taken, and 80 µL of Folin Ciocalteau reagent and 200 µL sodium carbonate (20%) were added, made up to 700 µL using distilled water and incubated at ambient temperature (25-27°C) for 90 min. The colour developed was measured at 760 nm using a multimode

reader (Biotek, USA). The phenolic contents were calculated using a standard curve for gallic acid, and results were expressed as mg gallic acid equivalents (GAE) per gram dry weight of fraction (mg GAE/g). All measurements were performed in triplicates.

2.2.8. Determination of Total flavonoid content (TFC)

The TFC was estimated using standard procedures described by Chang *et al*., (2002) with slight modifications. Different concentrations of fractions were diluted with 150 µL of ethanol. Further, 10 µL of 10% aluminium chloride solution and 1 M potassium acetate (10 µL) were added and made up to 280 µL using distilled water. The final solution was mixed well and incubated at room temperature for 40 min. The absorbance was measured at 415 nm using a multimode reader (Biotek, USA). Quercetin was used as a standard, and results were expressed as mg quercetin equivalents (QE) per gram dry weight of fraction (mg QE/g).

The whole work of the thesis was performed using the single lot of extracts stored at 4°C; the stability of these extracts were checked at every 6 months by TPC and TFC analysis. There was no significant difference in the TPC and TFC quantities throughout the period of the work.

2.2.9. HPLC-DAD analysis of phenolic compounds

The identification and quantification of phenolic compounds present in different fractions of *Syzygium cumini* were performed with a Shimadzu HPLC system containing two LC-8A preparative liquid chromatography pump units, a C18 reverse phase column (Phenomenex, 5 µm, 250 x 4.6mm2 dia.) and a diode array detector (DAD; SPD-M10A VP) with a wavelength range of 200-450 nm. The fractions and 13 reference standards viz. gallic acid, chlorogenic acid, caffeic acid, syringic acid, coumaric acid, ferulic acid, ellagic acid, cinnamic acid, catechol, myricetin, quercetin, kaempferol, apigenin were prepared in HPLC grade methanol at a concentration of 1 mg/mL and filtered through a

0.45 μm filter. Each sample (20 μL) were injected, and the HPLC analysis was done according to the standard method (Chen *et al*., 2001) with slight modifications. The mobile phase used was water:acetic acid (98:2, v/v) as solvent A and methanol: acetic acid (98:2, v/v) as solvent B with a time program of 0– 15 min 15% B, 16–20 min 50% B, 21–35 min 70%B, 36–50 min 100% B. The flow rate was 1 mL/min and the column temperature was set at 30°C. Identification and quantification of the phenolic compounds was done by comparing the retention time and characteristic absorption spectra from the DAD with those of the authentic standards. In order to minimize variation in quantification, samples were taken in triplicates. Data acquisition and analysis were carried out using Shimadzu- Class-VP version 6.14 SP1 software.

2.2.10. LC-Q-ToF (MS/MS) analysis of phenolic compounds

The fractions were analyzed by the acquity H class Ultra Performance Liquid Chromatography and Xevo G2 Quadrapole – Time-of-Flight (Q-TOF) mass spectrometer (Waters, USA). Chromatographic separation was carried out on a BEH C18 column (50 mm × 2.1 mm × 1.7 µm). Polyphenols in each fractions were separated using a 10 min long gradient program using 95% mobile phase A (water containing 0.1% (v/v) formic acid) and 5% mobile phase B (LC- MS grade methanol) for 0.1 min, 95% B upto 6.0 min, 95% B upto 6.5 min, 5% B upto 8.0 min, and 5% B upto 9.0 min. The flow rate was 0.3 mL/min, and the column temperature was held at 40 °C. MS analysis was conducted using ESI ion source in a negative ionization mode with the following parameters: capillary voltage 2.5 kV, sample cone 30 V, extraction cone 1 kV, capillary temperature 350 °C, sheath gas (N_2) flow rate 900 L/Hr, auxiliary gas (N2) flow rate 50 L/Hr. The scan range was m/z 2–1000, the maximum injection time was 10 min with three microscans. Two scan events were arranged to run sequentially in the LCQ mass spectrometer. The first event was a full scan mass spectrum to acquire data on anions in

the designated scan range. The second scan event was an MS/MS experiment at normalized collision energy of 35%, performed on the most prominent $[M - H]^-$ ion acquired during the previous scan event.

2.2.11. Isolation and identification of compound from 70% methanol fraction

Acetone extraction was done for 10 gm of 70% methanol fraction (10 gm) by cold maceration, and the extract was concentrated by reduced pressure to yield crude acetone extract. The acetone soluble fractions of 70% methanolic extracts were subjected to column chromatography. The column was packed with silica (100-200 #) using hexane as solvent. The column was then eluted with increasing order of polarity from 100% hexane to 100% ethyl acetate. The fraction eluted at 50% hexane: ethyl acetate obtained as white amorphous solid. The fraction was characterized by spectroscopy techniques like $_1$H NMR, $_{12}$C NMR, and mass spectrum.

2.2.12. Statistical analysis

All experimental results were expressed as mean ± SD (standard deviation) of three different experiments. Data were subjected to one-way analysis of variance (ANOVA), and Duncan's multiple range tests were used to test the significant differences. Data are presented as mean ± SD, and $p \leq 0.05$ was considered to be significant. All statistical analyses were performed with SPSS 11.0 (Statistical Program for Social Sciences, SPSS Corporation, Chicago, IL) for Windows.

2.3. RESULTS

2.3.1. Yield

Plants are rich in medicinally active and economically important compounds. Solvent extraction helps in segregating and concentrating the active compounds. Initial extraction with hexane defatted the extract. Ethyl acetate, methanol, 70% methanol and

water were sequentially used for the extraction. The yield of more than 70% was found for the methanolic fraction followed by 10-20% by 70% methanolic fraction of all the geographical variants (Fig 2.2). Ethyl acetate, hexane, and water fraction together constituted for only 6-8% yield in all the geographical variants.

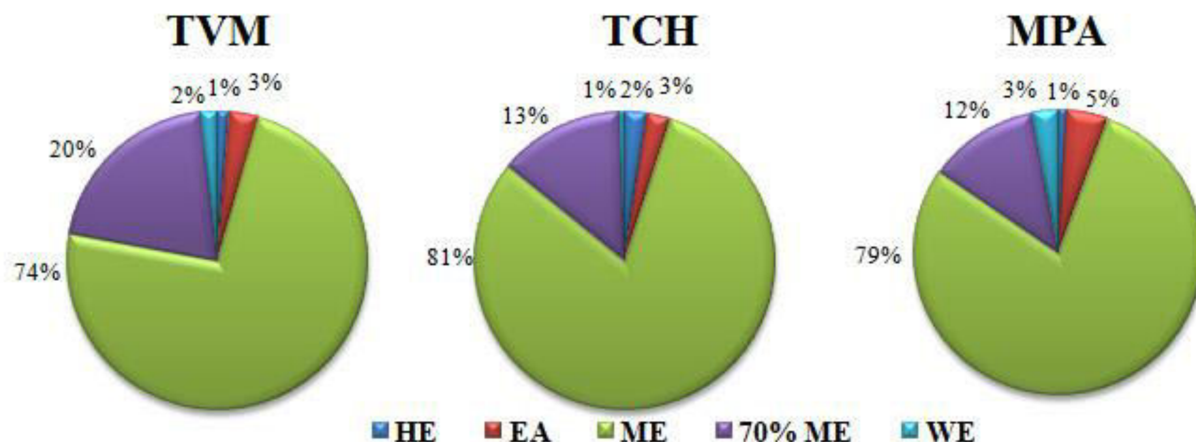


Fig. 2.2: Graph showing percentage yield in different fractions of three different geographical variants of Syzygium cumini seeds.
TVM: Trivandrum variant, TCH: Trichy variant, MPA: Malampuzha variant

2.3.2. DPPH radical scavenging activity

DPPH radical scavenging activity is one of the most widely used method to evaluate the antioxidant properties of natural products. DPPH• (1,1-Diphenyl-2-picrylhydrazyl radical) can accept an electron or hydrogen radical to become a stable diamagnetic molecule. The radical scavenging power of the sample was measured by the decrease in absorbance due to DPPH• at 517 nm, showing the formation of its reduced form, 1, 1-Diphenyl-1-2 picrylhydrazine (DPPH), which was yellow in color. The purple coloured methanolic solution shows a strong absorption band at 517 nm due to the presence of odd electron.

In all the three geographical variants, DPPH radical scavenging activity increased in the following order, water fraction < ethyl acetate fraction < methanol fraction < 70% methanol fraction. 70% methanol fraction exhibited highest DPPH scavenging activity.

The IC_{50} value of 70% methanol fraction was found to be 5.1 ± 0.96 µg/mL, 5.5 ± 0.65 µg/mL, 6.2 ± 0.43 µg/mL, respectively for TVM, TCH and MPA variants. However, the activities of extracts were less when compared to the standard, gallic acid (1.8 ± 0.77 µg/mL). Methanol extract also exhibited potential DPPH scavenging activity. The IC_{50} values for DPPH scavenging activity of ethyl acetate, methanol and water extracts of *Syzygium cumini* seeds are represented in Fig 2.3.

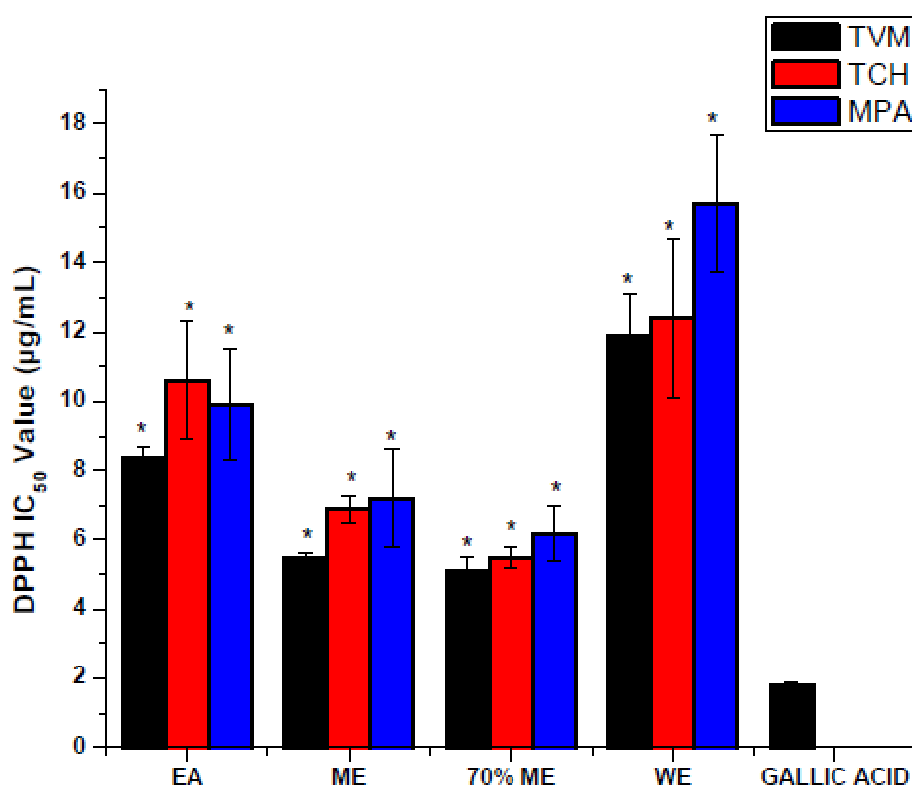


Fig. 2.3: DPPH radical scavenging activity of ethyl acetate, methanol, 70% methanol and water extracts of different geographical variants of Syzygium cumini.
EA-ethyl acetate fraction, ME-methanol fraction, 70% ME- 70% methanol fraction, WE-Water fraction, TVM-Trivandrum variant, TCH-Trichy variant, MPA- Malampuzha variant. Values are means ± SD; n = 6. * represents groups differ significantly from standard ($p \leq 0.05$).

2.3.3. Nitric oxide scavenging activity

Because of the presence of unpaired electron, nitric oxide is classified as a free radical and is a potent pleiotropic inhibitor of biological processes such as relaxation of smooth muscle, neuronal signaling, and regulation of cell mediated toxicity (Hagerman

et al., 1998). In addition to reactive oxygen species, nitric oxide is also involved in inflammation, cancer and other pathological conditions (Nabavi *et al*., 2008a; Nabavi *et al*., 2008b) and this free radical reacts with superoxide anion and form a potentially cytotoxic molecule, the peroxynitrite. Peroxynitrite causes nitration or hydroxylation of aromatic compounds especially tyrosine and also triggers adduct formation with dissolved carbon dioxide in body fluids and damages various proteins (Szabo *et al.*, 2007). NO is released from sodium nitroprusside (SNP) at physiological pH which reacts with oxygen to produce nitrite ions. The nitrite ions react with Griess reagent to form pink chromospheres whose absorbance was measured at 540 nm.

The results from the above assay illustrated that the 70% methanol extract of all geographical variants have better NO scavenging potential. The IC_{50} value of 70% methanol fractions were 4.23 ± 0.34 µg/mL, 5.23 ± 0.24 µg/mL, 6.24 ± 0.31 µg/mL, respectively for TVM, TCH and MPA geographical variants. The standard, curcumin demonstrated an IC_{50} value of 17.35 ± 2.3 µg/mL which was comparable to that of 70% methanol fraction and methanol fraction of TVM variant (IC_{50} value of 16.92 ± 2.3 µg/mL). The IC_{50} values for NO scavenging activity of ethyl acetate, methanol, 70% methanol and water fractions are represented in Fig 2.4.

2.3.4. Superoxide radical scavenging activity

Superoxide anion radical is biologically quite toxic and is one of the strongest ROS, which get converted to other harmful reactive oxygen species such as hydrogen peroxide and hydroxyl radical, damaging biomolecules which results in chronic diseases (Al-mamun *et al*., 2007). The biological toxicity of superoxide is due to its capacity to inactivate iron–sulfur cluster containing enzymes, generate the highly reactive hydroxyl radical PMS-NADH systems by oxidation of NADH and assayed by the reduction of

nitrobluetetrazolium (NBT) (Gulcin *et al.*, 2010). The consumption of superoxide anion in the reaction mixture is indicated by the decrease in absorbance at 560 nm.

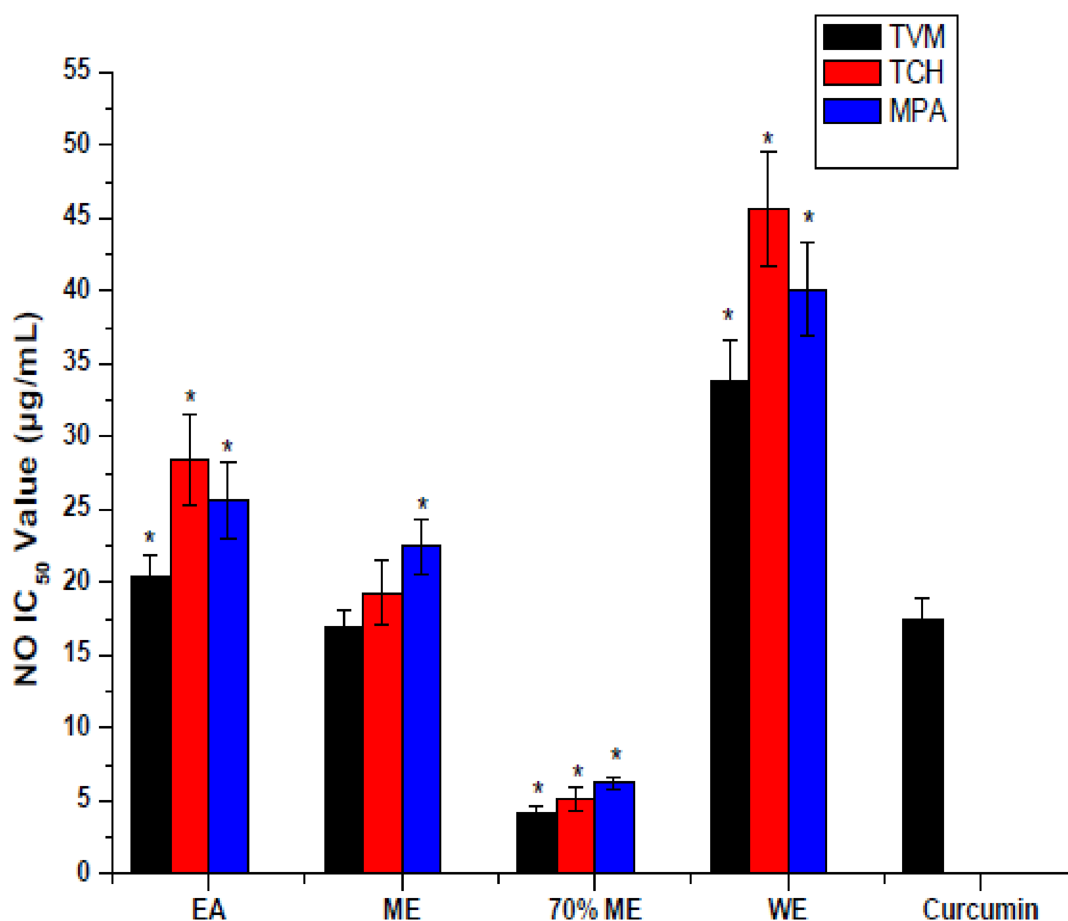


Fig. 2.4: NO radical scavenging activity of ethyl acetate, methanol, 70% methanol and water extracts of different geographical variants of Syzygium cumini.
EA-ethyl acetate fraction, ME-methanol fraction, 70% ME- 70% methanol fraction, WE-Water fraction, TVM-Trivandrum variant, TCH-Trichy variant, MPA- Malampuzha variant. Values are means ± SD; n = 6. * represents groups differ significantly from standard ($p \leq 0.05$).

The results from the assay showed that 70% methanol and methanol fractions of all geographical variants exhibited significant superoxide radical scavenging activity (Fig 2.5) which was comparable with the standard, catechin with an IC_{50} value 83.99 ± 2.34 µg/mL. The IC_{50} value of 70% methanol fractions were 28.83 ± 2.14 µg/mL, 34.72 ±

1.24 µg/mL, 39.46 ± 2.64 µg/mL, respectively for TVM, TCH and MPA geographical variants. The IC_{50} value for superoxide radical scavenging activity of all fractions of all geographical variants has been represented in Fig 2.5.

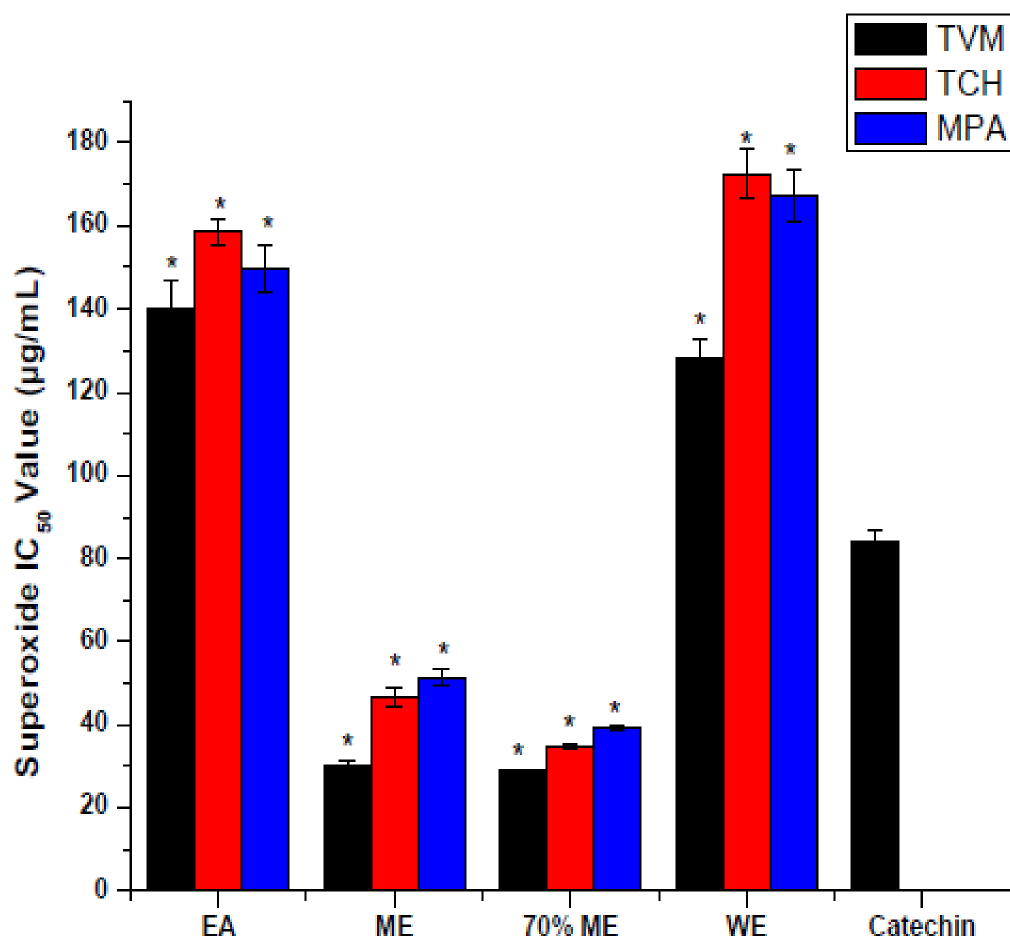


Fig. 2.5: Superoxide radical scavenging activity of ethyl acetate, methanol, 70% methanol and water extracts of different geographical variants of Syzygium cumini. EA-ethyl acetate fraction, ME-methanol fraction, 70% ME- 70% methanol fraction, WE-Water fraction, TVM-Trivandrum variant, TCH-Trichy variant, MPA- Malampuzha variant. Values are means ± SD; n = 6. * represents groups differ significantly from control group ($p \leq 0.05$).

2.3.5. ABTS scavenging potential

ABTS scavenging assay is an excellent tool for determining the antioxidant capacity of hydrogen-donating antioxidants as compared with a standard amount of Trolox. ABTS is a protonated radical which has a characteristic maximum at 734 nm,

and the interaction with the extract or standard Trolox suppresses the absorbance of ABTS radical, and the results were expressed as TEAC value (Fig 2.6).

The data indicated that both methanol and 70% methanol fractions of all geographical variants effectively scavenge ABTS radicals and the scavenging potential increased in a dose dependent manner. The standard, trolox, exhibited an IC_{50} value of 2.96 ± 0.87 μg/mL. Among the extracts, 70% methanol of TVM variant exhibited highest ABTS scavenging ability (IC_{50}: 1.43 ± 0.05 μg/mL).

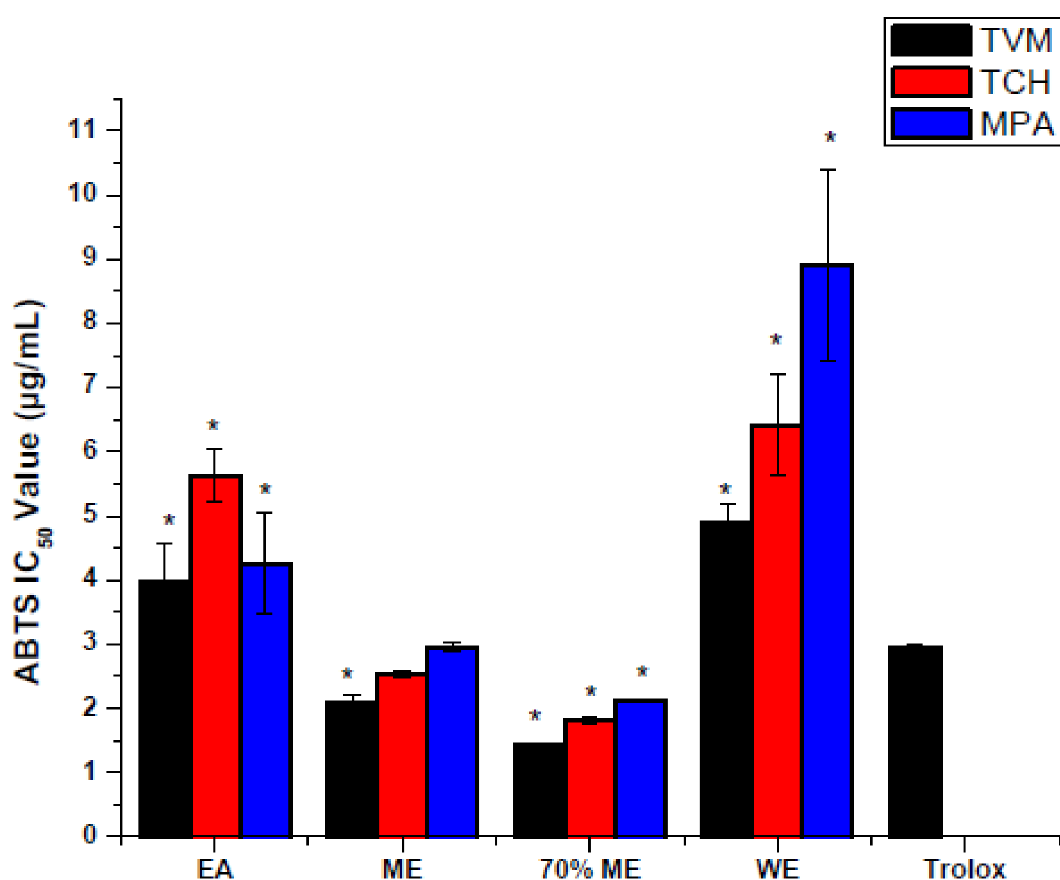


Fig. 2.6: ABTS scavenging activity of ethyl acetate, methanol, 70% methanol and water extracts of different geographical variants of Syzygium cumini.
EA-ethyl acetate fraction, ME-methanol fraction, 70% ME- 70% methanol fraction, WE-Water fraction, TVM-Trivandrum variant, TCH-Trichy variant, MPA- Malampuzha variant. Values are means ± SD; n = 6. * represents groups differ significantly from standard ($p \leq 0.05$).

2.3.6. Total phenolic content (TPC)

All the geographical variants of *Syzygium cumini* seeds were initially analyzed for their total phenolic and flavonoid contents, followed by their detailed phenolic profiling. The TPC of all fractions were expressed as milligram of gallic acid equivalents (GAE) per gram dry weight of fractions as represented in Table 2.1. All the fractions contained a significant amount of phenolic compounds. The highest phenolic content was shown by 70 % methanolic extract for all the three geographical variants (TVM - 906 ± 7.2, TCH - 808.5 ± 3.9, MPA - 984.4 ± 5.6 mg GAE/g dry weight of extracts) and methanol extract of MPA (935.4 ± 6.2 mg GAE/g dry weight of extracts). TPC increased in the following order for all the three geographical variants: hexane fraction < aqueous fraction < ethyl acetate fraction < methanol fraction < 70% methanol fraction (Table: 2.1).

2.3.7. Total flavonoid content (TFC)

TFC for all the three geographical variants of *Syzygium cumini* has been presented in Table 2.1. Our analysis revealed that for all the variants, 70% methanol possessed the highest flavonoid content (TVM - 233.8 ± 5.5, TCH - 222.8 ± 5.1, MPA - 198.3 ± 4.1 mg QE/g DW). TFC increased in the following order for all the three geographical variants: hexane fraction < aqueous fraction < ethyl acetate fraction < methanol fraction < 70% methanol fraction. Methanol fraction of all variants also showed significant flavonoid content (TVM - 137.9 ± 6.9, TCH - 120.2 ± 3.0, MPA - 106.4 ± 2.6 mg QE/g DW). TFC analysis showed that among all the three geographical variants, flavonoid content was highest in TVM variant (Hexane fraction - 6.5 ± 0.9, ethyl acetate fraction - 50.04 ± 1.1, methanol fraction - 137.9 ± 6.9, 70% methanol fraction 233.8 ± 5.5, water fraction - 19.4 ± 2.1 mg QE/g DW).

Table 2.1: Total phenolic content (mg GAE/g dry wt.) and total flavonoid content (mg QE/g dry wt.) of three different geographical variants of Syzygium cumini seeds

		Hexane	**Ethyl acetate**	**Methanol**	**70% methanol**	**Water**
TPC	TVM	33.73 ± 0.5[a]	641.66 ± 3.1[A]	757.33 ± 4.5[*]	906 ± 7.1[#]	253.33 ± 9.9[^]
	TCH	9.03 ± 0.4[b]	628.66 ± 4.5[B]	712.46 ± 4.2[**]	808.5 ± 3.9[##]	134 ± 7.5[^^]
	MPA	24.56 ± 1.0[c]	615.63 ± 7.2[C]	735.4 ± 6.2[***]	864.4 ± 5.6[###]	148.02 ± 1.8[^^^]
TFC	TVM	6.5 ± 0.9[a]	50.04 ± 1.1[A]	137.9 ± 6.9[*]	233.8 ± 5.5[^]	19.4 ± 2.1[#]
	TCH	2.5 ± 0.5[b]	41.5 ± 5.3[A]	120.2 ± 3.0[**]	222.8 ± 5.1[^^]	18.5 ± 1.3[#]
	MPA	3.5 ± 0.8[b]	48.1 ± 3.6[A]	106.4 ± 2.6[***]	198.3 ± 4.1[^^^]	14.4 ± 1.7[##]

Values are the means ± SD of three replicated samples. Duncan's multiple range test was conducted, and data in the same column with different letters/symbols indicate statistically significant differences among groups at $p < 0.05$. TVM - Trivandrum variant, TCH- Trichy variant, MPA - Malampuzha variant.

2.3.8. HPLC – DAD analysis of phenolic compounds

The results of antioxidant assays, TPC and TFC showed that TVM variant was found to be the most active among all the geographical variants. Thus, all fractions of TVM variant were subjected for phenolic profiling (Fig. 2.7) and quantified using HPLC-DAD (Table 2.2). All fractions were individually spiked with each standard and recorded an increased peak height at almost same retention time, indicating the presence of those compounds. The results showed that 70% methanol extract, methanol, and ethyl acetate extracts possessed the major quantity of phenolic acids and flavonoid compounds. Among this, 70% methanol extract of TVM showed the highest concentration of ellagic acid (222.2 mg/g dry wt) and gallic acid (272.2 mg/g dry wt) (Table 2.2, Fig. 2.7). Methanol fraction of TVM variant had increased concentration of gallic acid (531.1 mg/g DW), ellagic acid (199.1 mg/g dry wt) and myricetin (100.5 mg/g dry wt). Ethyl acetate fraction possessed high concentration of flavonoids (quercetin) and phenolic acids (cinnamic acid and gallic acid) (Table 2.2, Fig. 2.7). The phenolic acids, namely, caffeic acid, was found in least concentration which was lesser than the limit of quantification.

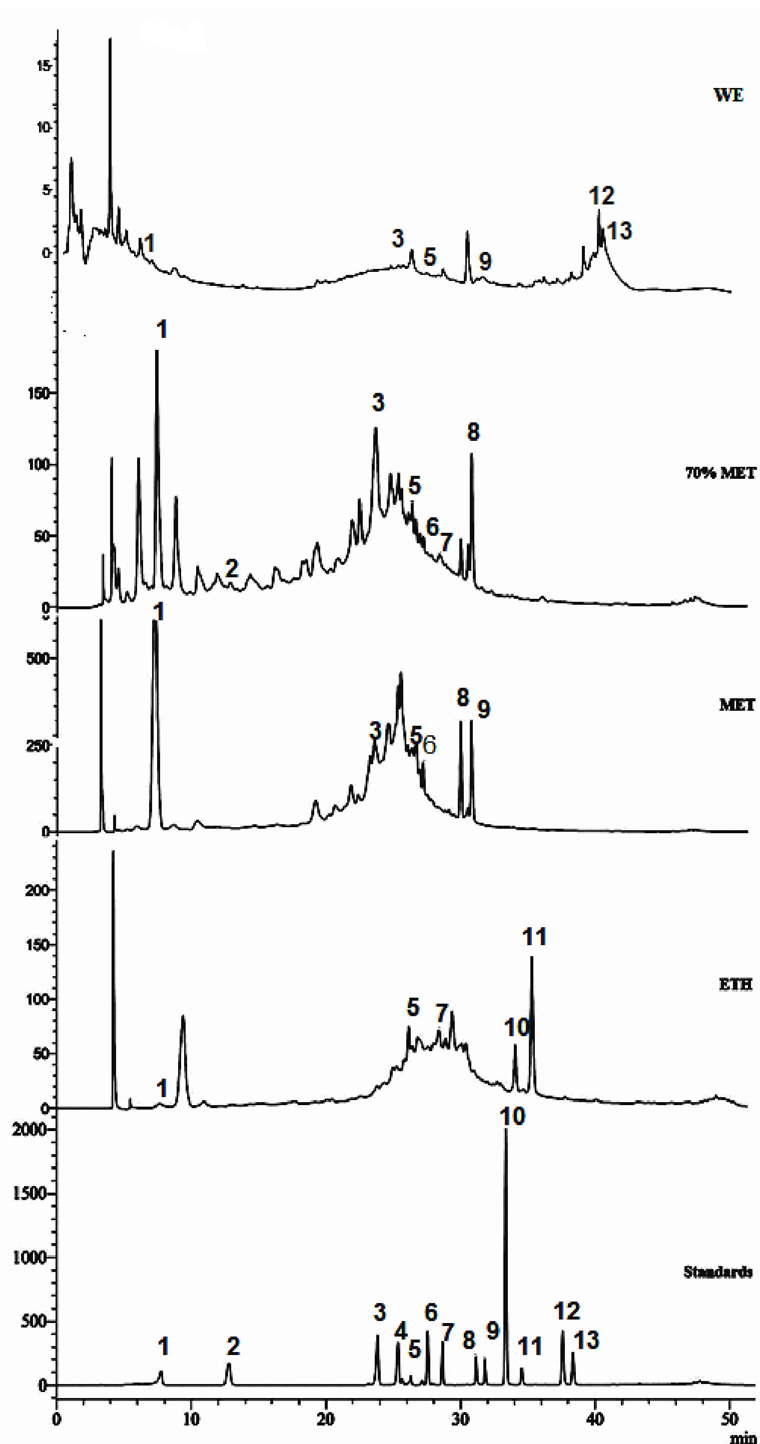


Fig. 2.7:. Representative HPLC–DAD chromatograms of mixed standards and different fractions of TVM, TCH, and MPA recorded at 280 nm.

Standards are (1) gallic acid, (2) catechol, (3) chlorogenic acid, (4) caffeic acid, (5) syringic acid, (6) coumaric acid, (7) ferulic acid, (8) ellagic acid, (9) myricetin, (10) cinnamic acid, (11) quercetin, (12) kaempferol, (13) apigenin.

Table 2.2: Quantification of phenolic compounds (mg/g dry wt.) by HPLC-DAD from ethyl acetate, methanol and 70% methanol and aqueous fractions of Syzygium cumini

Phenolics	**EA** mg/g dry wt	**ME** mg/g dry wt	**70% ME** mg/g dry wt	**WE** mg/g dry wt
GA	11.78 ± 0.3	531.08 ± 7.8	272.22 ± 8.0	34.34 ± 0.2
Ctl	nd	nd	1.72 ± 0.3	nd
ClA	nd	7.25 ± 0.7	42.46 ± 2.8	12.24 ± 0.4
CfA	nd	nd	nd	nd
SA	16.55 ± 1.0	5.08 ± 0.5	8.20 ± 0.7	4.23 ± 0.3
CoA	nd	10.77 ± 0.7	3.88 ± 0.5	nd
FA	21.37 ± 2.8	nd	10.79 ± 1.3	nd
EA	nd	199.12 ± 13.8	222.18 ± 10.4	nd
Mcn	nd	100.53 ± 4.8	nd	12.35 ± 0.6
CA	50.99 ± 3.6	nd	nd	nd
Qtn	114.49 ± 5.3	nd	nd	nd
Kmp	nd	nd	nd	12.25 ± 0.3
Apn	nd	nd	nd	3.45 ± 0.03

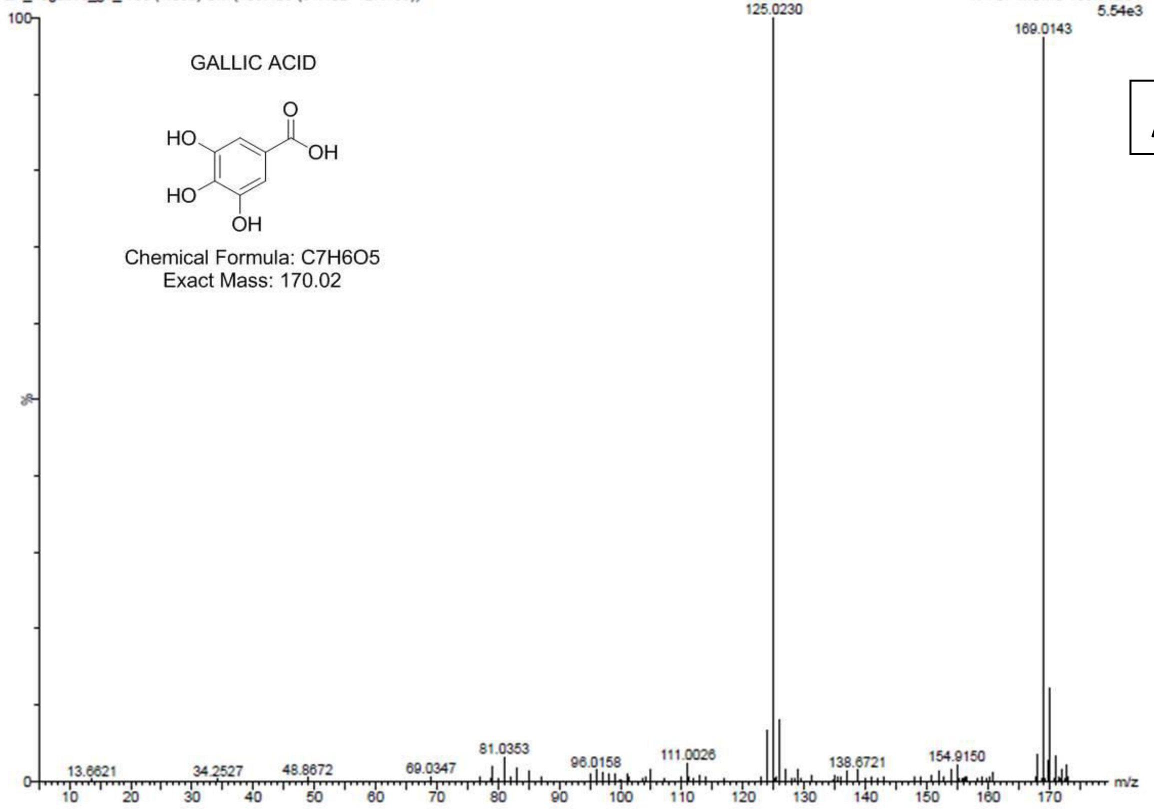
Values are the means ± SD of three replicated samples. GA – Gallic acid, Ctl – Catechol, ClA – Chlorogenic acid, CfA – Caffeic acid, SA – Syringic acid, CoA – Coumaric acid, FA – Ferulic acid, EA – Elagic acid, Mcn – Myricetin, CA – Cinnamic acid, Qtn – Quercetin, Kmp – Kaempferol, Apn – Apigenin, nd- not detected

2.3.9. LC- MS/MS analysis of phenolic compounds

TVM variant of *Syzygium cumini* seed which was found to be the most active was further analyzed by LC-MS/MS. LC-MS/MS data (Table 2.3) confirmed the presence of gallic acid [[M - H]$^-$ $C_7H_5O_5$ calcd for m/z 169.0, found 169.0], syringic acid [[M + Formate]$^-$ $C_{10}H_{11}O_7$ calcd for m/z 243.0, found 243.0], ferulic acid [[M - H]$^-$ $C_{10}H_9O_4$ calcd for m/z 193.0, found 193.0], cinnamic acid [[M + Formate]$^-$ $C_{10}H_9O_4$ calcd for m/z 193.0, found 193.0], and quercetin [[M + Formate]$^-$ $C_{16}H_{11}O_9$ calcd for m/z 347.0, found 347.0] in ethyl acetate fraction (Fig 2.8); gallic acid [[M - H]$^-$ $C_7H_5O_5$ calcd for m/z 169.0, found 169.0], chlorogenic acid [[M - H]$^-$ $C_{16}H_{17}O_9$ calcd for m/z 353.0, found 353.0], syringic acid [[M + Formate]$^-$ $C_{10}H_{11}O_7$ calcd for m/z 243.0, found 243.0], coumaric acid [[M + Formate]$^-$ $C_{10}H_9O_5$ calcd for m/z 209.0, found 209.0], ellagic acid [[M - H]$^-$ $C_{14}H_5O_8$ calcd for m/z 300.9, found 300.9], and myricetin [[M + Formate]$^-$ $C_{16}H_{11}O_{10}$ calcd for m/z 363.0, found 363.0] in methanol fraction (Fig 2.9); and gallic acid [[M - H]$^-$ $C_7H_5O_5$ calcd for m/z 169.0, found 169.0], catechol [[M + Formate]$^-$ $C_7H_7O_4$ calcd for m/z 155.0, found 155.0], chlorogenic acid [[M - H]$^-$ $C_{16}H_{17}O_9$ calcd for m/z 353.0, found 353.0], syringic acid [[M + Formate]$^-$ $C_{10}H_{11}O_7$ calcd for m/z 243.0, found 243.0], coumaric acid [[M + Formate]$^-$ $C_{10}H_9O_5$ calcd for m/z 209.0, found 209.0], ferulic acid [[M - H]$^-$ $C_{10}H_9O_4$ calcd for m/z 193.0, found 193.0], and ellagic acid [[M - H]$^-$ $C_{14}H_5O_8$ calcd for m/z 300.9, found 300.9] in 70% methanol fraction (Fig 2.10).

Table 2.3: Characterisation of polyphenol compounds in Syzygium cumini seed fractions using LC-Q_ToF analysis in negative mode

Fractions	Compounds	*$[M-H]^-$*	Major fragments (m/z)
Ethyl acetate	Gallic acid	169	125
	Syringic acid	197	153, 182, 138, 167
	Ferulic acid	193	134, 149
	Cinnamic acid	147	134
	Quercetin	301	151, 121, 179
Methanol	Gallic acid	169	125
	Chlorogenic acid	353	191
	Syringic acid	197	153, 182, 138, 167
	Coumaric acid	163	119
	Ellagic acid	301	284, 257, 229
	Myricetin	317	274, 257, 241
70% methanol extract	Gallic acid	169	125
	Chlorogenic acid	353	191
	Syringic acid	197	153, 182, 138, 167
	p-coumaric acid	163	119
	Ferulic aciEllagic	193	134, 149
	acid	301	284, 257, 229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EA_negative_ga_ 108 (1.062) Cm (103:126-(91:102+127:139))
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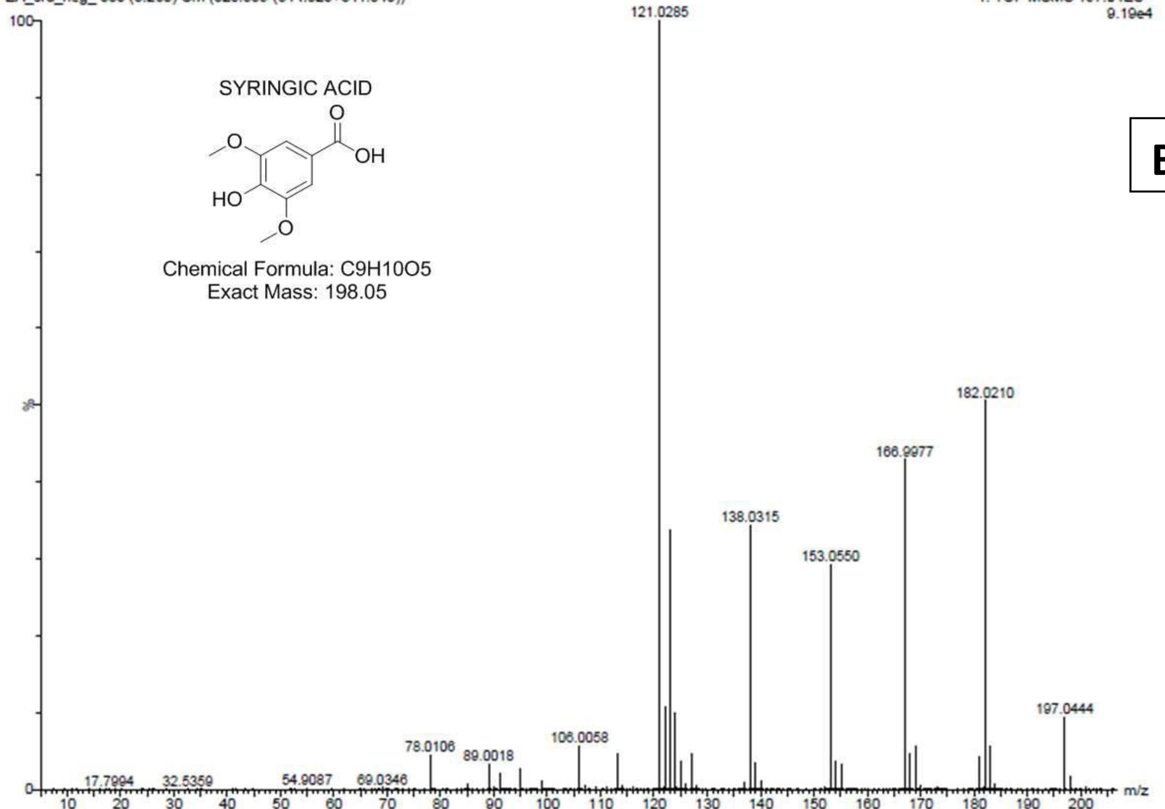
5.54e3
GALLIC ACID
Chemical Formula: C7H6O5
Exact Mass: 170.02
A
125.0230
169.0143
13.6621
34.2527
48.8672
69.0347
81.0353
96.0158
111.0026
138.6721
154.9150
m/z


negative
EA_crd_neg_ 335 (3.265) Cm (325:339-(314:323+341:343))
1: TOF MSMS 197.04ES-
9.19e4
SYRINGIC ACID
Chemical Formula: C9H10O5
Exact Mass: 198.05
B
121.0285
182.0210
166.9977
138.0315
153.0550
197.0444
106.0058
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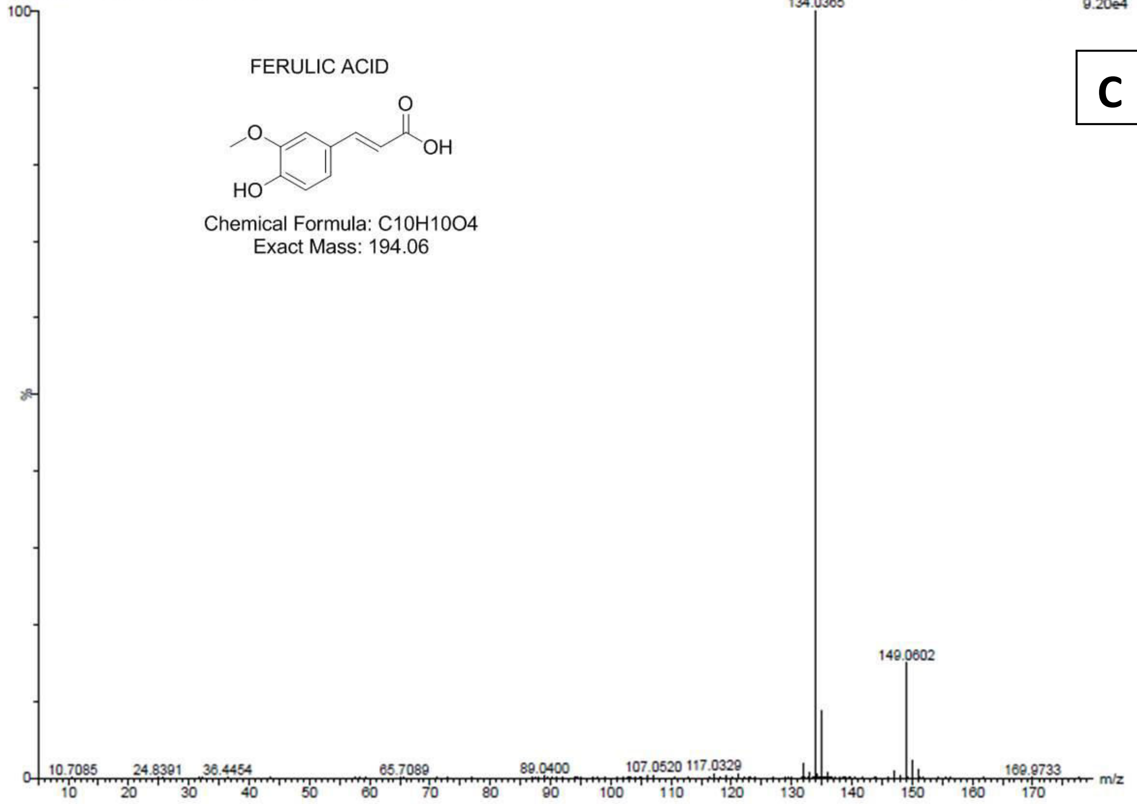
17.7994
32.5359
54.9087
69.0346
m/z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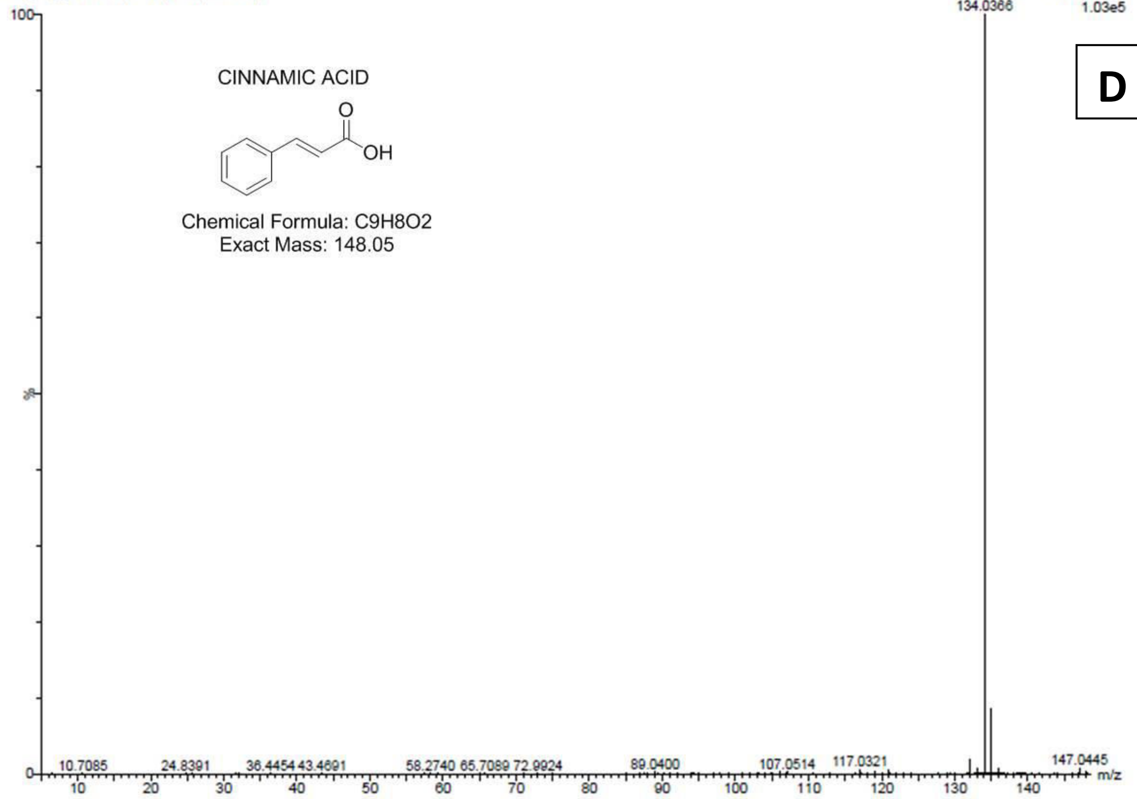
EA_neg_147 396 (3.847) Cm (387:396)
1: TOF MSMS 147.04ES-
9.20e4
134.0365
FERULIC ACID
Chemical Formula: C10H10O4
Exact Mass: 194.06
C
149.0602
10.7085
24.8391
36.4454
65.7089
89.0400
107.0520
117.0329
169.9733
m/z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1.03e5
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D
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24.8391
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117.0321
147.0445
m/z

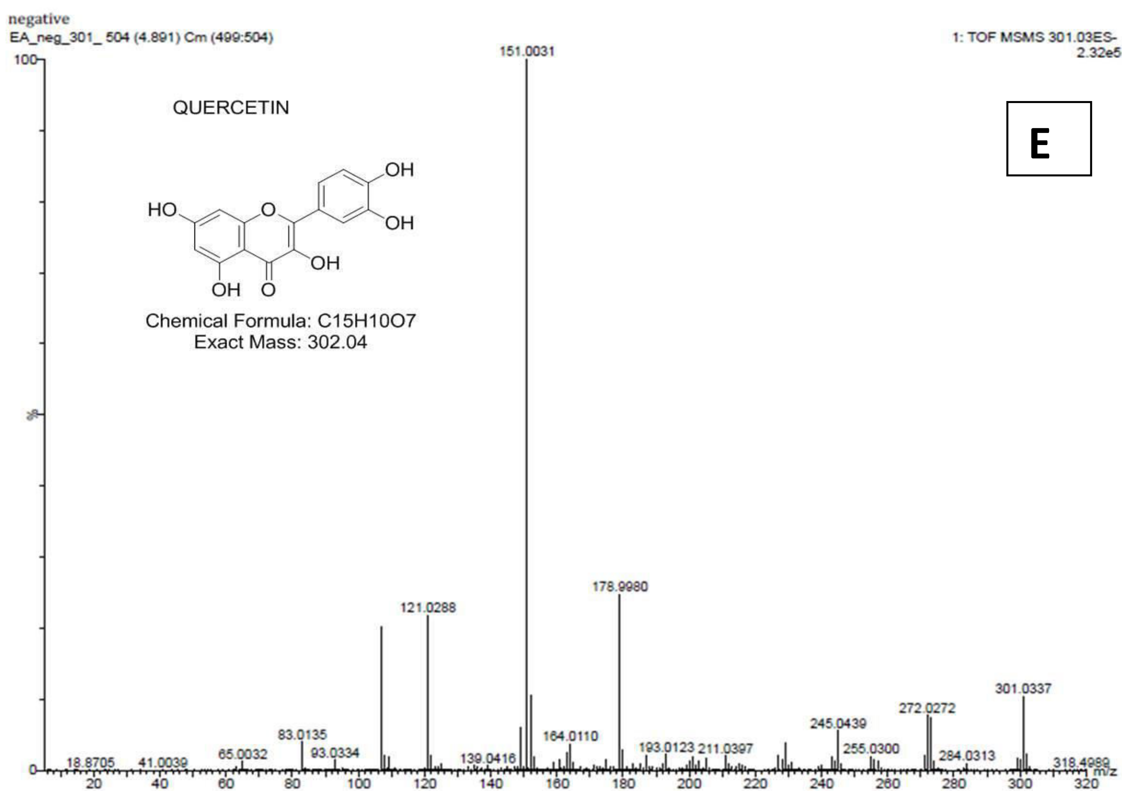
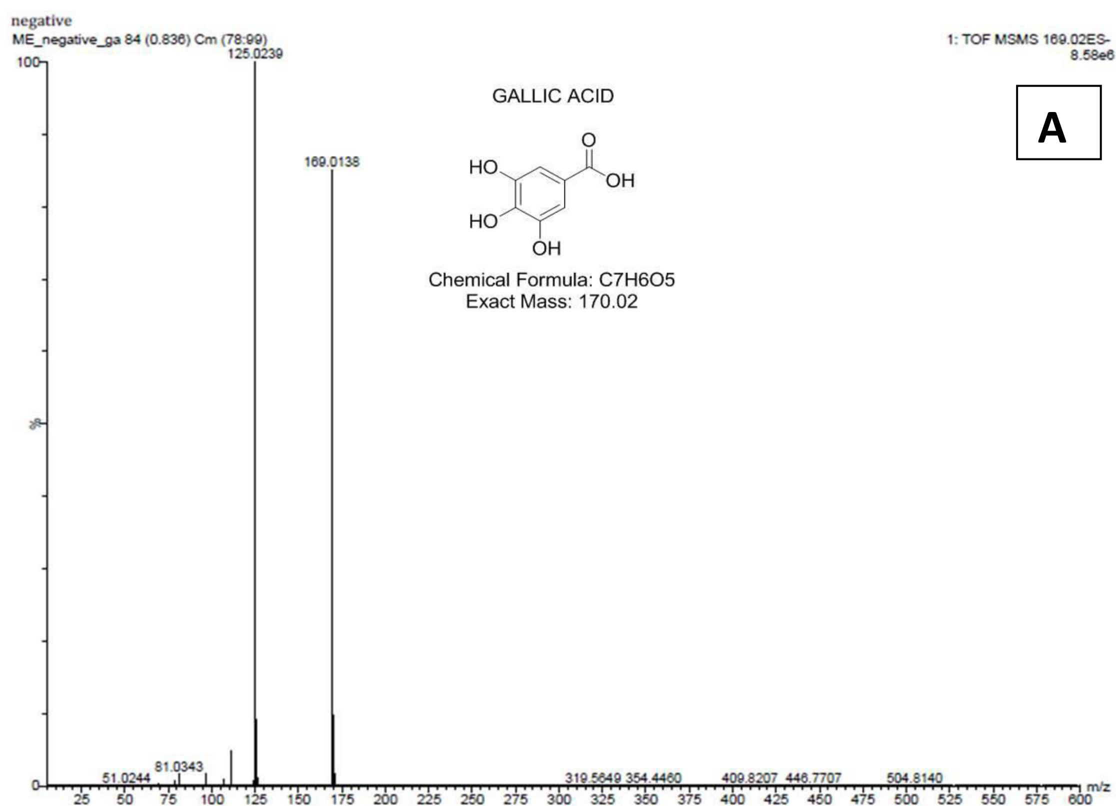
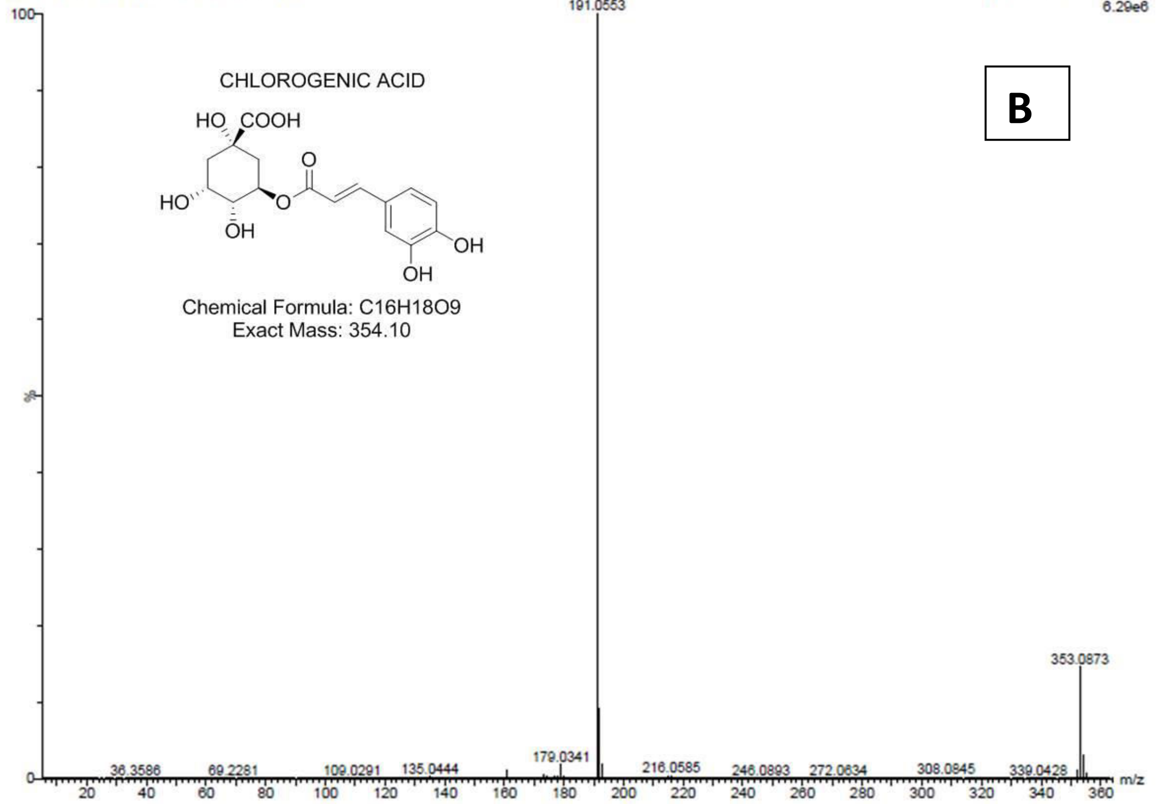


Fig. 2.8: LC-MS/MS spectra of compounds in ethyl acetate fraction.
A) Gallic acid; B) Syringic acid; C) Ferulic acid; D) Cinnamic acid; E) Quercetin

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6.29e6
CHLOROGENIC ACID
HO COOH
HO
OH
OH
OH
O
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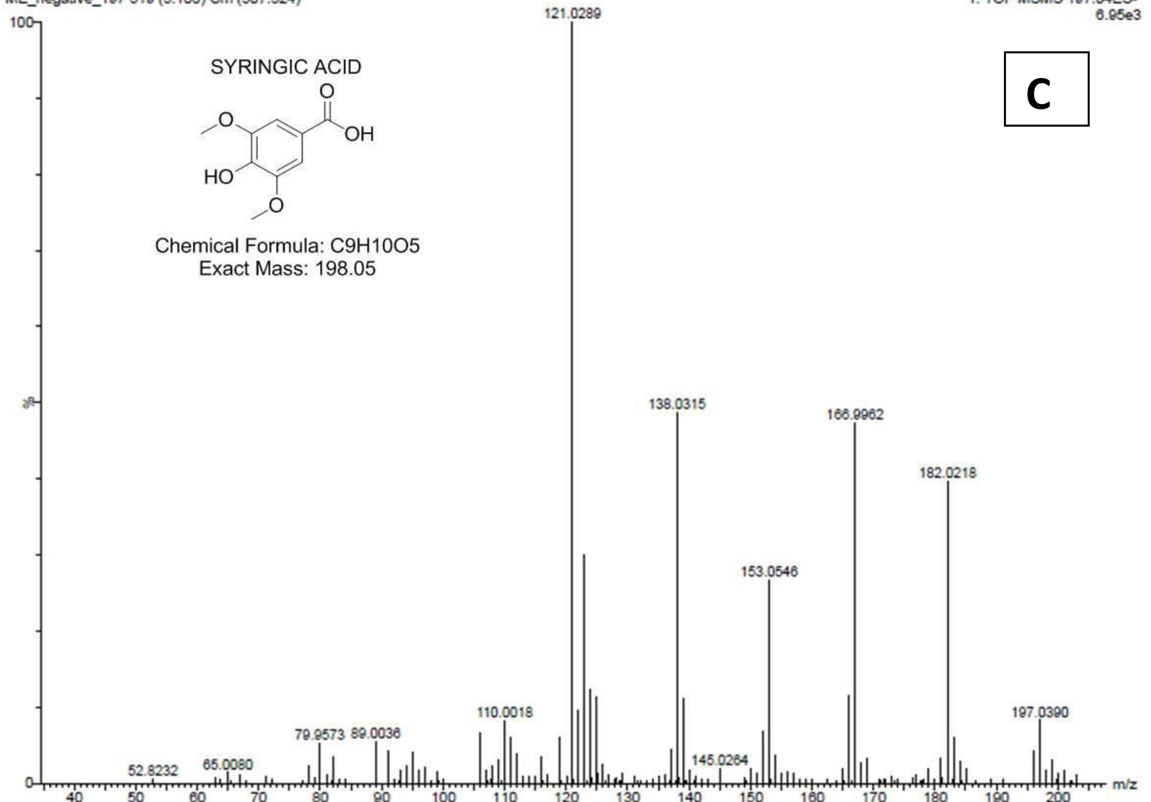
Exact Mass: 354.10
B
191.0553
353.0873
179.0341
216.0585
m/z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ME_negative_197 319 (3.108) Cm (307:324)
1: TOF MSMS 197.04ES-
6.95e3
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OH
HO
Chemical Formula: C9H10O5
Exact Mass: 198.05
C
121.0289
138.0315
166.9962
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153.0546
197.0390
110.0018
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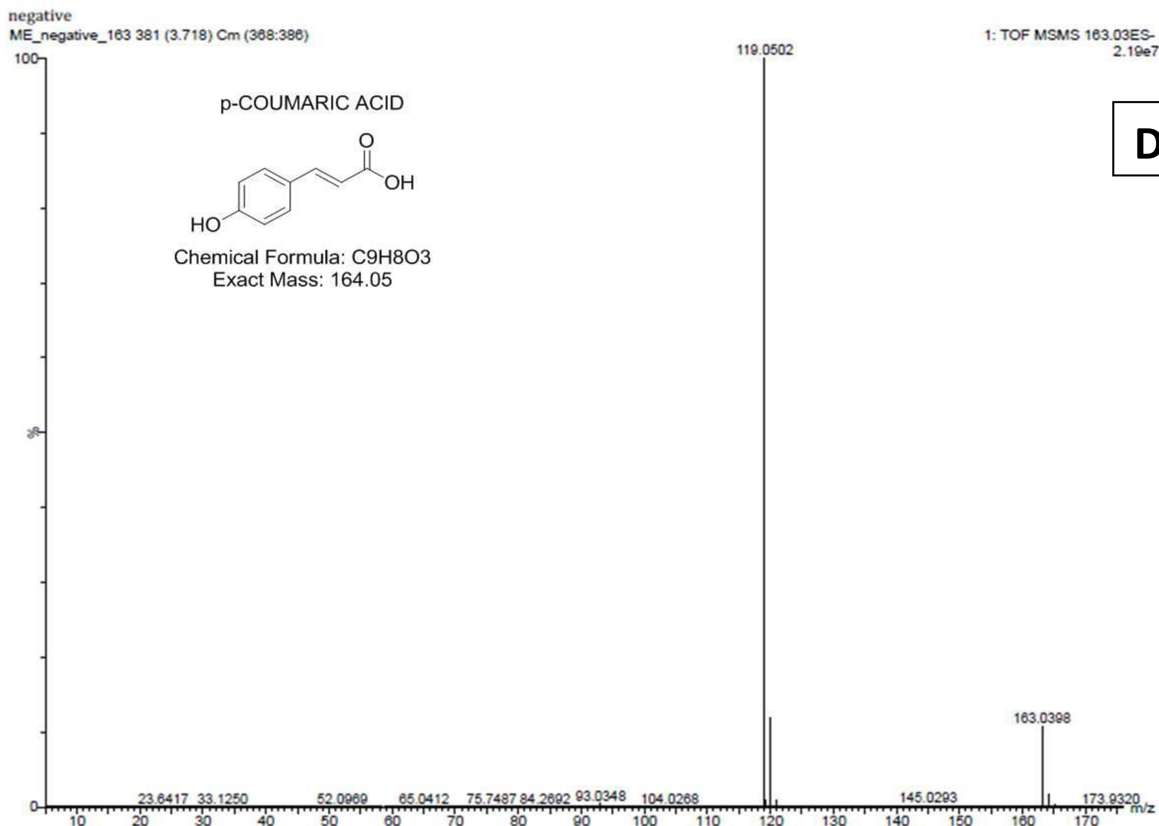
m/z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ME_negative_163 381 (3.718) Cm (368:386)
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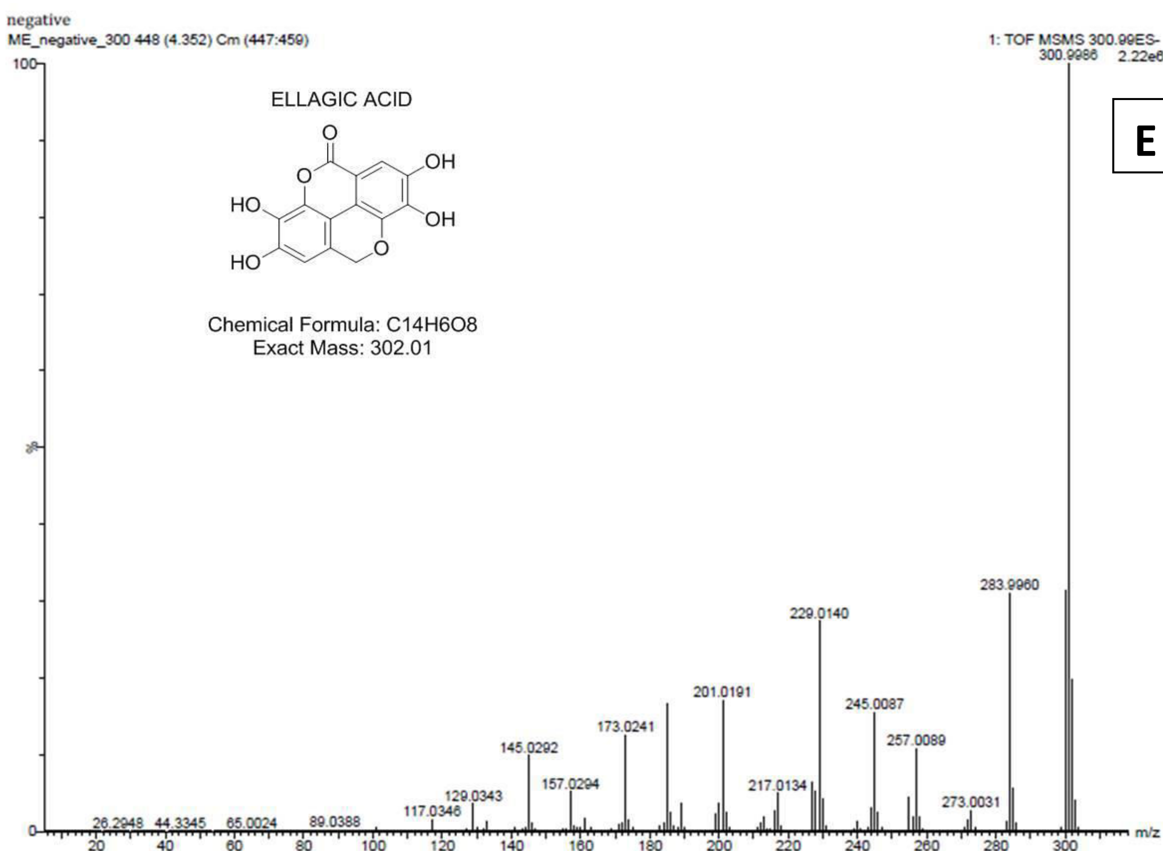
2.19e7
119.0502
p-COUMARIC ACID
O
OH
HO
Chemical Formula: C9H8O3
Exact Mass: 164.05
D
%
163.0398
23.6417 33.1250 52.0969 65.0412 75.7487 84.2692 93.0348 104.0268 145.0293 173.9320
m/z


negative
ME_negative_300 448 (4.352) Cm (447:459)
1: TOF MSMS 300.99ES-
300.9986 2.22e6
ELLAGIC ACID
O
OH
HO
OH
HO
O
O
Chemical Formula: C14H6O8
Exact Mass: 302.01
E
%
283.9960
229.0140
201.0191
245.0087
257.0089
173.0241
145.0292
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m/z

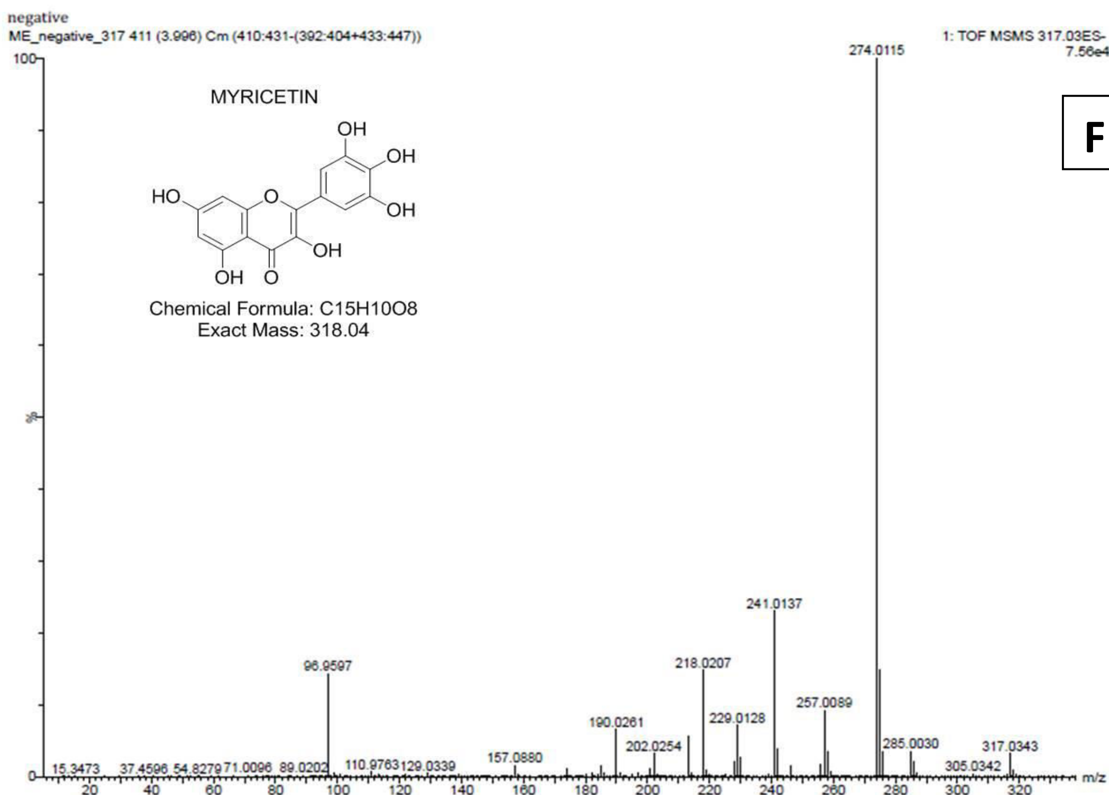
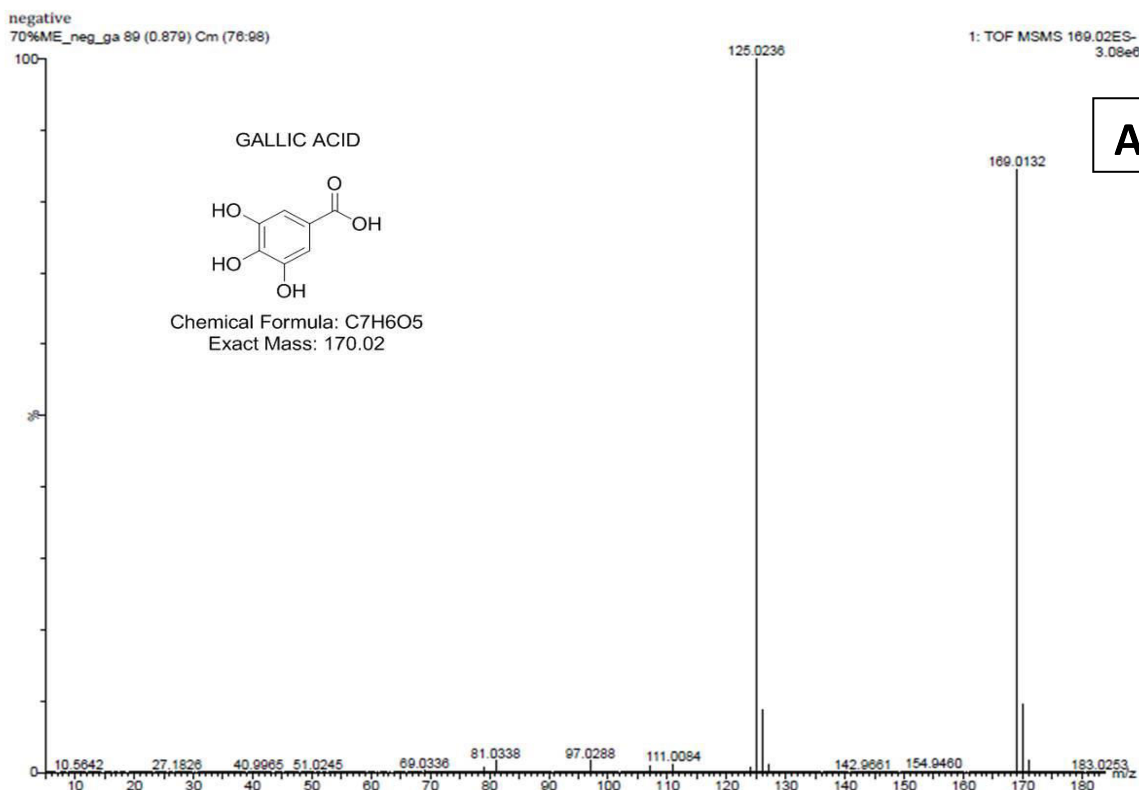
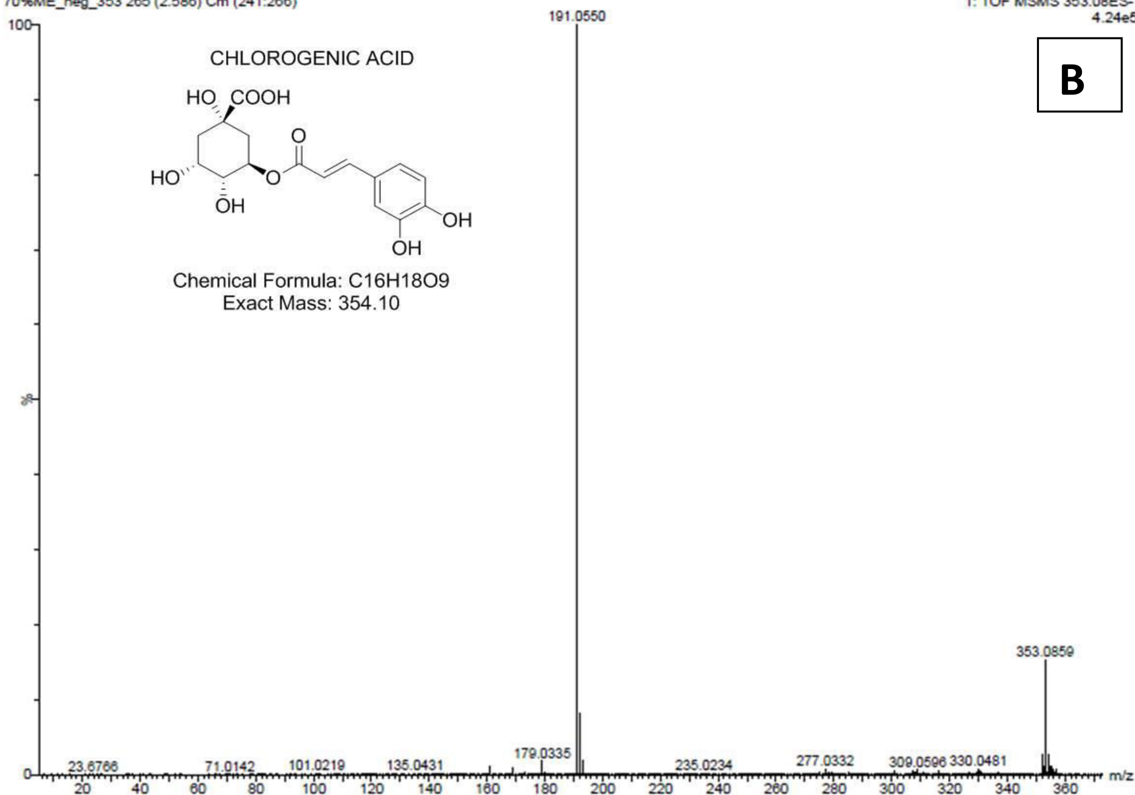


Fig. 2.9: LC-MS/MS spectra of compounds in methanol fraction

A) Gallic acid; B) Chlorogenic acid; C) Syringic acid; D) p-coumaric acid; E) Ellagic acid; F) Myricetin

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1: TOF MSMS 353.08ES-
4.24e5
B
CHLOROGENIC ACID
Chemical Formula: C16H18O9
Exact Mass: 354.10
191.0550
353.0859
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23.6766
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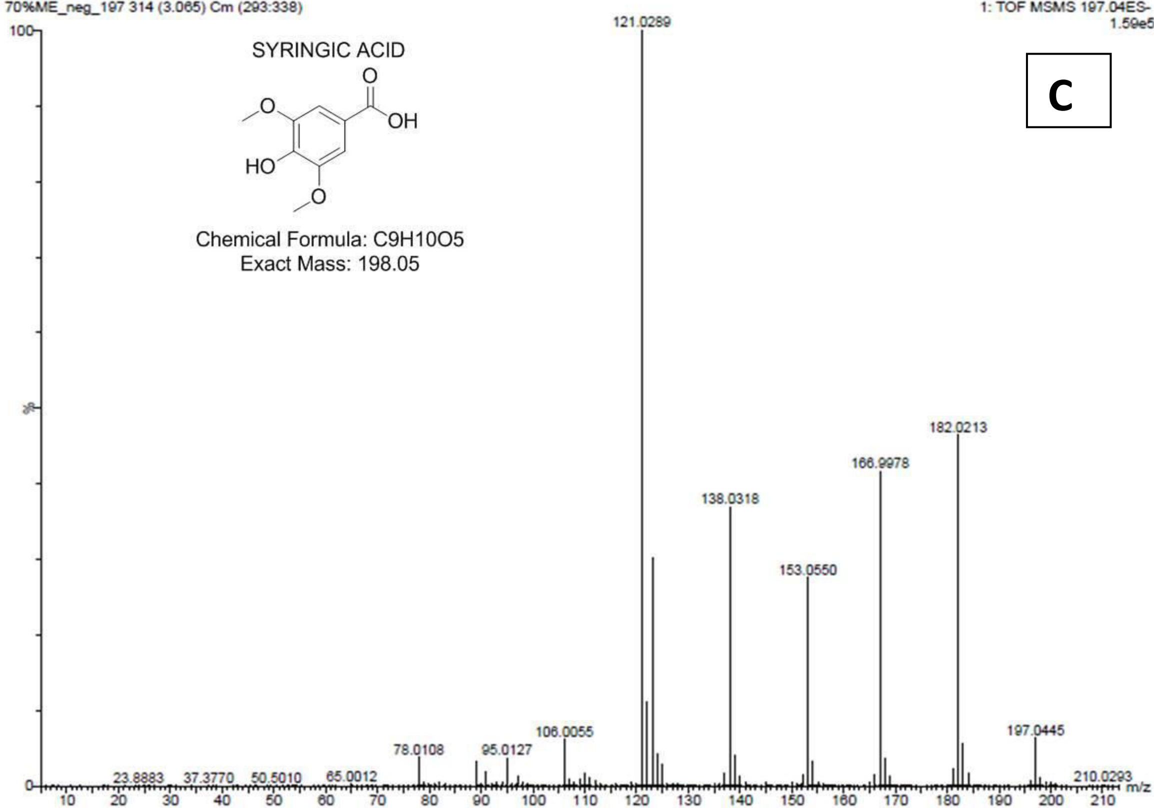
101.0219
135.0431
235.0234
277.0332
309.0596
330.0481
m/z
%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1.59e5
C
SYRINGIC ACID
Chemical Formula: C9H10O5
Exact Mass: 198.05
121.0289
182.0213
166.9978
138.0318
153.0550
106.0055
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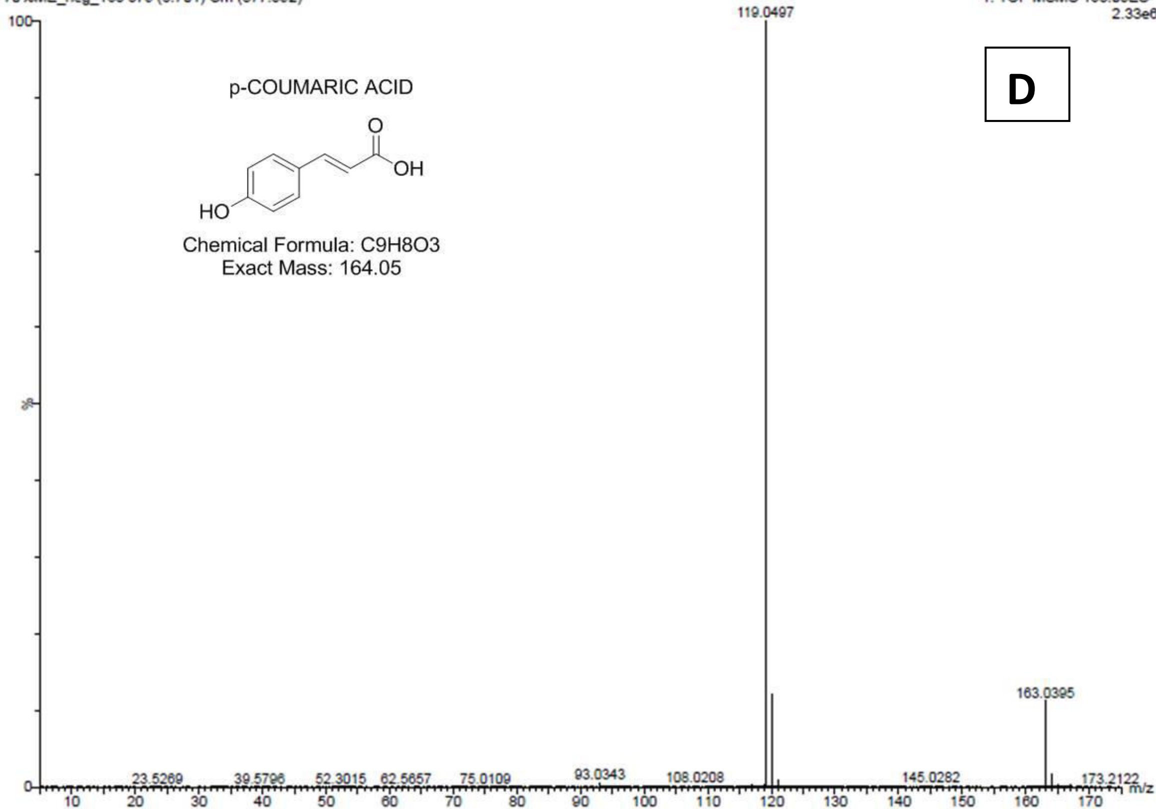
78.0108
95.0127
23.8883
37.3770
50.5010
65.0012
210.0293
m/z
%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70%ME_neg_163 379 (3.701) Cm (377:392)
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2.33e6
D
p-COUMARIC ACID
HO
OH
O
Chemical Formula: C9H8O3
Exact Mass: 164.05
119.0497
163.0395
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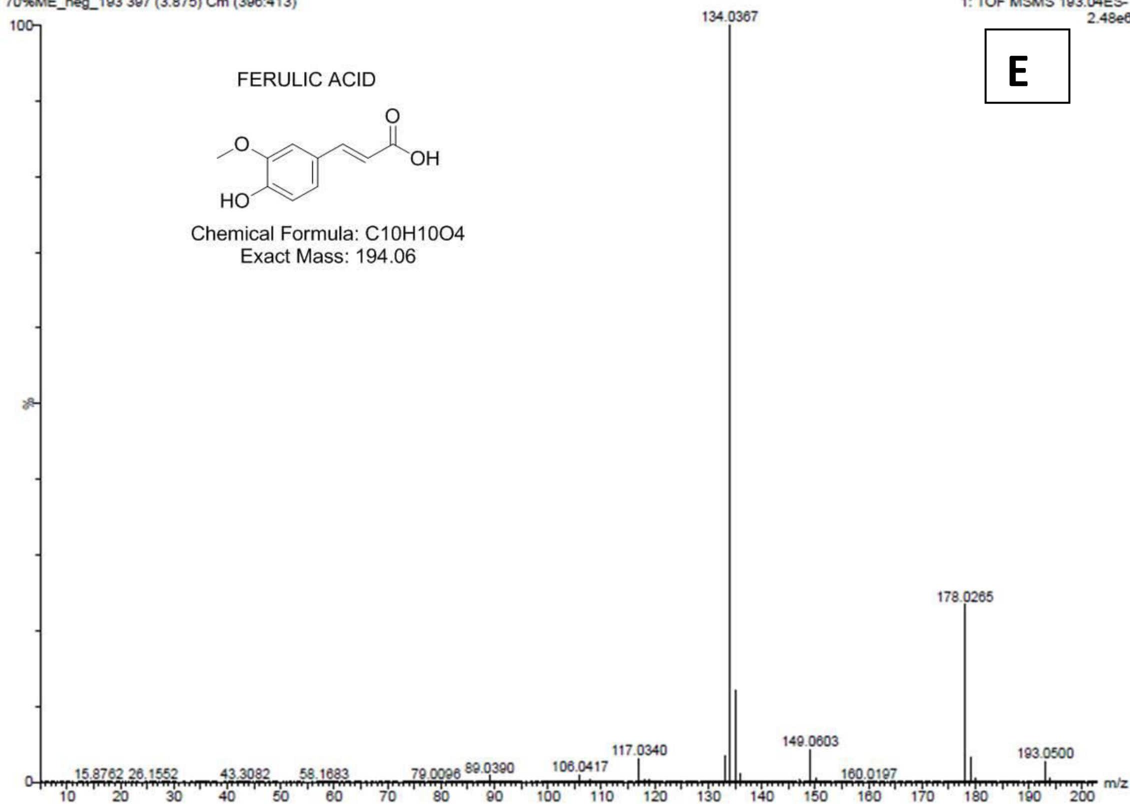
75.0109
93.0343
108.0208
145.0282
173.2122
m/z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HO
OH
O
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Exact Mass: 194.06
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m/z

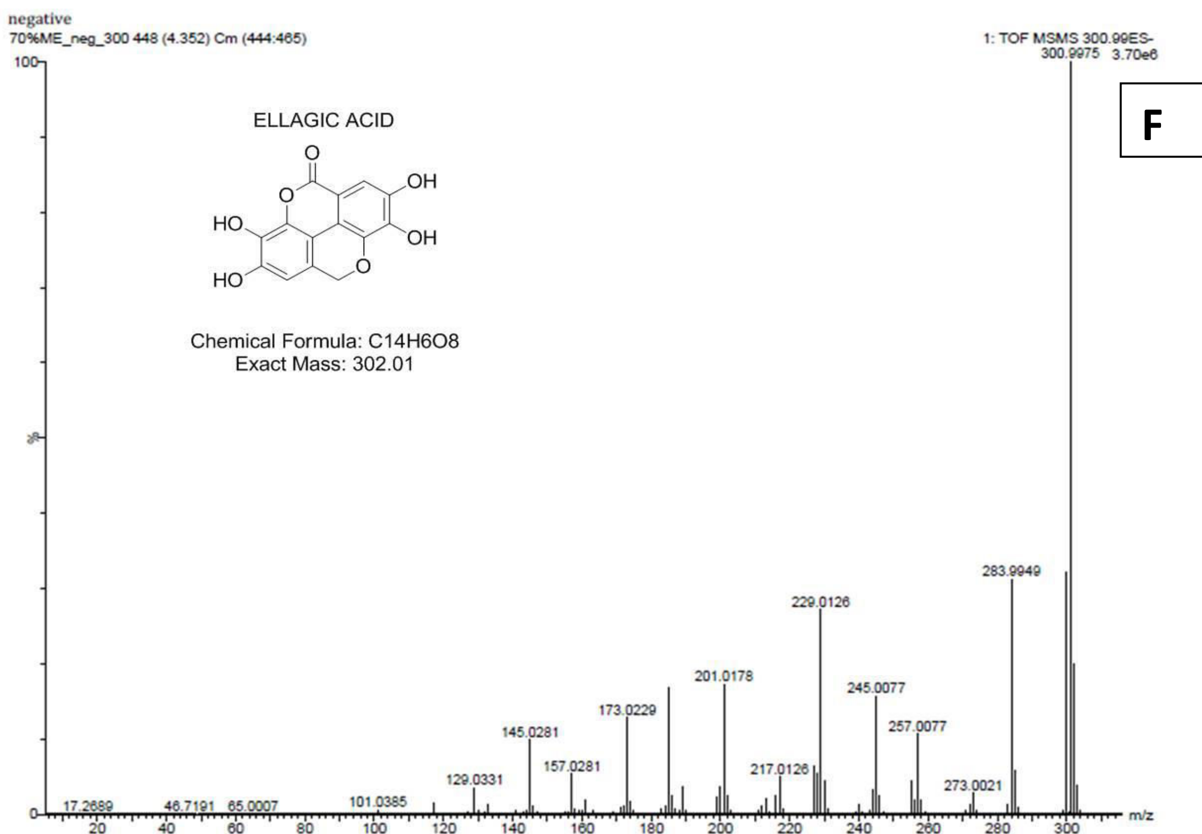

Fig. 2.10: LC-MS/MS spectra of compounds in 70% methanol fraction
A) Gallic acid; B) Chlorogenic acid; C) Syringic acid; D) p-coumaric acid; E) Ferulic acid; F) Ellagic acid

2.3.10. Identification of compound from 70% methanol fraction

The results clearly showed that among all the fractions of TVM variant, 70% methanol fraction was found to be the most active. Thus, 70% methanol fraction was selected for compound isolation studies.

The ^{1}H NMR spectrum of white amorphous solid showed six signals at δ 0.58, 0.68, 0.71, 0.75, 0.83 and 0.90 ppm for methyl hydrogen ($-CH_3$) at C-18, C-29, C-27, C-26, C-21, C-19, respectively (Fig 2.11). The proton at C-3 appeared as a multiplet at 3.66 ppm. A doublet at 5.26 ppm was the characteristics of the double bond present in the ring in between quaternary carbon and aromatic carbon C-5 and C-6. ^{13}C NMR indicated that the compound consisted of 29 carbons. The glucose unit contained six carbons out of which oxygenated carbon C-1 appeared at 100.92 ppm, and methylene carbon C-6

appeared at 61.66 ppm (Fig 2.12). The other four carbons of the glucose molecule appeared at 70.01, 75.60, 76.7 and 77.03 ppm.

Spectral data of compound

The spectral data of isolated compound has been described below:

Molecular formula	:	$C_{29}H_{50}O$
^{1}H NMR (500 MHz, DMSO-d_6)	:	δ 5.34 (d, 1H, *J*=5Hz), 4.88 (m, 3H), 4.44(m, 1H), 4.23 (d, 1H, J=8Hz), 3.65 (m, 1H), 3.46 (m, 1H, *J*=8Hz), 3.13 (m, 1H), 3.08 (m, 1H), 3.02 (m, 1H), 2.89 (m, 1H), 2.36 (m, 1H), 2.13 (m, 1H), 1.94(m, 2H), 1.80 (m, 3H), 1.64 (m, 1H), 1.51-1.40 (m, 6H), 1.28-1.23 (m, 6H), 1.16 (m, 4H), 0.96 (s, 3H), 0.91 (s, 5H), 0.82 (m, 9H), 0.66 (s, 3H) ppm
^{13}C NMR (125 MHz, DMSO-d_6)	:	δ 140.4, 121.2, 100.7, 99.5, 76.9, 76.7, 73.4, 70.1, 61.1, 56.1, 55.4, 49.6, 45.1, 41.8, 38.3, 36.8, 36.2, 35.4, 33.3, 31.4, 31.3, 29.2, 28.7, 27.8, 25.4, 23.8, 22.6, 20.6, 19.7, 19.1, 18.9, 18.6, 11.8, 11.6 ppm
m/z	:	577.4013$(M+1)^+$

Fig. 2.11: ^{1}H NMR in DMSO-d_6

Fig. 2.12: ^{13}C NMR in DMSO-d_6

^{1}H NMR and ^{13}C NMR data revealed that the isolated compound wasβ-sitosterolglucoside, which was further confirmed by HRMS analysis. Based on the NMR and HRMS data the structure of the compound is elucidated as shown in Fig 2.13.

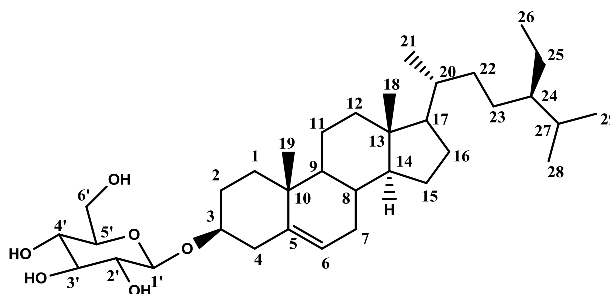

Fig. 2.13: Structure of isolated compound

2.4. DISCUSSION

In the present study, the antioxidant potential of *Syzygium cumini* fractions of three geographical variants was assessed in terms of their phenolic and flavonoid content in addition to their free radical scavenging efficacies. Bajpai *et al.*, 2005 reported that 50% methanolic extract of seeds possessed 108.7 mg/g of total phenolic content. An independent study using methanolic fraction of leaves had reported the presence of 610.32 mg/g of total phenolic content and 451.5 mg/g of total flavonoid content (Ruan *et al.*, 2008). In contrast, our results suggested higher total phenolic content in 70% methanolic fraction of *Syzygium cumini* seeds (906 ± 7.1 mg GAE/g dry wt. for TVM variant). This may be due to the fact that sequential extraction of seeds using solvents would have fractionated phenolic and flavonoid compounds in 70% methanol.

The radical scavenging activities such as DPPH, ABTS, nitric oxide, superoxide radical scavenging potential of the fractions were analyzed. The electron donating ability of *Syzygium cumini* seed fractions were measured by the bleaching action of 1,1-Diphenyl-2-picrylhydrazyl radical purple colored solution. A study by Banerjee *et al.*,

(2005) reported that ethanolic and methanolic fractions of *Syzygium cumini* seeds showed a DPPH radical scavenging activity of 140 mg/GAE/g dry weight and 19.1 mg/g dry weight, respectively. From the results of the present study, the exhibition of higher DPPH radical scavenging activity demonstrated by 70% methanol and methanol fractions (5.1 ± 0.96 µg/mL, 5.2 ± 0.85 µg/mL for TVM variants) may be due to the presence of phenolic constituents that are more capable of donating hydrogen to a free radical and scavenge the radicals. In addition, 70% methanol and methanol fractions of all geographical variants showed significant ABTS and nitric oxide radical scavenging potential which was significantly higher than the results reported by Nair *et al*., (2013) using ethyl acetate fraction of *Syzygium cumini* seeds. Though superoxide is a weak oxidant, powerful and dangerous hydroxyl radicals that can contribute to oxidative stress that are generated from superoxides. Earlier reports on *Syzygium cumini* fruit skin demonstrated IC_{50} value of 260 µg/mL for scavenging superoxide radicals (Mohammad *et al*., 2007). The results from the present study demonstrated significant superoxide radical protection by 70% methanol and methanol fractions of all geographical variants. The demonstration of significant radical scavenging activities (DPPH, ABTS, nitric oxide and superoxide radicals) especially by the 70% methanolic fractions of all the geographical variants of *Syzygium cumini* seeds may be due to the presence of higher content of phenolic compounds in the fraction.

The phenolic profiling of active fractions of all the geographical variants indicated the presence of prominent phenolic compounds (gallic acid, ellagic acid, chlorogenic acid, syringic acid, etc.). These phenolic compounds have been reported for their antioxidant potential (Kahkonen *et al*., 2001; Srinivasan *et al*., 2007). Gallic acid, ellagic acid, chlorogenic acid and ferulic acid were found predominantly in 70% methanol fraction of TVM variant. These phenolic acids have been very well known for

the antioxidant activity (Kono *et al*., 1997) and these compounds were found in 70% methanolic fraction of all geographical variants of *Syzygium cumini* seeds. This difference in the composition of phenolics in 70% methanolic fractions of TVM variant may be responsible for its higher antioxidant efficacy when compared with the other geographical variants. A positive correlation was reportedly observed between total phenolic content and antioxidant activity (Piluzza *et al*., 2011; Saeed *et al*., 2012). A similar correlation between total phenolic content and antioxidant activity was also reflected among the fractions in the present study.

In summary, 70% methanol fraction of three geographical variants of *Syzygium cumini* seeds demonstrated significant radical scavenging activities which may be attributed to the higher levels of total phenolic compounds. TVM variant showed the best activity which can be attributed to the presence of highest phenolic content and relative composition of phenolics and flavonoids among all the geographical variants.

Thus, among all the geographical variants, since TVM variant was found to be the most active, its fractions were subjected to HPLC profiling. The presence of phenolic acids and flavonoids in these fractions were confirmed by LC-MS/MS analysis. When compared with all fractions of TVM variant, 70% methanol fraction demonstrated the highest antioxidant activity. Hence this fraction was subjected to fractionation and compound isolation which resulted in isolation of a pure compound, *β*-sitosterolglucoside, which was confirmed by its HRMS and NMR data.

The compound *β*-sitosterolglucoside, which was isolated from 70% methanol fraction in the present study, has been reported for its antioxidant efficacy. β-sitosterol has been reported to activate estrogen receptor/PI3-kinase dependent pathway, and thus it stimulates the level of antioxidant enzymes. β-sitosterol can recover GSH and GSH/total glutathione ratio which explains the reactive oxygen species scavenging ability of

tµµµhis compound suggesting that this phytosterol could be a ROS scavenger (Vivancos *et al*., 2005). Since the yield of *β*-sitosterolglucoside is very less, further biological activity studies using this compound is limiting.

2.5. SUMMARY

The antioxidant potential in terms of DPPH, ABTS, nitric oxide scavenging potential and superoxide scavenging potential was evaluated for the fractions of different geographical variants of *Syzygium cumini* seeds. The study showed that total phenolic and flavonoid contents were higher in ethyl acetate, methanol, and 70% methanol fractions of all geographical variants of *Syzygium cumini* seeds which correlated with their antioxidant capacity. The phyto-chemical constituents in each geographical variants differed which may be due to geographical, genetic, environmental, degree of maturity at the time of harvest, variety, geo-agro-climatic conditions, agronomical practices, post harvest handling, etc. Thus the active principle can vary enormously and that in turn would affect the biopotency. Among the variants, the best efficacy was demonstrated by the fractions (ethyl acetate, methanol and 70% methanol) of TVM variant. The phenolic compounds in all fractions of TVM variant were identified and quantified using HPLC which was further confirmed by LC-MS/MS. The results showed that 70% methanol fraction was rich in polyphenols. Further exploration on this fraction resulted in the identification of β- sitosterolglucoside.

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CHAPTER 3

Cardioprotective effect of polyphenol rich Syzygium cumini seed against tertiary butyl hydrogen peroxide induced oxidative stress in H9c2 cell lines and modulation of key enzymes involved in hypertension and cholesterol biosynthesis

3.1. INTRODUCTION

Cardiovascular disease (CVD) is the leading global cause of death, accounting for 17.3 million deaths per year, a number that is expected to grow to more than 23.6 million by 2030 (Mozaffarian *et al*., 2015). Therefore, preventive strategies focusing factors associated with CVD are the major tasks of health care professionals and researchers.

Hypertension is quantitatively the most independent risk factor for the development of cardiac diseases for all age/race/sex groups. The use of synthetic hypotensive drugs like captopril, enalapril and lisinopril are reported to have adverse effects such as developing a dry cough, taste disturbances, skin rashes, as well as alterations in serum lipid metabolism (Israili & Hall, 1992). Therefore, search for natural products with minimum side effects as an alternative approach to synthetic drugs is on rise worldwide.

The relationship between dietary factors and cardiovascular disease (CVD) was exclusively dependent on lipid consumption and metabolism leading to increased serum cholesterol; in particular, low-density lipoprotein (LDL) levels resulting in atherosclerotic problem (Schroeder *et al*., 2015). High fruit and vegetable consumption have been reported to reduce cardiovascular and cerebrovascular diseases due to the antioxidant potential of flavonoids and phenolic acids present in them (Hertog *et al*., 1993; Prior *et al*., 2000). Therefore, use of plant fractions as natural cardioprotective agents offers enormous hope for the prevention of chronic human diseases and can enhance the long-term health of a person with atherosclerotic dysfunctions.

Syzygium cumini (commonly known as *black plum* or *Jamun*) is a well-known Indian medicinal plant tested for many therapeutic properties and has been

extensively used for the treatment of diabetes (Ayyanar *et al*., 2013). The plant is reported to possess acetyl oleanolic acid, triterpenoids, ellagic acid, isoquercetin, quercetin, kaempferol and myricetin (Ayyanar *et al*., 2012). Antimicrobial, anti-diabetic, anti-inflammatory, hepatoprotective, and diuretic properties of leaves, stem, bark and fruit pulp of *S. cumini* have been evaluated extensively (Saroj *et al*., 2015). Compounds such as jambosine, gallic acid, ellagic acid, corilagin, 3,6 hexahydroxydiphenoylglucose, 3-galloylglucose, quercetin, kaempferol, myricetin, and β – sitosterol were identified from *Syzygium cumini* seeds (Baliga *et al*., 2011).

However, to date, research on the beneficial effect of different fractions of *Syzygium cumini* seed against cardiovascular diseases is limited. A very few studies supporting antihyperlipidaemic effect of *Syzygium cumini* seeds in STZ induced diabetic rat and against glucose induced oxidative stress were reported (Patel et al., 2010). The present study has been designed to study the activity of *Syzygium cumini* seeds fractions against tertiary butyl hydrogen peroxide induced oxidative stress and key enzymes involved in hypertension, cholesterol biosynthesis. This is the first reported study that compares the activity of ethyl acetate, methanol, 70% methanol and water fractions of *Syzygium cumini* seeds.

3.2. MATERIALS AND METHODS

3.2.1. Chemicals

Low density lipoprotein (LDL) from human plasma, thiobarbituric acid (TBA), trichloro acetic acid (TCA), angiotensin converting enzyme (ACE) from rabbit lung, Furylacryloyl-Phenylalanyl-Glycyl-Glycine (FAPGG) as a substrate peptide of ACE, HMG-CoA reductase assay kit, Dulbecco's modified Eagle's

media (DMEM), antibiotic-antimycotic mix, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and ter-butyl hydrogen peroxide (TBHP) were purchased from Sigma–Aldrich Chemicals (St. Louis, MO, USA); foetal bovine serum (FBS) was purchased from Gibco-BRL (Auckland, NZ); glutathione assay kit, catalase assay kit (Cat No. 706002) and super oxide dismutase (SOD) activity kit were procured from Cayman Chemicals, USA; glutathione peroxidase assay kit (K762-100) from Biovision (USA); methanol and acetic acid of HPLC grade were supplied by Merck, Germany; H9c2 rat cardiac myoblast cells were purchased from the American Type Culture Collection (Rockville, MD, USA); BCA protein assay kit was procured from Pierce Biotechnology, Rockford, USA. All other chemicals used were of the standard analytical grade.

3.2.2. Experimental design

The work flow of this chapter is illustrated in the schematic representation in Fig 3.1.

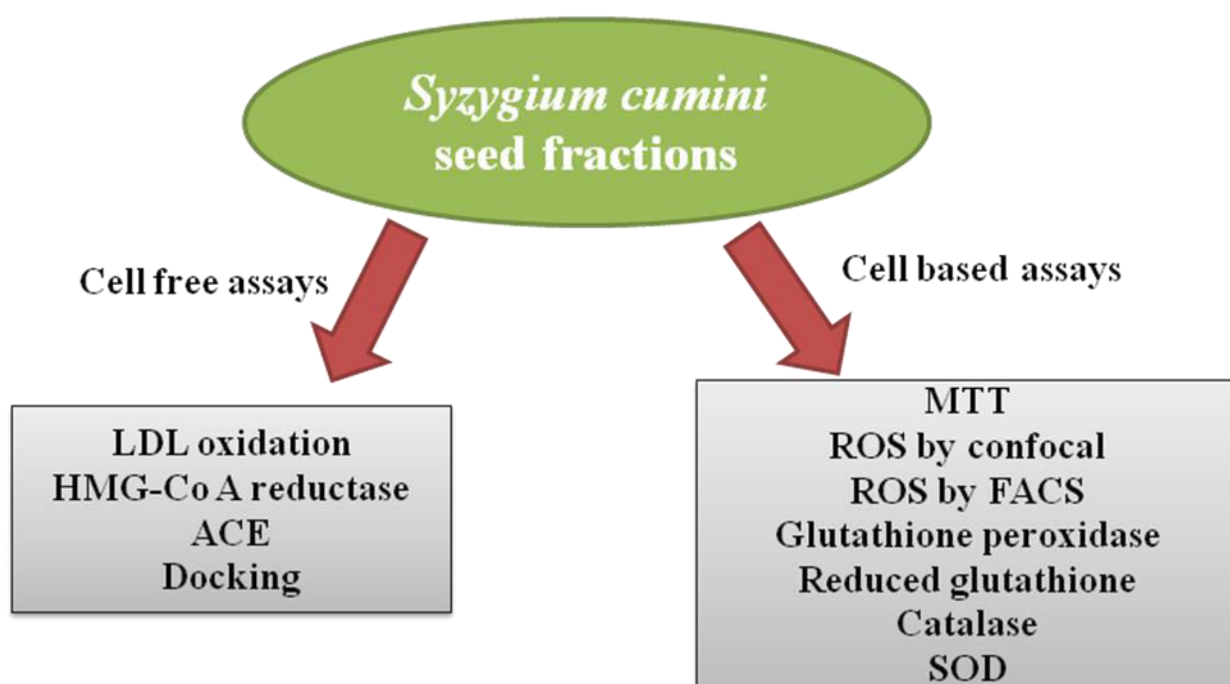


Fig. 3.1: Schematic representation of experimental design

3.2.3. *In vitro* cell based assay

3.2.3.1. Maintenance of cell lines

H9c2 rat cardiac myoblast cells were cultured in DMEM supplemented with 10% FBS, 1% antibiotic–antimycotic mix at 37°C under 5% CO_2 atmosphere. After attaining 70-80% confluency, cells were washed with phosphate buffered saline (PBS) and trypsinised using 1% trypsin and were subcultured and seeded in 24 well plates.

3.2.3.2. Cytotoxicity assay

The cytotoxicity of H9c2 was assessed by MTT assay (Mosmann, 1983). The H9c2 cells were seeded (1×10^4 cells/well) in a 96-well plate. The cells were treated with various concentrations of fractions (1, 10, 100, 250, 500 µg). After 24 h incubation, cells were washed and 100 µL of MTT (5 mg/mL), dissolved in DMEM, was added to each well and incubated at 37°C in a CO_2 incubator. After 4 h incubation, DMSO was added to each well, and the plate was kept on a shaker at 12 rpm for 45 min. The change in colour was monitored using a micro-plate reader (BIOTEK-USA) at 570 nm. Results were expressed as percentage of cytotoxicity:

Percentage toxicity = $((A_c-A_s)/A_c) \times 100$

where A_c-absorbance of control, A_s-absorbance of the sample.

3.2.3.3.Detection of reactive oxygen species (ROS) production by flow cytometry

The intracellular level of ROS was determined using the fluorescent probe 2,7-dichlorodihydrofluorescein diacetate (LeBel et al., 1992) (DCFH-DA). H9c2 cells were pre-treated with different concentrations of fractions (1, 10 and 100 µg). After 24 h, the culture medium was removed, and 100 µM TBHP in PBS was added and incubated for 20 min. The cells were then washed with PBS, and 20 µM DCFH-DA in PBS was added and incubated for 20 min. The cells were then

washed twice with cold PBS, trypsinised, resuspended in ice-cold PBS and subjected to flow cytometry. Samples were analyzed using BD FACS Aria II (BD Biosciences) at FITC range (excitation 490 nm, emission 525 nm band pass filter). The mean fluorescence intensity of different groups was analyzed by BD FACS Diva software and corrected for autofluorescence from unlabelled cells.

3.2.3.4.Antioxidant assays

H9c2 cells cultured in six-well plates at a density of 1x 10^6 cells/well were pre-treated with 100 µg concentration of fractions for 24 h, followed by TBHP (100 µM) for 15 min at 37°C. After incubation, cells were washed with PBS and lysed using respective enzyme specific buffer. The lysed cells were used to determine the antioxidant activity. Cells without fractions treatment were used as a control, and the cells with only TBHP serves as TBHP control.

3.2.3.4.1. Catalase

Catalase enzyme helps in promoting the conversion of hydrogen peroxide to water and molecular oxygen. After incubation, as in 3.2.3.4, cells were washed with PBS and lysed using cold catalase assay buffer. The supernatant was used to determine the intracellular catalase activity using catalase activity colorimetric assay kit according to the manufacturer instructions. The results were expressed as mU/mL.

3.2.3.4.2. Superoxide dismutase assay (SOD assay)

Superoxide is one of the most effective intracellular enzymatic antioxidants, and it catalyzes the conversion of superoxide anions to dioxygen and hydrogen peroxide. After incubation, as in 3.2.3.4, cells were washed with PBS and lysed using cold HEPES buffer (20 mM, pH 7.2). The supernatant obtained after cell lysis was used for assaying SOD activity and was done using Cayman kit

according to the manufacturer instructions. The results were expressed as units/mL.

3.2.3.4.3. Glutathione peroxidase assay

Glutathione peroxidase acts in association with tripeptide glutathione (GSH), which is present in high concentrations in cells and catalyzes the conversion of hydrogen peroxide or organic peroxide to water or alcohol while simultaneously oxidizing GSH. It also competes with catalase for hydrogen peroxide as a substrate and is the major source of protection against low levels of oxidative stress. After incubation, cells were washed with PBS and homogenized using cold glutathione peroxidase assay buffer. Glutathione peroxidase activity was evaluated as per manufacturer's instructions (Biovision). The results were expressed as mU/mL.

3.2.3.4.4. Glutathione reductase assay

Glutathione reductase can recycle back to glutathione. After incubation as in 3.2.3.4, cells were washed with PBS and lysed using cold glutathione buffer. The lysed cells were used to determine the reduced glutathione. The assay was performed using the kit according to the manufacturer instructions. The results were expressed in microgram.

3.2.4. *In vitro* chemical assays

3.2.4.1. Inhibition of human LDL oxidation

Oxidation of LDL leads to the production of malondialdehyde (MDA) which was measured by reaction with TBA according to the method of Chidambara, 2002with slight modification. LDL (50 μg/mL) was incubated with different concentrations of the fractions, and the oxidation of LDL was initiated by the addition of 50 μL copper sulphate (2 mM) at 37°C for 2 h. The final volume of

the reaction mixture was made up to 1.5 mL with phosphate buffer (pH 7.4). Reaction mixture (500 μL) was mixed with 250 μL of TBA (1% in 50 mM of NaOH) and TCA (0.28%). Samples were again incubated at 95°C for 45 min. After cooling and centrifugation at 2000 rpm (10 min), the fluorescence of the supernatant was taken at 515 nm excitation and 553 nm emission. The result was expressed as percent of inhibition of LDL oxidation.

Percentage inhibition = $((A_c - A_s)/A_c) \times 100$

where A_c-absorbance of control, A_s-absorbance of sample.

3.2.4.2. Determination of HMG-Co A reductase inhibitory activity

The assay is based on the spectrophotometric measurement of the decrease in absorbance at 340 nm, which represents the oxidation of NADPH by the catalytic subunit of HMGR in the presence of the substrate HMG-CoA. The HMG-CoA reductase assay kit from Sigma-Aldrich (St. Louis, MO, USA) with the catalytic domain of the human enzyme (recombinant GST fusion protein expressed in *E. coli*) was used, under conditions recommended by the manufacturer, to identify the most effective fraction of plant fractions. The concentration of the purified human enzyme stock solution (Sigma) was 0.52 – 0.85mg protein/mL. Reference statin drug, pravastatin from Sigma, was used as positive control. To characterize HMG-CoA reductase inhibition under defined assay conditions, reactions containing 4 μL of NADPH (to obtain a final concentration of 400 μM) and 12 μL of HMG-CoA substrate in a final volume of 0.2mL of 100mM potassium phosphate buffer (pH 7.4) were added. The reaction was initiated by the addition of 2 μL of the catalytic domain of human recombinant HMG-CoA reductase and incubated at 37°C in the presence or absence (control) of 1 μL aliquots of fractions dissolved in DMSO. The rates of

NADPH consumed were monitored every 20 sec for up to 15 min by scanning spectrophotometrically.

IC_{50} was calculated as:

Percentage inhibition = $[(A_c-A_s)/A_c] \times 100$

where A_c-absorbance of control, A_s-absorbance of the sample.

3.2.4.3. Determination of ACE inhibition using HPLC method

To 10 µg of inhibitor solution/fractions, 30 µL of buffer (50 mMTris–HCl pH 7.5 containing 0.3 M NaCl) and 100 µL of substrate solution (0.25 mM FAPGG in the same buffer) were added. The reaction was started by addition of 20 µL of ACE enzyme (0.3 U/mL), and the mixture was incubated in a water bath at 37°C for 45 minutes. The reaction was then stopped by addition of 100 µL of methanol, and the reaction mixture was then analyzed by HPLC. Suitable blank FAPGG alone (blank) and ACE reacted with FAPGG without inhibitors (control) was performed under same conditions. ACE inhibition was determined by measuring the level of FAP with and without inhibitor under same conditions. The analysis was performed with a Shimadzu HPLC system containing two LC-8A preparative liquid chromatography pump units, a C18 reverse phase column (Phenomenex, 5 µm, 250 x 3.6mm^2 dia.), and a diode array detector (DAD; SPD-M10A VP). FAP and FAPGG were separated using the mobile phases water containing 0.1 % TFA as solvent A and acetonitrile containing 0.1 % TFA as solvent B with a time program of 0 % B to 100% B for 25 minutes. The flow rate was 1 mL/min and the column temperature was set at 35°C. Data acquisition and analysis were carried out using SHIMADZU- CLASS-VP version 6.14 SP1 software. ACE inhibition was evaluated based on the comparison between the concentration of FAP in the presence or absence of an inhibitor. The FAP peak areas obtained in the two cases were measured, and thus the percentage of relative activity of ACE was measured.

3.2.4.4. Molecular Docking Studies

The inhibitory potential of *Syzygium cumini* fractions against HMG-CoA and ACE were performed. Following these assays, molecular docking studies were carried out to understand the binding interaction of major phenolics in *Syzygium cumini* seeds with angiotensin converting enzyme and HMG-CoA reductase enzyme. Docking studies were performed using Autodock 3.2 (Mahindroo *et al*., 2006; Morris *et al*., 2009; The PyMOL Molecular Graphics System, 2015). The 3D model of angiotensin converting enzyme (PDB ID: 4CA6) and HMG-CoA reductase enzyme (PDB ID: 1DQ8) were retrieved from the Brookhaven Protein Data Bank (PDB) (http:// www.rcsb.org/pdb/). Gallic acid (ChemSpider ID: 361), ellagic acid (ChemSpider ID: 4445149), cinnamic acid (ChemSpider ID: 392447), quercetin (ChemSpider ID: 4444051), ferulic acid (ChemSpider ID: 393368), syringic acid (ChemSpider ID: 10289), pravastatin (ChemSpider ID: 54687) and captopril (ChemSpider ID: 40130) structures were downloaded from Chemspider (http://www.chemspider.com/) and converted to PDB file using Chem3D Pro 10.

3.2.5. Statistical analysis

All experimental results were expressed as mean ± SD (standard deviation) of three different experiments. Data were subjected to one-way analysis of variance (ANOVA), and Duncan's multiple range tests were used to test the significant differences. Data are presented as mean ± SD, and $p \leq 0.05$ was considered to be significant. All statistical analyses were performed with SPSS 11.0 (Statistical Program for Social Sciences, SPSS Corporation, Chicago, IL) for Windows.

3.3. RESULTS

3.3.1. Assessment of cytotoxicity

The cytotoxicity of each fractions in H9c2 cell lines were determined by MTT assay. The concentration of fractions up to 100 µg was found to be less toxic for a period of 24 h (Fig 3.2). Percentage cytotoxicity of all fractions has been represented in Table 3.1.

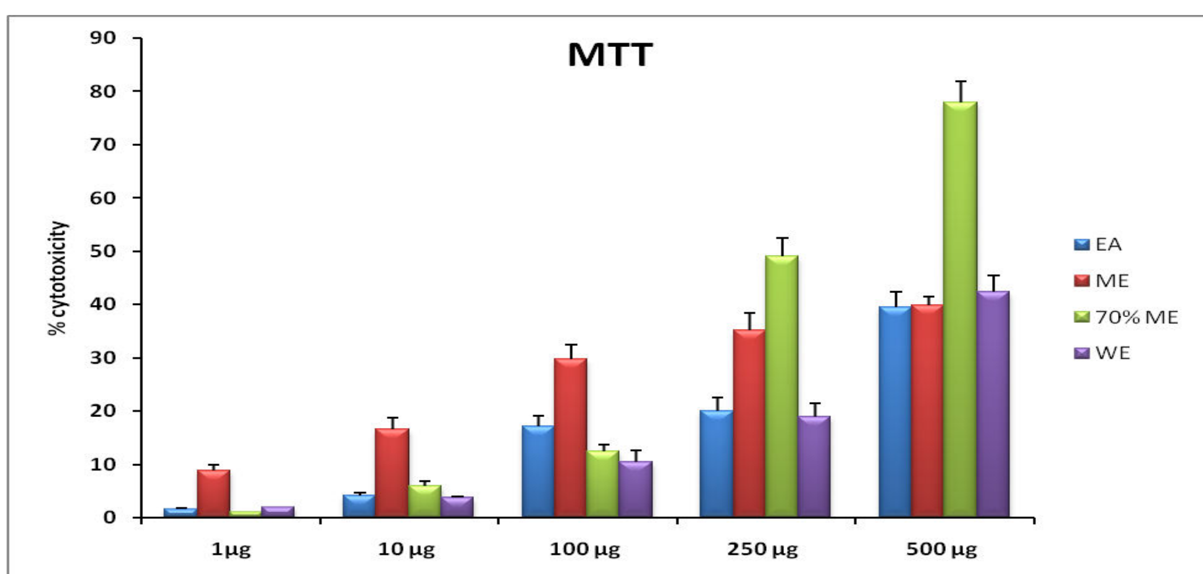


Fig. 3.2: Percentage of cytotoxicity of fractions in H9c2 cell lines.
EA- Ethyl acetate fractions, ME- Methanol fractions, 70% ME- 70% methanol fractions, WE- Water fractions

Table 3.1: Percentage cytotoxicity of extracts in H9c2 cell lines

Concentration	EA	ME	70% ME	WE
1 µg	1.56±0.2	8.75±1.2	1.09±0.04	1.98±0.05
10 µg	3.22±0.5	16.63±2.2	5.94±0.9	3.76±0.12
100 µg	17.05±2.1	29.76±2.7	12.51±1.21	10.45±2.23
250 µg	20.03±2.5	35.21±3.2	48.98±3.5	18.87±2.56
500 µg	39.42±2.9	39.9±1.6	77.93±3.9	42.34±3.2

EA - ethyl acetate fraction, ME - methanol fraction, 70% ME - 70% methanol fraction, WE Water fraction. Values are the means ± SD of three replicated samples. Duncan's multiple range test was conducted, and data in the same column with different letters/symbols indicate statistically significant differences among groups at $p < 0.05$.

3.3.2. Determination of ROS generation using DCFH-DA

Inorder to check the protective effect of *Syzygium cumini* seed fractions against TBHP induced toxicity, H9c2 cells were treated with 100 µg concentration of extracts for 24 h followed by TBHP at a concentartion of 100 µM for 20 min. The level of ROS production was estimated by analyzing the fluorescent intensity derived from oxidized DCFDA. The fluorescent intensity analyzed by confocal microscopy has been depicted in Fig 3.3. The results showed that ROS level was increased significantly on treatment with 100 µM TBHP as compared to control. Pre-treatment with *Syzygium cumini* seed fractions especially methanol and 70% methanol fractions reduced ROS production and thus protecting the cells from TBHP induced cytotoxicity.

To confirm the extent of cellular oxidative stress, fluorescent intensity of intracellular ROS production was also estimated by flow cytometry (Fig 3.4). Cells treated with TBHP (100 µM) for 20 min showed a significant increase in ROS generation (47.2%) as compared to untreated control (5.2%). H9c2 cells pre-treated with *Syzygium cumini* seed fractions showed significant ($p<0.05$) reduction in ROS generation in presence of TBHP. Methanol (100 µg) and 70% methanol fractions (100 µg) showed three fold and two fold reduction in oxidative stress when compared to TBHP treated cells as shown in Fig 3.4 D and E, respectively. The highest reduction of ROS generation was demonstrated by cells pre-treated with methanol fraction (100 µg). In addition, 70% methanol and ethyl acetate fractions also significantly prevented ROS generation in TBHP treated cells.

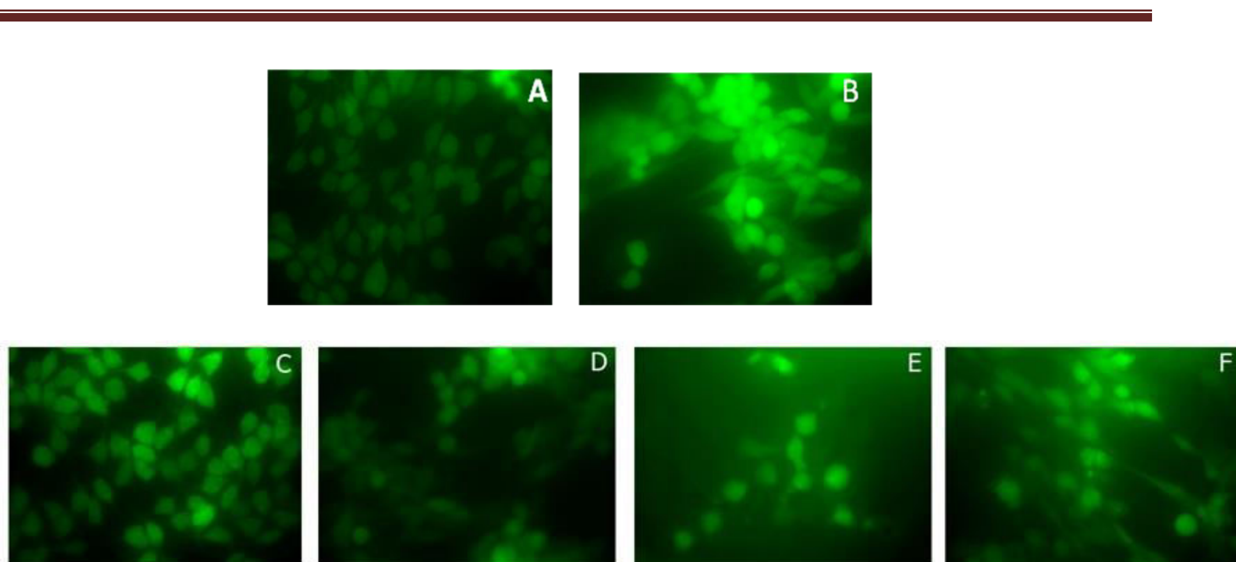


Fig. 3.3: Fluorescent images (20X magnification) of intracellular ROS production by confocal microscopy

Intracellular ROS production in H9c2 cell lines (A) control cells (B) cells treated with Tertiary butyl hydrogen peroxide (TBHP) - 100 µM (C) Ethyl acetate fractions (100 µg) (D) Methanol fractions (100 µg) (E) 70% methanol fractions (100 µg) (F) Water fractions (100 µg)

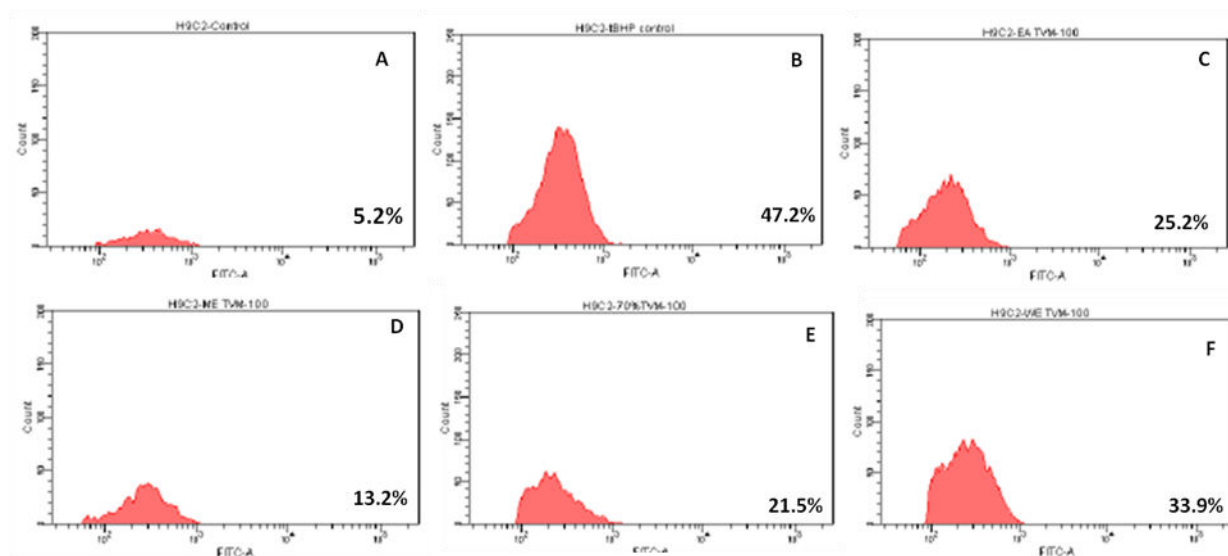


Fig. 3.4: Fluorescent analysis of ROS by flow cytometry

FACS analysis of intracellular ROS production in H9c2 cell lines by plotting cell count against FITC. The groups contained (A) control cells (B) cells treated with Tertiary butyl hydrogen peroxide (TBHP) (C) Ethyl acetate fractions (100 µg) (D) Methanol fractions (100 µg) (E) 70% methanol fractions (100 µg) (F) Water fractions (100 µg). X axis showed the log scale of fluorescent intensity and Y axis showed the cell count.

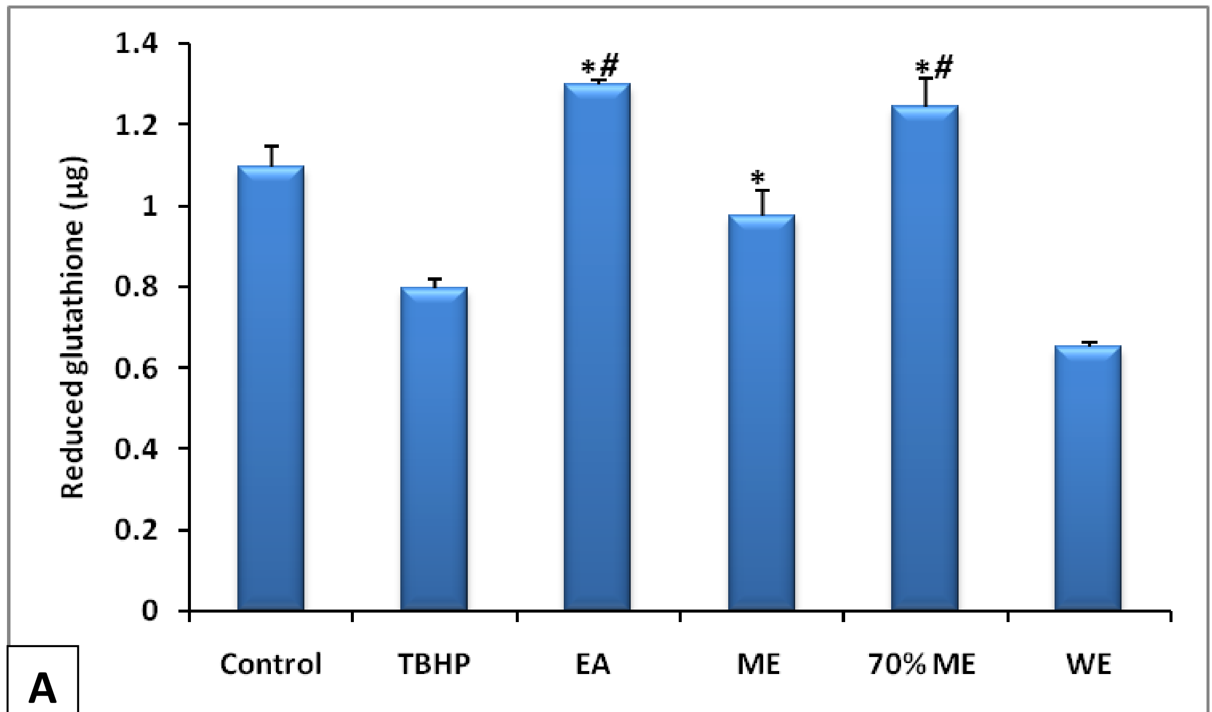
3.3.3. Antioxidant enzyme levels

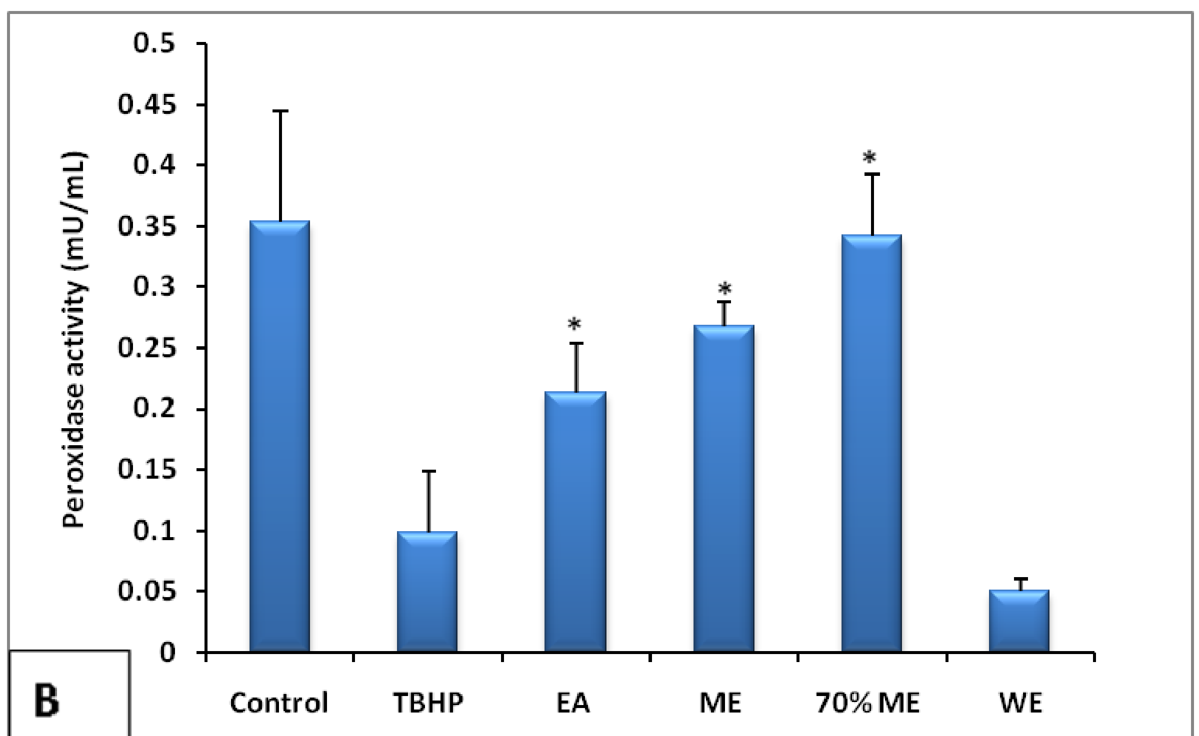
The most evident enzymatic antioxidants catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase levels in H9c2 cells were determined by pretreating the fractions and compared with cells treated with only TBHP (control) (Fig 3.5).

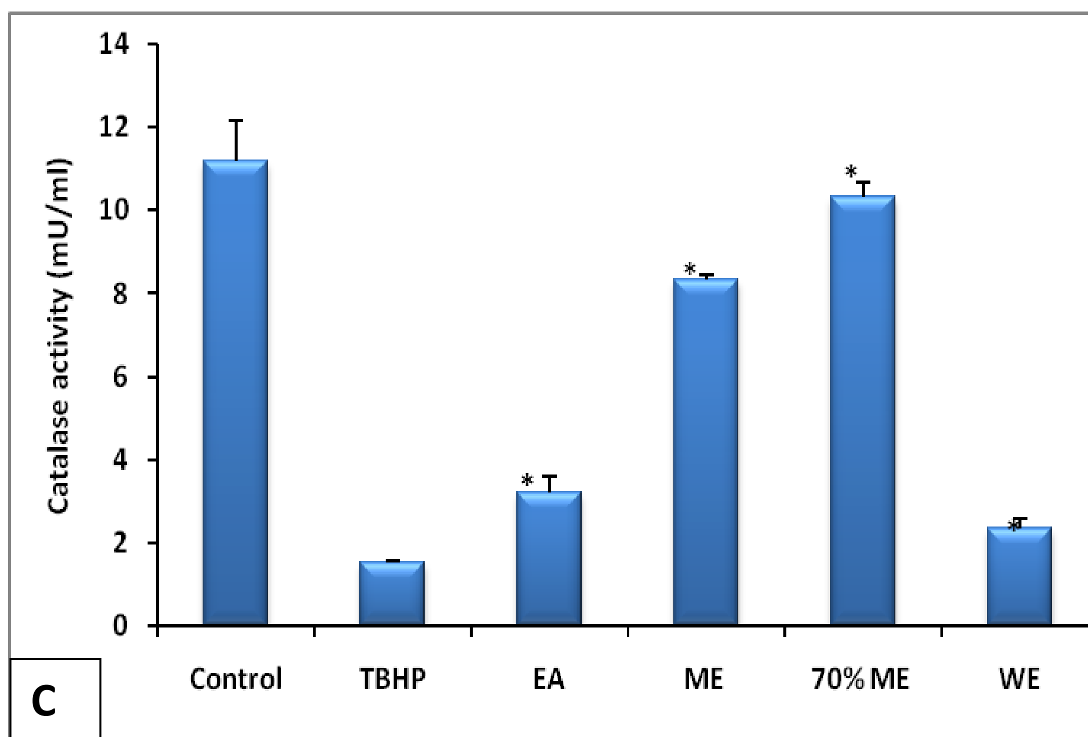
Treatment of fractions with TBHP reduced the level of antioxidant enzymes. Pre-treatment with 70% methanol, methanol and ethyl acetate fractions demonstrated significant increase ($p<0.05$) in reduced glutathione and peroxidase activity as compared to TBHP group. Pre-treatment with 70% methanol, methanol, and ethyl acetate fractions increased the level of reduced glutathione to 55.38%, 22.43% and 50.2% respectively when compared with TBHP treated group (Fig 3.5A). Pre-treatment with ethyl acetate and methanol fraction showed a two fold increase in gluathione peroxidase, whereas pre-treatment with 70% methanol showed a threefold increase in the level of peroxidase enzyme (Fig 3.5B).

The catalase and SOD activity also showed a similar trend in *Syzygium cumini* seed fractions pre-treated cells. The level of catalase and SOD was decreased significantly in TBHP treated cells, but pre-treatment with different fractions helped the cells in reverting the glutathione levels to near normal ($P>0.05$). The results showed that treatment with ethyl acetate, methanol, and 70% methanol fractions increased the level of catalase enzyme by two fold, 5 fold and 6 fold respectively (Fig 3.5C). There was a marked increase in the level of SOD on pre-treatment with *Syzygium cumini* seed fractions (Fig 3.5D). The level of reduced glutathione, peroxidase, catalase and SOD activity in 70% methanol pre-treated fractions were comparable to control. The results from the present study demonstrated that 70% methanol, methanol and ethyl acetate fractions of *Syzygium*

cumini can retrieve the natural antioxidant enzymes which were drastically reduced under TBHP exposure which signifies the potent antioxidant property of the fractions of *Syzygium cumini.*







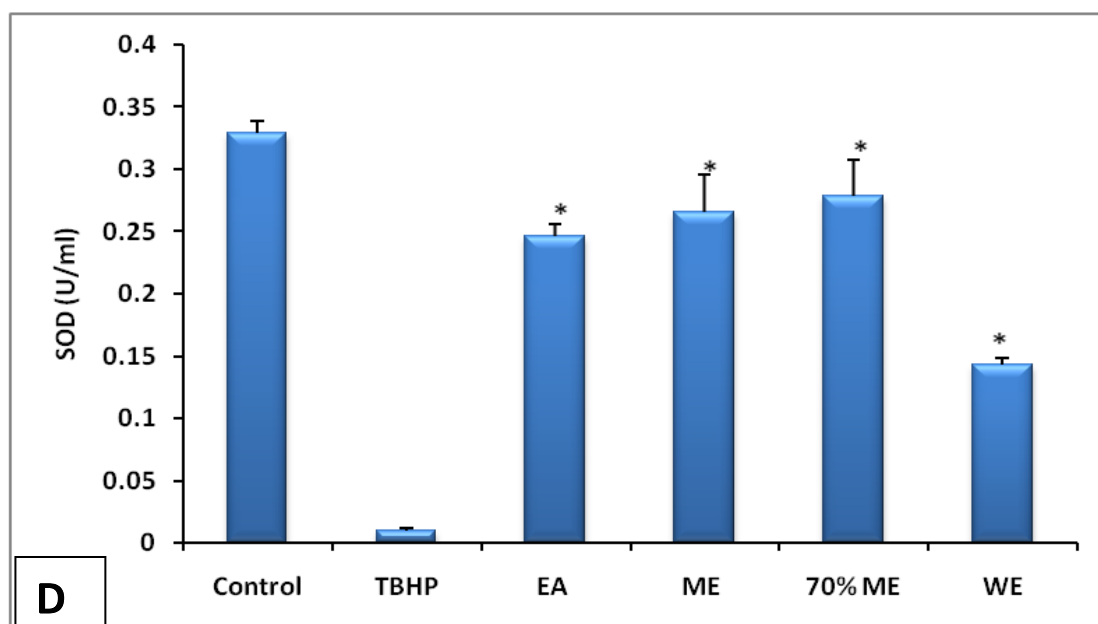


Fig. 3.5 (A-D): Antioxidant potential of different fractions of Syzygium cumini (100 µg) in H9c2 cell lines

Values are mean ± SD (standard deviation) of triplicate samples. Significance test compared with TBHP treated cells was determined by using one way ANOVA followed by Duncan's multiple range test and the significance accepted at $p \leq 0.05$. A) Catalase activity B) Superoxide dismutase activity C) Glutathione peroxidase activity D) Reduced glutathione, *$p \leq 0.05$ versus TBHP, # $p \leq 0.05$ versus control (EA – ethyl acetate fractions, ME – methanol fractions, 70% ME – 70% methanol fractions, WE-Water fractions)

3.3.4. LDL oxidation inhibition

LDL oxidation is a key mechanism behind the development of atherosclerosis. Oxidized LDL increases foam cell formation that eventually leads to atherosclerosis. Compared with the standard, ascorbic acid (IC_{50} – 28.7 µg/mL), our results demonstrated that 70% methanol fractions (0.156 µg/mL) and methanol fractions (15.46 µg/mL) of *Syzygium cumini* significantly ($p<0.05$) inhibited LDL oxidation (Fig 3.6). The results thus showed that *Syzygium cumini* seed and its fractions play an important role in preventing LDL oxidation.

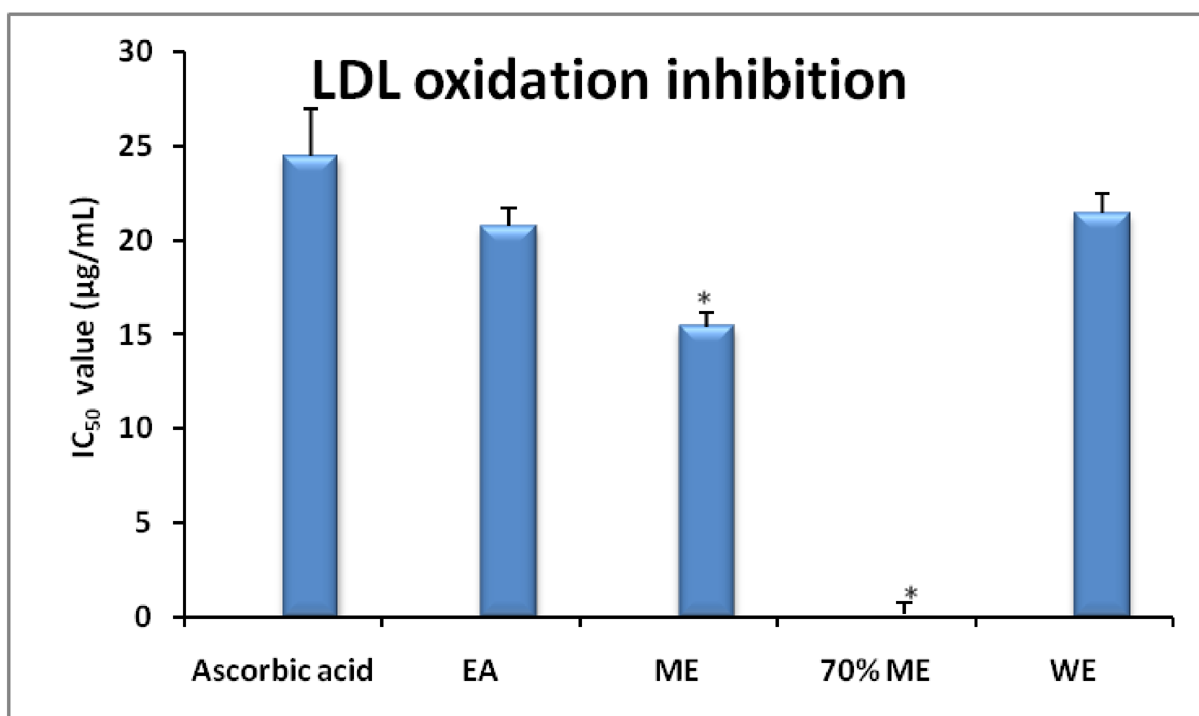


Fig. 3.6: LDL oxidation inhibitory potential of Syzygium cumini
Values are mean ± SD (standard deviation) of triplicate samples. Significance test compared with the standard was determined by using one way ANOVA followed by Duncan's multiple range test and the significance accepted at $p \leq 0.05$. *$p \leq 0.05$ versus ascorbic acid. (EA – ethyl acetate fractions, ME – methanol fractions, 70% ME – 70% methanol fractions, WE- Water fractions)

3.3.5. HMG-CoA reductase inhibition

HMG-CoA reductase inhibition of methanol fractions (0.2 units/mg) was highly significant when compared with positive control, pravastatin (Fig 3.7). Ethyl acetate and 70% methanol fractions also exhibited marked HMG-CoA reductase inhibitory potential. Methanol fraction showed 24% reduction in HMG-CoA reductase when compared with positive control pravastatin. Hypercholesterolemia is considered as a risk factor for the development of atherosclerosis and other cardiovascular complications. Cholesterol biosynthesis can be blocked by inhibiting HMG-CoA reductase which is the key enzyme in cholesterol biosynthetic pathway. Thus the results showed that all fractions of *Syzygium cumini* seeds possess significant HMG-CoA inhibitory activity.

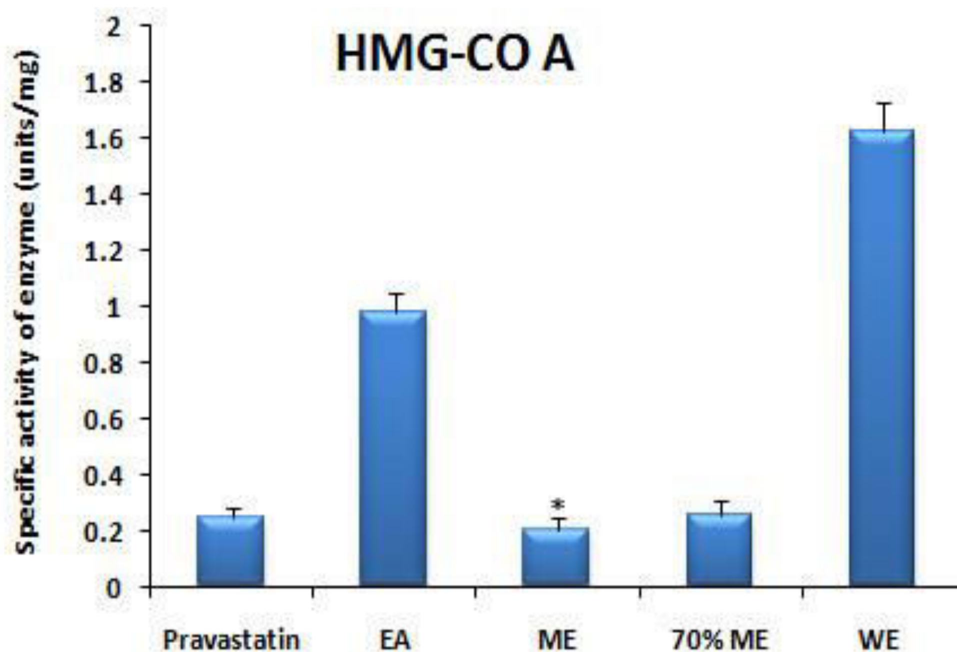


Fig. 3.7: HMG-CoA reductase potential of Syzygium cumini

Values are mean ± SD (standard deviation) of triplicate samples. Significance test compared with positive control pravastatin for HMG-CoA reductase was determined by using one way ANOVA followed by Duncan's multiple range test and the significance accepted at $p \leq 0.05$. *$p \leq 0.05$ versus positive control. (EA – ethyl acetate fractions, ME – methanol fractions, 70% ME – 70% methanol fractions, WE – Water fractions)

3.3.6. ACE inhibition

In the present study, the role of *Syzygium cumini* seed fractions in inhibiting the activity of rabbit lung ACE was investigated. HPLC analysis demonstrated that *Syzygium cumini* seed fractions effectively inhibited ACE activity. The area of FAPGG peak was used to calculate the relative activity of ACE (Fig 3.8 A).

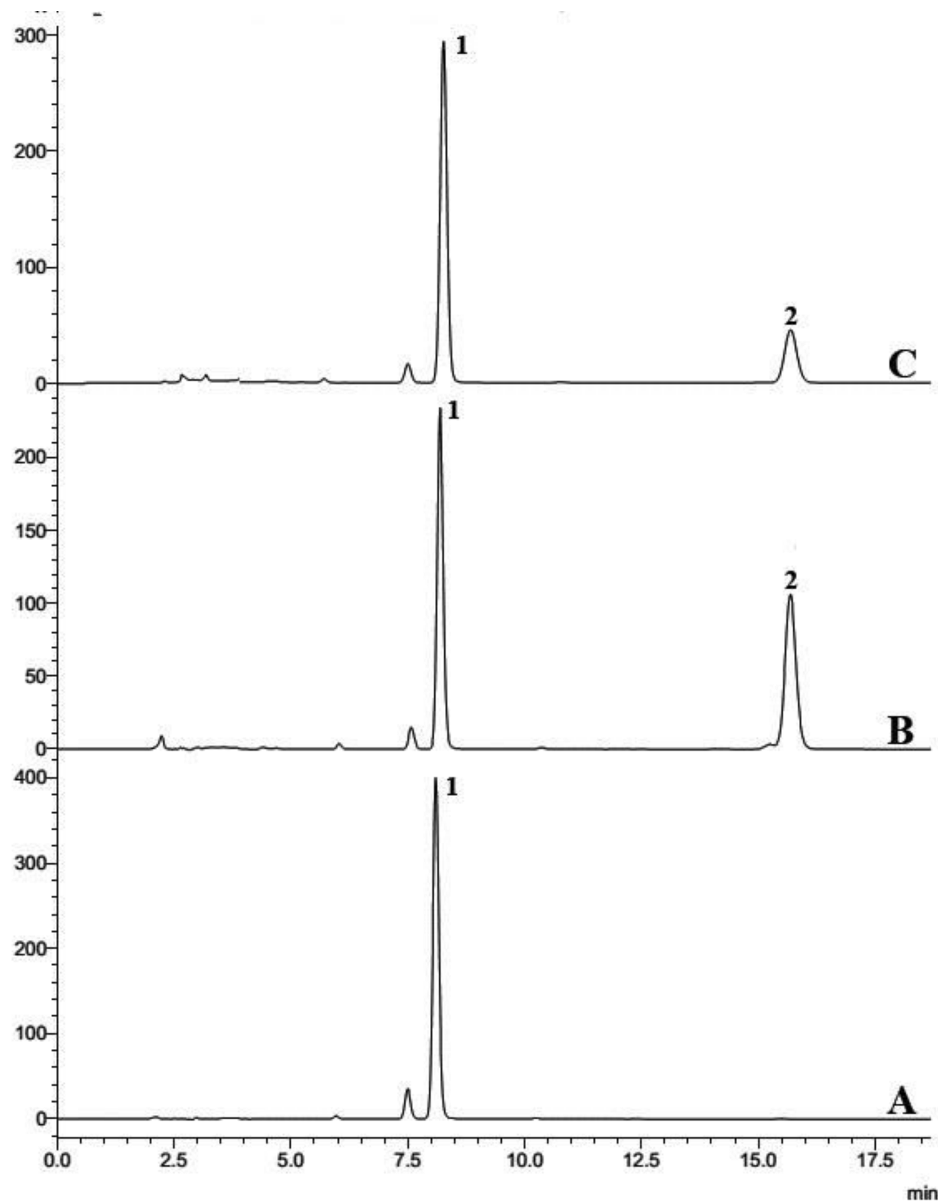


Fig. 3.8: Chromatogram from HPLC for determining ACE activity
(A) Blank without enzyme, (B) Control without inhibitor, (C) Captopril-standard ACE inhibitor (0.25 μM)

The standard, captopril (0.25 μM) exhibited a relative activity of 20.35 ± 0.56% whereas the same for control sample was 42.50 ± 0.23%. However, pre-treatment with ethyl actate, methanol, and 70% methanol fractions of the seeds of *Syzygium cumini* decreased ACE activity (29.32 ± 0.4%, 21.89 ± 0.87 and 23.96 ± 0.6% respectively) as compared to that of positive control, captopril (20.35 ± 0.56%) (Fig 3.9). Thus the results showed that all fractions of *Syzygium cumini* seeds could play an important role in regulating blood pressure.

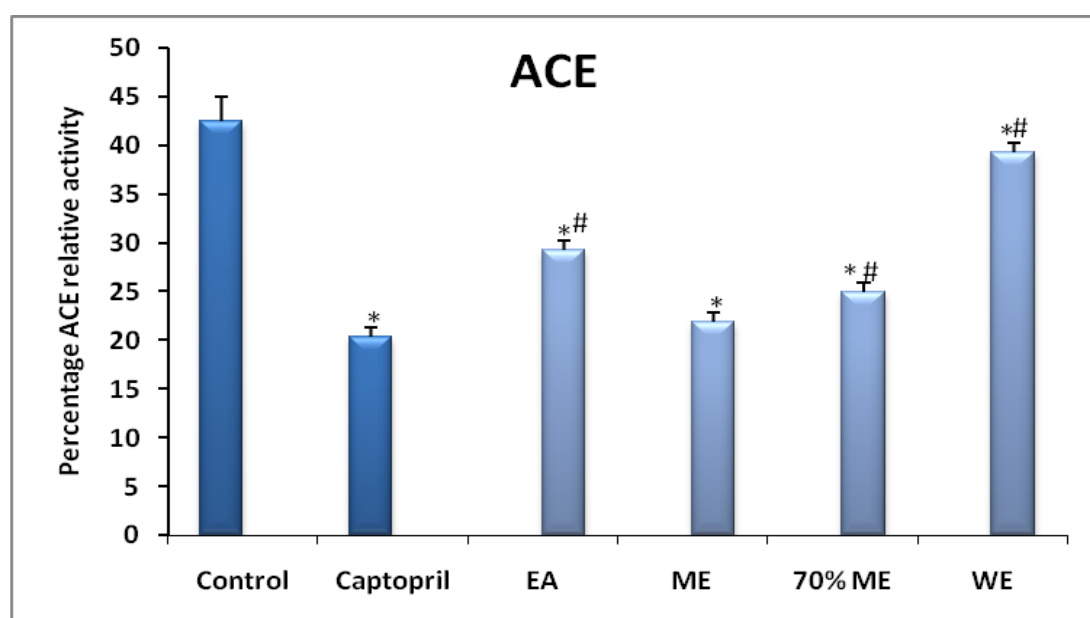


Fig. 3.9: ACE inhibitory potential of Syzygium cumini

ACE inhibitory potential of *Syzygium cumini* (100 μg).Values are mean ± SD (standard deviation) of triplicate samples. Significance test compared with positive control captopril for ACE inhibition was determined by using one way ANOVA followed by Duncan's multiple range test and the significance accepted at $p \leq 0.05$. *$p \leq 0.05$ versus positive control. (EA – ethyl acetate fractions, ME – methanol fractions, 70% ME – 70% methanol fractions, WE – Water fractions)

3.3.7. Molecular docking

In order to confirm the HMG-Co A and ACE inhibitory potential of *Syzygium cumini* seed fractions, docking studies with HMG-Co A reductase (Fig 3.10A) and ACE (Fig 3.10B) were conducted to identify the binding interactions of the major identified compounds that we identified in *Syzygium cumini* fractions.

The minimum binding energy from the docking studies indicated that the ACE and HMG-CoA reductase (target enzymes) were successfully docked with the compounds, gallic acid, ellagic acid, quercetin, cinnamic acid, syringic acid and ferulic acid. The best conformation of the ligand binding to the receptor and also the residues on the receptor to which the ligand can bind in Autodock are represented in Fig 3.10.

Fig.3.10: Molecular docking images of predominant phenolics with HMG- Co A and ACE

A) Representing the residues on HMG-CoA reductase enzyme B) Representing the residues on ACE enzyme binding to i) Gallic acid ii) Ellagic acid iii) cinnamic acid iv) quercetin v) syringic acid vi) ferulic acid vii-a) pravastatin vii-b) captopril using Autodock 3.2.

Table 3.2: The free binding energy (kcal/mol) of compounds with ACE and HMG-CoA reductase proteins

	GA	EA	CA	Qtn	SA	FA	Cap	PS
ACE	-3.65	-7.46	-6.27	-6.92	-5.54	-5.86	-7.12	
HMG CoA reductase	-7.92	-7.26	-7.11	-7.94	-7.22	-7.59		-5.46

GA – Gallic acid, EA – Elagic acid, CA – Cinnamic acid, Qtn – Quercetin, SA – Syringic acid, FA – Ferulic acid, Cap – captopril, PS –Pravastatin

All the predominant compounds showed relatively higher binding affinity to HMG CoA reductase protein when compared to the positive control, pravastatin (minimum binding energy of −5.46 kcal/mol) (Table 3.2). The lowest binding energy was shown by gallic acid (-7.92 kcal/mol) and quercetin (-7.94 kcal/mol) that may attribute to the HMG-CoA reductase inhibitory property of methanol fractions of *Syzygium cumini* (Fig 3.10A-i, iv). Against ACE, the compounds, namely ellagic acid (-7.46 kcal/mol) and quercetin (-6.92 kcal/mol) demonstrated higher binding affinity which proves the ACE inhibitory potential of methanol fractions (Fig 3.10B ii, iv).

3.4. DISCUSSION

Cardiovascular diseases are currently one of the leading cause of morbidity and mortality globally. Oxidation of LDL, excess of cholesterol deposits and increased blood pressure play vital role in the development of cardiovascular diseases. Natural products are emerging as a promising target to address the increasing risk of this disease. Any natural product that can act against these three major factors can provide immense benefit to mankind that can overcome the side

effects of synthetic drugs. *Syzygium cumini* is widely used in traditional medicine to treat various diseases. However, apart from a few isolated studies on the cardioprotective effects of *Syzygium cumini*, the scientific evidence is limited. Thus in the present study, we explored the cardio protective efficiency of *Syzygium cumini* seeds.

ROS is a connective term used for a group of oxidants, which are either free radicals or molecular species capable of generating free radicals (Varsha et al., 2014). Oxidative stress reflects an imbalance between the systemic manifestation of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Oxidative stress, or the generation of ROS, are known to be a contributing factor to the progression of many cardiovascular diseases (Taibur et al., 2012). Antioxidants are employed to protect biomolecules from the damaging effects of such ROS. Low intake of antioxidants has been suggested to be an increased risk factor for the development of CVD (Kris-Etherton *et al*., 2004). Polyphenols present in fruits and vegetables provide protection against oxidative stress, and thus they can act as powerful antioxidants.

Atherosclerosis is the hardening and narrowing of arteries which leads to heart attacks, stroke and other cardiovascular diseases. Evidence suggests that LDL oxidation and ROS play a crucial role in the pathogenesis of atherosclerotic complications including coronary heart diseases (Anderson *et al*., 2001; Stocker et al., 2004; Kuo *et al*., 2011). Oxidised LDL accumulates near blood vessels, and vascular plaques are formed which ultimately leads to cardiovascular diseases. Our result suggests that 70% methanol and methanol fractions of *Syzygium cumini* have a significant inhibitory action on LDL oxidation. The inhibitory potential

shown by the fractions may be due to the presence of pharmacologically valuable phenolics like quercetin, myricetin, gallic acid, ellagic acid and cinnamic acid in *Syzygium cumini* seeds. The presence of antioxidants like phenolics and flavonoids in plants are known to suppress LDL oxidation and delay the development of heart diseases (Ghosh et al., 2006; Itabe et al., 2011). Polyphenols such as ellagic acid and gallic acid found to be present in the fractions of *Syzygium cumini* in the present study were independently reported to inhibit lipid peroxidation, while ellagic acid has been shown to inhibit LDL oxidation, specifically (Safari et al., 2003; Baba *et al*., 2007). In the present study, the gallic acid concentration was markedly high in methanol and 70% methanol fractions which might have attributed to their relatively high LDL oxidation inhibitory potential. Based on the earlier reports and the results from the present study, it can be suggested that *Syzygium cumini* fractions possess significant potential against LDL oxidation which could ameliorate its deleterious effects in the vessel wall.

Hypercholesterolemia is another risk factor for the development of atherosclerosis and attempts have been made for blocking the biosynthesis of cholesterol by inhibiting the activity of the key enzyme of cholesterol biosynthetic pathway, the 3-hydroxy-3-methyl glutaryl Coenzyme A (HMG-CoA) reductase. HMG-CoA reductase is the rate controlling enzyme of the mevalonate pathway, the metabolic pathway that produces cholesterol and other isoprenoids. In the present study, 70% methanol and methanol fractions inhibited HMG-CoA reductase activity demonstrating their ability to reduce the cholesterol level. The presence of phenolics (such as, ferulic acid, gallic acid, ellagic acid, quercetin and myricetin) in *Syzygium cumini* fractions may be attributing to high HMG-CoA

reductase inhibitory potential, since these phenolics have been reported for the inhibition of HMG-CoA reductase (Santhosh et al., 2008; Paival et al., 2013).

ACE is a dipeptidylcarboxypeptidase that plays a crucial role in the regulation of blood pressure. ACE promotes the conversion of angiotensin-I to the potent vasoconstrictor angiotensin-II as well as inactivates the vasodilator bradykinin, which has a depressor action in the renin-angiotensin system. The results demonstrated that methanol and 70% methanol significantly inhibited ACE. The inhibitory potential of the fractions might be due to the presence of apigenin, chlorogenic acid, catechin, and quercetin, as these compounds are reported to have potent ACE inhibitory activity (Kumar et al., 2010). The presence of these compounds in *Syzygium cumini* seeds may attribute to the inhibition of rabbit lung ACE by competing with the substrate for their active sites.

The inhibitory studies of the phenolics, detected in the *Syzygium cumini* seed fractions, against HMG-CoA reductase and ACE were confirmed through molecular docking studies. Molecular docking experiments with major phenolic acids and flavonoids reveal the binding interaction of these compounds with HMG-CoA and ACE which indirectly stating that these phenolics and flavonoids may be the attributing factor for HMG-CoA and ACE inhibitory potential of *Syzygium cumini* seed fractions.

Our findings shed light into the cardioprotective benefits of *Syzygium cumini* seeds, by exploring the phenolics present in different fractions and the mode of action of these fractions in preventing cardiovascular diseases. Our molecular docking studies demonstrated a positive correlation between the phenolics and key enzymes in preventing cardiovascular diseases. These findings are of greatest

importance as it provides a novel therapeutic strategy to overcome the risk of cardiovascular disease.

3.5. SUMMARY

The present study is one of the first study that reports the importance of sequential fractions of *Syzygium cumini* seeds in demonstrating cardioprotective potential. The present study also highlighted the mode of action of these fractions for their cardioprotective effect. The study also showed the importance of ethyl acetate, methanol and 70% methanol fractions in preventing cardiovascular diseases. The presence of phenolic acids and flavonoids which were reported in Chapter 2 may be the reason for potent cardioprotective action shown by these fractions. This study clearly showed the role of different fractions of *Syzygium cumini* seeds in modulating the level of various antioxidant enzymes, cholesterol biosynthesis, blood pressure thus demonstrating cardioprotective potential.

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CHAPTER 4

Anti-diabetic effects of Syzygium cumini seed

4.1. INTRODUCTION

Diabetes mellitus is a complex and a fast growing medical problem throughout the globe and one of the principal causes of morbidity and mortality among the human race. The prevalence of diabetes in 2014 was 386.7 million, representing 8.5% of the world's adult population, with a prediction that by 2035, the number of people with diabetes would shoot up to 591.9 million (IDF Diabetes Atlas; 2015). Diabetes is a chronic metabolic disorder characterised by changes in carbohydrate, protein and fat metabolism. This leads to an absolute or relative lack of the hormone insulin, and the later's inefficiency would lead to increased level of glucose in the body of diabetic persons. This increase in the level of glucose (hyperglycemia) can react with plasma proteins through a non-enzymatic process to form advanced glycated end products like glycated hemoglobin and glycated albumin. Advanced glycated end products have been reported to accumulate in kidney, retina and other sites which would lead to diabetic complications.

Currently, antidiabetic therapies are based on synthetic drugs that are known to have adverse side effects such as kidney complications, stomach upset, diarrhoea, dizziness, skin rashes, etc. (Krentz *et al*., 2005). There is a continuous drive globally to investigate more effective agents as alternatives with comparatively lesser side effects for the management of diabetes. Natural hypoglycemic compounds from traditional plants are the attractive alternatives for synthetic drugs, which can efficiently counter the side effects, high cost, and poor availability.

Syzygium cumini Linn (family Myrtaceae) commonly known as black plum or Jamun is used extensively in various traditional systems of medicine in India, Thailand, Philippines, Madagascar and some other countries.

Eventhough studies had shown that fibre content in *Syzygium cumini* seeds (4.19%) are more when compared with pulp (1.76%) (Raza *et al*., 2015), the seeds are often discarded as waste following pulp consumption. The seeds of *Syzygium cumini* are claimed to contain alkaloids, such as jambosine and glycoside jambolin or antimellin (Ayyanar *et al*., 2012). The therapeutic importance of *Syzygium cumini* seeds had been attributed partly due to the presence of phenolic compounds in it (Priya *et al*., 2017). Polyphenols present in the plant has been reported for antidiabetic potential by inhibiting carbohydrate hydrolysing enzymes (Olaokun *et al*., 2013), antiglycation (Wang *et al*., 2011) dipeptidyl peptidase-IV inhibition (Bower *et al*., 2014) and through modulation of several other key enzymes linked to diabetes. Studies had shown that *Syzygium cumini* seed exhibited antihyperglycemic effect in streptozotozin induced diabetic rats. However, scientific evidence is limited on the mechanism of action of *Syzygium cumini* seeds as an antidiabetic agent. The present study has been designed to explore the mechanism of antidiabetic potential of different fractions of *Syzygium cumini* seed using various *in vitro* and cell based assays.

4.2. MATERIALS AND METHODS

4.2.1. Chemicals

Porcine pancreatic α-amylase (E.C. No:3.2.1.1), α-glucosidase from baker's yeast (E.C. No: 3.2.1.20), acarbose, polyphenol standards, Dulbecco's Modified Eagle's Media (DMEM), bovine serum albumin, streptomycin ampicillin–amphotericin B mix, Rosiglitazone and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) were procured from Sigma-Aldrich Chemicals (St. Louis, MO, USA); Dipeptidyl peptidase-IV (DPP-IV) (E.C. No: 3.4.14.5) inhibition assay kit was from Cayman Chemicals (Ann Arbor, USA)., Folin Ciocalteau reagent was procured from Sisco

Research Laboratories, India; soluble starch extrapure, methanol and acetic acid of HPLC grade were supplied by Merck, Germany; Foetal bovine serum (FBS) was purchased from Gibco-BRL (Auckland, NZ); Horse serum was purchased from PAN Biotech (Aidenbach, Germany); 2-(7-Nitrobenz-2-oxa-1,3-diazol- 4-yl) amino-2-deoxy-D-glucose (2-NBDG) was purchased from Molecular Probe (Invitrogen Life Technologies, Carlsbad, CA, USA); L6 myoblast was obtained from National Centre for Cell Sciences, Pune, India; All other chemicals used were of standard analytical grade.

4.2.2. Experimental design

The work flow of this chapter is illustrated in the schematic representation in Fig 4.1.

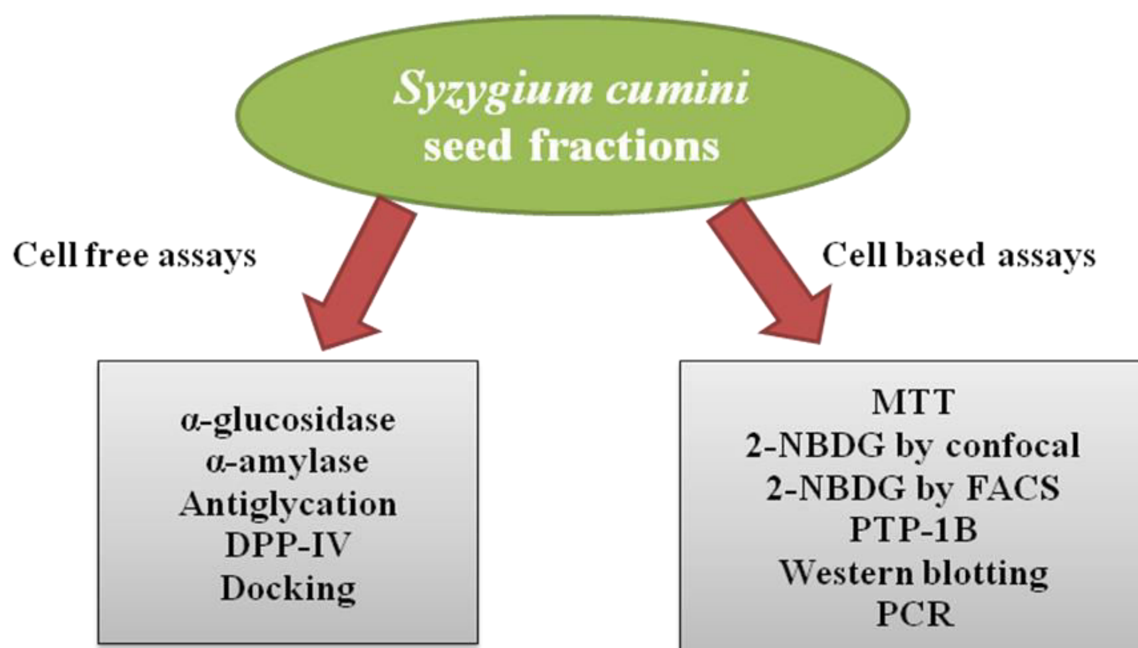


Fig. 4.1: Illustration of experimental design

4.2.3. α -amylase inhibition assay

Screening of plant material for α-amylase inhibition was performed according to the standard method (Xiao *et al*., 2006) with slight modifications. The amylase activity was determined by adding 40 µL of starch (2g/L) and 40 µL of amylase enzyme (0.02 U in 0.1 M phosphate buffer of pH 7) to different concentration of fractions and incubated at 50°C for 30 min. Following incubation, 20 µL of 1 M HCl and 100 µL of iodine

reagent were added. The colour change was noted, and the absorbance was read at 580 nm on a microplate reader (Biotek, USA). The control reaction representing 100% enzyme activity did not contain any plant fractions. To eliminate the absorbance produced by plant fractions, appropriate fraction controls without the enzymes were also included. Acarbose was used a positive control.

The results were expressed as percentage relative activity of enzyme activity and calculated according to the following equation:

$$\% \text{ Relative activity of Enzyme} = \frac{\text{Absorbance of control- Absorbance of fraction}}{\text{Absorbance of control}} \times 100$$

4.2.4. α- glucosidase inhibition assay

α-glucosidase (100 µL, 0.6 U/ mL) was mixed with different concentration of fractions made up to 50 µL with distilled water and incubated for 5 min at room temperature. 250 µL of 1mM para-nitrophenyl- α-D glucopyranoside was added to initiate the reaction, and the mixture was further incubated at 37°C for 20 min. The reaction was stopped by adding 500 µL of 1% Na_2CO_3, and the final volume was made up to 1500 µL using distilled water. The para-nitrophenol formed was measured at 405 nm (Biotek, USA). Acarbose was used as the positive control. Percentage inhibition was plotted against corresponding concentrations to obtain IC_{50} value.

4.2.5. Antiglycation assay

The assay was performed according to the methods reported by Jedsadayanmata with slight modifications (Jedsadayanmata *et al*., 2005). Briefly, BSA (1 mg/mL) was non-enzymatically glycated with 500 mM glucose, and different concentrations of fraction were added to it, incubated at 60°C for 24 hrs. After incubation, 10 µL of 100 % TCA was added to stop the reaction and kept at 4°C for 10 min. The reaction was immediately quantified for the relative amount of glycated BSA based on fluorescence

intensity at 370 nm (excitation) and 440 nm (emission) (Biotek, USA). The standard used was ascorbic acid. Percentage inhibition was plotted against corresponding concentrations to obtain IC_{50} value.

4.2.6. Dipeptidyl peptidase-IV (DPP-IV) inhibition assay

DPP-IV inhibition was measured using a commercially available kit from Cayman (Ann Arbor, USA). Briefly, the test samples (10 µg) were pipetted into a 96 well solid plate containing the diluted enzyme DPP-IV (10 µL) and 1X assay buffer (30 µL). The reaction was initiated by adding 50 µL of the diluted substrate solution to all wells. The plates were covered with plate cover and incubated for 30 min at 37°C. Diprotin A served as a positive control. The wells without DPP-IV enzyme serve as background wells. The wells with DPP-IV and substrate solution without any inhibitors act as 100% initial activity wells. The results were expressed as percentage inhibition and calculated according to the following equation:

$$\% \text{ Inhibition} = \left(\frac{\text{Initial Activity} - \text{Inhibitor}}{\text{Initial Activity}} \right) \times 100$$

4.2.7. Cell culture and treatment

L6 myoblasts, rat skeletal muscle cell line, were cultured in DMEM supplemented with

10% FBS, 1% antibiotic–antimycotic mix at 37° C under 5% CO_2 atmosphere.

4.2.7.1. Cytotoxicity assay

The viability of L6 myoblasts was assessed by MTT assay (Mosmann *et al*., 1983). The cells were treated with various concentrations of ethyl acetate, methanol and 70% methanol fractions. After 24 h incubation, cells were washed and 100 µL of MTT (5 mg/mL), dissolved in DMEM, was added to each well and incubated at 37°C in a CO_2

incubator. After 4 h incubation, DMSO was added to each well, and the plate was kept on a shaker at 12 rpm for 45 min. The change in colour was monitored using a micro-plate reader (Biotek-USA) at 570 nm. Results were expressed as percentage of cytotoxicity using the following equation:

$$\text{Percentage of Toxicity} = \frac{\text{Absorbance of Control - Absorbance of Sample}}{\text{Absorbance of Control}} \times 100$$

4.2.7.2. 2-NBDG uptake in L6 myotubes

L6 myoblasts, seeded at a density of $1X10^4$ cells per well on 96 well black plates (BD Biosciences, Franklin Lakes, BJ) for confocal imaging were differentiated for 5 days in DMEM containing 2% horse serum. The cells were pretreated with 10 µg concentration of fractions for 24 h in low glucose medium. For experiments, the culture medium was removed from each well and replaced with fresh culture medium in the presence or absence of 10 mM fluorescent 2-NBDG (Molecular Probes-Invitrogen), a fluorescent analog of glucose and incubated for 30 min. The cells treated with rosiglitazone served as positive control. The fluorescence retained in cells were imaged by a fluorescent microscope (Pathway 855, BD Bioscience, San Jose, CA, USA) set at an excitation wavelength of 490 nm and an emission wavelength of 525 nm.

4.2.7.3. Fluorescence analysis of 2-NBDG uptake by flow cytometry

L6 cells were seeded in 12 well plates (Costar, USA) for glucose uptake analysis. Briefly, after differentiation for 5 days, cells were pretreated with fractions at a concentration of 10 µg in low glucose medium. After 24 h, the culture medium was removed from each well and replaced with fresh culture medium in the absence or presence of 10 mM fluorescent 2-NBDG and incubated for 30 min. The cells were then washed twice with cold PBS, trypsinized, resuspended in ice-cold PBS and subjected to flow cytometry. Samples were analyzed using BD FACS Aria II (BD Biosciences, USA)

at FITC range (excitation 490 nm, emission 525 nm band pass filter). The mean fluorescence intensity of different groups was analyzed by BD FACS Diva software and corrected for autofluorescence from unlabeled cells.

4.2.8. Molecular Docking Studies

Following inhibitory studies of *Syzygium cumini* fractions against α-amylase, α-glucosidase, and DPP-IV enzymes, molecular docking studies were performed to understand the binding interaction of major phenolics in *Syzygium cumini* seeds. Docking studies were performed using AutoDock 1.4.6, free version. The 3D model of α-amylase (PDB ID: 4XON), α-glucosidase (PDB ID: 4J5T) and DPP-IV (PDB ID: 3WQH) were retrieved from the Brookhaven Protein Data Bank (PDB) (http:// www.rcsb.org/pdb/). Gallic acid (PubChem ID: 370), ellagic acid (PubChem ID: 5281855), cinnamic acid (PubChem ID: 444539), quercetin (PubChem ID: 5280343), ferulic acid (PubChem ID: 445858), syringic acid (PubChem ID: 10742), acarbose (PubChem ID: 41774) and diprotin A (PubChem ID: 3107) structures were downloaded from PubChem (http://pubchem.ncbi.nlm.nih.gov) and converted to PDB file using Pymol 1.7.0.0.

4.2.9. Protein Tyrosine Phosphatase 1B (PTP1B) assay

PTP1B is a non trans membrane tyrosine phosphatase which acts as a negative regulator of the insulin signaling pathway and is a promising potential therapeutic target, in particular for treatment of type II diabetes (Combs *et al*., 2009). PTP1B is localized to the cytoplasmic face of the endoplasmic reticulum. PTP1B can dephosphorylate the phosphotyrosine residues of the activated insulin receptor kinase (Cicirelli *et al*., 1990). Cells were pre-treated with different fractions of *Syzygium cumini* seeds and were incubated for 24 h. After incubation, cells were lysed using Ripa buffer and lysates were collected. PTP1B activity was checked with 100-μL p-nitrophenyl phosphate (PNPP)

buffer (25 mM Hepes, pH 7.2, 50 mM NaCl, 5 mM dithiothreitol, 2.5 mM EDTA). Phosphatases catalyze the hydrolysis of PNPP to p-nitrophenol, a chromogenic product and the intensity of the colour reaction was measured at 410 nm on a microplate reader (Biotek, USA). Results were expressed as percentage activity relative to control.

4.2.10. Isolation of total RNA and quantitative real time PCR analysis

Total RNA was isolated from pre-treated L6 cell lines using Trizol (Invitrogen Corp., Grand Island, NY, USA) according to the manufacturer's protocol. Reverse transcription was carried out with 1 µg RNA using Superscript VILO cDNA Synthesis kit. The primer sequences used were as follows:

Genes	Oligonucleotide primers
Glut4	Forward 5′-GTGCCTATGTATGTGGGAGAAA-3′
	Reverse 5′-TCGTGTGGCAAGATGTGTAT-3'
IRS-1	Forward 5'- GAGTTGAGTTGGGCAGAGTAG-3'
	Reverse 5'- CATGTAATCACCACGGCTATTTG-3'
PI3K	Forward 5'- GTGGACAAAGCAGAAGCATTAC-3'
	Reverse 5'- ACCCTGTGTTCTTTGTCTAGTG-3'
Akt	Forward 5′-GAGCTGTGAACTCCTCATCAA-3′
	Reverse 5′-TCTCCATAGTCCTCTGGGTAAG-3′
ppia	forward 5′-CAAAGTTCCAAAGACAGCAGAAA-3′
	reverse 5′-CTGTGAAAGGAGGAACCCTTATAG-3′

Quantification was performed using a real-time PCR system (Bio-Rad, Hercules, CA, USA) with SYBR green. The PCR conditions for cycling parameters were as follows: initial denaturation at 95°C for 10-15 sec, followed by 40 cycles of amplification with denaturation at 95°C for 10-15 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec. The samples were normalized to the expression level of house keeping gene - ppia, and the results were expressed as the fold changes relative to the treated group using the $2^{-\Delta\Delta CT}$ method. All analysis were carried out in triplicates.

4.2.11. Western Blot Analysis

Expression level of proteins namely, IRS-1, PI3K, PDK-1, p-Akt, Glut-4 were evaluated by Western blotting. L6 myotubes were treated with different fractions of *Syzygium cumini* seeds. After incubation, the cells were lysed in ice-cold lysis buffer (50 mM Tris-HCl, 150 mM sodium chloride, 1.0% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% Triton X-100 and protease inhibitor cocktail, pH 8.0) for 30 min on ice and were centrifuged at 12000 x g for 10 min. The protein content was then measured using BCA protein assay kit. The lysates (40µg) were subjected to SDS-PAGE on 10% gel and transferred on to a poly vinylidene di fluoride (PVDF, Immobilon P™, Millipore®, USA) membrane by using Trans-Blot Turbo™ (Bio-Rad). The membranes were blocked by incubating in blocking buffer (5% skim milk in PBST, PBST-PBS buffer containing 0.1% Tween 20), for 1h at room temperature, washed three times with PBST and probed over night at 4°C with appropriate phospho-specific or pan-specific antibodies against IRS-1, PI3K, PDK-1, p-Akt, Glut-4 (each at 1: 500). Membranes were washed 3 times and incubated for 1 h at room temperature with horse radish peroxidase (HRP) conjugated secondary antibody at 1:1000 dilution and again washed three times in PBST. The bound antibodies were detected using an enhanced chemiluminescence substrate (Biorad, USA) and measured

by densitometry using a Chemi Doc XRS digital imaging system and the Multi Analyst software from Bio-Rad Laboratories (USA).

4.2.12. Statistical analysis

All experimental results were expressed as mean ± SD (standard deviation) of three different experiments. Data were subjected to one-way analysis of variance (ANOVA), and Duncan's multiple range tests were used to test the significant differences. Data are presented as mean ± SD, and $p \leq 0.05$ was considered to be significant. All statistical analyses were performed with SPSS 11.0 (Statistical Program for Social Sciences, SPSS Corporation, Chicago, IL) for Windows.

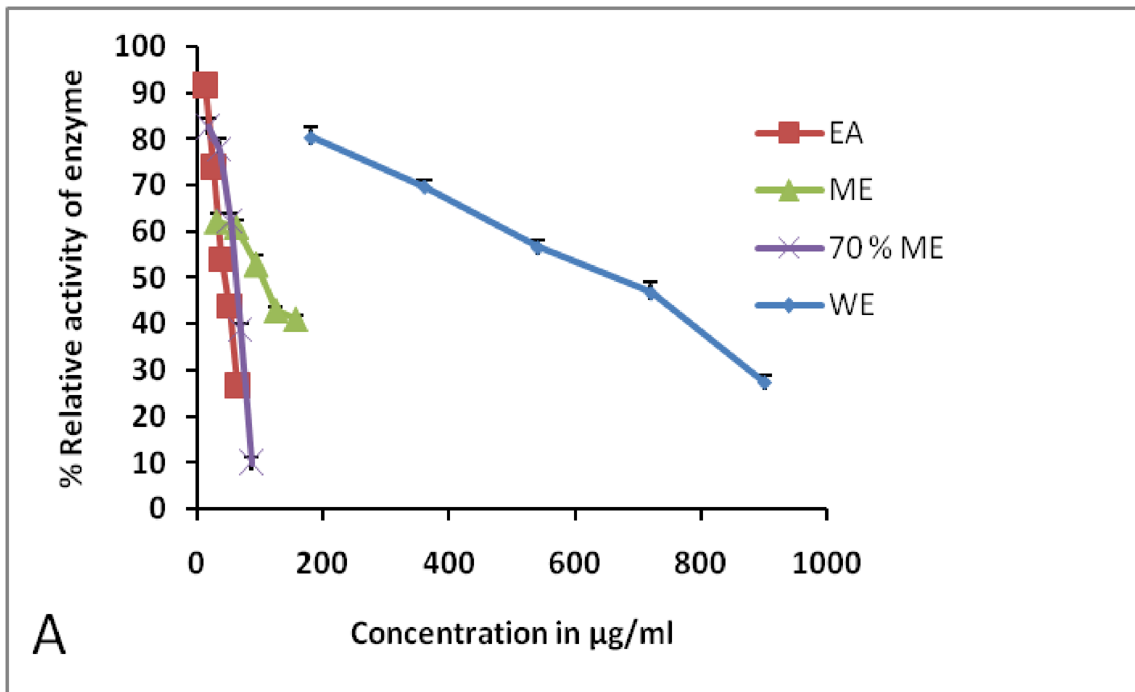
4.3. RESULTS

4.3.1. α-amylase inhibition

The α-amylase inhibitory potential of all the fractions except, aqueous fraction, were highly significant and was comparable with the positive control, acarbose (IC_{50}-124.5 ± 6.7 µg/mL) (Fig. 4.2A). The α-amylase inhibitory potential was in the order: ethyl acetate fraction (IC_{50}-43.2 ± 1.2 µg/mL; corresponding to 27.7 µg of TPC) > 70% methanol fraction (IC_{50}-60 ± 1.8 µg/mL; corresponding to 54.3 µg of TPC) > methanol fraction (IC_{50}-101.2 ± 2.6 µg/mL; corresponding to 76.6 µg of TPC) > aqueous fraction (IC_{50}-658.3 ± 13.43 µg/mL; corresponding to 166.7 µg of TPC) (Fig. 4.2B).

Molecular docking studies

To determine the putative binding mode and the potential ligand-target interactions, the predominant polyphenols which were present in *Syzygium cumini* seed fractions as reported in Chapter 2 (2.3.8) were docked with α-amylase using Autodock 1.4.6. Autodock estimated the free binding energy of quercetin (-6.96 kcal/mol), cinnamic acid (-4.88 kcal/mol), syringic acid (-4.85 kcal/mol), ferulic acid (-4.09 kcal/mol), ellagic acid (-8.14 kcal/mol), gallic acid (-4.36 kcal/mol), positive



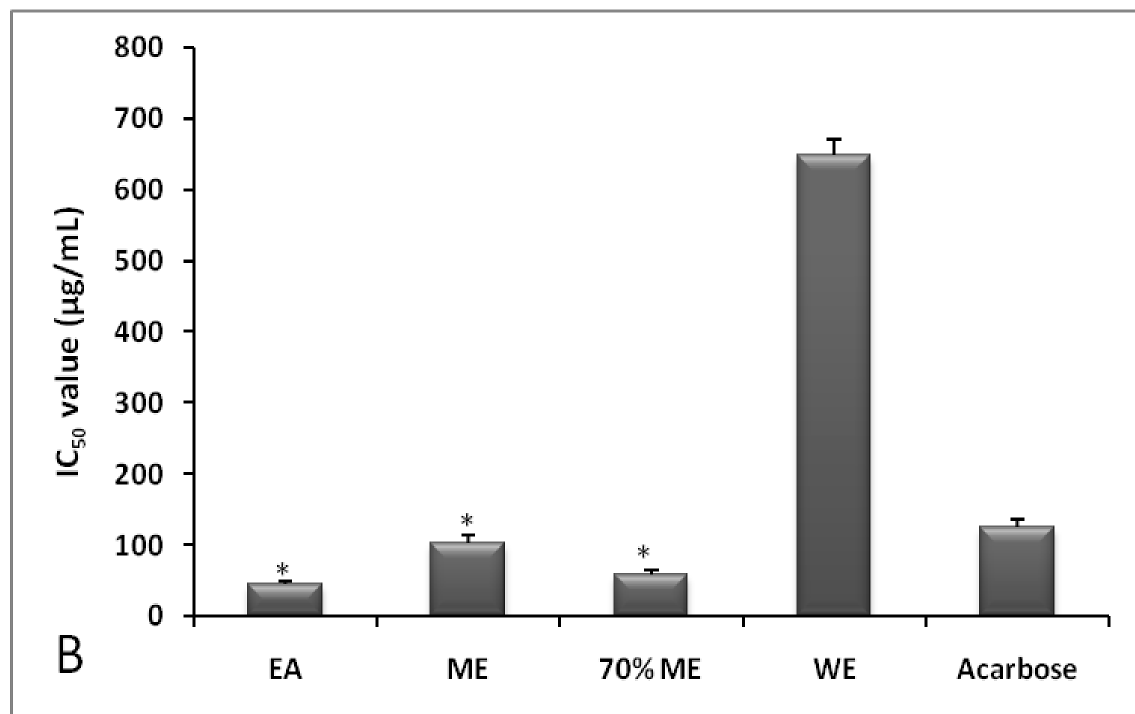


Fig. 4.2: α-amylase inhibition potential of Syzygium cumini seed

A) Graph showing percentage relative activity of α-amylase inhibitory potential of different fractions of *Syzygium cumini* seed. (EA – ethyl acetate, ME – methanol fraction, 70% ME – 70% methanol fraction, WE – aqueous fraction). B) Graph showing the IC_{50} value of the α-amylase inhibitory potential of different fractions of *Syzygium cumini* seed. Each value represents mean ± SD (standard deviation) of triplicate measurements (n=3) and the significance accepted at P≤0.05; * P≤0.05 versus acarbose.

control (acarbose) (-10.84 kcal/mol) showing moderate binding affinity into the binding site (Table 4.1). The best docking pose of polyphenols as obtained from Autodock has been represented in Fig. 4.3.

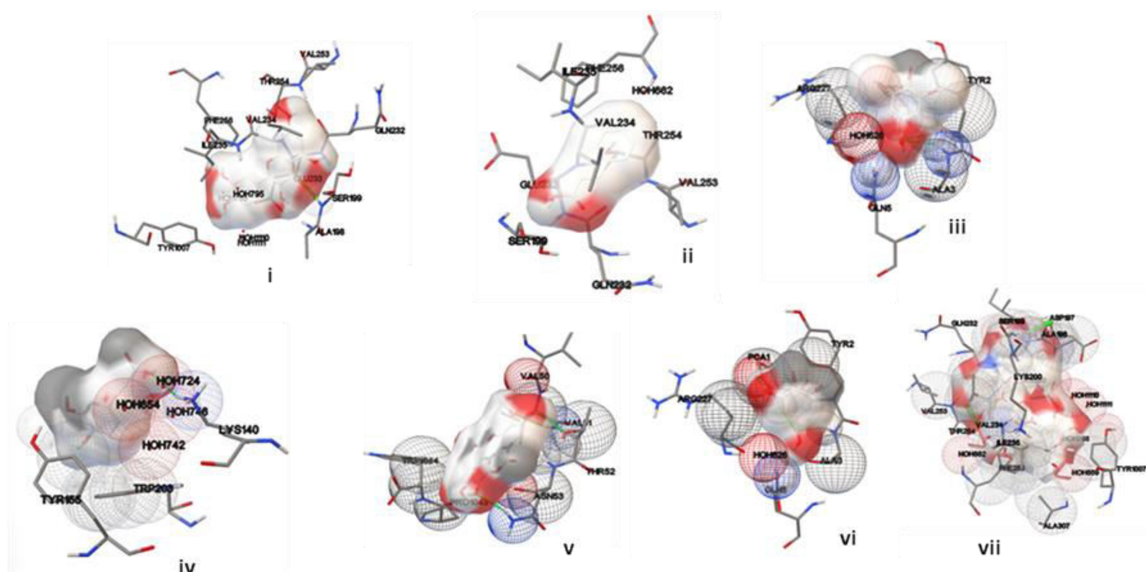


Fig. 4.3: Docking of phenolics with α-amylase enzyme
Non-covalent interaction with residues of α-amylase which may reflect the α-amylase inhibitory activities of phenolics. i) quercetin, ii) cinnamic acid, iii) syringic acid, iv) ferulic acid, v) ellagic acid, vi) gallic acid and vii) acarbose.

Table 4.1: Free binding energy (kcal/mol) of major phenolics with α-amylase

	Qtn	CA	SA	FA	EA	GA	Positive control
α-amylase	-6.96	-4.88	-4.85	-4.09	-8.14	-4.36	-10.86

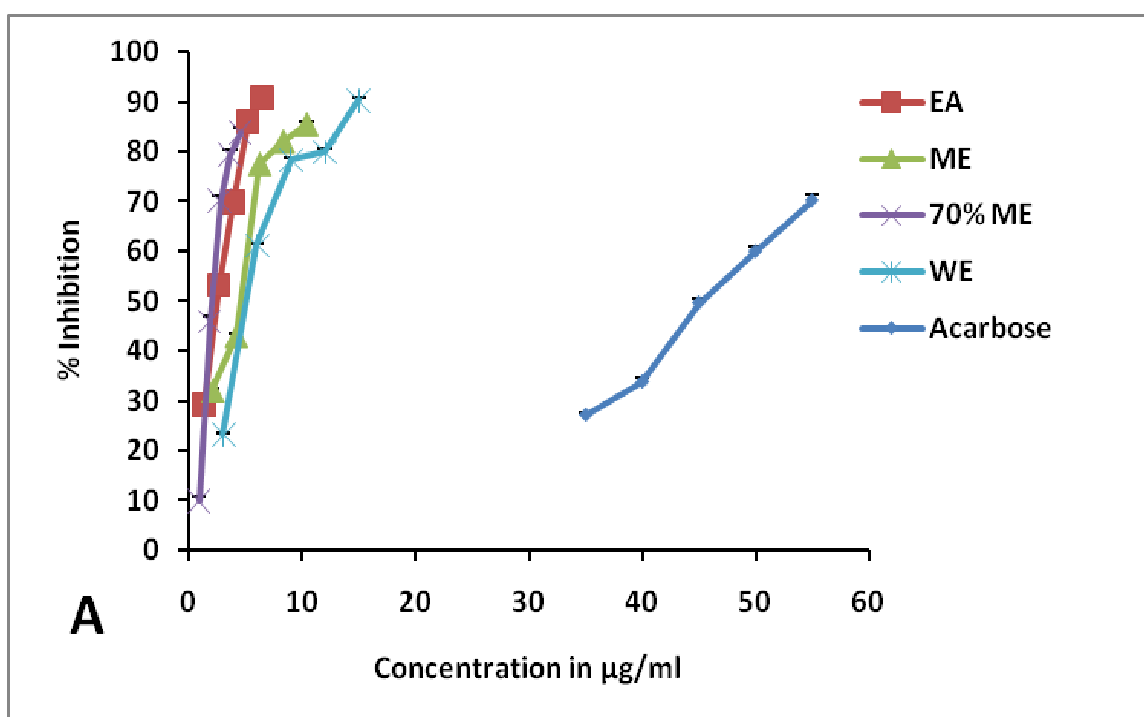
Qtn – Quercetin, CA – Cinnamic acid, SA – Syringic acid, FA – Ferulic acid, EA – Ellagic acid, GA – Gallic acid, Acarbose (Positive control for α-amylase and α-glucosidase) Diprotin A (Positive control for DPP-IV inhibition).

The results demonstrated that except syringic acid, all docked polyphenols showed hydrogen bonding between polyphenols and α-amylase. The lower binding energy of these polyphenols (quercetin, cinnamic acid, and ferulic acid) with α-amylase

may be responsible for the inhibitory potential of ethyl acetate fraction among all the fractions (Fig. 4.3).

4.3.2. α-glucosidase inhibition

The α-glucosidase inhibitory potential of all the *Syzygium cumini* seed fractions were analysed. Except hexane fraction, all the other fractions demonstrated significant α-glucosidase inhibitory potential with IC_{50} values ranging between 1.6–4.2 µg/mL (Fig. 4.4A). The activity of these fractions was highly significant as compared with the positive control, acarbose (44.2 ± 1.1 µg/mL). The α-glucosidase inhibitory potential was found to be in the following order: 70% methanol fraction (IC_{50}-1.9 ± 0.07 µg/mL) > ethyl acetate fraction (IC_{50}-2.3 ± 0.12 µg/mL) > methanol fraction (IC_{50}-4.7 ± 0.6 µg/mL) > aqueous fraction (IC_{50}-4.2 ± 0.97 µg/mL) (Fig. 4.4B). The 70% methanol fraction, which demonstrated highest α-glucosidase inhibitory potential, was high in polyphenols, such as ellagic acid, gallic acid, and quercetin.



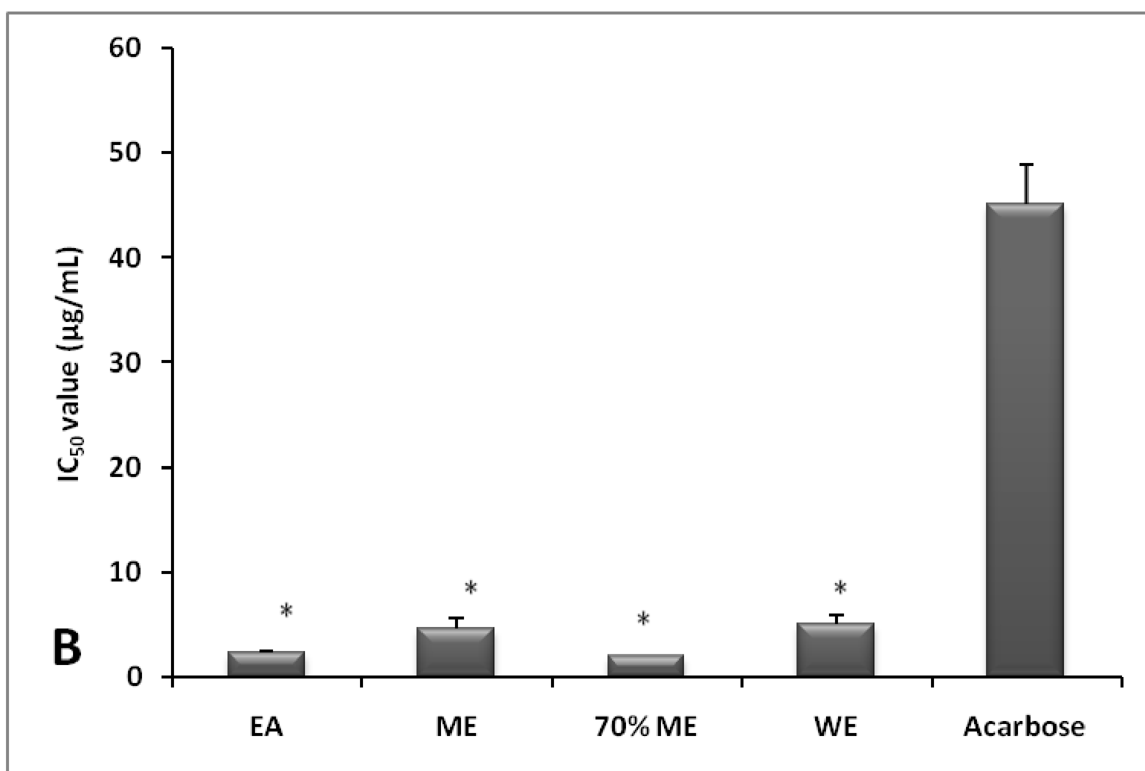


Fig. 4.4: α-glucosidase inhibition potential of Syzygium cumini

A) Graph showing percentage inhibition of α-glucosidase inhibitory potential of different fractions of *Syzygium cumini* seed. (EA – ethyl acetate, ME – methanol fraction, 70% ME – 70% methanol fraction, WE – aqueous fraction). B) Graph showing the IC_{50} value of the α-glucosidase inhibitory potential of different fractions of *Syzygium cumini* seed. Each value represents mean ± SD (standard deviation) of triplicate measurements (n=3) and the significance accepted at P≤0.05; * P≤0.05 versus acarbose.

Molecular docking studies

The predominant polyphenols were docked with α-glucosidase using Autodock software. The lesser binding energies of polyphenols (ellagic acid -8.48 kcal/mol, quercetin -4.7 kcal/mol, gallic acid –4.77 kcal/mol) with α- glucosidase clearly explains the higher inhibitory potential demonstrated by ethyl acetate and 70% methanol fractions (Table 4.2). The best docking pose of the polyphenols has been represented in Fig. 4.5. All docked polyphenols demonstrated intramolecular hydrogen bonding between the ligand and target.

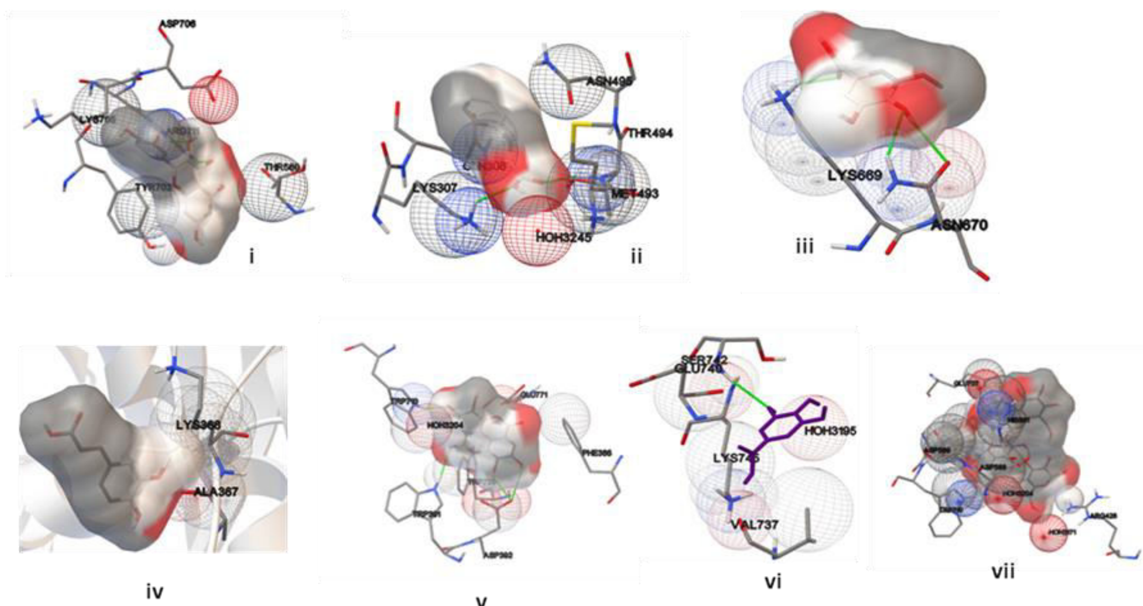


Fig. 4.5: Docking of phenolics with α-glucosidase enzyme
Non-covalent interaction with residues of α-glucosidase which may reflect the α-amylase inhibitory activities of phenolics. i) quercetin, ii) cinnamic acid, iii) syringic acid, iv) ferulic acid, v) ellagic acid, vi) gallic acid and vii) acarbose

Table 4.2: Free binding energy (kcal/mol) of major phenolics with α-glucosidase

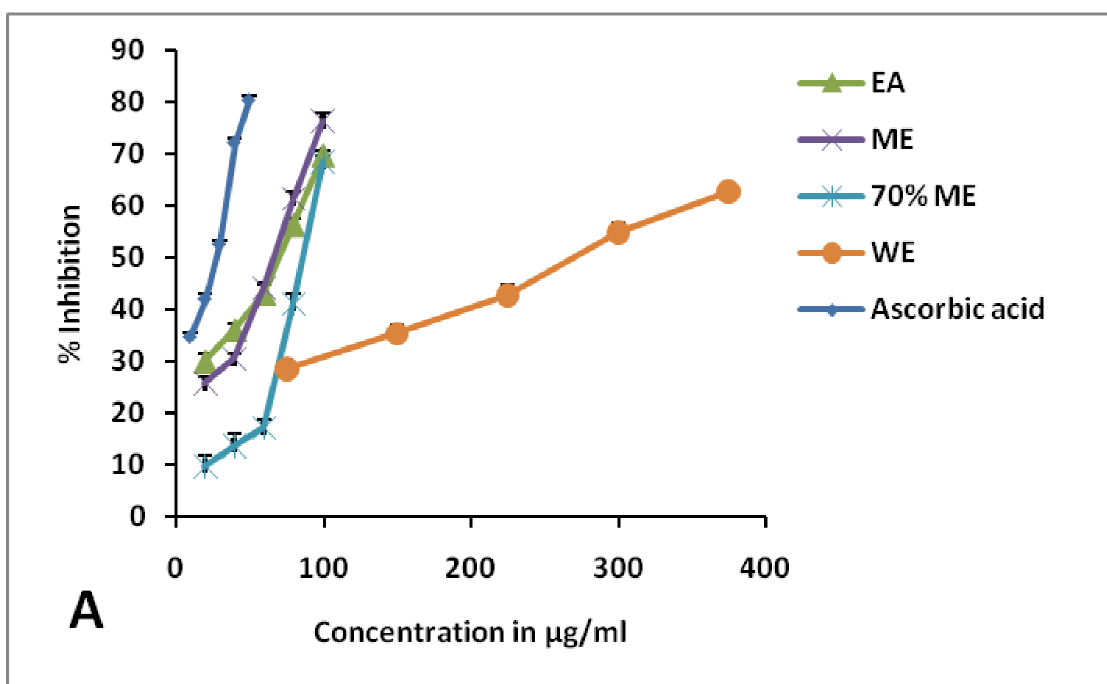
	Qtn	CA	SA	FA	EA	GA	Positive control
α-glucosidase	-4.7	-4.4	-3.58	-4.03	-8.48	-4.77	-13.82

Qtn – Quercetin, CA – Cinnamic acid, SA – Syringic acid, FA – Ferulic acid, EA – Ellagic acid, GA – Gallic acid, Acarbose (Positive control for α-amylase and α-glucosidase) Diprotin A (Positive control for DPP-IV inhibition).

4.3.3. Antiglycation

The positive control, ascorbic acid, showed an IC_{50} value of 48.5 µg/mL. When compared with the positive control, all fractions exhibited significant antiglycating property (Fig. 4.6) which may be due to the presence of phenolic compounds, such as, gallic acid, ellagic acid, quercetin and chlorogenic acid. The antiglycating potential of

Syzygium cumini seed fractions increases in the following order: water fraction < 70% methanol fraction < methanol fraction < ethyl acetate fraction.



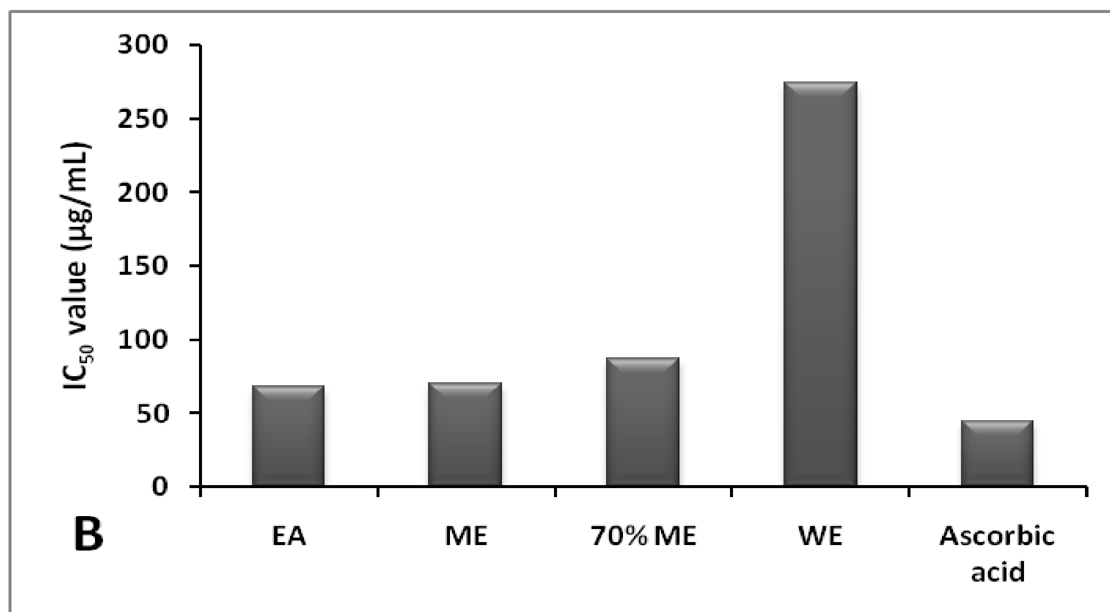


Fig. 4.6: Antiglycation activity of Syzygium cumini seed

A) Graph showing percentage antiglycation potential of different fractions of *Syzygium cumini.* (EA – ethyl acetate, ME – methanol fraction, 70% ME – 70% methanol fraction, WE – aqueous fraction). B) Graph showing the IC_{50} value of antiglycation potential of different fractions of *Syzygium cumini.* Each value represents mean ± SD (standard deviation) of triplicate measurements (n=3).

4.3.4. DPP-IV inhibition

In the present study, all the fractions, except aqueous fraction, of *Syzygium cumini* seeds at a concentration of 10 µg exhibited marked DPP-IV inhibitory potential compared with positive control, Diprotin A (42.8 ± 2.3 %) (Fig. 4.7). Among the fractions, methanol fraction demonstrated the highest DPP-IV inhibition activity (88.1± 3.1%). Methanol fraction exhibited more than two fold increase in DPP-IV inhibition potential when compared with positive control diprotin A. Ethyl acetate, and methanol fractions demonstrated 73.46% and 68.81% increase in DPP-IV inhibition when compared with positive control.

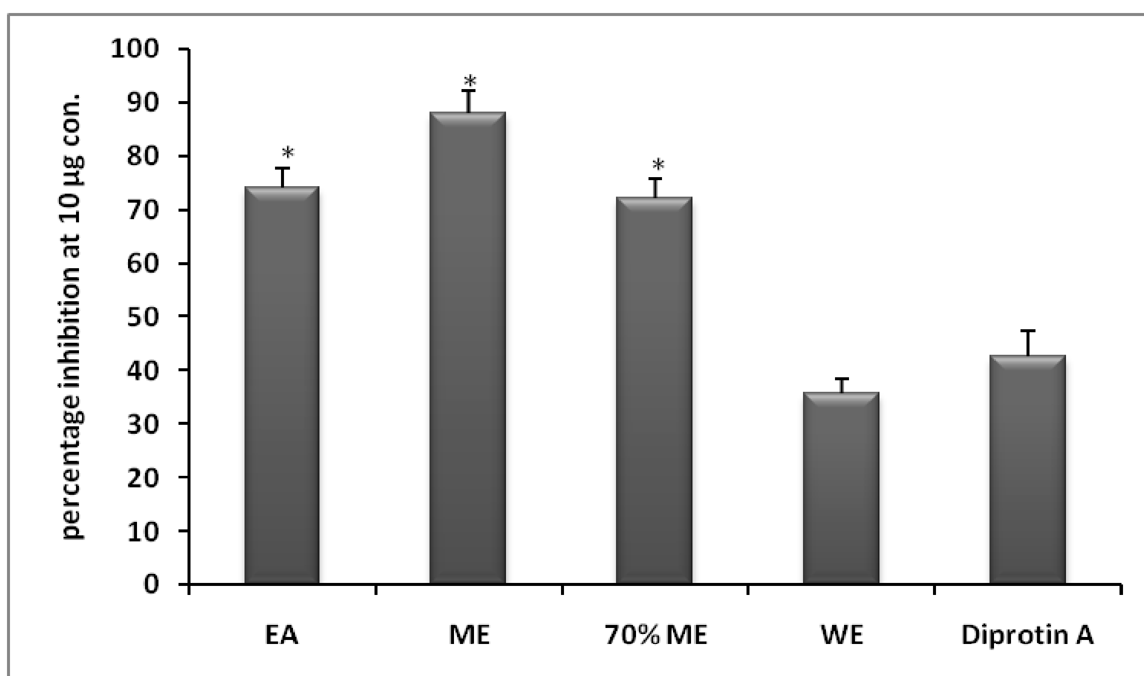


Fig. 4.7: DPP–IV inhibitory potential of Syzygium cumini seed
Bar diagram showing percentage inhibition of *Syzygium cumini* fractions at 10 µg concentration. EA – ethyl acetate, ME – methanol fraction, 70% ME – 70% methanol fraction, WE – aqueous fraction. Each value represents mean ± SD (standard deviation) of triplicate measurements (n=3) and the significance accepted at P≤0.05; * P≤0.05 versus diprotin A.

Molecular docking studies

The ligand-target interactions of polyphenols with DPP-IV were studied using Autodock (Fig. 4.8). The binding energies obtained by computational docking studies were compared among the polyphenols identified (Table 4.3). Hydrogen bonds formed between polyphenols and DPP - IV enzyme are shown in green lines in Fig. 4.8. The lower binding energy of DPP-IV with ellagic acid (-7.73 kcal/mol) and quercetin (-6.49 kcal/mol) supports higher DPP-IV inhibitory potential of methanol, ethyl acetate, and 70% methanol fractions.

Fig. 4.8: Docking of phenolics with Dipeptidyl peptidase-IV enzyme
Non-covalent interaction with residues of Dipeptidyl peptidase-IV enzyme which may reflect the DPP-IV inhibitory activities of phenolics. i) quercetin, ii) cinnamic acid, iii) syringic acid, iv) ferulic acid, v) ellagic acid, vi) gallic acid and vii) acarbose

Table 4.3: Free binding energy (kcal/mol) of major phenolics with dipeptidyl peptidase-IV proteins

	Qtn	CA	SA	FA	EA	GA	Positive control
Dipeptidyl peptidase-IV	-6.49	-4.54	-4.05	-4.26	-7.73	-3.62	-8.23

Qtn – Quercetin, CA – Cinnamic acid, SA – Syringic acid, FA – Ferulic acid, EA – Ellagic acid, GA – Gallic acid, Acarbose (Positive control for α-amylase and α-glucosidase) Diprotin A (Positive control for DPP-IV inhibition).

4.3.5. MTT Assay

The cytotoxicity of *Syzygium cumini* seed fractions was determined in L6 myoblast cells by MTT assay at different concentrations. All the fractions exhibited less than 20% toxicity at a concentration 10 µg (Fig. 4.9), and thus the concentration of fractions up to 10 µg were used for glucose uptake studies.

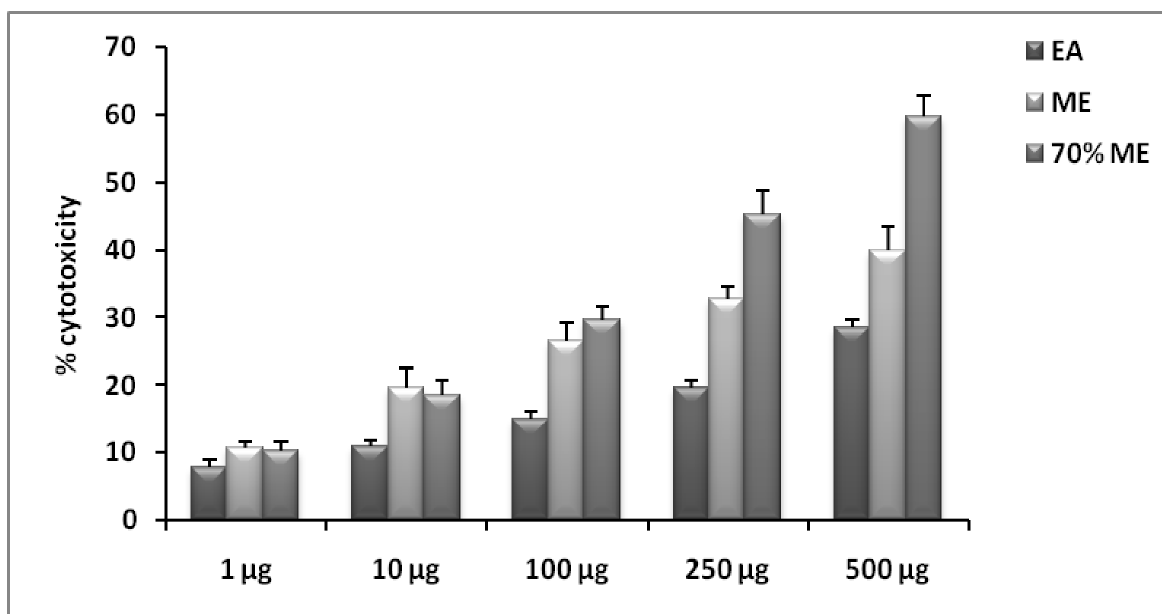


Fig. 4.9: Cytotoxicity of fractions in cultured L6 myoblasts
Effect of fractions on cell viability was standardized based on concentration. Each value represents mean ± SD (standard deviation) from triplicate measurements (n = 3) of three different experiments. EA – ethyl acetate, ME – methanol fraction, 70% ME – 70% methanol fraction.

4.3.6. Fluorescence analysis of 2-NBDG

Monitoring the level of glucose uptake ability in cells play a crucial role in understanding the efficacy of *Syzygium cumini* seed in the management of diabetes mellitus. Antidiabetic potential of different fractions of *Syzygium cumini* seed was evaluated in L6 myotubes with a fluorescent D-glucose analog 2-NBDG. The uptake of 2-NBDG in L6 myotubes after pre-treatment with fractions was monitored using confocal microscopy by detecting the fluorescence within the cells. An increased intracellular fluorescence indicating increased glucose uptake by the cells were observed

in different groups of cells pre-treated with different fractions of *Syzygium cumini* seed (Fig 4.10). At 24 h pre-treatment with 70% methanol fraction (10 µg), the glucose uptake in L6 myotubes remarkably increased (Fig 4.10E) when compared with positive control, Rosiglitazone (Fig 4.10B). Ethyl acetate (Fig 4.10C) and methanol fractions (Fig 4.10D) also showed an increase in glucose uptake when compared with control cells.

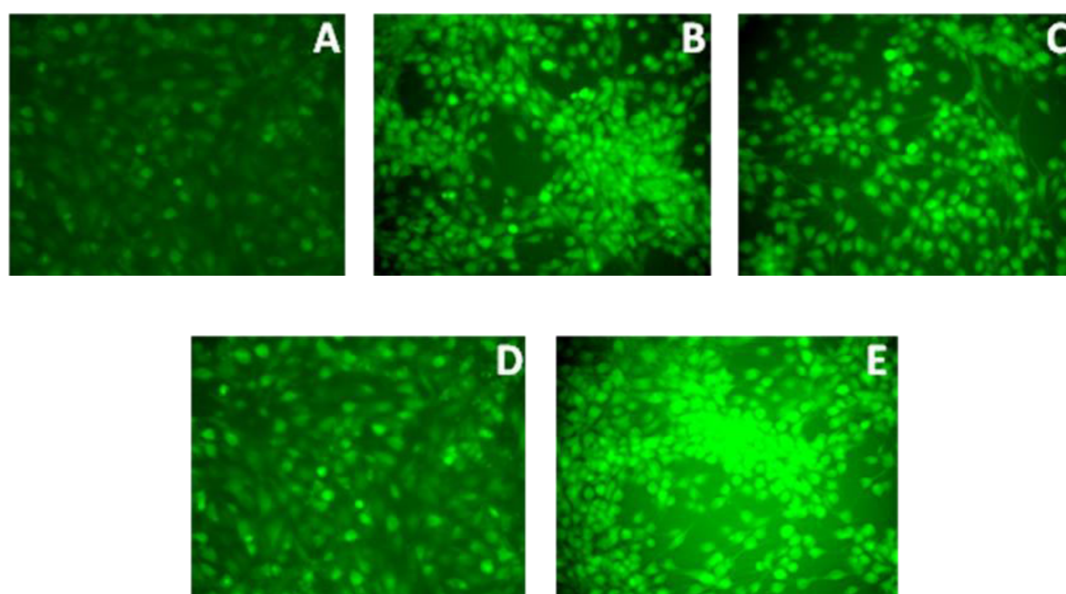


Fig. 4. 10: 2-NBDG uptake of Syzygium cumini seed fractions in L6 myotubes
Intracellular 2-NBDG uptake in L6 myotubes on pretreatment with *Syzygium cumini* seed fractions. (A) Control (B) Rosiglitazone (C) Ethyl acetate fraction (D) Methanol fraction (E) 70% methanol fraction

4.3.7. Fluorescence analysis of 2-NBDG uptake by flow cytometry

The ability to stimulate glucose uptake in the L6 myotubes pretreated with fractions were also quantified using 2-NBDG through flow cytometric analysis. Flow cytometric analysis revealed the uptake of 4.7% and 22.5% uptake of 2-NBDG in control and rosiglitazone treated cells (Fig. 4.11). After pretreatment of L6 myotubes with 70% methanol fraction, a four-fold increase in glucose uptake (26.9%) was observed (Fig 4.11E) when compared with control (4.7%) and was comparable to the positive control,

rosiglitazone (22.5%). Ethyl acetate (Fig 4.11C) and methanol fractions (Fig 4.11D) showed more than two-fold increase in glucose uptake compared to the control.

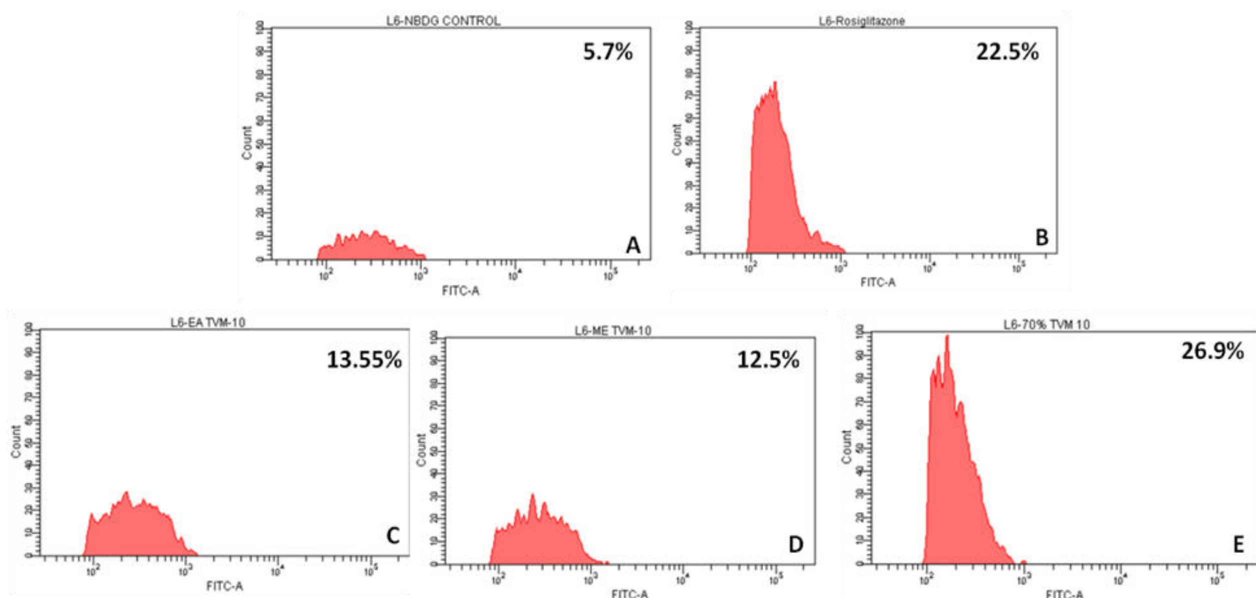


Fig. 4.11: Flow cytometry analysis of 2-NBDG uptake in L6 myoblasts
FACS analysis of 2-NBDG uptake in differentiated L6 cells by plotting cell count against FITC. (A) Control, (B) Rosiglitazone, (C) Ethyl acetate fraction, (D) Methanol fraction and (E) 70% methanol fraction

4.3.8. Effect on protein tyrosine phosphatase 1B (PTP1B) activity

PTP1B reduces insulin receptor tyrosine kinase (IRTK) activity by dephosphorylating specific phosphotyrosine residues. Therefore inhibition of PTP-1B is considered as an important target in the treatment of type II diabetes. All fractions of *Syzygium cumini* seeds (10 µg) were tested for their PTP1B inhibition potential. Ethyl acetate, methanol and 70% methanol fractions exhibited 68.28 ± 3.3, 72.42 ± 4.2% and 82.43 ± 4.8% inhibition which was higher than that of positive control (Sodium orthovanadate) (54.81 ± 3.98%) as shown in Fig 4.12.

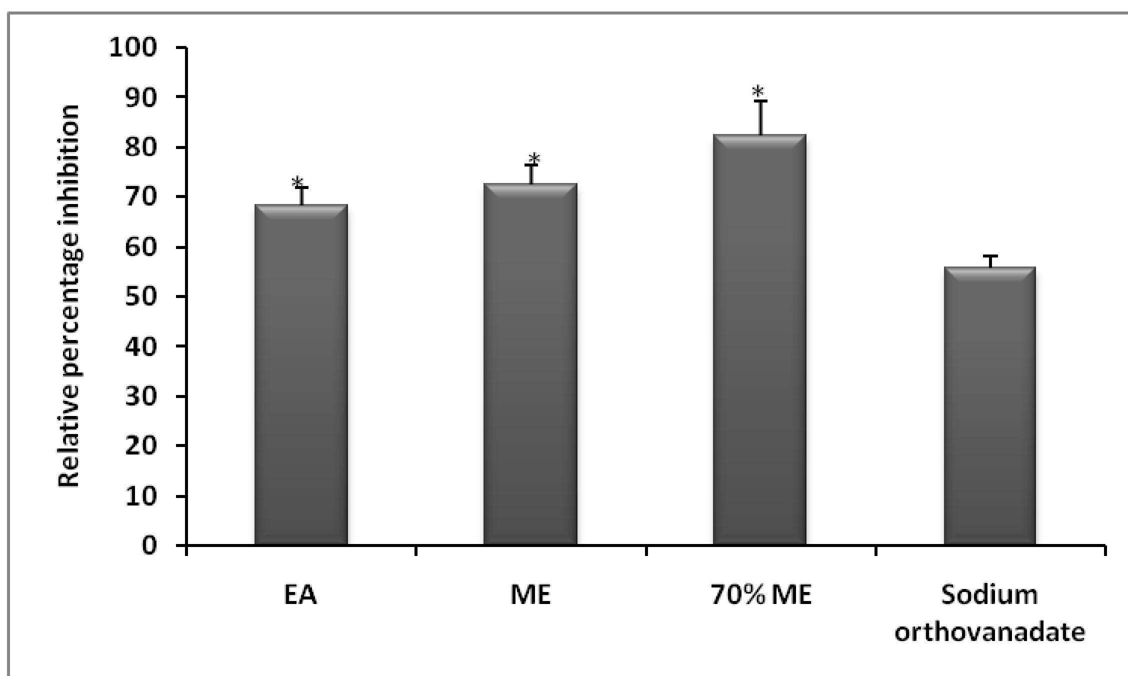


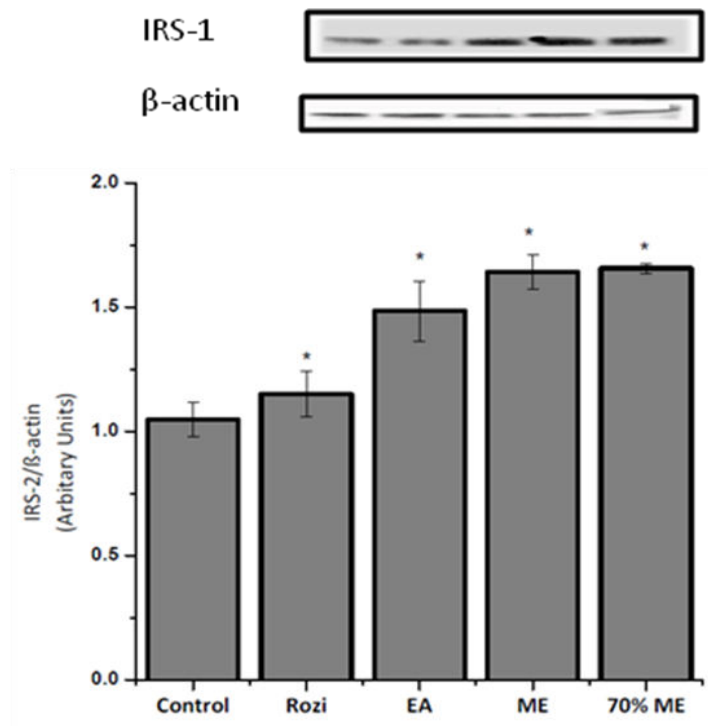
Fig. 4.12. Effect of Syzygium cumini seed fractions on PTP1B activity
Ethyl acetate, methanol, and 70% methanol fractions(10 µg) exhibited higher percentage inhibition than that of positive control (Roziglitazone). Each value represents mean ± SD (standard deviation) from triplicate measurements (n=3) of three different experiments. Significance test between different groups were determined by using one way ANOVA followed by Duncan's multiple range test and the significance accepted at P≤0.04. *P≤0.05 verses sodium orthovanadate.

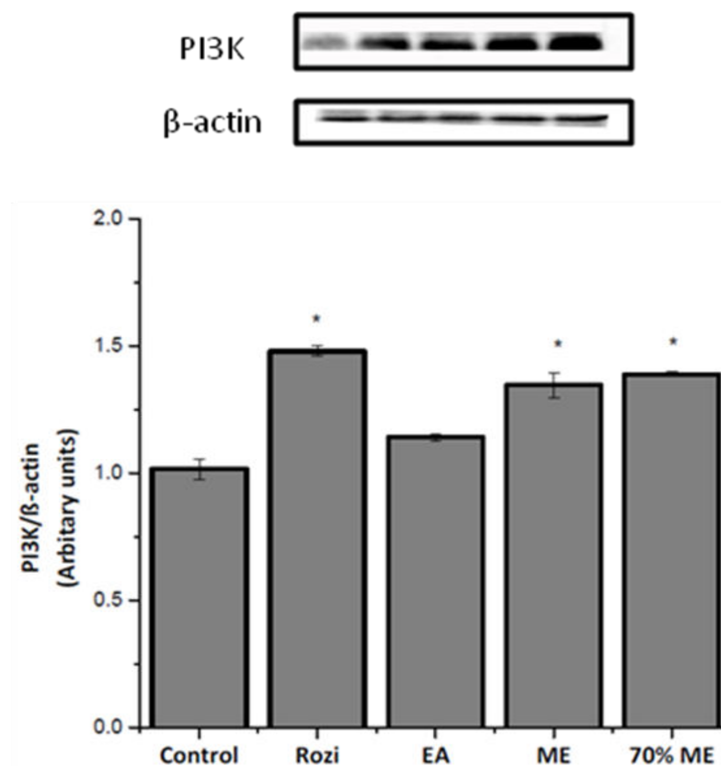
4.3.9. Western blot analysis

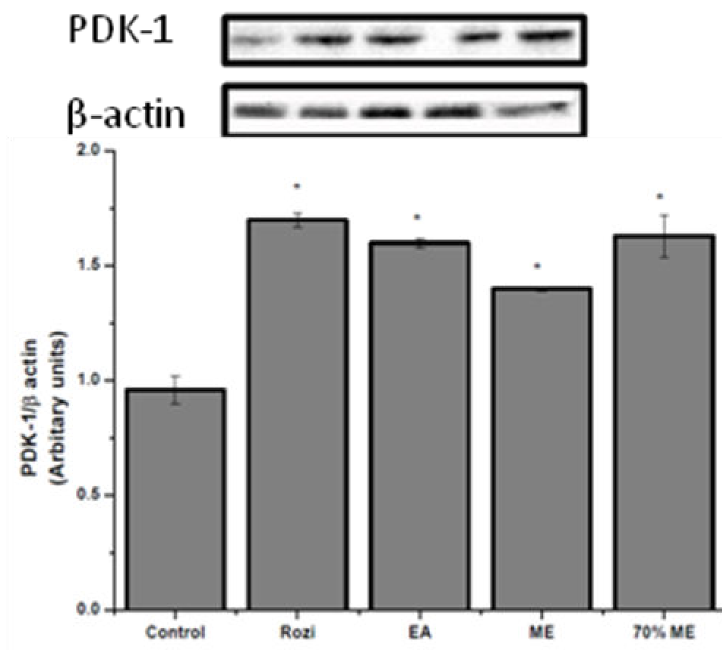
The results obtained from various experiments proved that *Syzygium cumini* seed fractions exhibited significant antidiabetic potential. Inorder to understand the mechanism of action of *Syzygium cumini* seed fractions, the expression of some of the key proteins involved in insulin signaling were investigated after pre-treating L6 cells with *Syzygium cumini* seed fractions (10 µg) for 24 h.

Various proteins involved in insulin signaling like IRS-1, PI3K, PDK-1, Akt, p-Akt, and Glut-4 were analyzed. Insulin-receptor substrate 1 (IRS-1) is an important substrate of the receptor tyrosine kinase for insulin and insulin-like growth factor 1 (LeRoith *et al*., 1995). Understanding the intricacies of PI3K pathway may provide new avenues for therapeutic intervention in diabetes and cancer (Cantley at al., 2002). The

results indicate that *Syzygium cumini* seed fractions especially ethyl acetate, methanol and 70% methanol fractions significantly upregulated the level of IRS-1, PI3K, PDK-1, Akt, p-Akt and Glut-4 proteins (Fig 4.13). The presence of phenolic acid and flavonoids in *Syzygium cumini* increased the expression of these proteins.






PDK-1
β-actin
PDK-1/β actin
(Arbitary units)
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1.5
1.0
0.5
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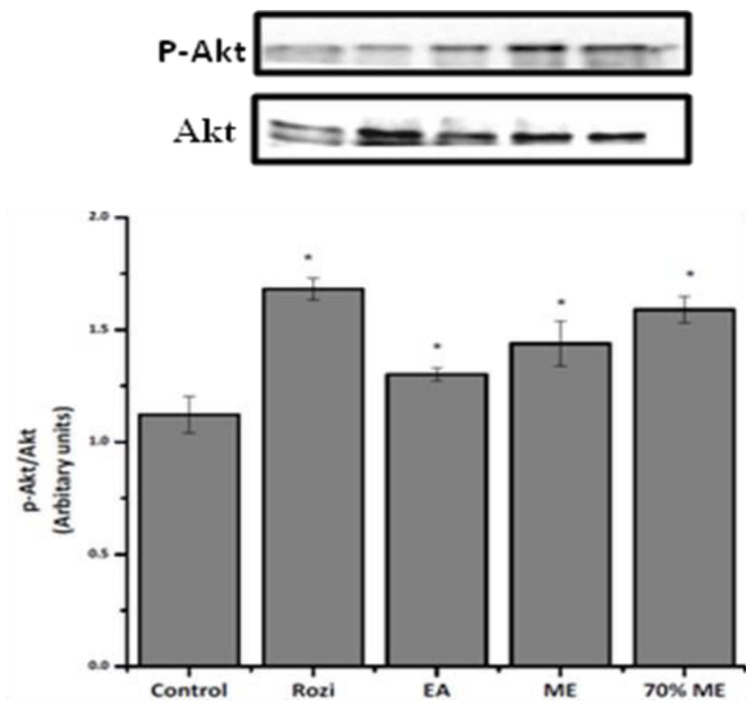
Control
Rozi
EA
ME
70% ME


P-Akt
Akt
p-Akt/Akt
(Arbitary units)
2.0
1.5
1.0
0.5
0.0
Control
Rozi
EA
ME
70% ME

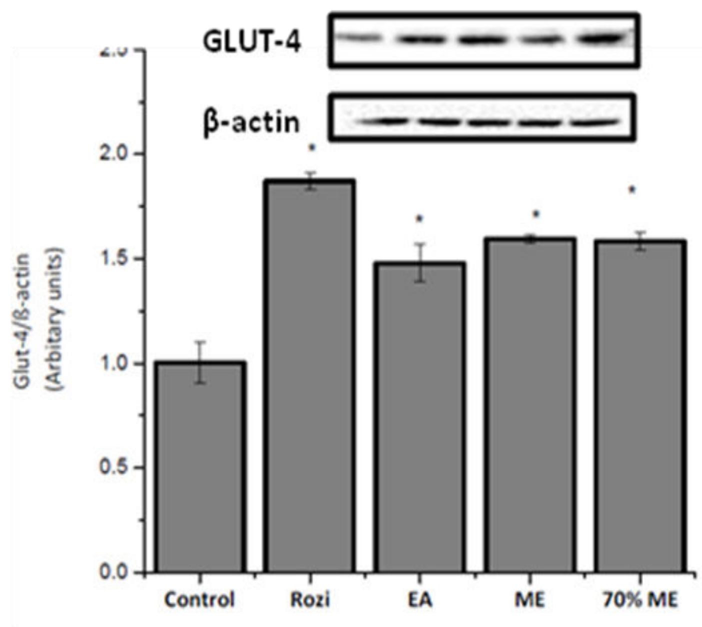
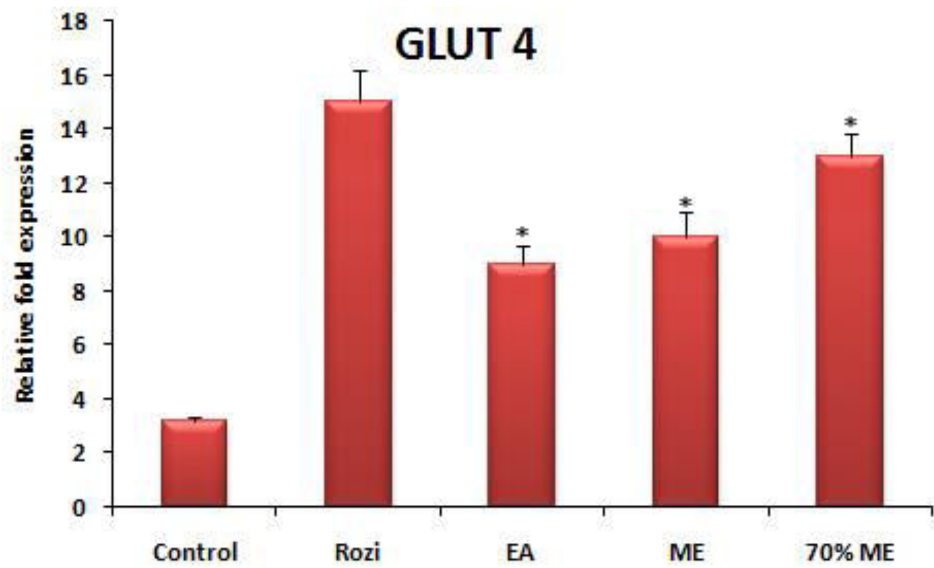
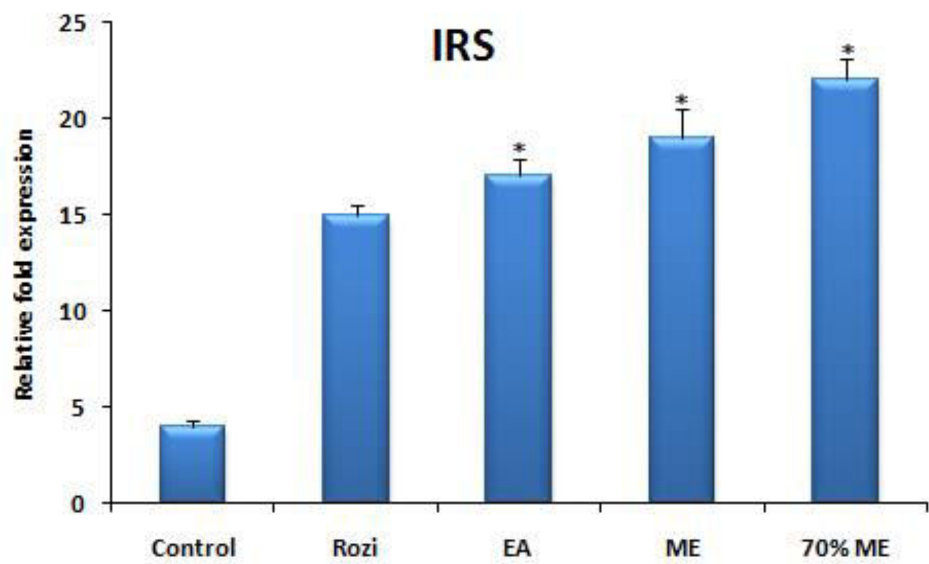


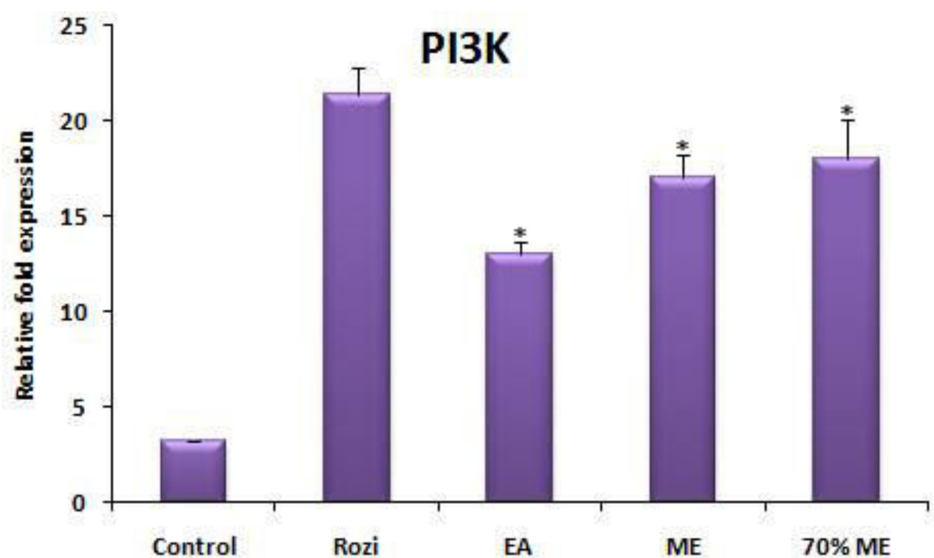
Fig. 4.13: IRS-1, PI3K, PDK-1, p-Akt, Glut-4 expression in L6 myotubes on pretreatment.
The effect of *Syzygium cumini* seed fractions on pre-treatment for 24h was comparable with that of the positive control rosiglitazone (100 nM).

4.3.10. Quantitative real time PCR

Inorder to confirm the role of *Syzygium cumini* seeds in glucose transport in L6 myotubes, mRNA levels of GLUT- 4 was examined. Pre-treatment with 70% methanol, methanol and ethyl acetate fractions at a concentration of 10 µg significantly upregulated the mRNA levels of GLUT- 4 when compared with control cells. To further elucidate the signaling pathway, the mRNA levels of IRS-1, PI3K, and PDK-1in L6 myotubes were analyzed. The results showed that ethyl acetate, methanol, and 70% methanol fractions significantly increased the level of IRS-1 and PDK-1(Fig 4.14) when compared with the positive control, roziglitazone. The results thus showed that *Syzygium cumini* seed fractions stimulated glucose uptake and induced GLUT-4 upregulation by activating insulin dependent pathway.


GLUT 4
Relative fold expression
18
16
14
12
10
8
6
4
2
0
*
*
*
Control
Rozi
EA
ME
70% ME


IRS
Relative fold expression
25
20
15
10
5
0
*
*
*
Control
Rozi
EA
ME
70% ME


PI3K
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20
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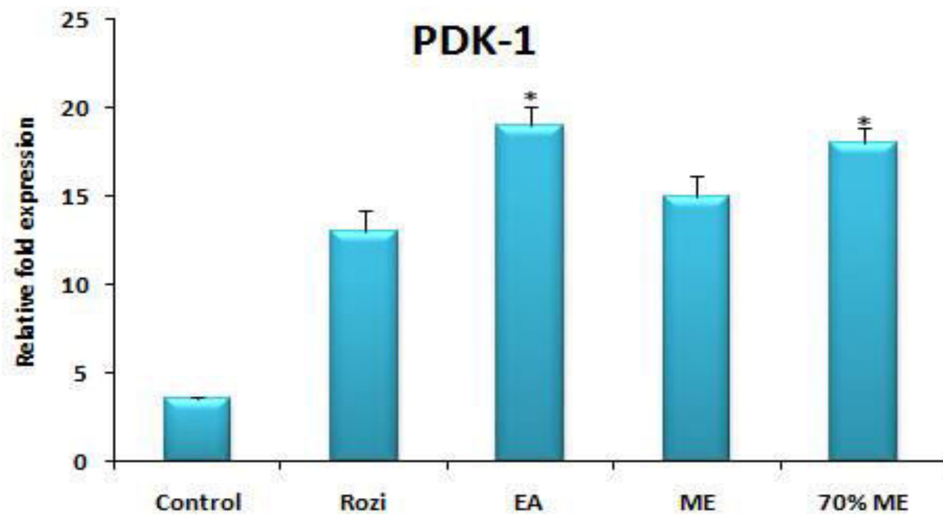

Fig. 4.14: Bar graphs show the mRNA levels (mean ±SE) of GLUT-4, IRS-1, PI3K & PDK-1 in L6 myotubes on pre-treatment of Syzygium cumini seed fractions.
The effect of *Syzygium cumini* seed fractions on pretreatment for 24h was comparable with that of the positive control rosiglitazone (100 nM).

4.4. DISCUSSION

Phenolic acids and flavonoids constitute one of the most pervasive groups of plant phenolics. These compounds play positive roles in maintaining blood glucose levels, glucose uptake, and insulin secretion and modulating immune function to prevent diabetes mellitus. This paves the way to search for more natural products for the prevention and management of diabetes.

Carbohydrate digesting enzymes like α-amylase and α-glucosidase undoubtedly plays a major role in managing type II diabetes. α-amylase breaks down long chain carbohydrates and α-glucosidase breaks down starch and disaccharides to glucose. DPP-IV inhibitors increase incretin level which in turn increases insulin secretion, decreases gastric emptying, and thereby decreases blood glucose levels. Nowadays, DPP-IV inhibitors are gaining much importance due to their effectiveness in treating diabetes when given as antidiabetic drugs. The antidiabetic potential of various fractions of *Syzygium cumini* was assessed in terms of inhibition of enzymes (α- amylase, α-glucosidase and DPP-IV), antiglycation and glucose uptake potential using L6 myotubes.

In addition, the major phenolics identified from all fractions, namely, quercetin, cinnamic acid, syringic acid, ferulic acid, ellagic acid and gallic acid were docked with predominant enzymes that are the targets in the management of diabetes, to confirm their biological potential. The compounds with lesser binding energy indicate that they have a higher affinity towards the enzyme and tend to bind to the active sites, thus making the enzyme sites unavailable for their substrates.

The enzymes, α-amylase, and α-glucosidase, are known to act synergistically in the digestion process of starch inside the human body and produce glucose. An effective strategy for the management of type II diabetes mellitus has been done through the inhibition of α-amylase and α-glucosidase enzymes (Krentz *et al*., 2005). As observed, the ethyl acetate fraction, which possessed lowest TPC, demonstrated highest α-amylase inhibitory potential among the extracts. This may be due to the presence of higher content of quercetin, gallic acid, and ellagic acid as they have been reported for their potent α-amylase inhibitory activity (Sales *et al*., 2012). The highest α-glucosidase inhibition potential was exhibited by 70% methanol fraction which possessed higher concentration of gallic acid, ellagic acid, and quercetin. These compounds have been independently reported to exhibit α-glucosidase inhibition activity (Yin *et al*., 2014).

The generation of advanced glycated end products increases with age and is known to be accelerated in diabetes. The formation of advanced glycated end products (AGEs) play an important role in the pathogenesis of diabetic complications like retinopathy, nephropathy, neuropathy, cardiomyopathy along with some other diseases such as rheumatoid arthritis, osteoporosis and aging (Singh *et al*., 2014). The most efficient way to reduce diabetic complications is to prevent the process of glycation which is possible by using compounds or agents that have antiglycation property (Siddiqui *et al*., 2014). Currently, none of the commercial drugs are available to prevent antiglycation, and

natural products can be a very good source for molecules with antiglycating properties. Hence in the present study, antiglycating efficacy of *Syzygium cumini* seed fractions were evaluated. The antiglycation potential of phenolic acids, like, quercetin and gallic acid has been reported earlier for their potent antiglycation potential whose presence in the ethyl acetate, methanol and 70% methanol fractions of *Syzygium cumini* (Wu *et al*., 2009) could have played an important role in demonstrating antiglycation efficacy in the present study. Earlier, Deve *et al*., (2014) had shown in their study that *Syzygium cumini* seeds aqueous fraction (500 mg) exhibited 78.1% inhibition in glycating property. The difference in the efficacy between the reported and the present study may be due to the presence of more bioactive polyphenols in different fractions of *Syzygium cumini* seed by sequential extraction.

DPP-IV is a serine protease that localizes on cell surfaces and plays an important role in regulating the action of glucagon-like peptide 1 (GLP-1) and a gastric inhibitory peptide which are the key enzymes responsible for maintaining glucose metabolism. DPP-IV acts as an attractive target for drug discovery research due to its strong correlation with various diseases like diabetes, obesity and tumor progression. Using natural resources, various studies have been recently reported exploring the polyphenols for the inhibition of DPP-IV (Fan et al., 2013). The results from the present study showed that methanol, 70% methanol and ethyl acetate fractions of *Syzygium cumini* seeds possessed significant DPP-IV inhibition potential. The phenolic compounds in different fractions of *Syzygium cumini* seeds may attribute to their increased DPP-IV inhibitory activity.

Antidiabetic potential of *Syzygium cumini* seeds was further confirmed using cell based studies. Insulin stimulates glucose uptake into muscle through increased glucose transporter activity, and this process involves insulin-stimulated translocation of

intracellular glucose transporters to the T-tubules or plasma membrane. Inducing glucose uptake in L6 myotubes on the treatment of extracts have a significant effect on lowering blood glucose level. In the present study, glucose uptake was significantly induced in cells pretreated with 70% methanol fraction, confirming the antidiabetic potential of *Syzygium cumini* seeds. Polyphenols, such as quercetin (Fang *et al*., 2008), ellagic acid (Paulose *et al*., 2011) and gallic acid (Vishnuprasad *et al*., 2010) have been individually reported for their glucose uptake efficiency in *in vitro* cell line studies. All these evidence strongly suggest that in the present study, the presence of these polyphenols in the fractions of *Syzygium cumini* seed might be the attributing factor for the increased glucose uptake in skeletal muscle cells.

PTP-1B is a key component in intracellular signaling and metabolism by dephosphorylating tyrosine residues. It plays a critical role in the insulin receptor (IR) signaling pathway and cellular activity. PTP1B is an effective target for the treatment of both type II diabetes and obesity. The present study showed that 70% methanol, methanol and ethyl acetate fractions of *Syzygium cumini* seeds significantly demonstrated PTP-1B inhibition potential, suggesting that they may be promising candidates for the future discovery of novel PTP1B inhibitors.

Skeletal muscle cells has been known to constitute 50 percent of the body cells. Therefore the role played by these cells in glucose uptake is highly significant for the body in maintaining glucose homeostasis. In the present study, the fractions of *Syzygium cumini* seeds had significantly demonstrated an induction in glucose uptake in the skeletal muscle cells. Inorder to understand the mechanism of the increased stimulation of glucose uptake, insulin signaling pathway protein and mRNA level expression studies were performed. The protein and mRNA level of IRS-1, PI3K, PDK-1, and GLUT-4 were increased significantly on pretreatment with *Syzygium cumini* seed fractions,

especially, 70% methanol fraction. The protein expression of p-Akt was also upregulated during pre-treatment with *Syzygium cumini* seed fractions. Except the level of PDK-1, the mRNA levels of all the key proteins (involved in insulin signaling), following *Syzygium cumini* pre-treatment, increased and were in the following order: 70% methanol > methanol > ethyl acetate fraction treated cells. The results confirmed that *Syzygium cumini* seed fractions acted by upregulating GLUT-4 expression and through insulin dependent pathway. Earlier reports have suggested that phenolic acids, like, chlorogenic acid enhance glucose uptake by increasing GLUT-4 expression whereas ferulic acid increases glucose uptake by PI3K dependent pathway (Prabhakar *et al*., 2009). The presence of these individual phenolic acids and flavonoids might be the reason for the increased glucose uptake potential of the *Syzygium cumini* seed fractions.

To summarize, ethyl acetate, methanol and 70% methanolic fractions exhibited significant antidiabetic activity in terms of inhibition of carbohydrate digesting enzymes, DPP-IV and glycation, in addition to induction of glucose uptake in skeletal muscle cells. This may be due to the richness of polyphenols present in these fractions which were identified and reported in chapter 2. The results obtained from this study indicate the potential of *Syzygium cumini* seeds in the management of diabetes mellitus.

4.5. SUMMARY

The results from the present study revealed that 70% methanol, methanol and ethyl acetate fractions of *Syzygium cumini* seeds were rich in phenolic compounds with potential antidiabetic efficacy. *Syzygium cumini* seeds can potentially inhibit α-amylase, α-glucosidase, DPP-IV enzymes and glycation. The predominant compounds present in these fractions which were reported in chapter 2, like, quercetin, cinnamic acid, syringic acid, ferulic acid, ellagic acid, and gallic acid were docked with different enzymes to

understand the affinity of phenolic compounds to these enzymes. Docking studies confirmed that these polyphenols block the substrate from binding to the enzymes. The enhancement in the glucose uptake by L6 myoblasts further confirmed the antidiabetic potential of the fractions. Protein expression studies using various different proteins involved in insulin signaling pathway further confirmed the antidiabetic potential of the fractions of *Syzygium cumini* seeds. Hence the present study demonstrated the antidiabetic efficacy of the *Syzygium cumini* seed fractions using different diabetic targets and explained their mode of action. The study provides significant evidence to use *Syzygium cumini* seeds as an important natural source of phenolic compounds and as a natural antidiabetic agent.

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CHAPTER 5

Anti-inflammatory effect of Syzygium cumini seeds on LPS treated murine macrophage RAW 264.7 cell lines through inhibiting TLR 4/ NF-κB pathway

5.1. INTRODUCTION

Inflammation and its markers act as mediators for diabetes and cardiovascular diseases. Chronic inflammation plays an important role in endothelial dysfunction, oxidative stress and insulin resistance (Adela *et al*., 2015). Glucotoxicity, lipotoxicity, and inflammation along with oxidative stress may contribute independently to both insulin resistance and endothelial dysfunction. Chronic inflammation is thus a pathogenic feature of atherosclerosis and cardiovascular disease mediated by various substances including pro-inflammatory cytokines, free fatty acids, Ang II, etc. (Maury *et al*., 2010). A new set of problems and complications arise with the advent of this chronic metabolic overload that causes endothelial dysfunction which leads to inflammation, lipoproteins oxidation, proliferation of smooth muscle, accumulation of lipid-rich material and insulin resistance. Insulin resistance and endothelial dysfunction together can contribute to the progression of cardiovascular diseases and type II diabetes (Van Gaal *et al*., 2006). In diabetic and cardiovascular disease conditions, there will be alterations in the level of cytokines, and this may be used as diagnostic biomarkers for tracking the progression of the disease.

Several synthetic drugs are globally practiced for the treatment of acute inflammatory disorders. Though their use is associated with detrimental side effects, they are ineffective in the treatment of chronic inflammatory disorders. The long-term use of these drugs may result in gastric ulcer, bleeding, cardiac abnormalities, bone marrow depression, renal dysfunction, kidney damage, bronchospasm, etc. (Hawkey *et al*., 2003). Therefore, it is a clinical requisite to identify more efficacious and safer drugs for the prevention and treatment of inflammatory diseases. In contrast to the limitations of synthetic drugs, medicinal plants have a more favorable pharmacological profile with potential therapeutic effects. Moreover, natural products are biocompatible and cost-

effective alternatives for the treatment of various inflammatory diseases. There is a great perspective to develop novel anti-inflammatory drugs through the integration of indigenous resources and traditional knowledge. The research on natural products with anti-inflammatory potential should, therefore, be viewed as a fruitful and logical research strategy, in the search for new anti-inflammatory drugs. Supporting this, several studies has been reported by various researchers regarding the role of phytochemicals against inflammation (Yoon *et al*., 2005; Rahman *et al*., 2006). The anti-inflammatory activities of phytochemicals can be attributed to their effect on modulating cellular activities of inflammation related cells including macrophages (Bellik *et al*., 2012).

Traditionally, *Syzygium cumini* has been used for treating several ailments. Different parts of *Syzygium cumini* are known to possess multiple biological activities like anti diabetic, antioxidant and cardioprotective effects (Ramya *et al*., 2012). Except a few citations, the report on anti-inflammatory activity of *Syzygium cumini* seeds is scarce and limited. Anti-inflammatory activity has been reported in water extracts of *Syzygium cumini* leaves, oil extracts of leaves and bark (Roy *et al*., 2007; Siani *et al*., 2013; Muruganandan *et al*., 2001 respectively). Kumar *et al*., (2008) reported that ethyl acetate and methanol extracts of *Syzygium cumini* seed showed anti-inflammatory activity in animal models. Despite the fact that *Syzygium cumini* bark and leaves oil were shown to have potent anti-inflammatory activities in previous research studies there is no thorough investigation for *Syzygium cumini* seed attenuation of the bacterial endotoxin lipopolysaccharide (LPS)-induced inflammatory responses for macrophages and their comprehensive mechanisms. The present study, therefore, aims to provide a molecular basis for the anti-inflammatory potential of different fractions *Syzygium cumini* seeds using LPS-stimulated RAW264.7 macrophages.

Thus, the present study focuses on anti-inflammatory effects of *Syzygium cumini* seeds through the modulation of key inflammatory mediators, effects on pro-inflammatory molecule expression like cyclooxygenase (COX), lipoxygenase (LOX) nitric oxide synthase (iNOS), tumor necrosis factor-α (TNF- α), interleukin-1β (IL-1β), interleukin-10 (IL- 10) and other cytokines. Moreover, we also monitored the signaling pathways including nuclear factor- κB (NF-κB), mitogen-activated protein kinases (MAPKs), extra cellular signal-regulated kinase (ERK), Jun N-terminus kinase (JNK), p38, to explicate the mechanism of action of *Syzygium cumini* seeds in LPS induced macrophages.

5.2. MATERIALS AND METHODS

5.2.1. Chemicals

Dulbecco's Modified Eagle's Media (DMEM), a streptomycin ampicillin–amphotericin B mix, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), Griess reagent, lipopolysaccharide, dexamethasone and oligonucleotide primers were synthesized from Sigma–Aldrich Chemicals (St. Louis, MO, USA); Foetal bovine serum (FBS) was purchased from Gibco-BRL (Auckland, NZ); IL-6, Il-10, INF-γ, IL-β, TNF-α mouse ELISA kits were obtained from BD Biosciences (San Jose, USA); BCA protein assay kit was procured from Pierce Biotechnology (Rockford, USA); Primary antibodies (p38, p-p38, JNK, p-JNK, ERK, p-ERK) and corresponding secondary antibodies for western blot analysis were purchased from Santa Cruz Biotechnology, USA; Clarity Western ECL substrate was purchased from Biorad, USA; Lipoxygenase assay kit, nuclear extraction kit and NFκB translocation assay kit were procured from Cayman Chemicals, USA; Trizol was obtained from Invitrogen Corp., (Grand Island, NY, USA); Invitrogen SuperScript VILO cDNA

Synthesis Kit from Thermo Fisher, USA; RAW 264.7 murine macrophage cell line was obtained from National Centre for Cell Sciences, Pune, India. All other chemicals used were of standard analytical grade.

5.2.2 Experimental design

The work flow in this chapter is schematically represented as in Fig. 5.1.

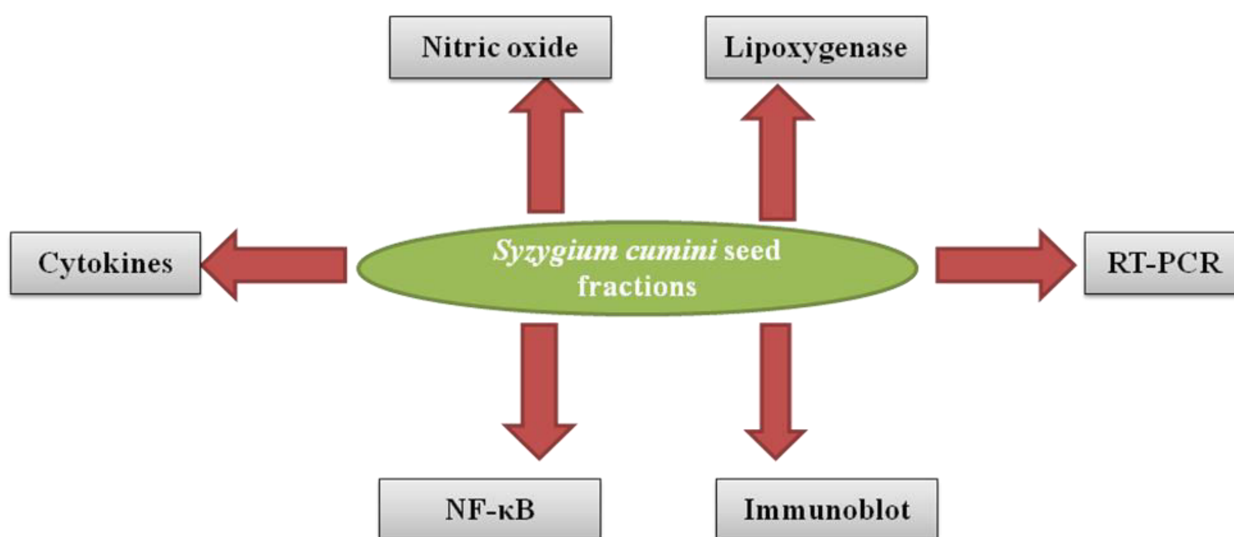


Fig. 5.1: Experimental design

5.2.3. Cytotoxicity assay

Viability of RAW 264.7 was assessed by MTT assay (Mosmann, 1983). The RAW 264.7 cells were seeded (1×10^4 cells/ well) in a 96-well plate. The cells were treated with various concentrations (10,100, 250, 500) of *Syzygium cumini* seed fractions. After 24 h incubation, cells were washed and 100 µL of MTT (5 mg/mL) dissolved in DMEM, was added to each well and incubated at 37 °C in a CO_2 incubator. After 4 h incubation, DMSO was added to each well, and the plate was kept on a shaker at 12 rpm for 45 min. The change in color was monitored using a micro-plate reader (BIOTEK-USA) at 570 nm. Results were expressed as percentage of cytotoxicity:

$$\text{Percentage of Toxicity} = \frac{\text{Absorbance of Control - Absorbance of Sample}}{\text{Absorbance of Control}} \times 100$$

5.2.4. Determination of nitric oxide (NO) production

After pre-incubation of RAW 264.7 cells (2×10^6 cells/mL) with different fractions of *Syzygium cumini* seed (100 µg) and LPS (1 µg/mL) for 24 h, the quantity of nitrite accumulated in the culture medium was measured as an indicator of NO production. Briefly, 100 µL of Griess reagent (1% sulfanilamide and 0.1% naphthyl ethylenediamine dihydrochloride in 2.5% phosphoric acid), was mixed with an equal volume of cell supernatant, the mixture was incubated at room temperature for 10 min, and the absorbance at 540 nm was measured in a microplate reader. Dexamethasone was used as positive control. The quantity of nitrite was determined based on a sodium nitrite standard calibration curve. All experiments were done in triplicates.

5.2.5. Quantification of inflammatory cytokines

RAW 264.7 cells were seeded in six well plates (1×10^6 cells/well). Different fractions of *Syzygium cumini* seed fractions and LPS (1 µg/mL) were treated for 24 h. After treatment, the culture medium was harvested, and the chemokine levels (TNF-α, IL-1ß, IL-6, INF-γ) in the supernatant were measured using ELISA kits according to the manufacturer's instructions. The inhibitory effect was measured based on the absorbance at 540 nm. Dexamethasone was used as positive control.

5.2.6. Lipoxygenase inhibition assay

Lipoxygenases are non-heme iron-containing dioxygenases that catalyze the addition of molecular oxygen to fatty acids containing a cis, cis-1,4-pentadiene system. Lipoxygenase inhibitor screening assay kit detects and measures the hydroperoxides produced in the liopxygenation reaction. RAW264.7 cells were seeded in six well plates (1×10^6 cells/well). Different fractions of *Syzygium cumini* seeds (100 µg/mL) and LPS (1 µg/mL) were treated for 24 h. After treatment, cell lysates were collected, and lipoxygenase inhibitor screening assay was done according to manufacturer's

instructions. The absorbance was read at 500 nm and percentage inhibition of lipoxygenase was calculated as:

$$\text{Percentage of inhibition} = \frac{\text{Absorbance of Control - Absorbance of Sample}}{\text{Absorbance of Control}} \times 100$$

5.2.7. NF-κ (p65) translocation assay

NF-κB (p65) transcription factor assay is a non-radioactive sensitive method for detecting specific transcription factor DNA binding activity in nuclear extracts. NF-κB contained in the nuclear extract, binds specifically to the NF-κB response element. After treatment, the cells were collected by centrifugation, and nuclear proteins were isolated using Cayman's nuclear extraction kit. The NF-κB (p65) level was detected after nuclear extraction. 10 µL samples were added to the wells and incubated overnight at 4 °C. All the wells were washed five times with 200 µL 1X wash buffer. Then to all the wells except blank, 100 µL NF-κB (p65) primary antibodies were added and incubated for 1 h at room temperature. The washing step was repeated with 1X wash buffer and to all the wells except blank 100 µL diluted goat anti-rabbit secondary antibody was added and incubated for 1 h at room temperature. After incubation, it was washed with 1X wash buffer. Then to all wells, 100 µL developing solutions was added and incubated for 30 min with gentle agitation. 100 µL of stop solution was added to all the wells and absorbance was measured at 450 nm.

5.2.8. Isolation of total RNA and quantitative real time PCR analysis

Total RNA was isolated from pretreated RAW 264.7 cell lines using Trizol (Invitrogen Corp., Grand Island, NY, USA) according to the manufacturer's protocol. Reverse transcription was carried out with 1 µg RNA using SuperScript VILO cDNA Synthesis kit. The primer sequences used were as follows:

Genes	Oligonucleotides
ppia	forward 5′-CAAAGTTCCAAAGACAGCAGAAA-3′,
	recerse 5′-CTGTGAAAGGAGGAACCCTTATAG-3′.
COX-2	forward 5′-ACTCACTCAGTTTGTTGAATCATTC-3′,
	reverse 5′-TTTGATTAGTACTGTAGGGTTAATG-3′,
iNOS	forward 5′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′
	reverse 5′-GGCTGTCAGAGCCTCGTGGCTTTGG-3′,

Quantification was performed using a real-time PCR system (Bio-Rad, Hercules, CA, USA) with SYBR green. The PCR conditions for cycling parameters were as follows: initial denaturation at 95°C for 10-15 s, followed by 40 cycles of amplification with denaturation at 95°C for 10-15 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. The samples were normalized to the expression level of ppia, and the results were expressed as the fold changes relative to the treated group using the $2^{-\Delta\Delta CT}$ method. All analysis were carried out in triplicates.

5.2.9. Western Blot Analysis

Expression level of proteins namely, p38, p-p38, JNK, p-JNK, ERK, p-ERK, were evaluated by Western blotting. RAW264.7 cell lines were treated with different fractions of *Syzygium cumini* seeds in the presence or absence of LPS for 24h. After incubation, the cells were lysed in ice-cold lysis buffer (50 mM Tris-HCl, 150 mM sodium chloride, 1.0% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% Triton X-100 and protease inhibitor cocktail, pH 8.0) for 30 min on ice and were centrifuged at 12000 x g for 10 min. The protein content was then measured using BCA protein assay kit. The lysates (40μg) were subjected to SDS-PAGE on 10% gel and transferred on to a poly vinylidene di fluoride (PVDF, Immobilon P™, Millipore®,

USA) membrane by using Trans-Blot Turbo™ (Bio-Rad). The membranes were blocked by incubating in blocking buffer (5% skim milk in PBST, PBST-PBS buffer containing 0.1% Tween 20) for 1hr at room temperature, washed three times with PBST and probed over night at 4°C with appropriate phospho-specific or pan-specific antibodies against p38, p-p38, JNK, p-JNK, ERK, p-ERK (each at 1: 500). Membranes were washed 3 times and incubated for 1 h at room temperature with horse radish peroxidase (HRP) conjugated secondary antibody at 1:1000 dilution and again washed three times in PBST. The bound antibodies were detected using an enhanced chemiluminescence substrate (Biorad, USA) and measured by densitometry using a Chemi Doc XRS digital imaging system and the Multi Analyst software from Bio-Rad Laboratories (USA).

5.2.10. Statistical analysis

All experimental results were expressed as mean ± SD (standard deviation) of three different experiments. Data were subjected to one-way analysis of variance (ANOVA) and Duncan's multiple range tests were used to test the significant differences. Data are presented as mean ± SD, and $p \leq 0.05$ was considered to be significant. All statistical analyses were performed with SPSS 11.0 (Statistical Program for Social Sciences, SPSS Corporation, Chicago, IL) for Windows.

5.5. RESULTS

5.5.1. Effect of *Syzygium cumini* seeds on cell viability

The cytotoxicity of *Syzygium cumini* seed fractions in RAW 264.7 cell lines was determined by MTT assay. The concentration of fractions up to 100 µg was found to be less than 25% toxic for 24 h (Fig. 5.2). Therefore, a concentration below 100 µg was used for subsequent experiments.

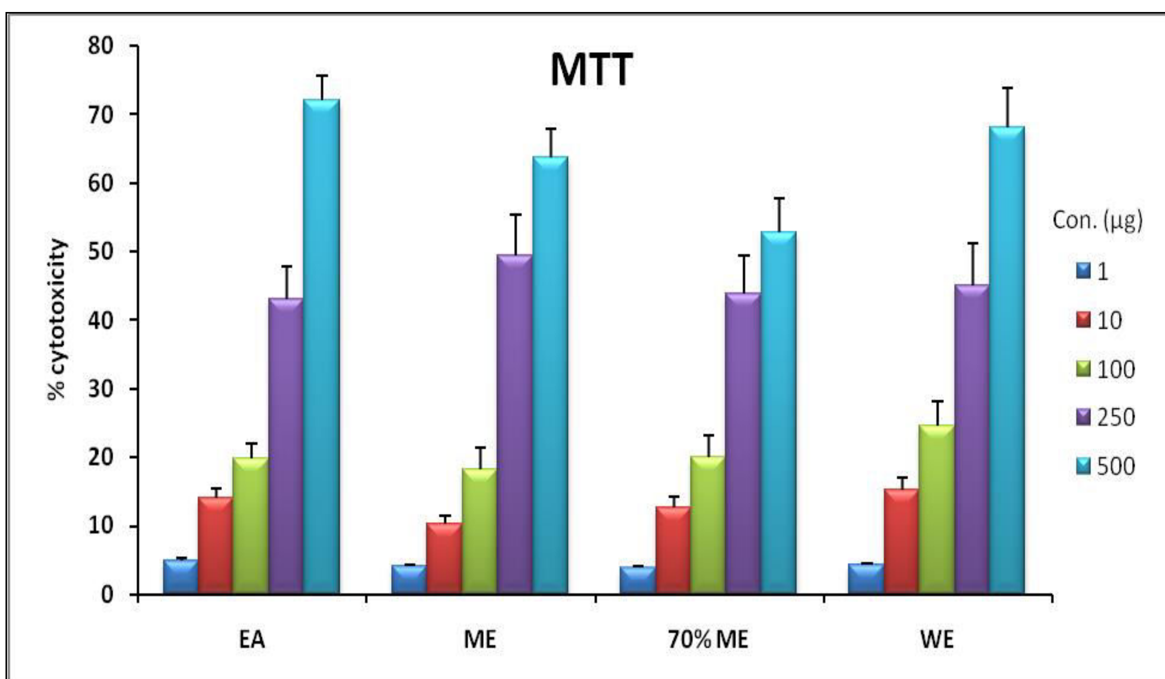


Fig. 5.2: Cytotoxicity of fractions in cultured RAW 264.7 cell lines
Effect of fractions on cell viability was standardized based on concentration. Each value represents mean ± SD (standard deviation) from triplicate measurements (n = 3) of three different experiments. EA – ethyl acetate, ME – methanol fraction, 70% ME – 70% methanol fraction, WE-water fraction.

5.5.2. Effect of *Syzygium cumini* seed fractions on NO production in LPS-stimulated RAW 264.7 cell lines

LPS is widely known as a stimulator of NO production. Furthermore, LPS can cause systemic inflammation if excessively stimulated. Therefore, the present study investigated whether *Syzygium cumini* seed fractions affected LPS-induced NO production in RAW 264.7 cells. The level of NO in cell supernatants was determined using Griess reagent. The results showed that NO production in RAW 264.7 cells remarkedly increased on stimulation with LPS. Cells pre-treated with *Syzygium cumini* seed fractions showed a remarkable decrease in NO production when compared with positive control, dexamethasone (Fig. 5.3).

Pre-treatment with ethyl acetate, methanol, 70% methanol, water and dexamethasone reduced NO production to 45.11 ± 2.4%, 46.65 ± 1.6%, 48.77 ± 2.5%, 15.51 ± 1.1 %, 40.92 ± 1.7 %, respectively when compared with LPS treated group.

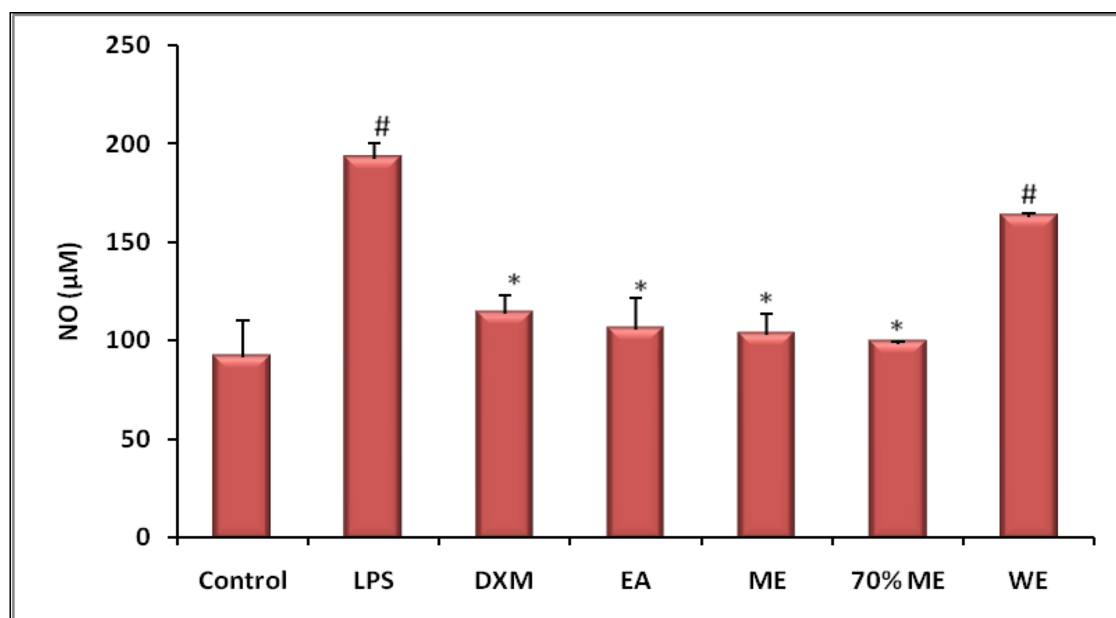


Fig 5.3: Effect of Syzygium cumini seed fractions on nitric oxide production.
Cells were pre-treated with different fractions of *Syzygium cumini* seed (100 µg) along with LPS challenge (1 µg/mL). Each value represents mean ± SD (standard deviation) from triplicate measurements (n=3) of three different experiments. Significance test between different groups were determined by using one way ANOVA followed by Duncan's multiple range test the significance accepted at P≤0.05. #P≤0.05 verses control, * P≤0.05 verses LPS treated group.

The results showed that pre-treatment with ethyl acetate, methanol and 70% methnaol fractions reduced the level of nitric oxide which were comparable with positive control, dexamethasone.

5.5.3. Inhibitory effects of *Syzygium cumini* seed fractions on the expression of inflammatory cytokines in RAW 264.7 cell lines

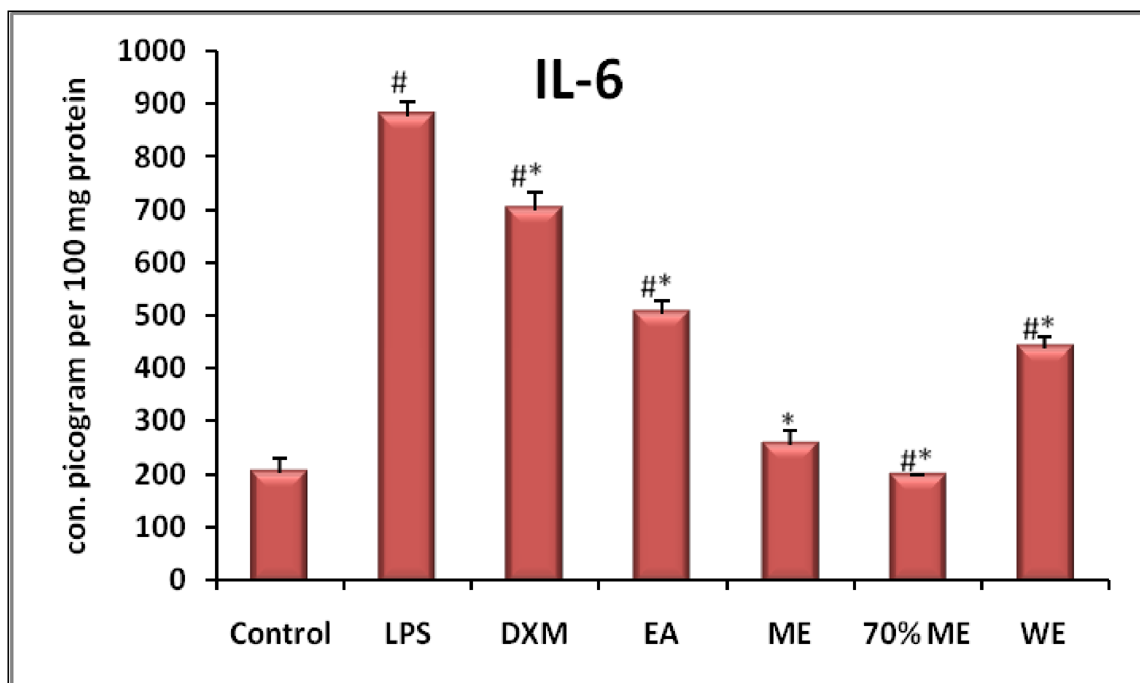
The level of proinflammatory cytokines such as TNF-α, IL-6, IL-β and INF-γ are elevated during the development of inflammatory diseases and play important roles in response towards inflammatory insult in cells. Therefore, we evaluated the effect of

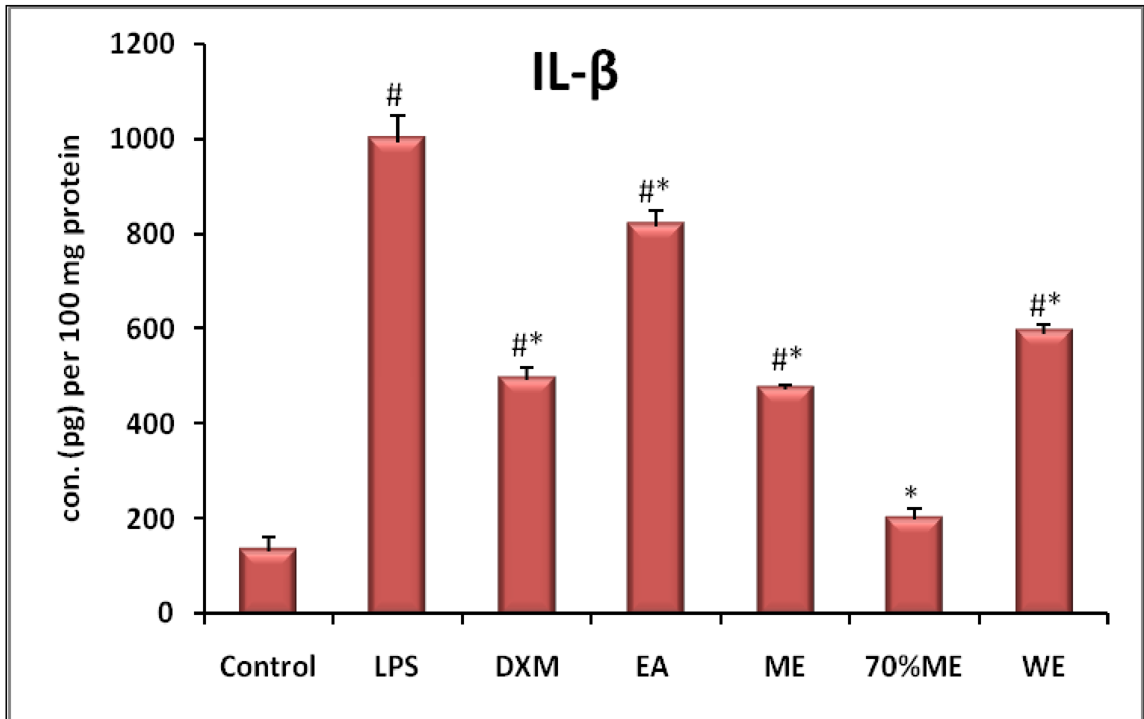
Syzygium cumini seed fractions on the generation of pro-inflammatory cytokines in LPS stimulated RAW 264.7 cells.

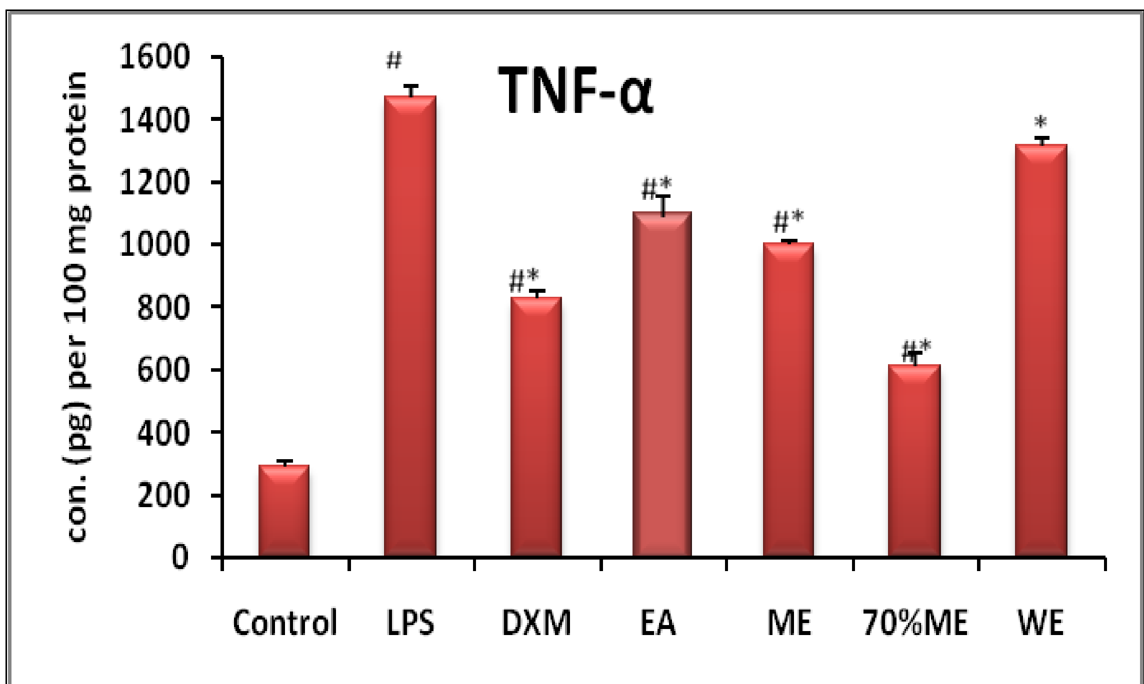
Following pre-treatment with *Syzygium cumini* seed fractions and LPS, the level of cytokines in culture supernatants was analyzed by ELISA. The level of pro-inflammatory cytokines were upregulated following LPS induction as compared to control. Pre-treatment with *Syzygium cumini* seed fractions, especially, 70% methanol, methanol, and ethyl acetate fractions, significantly reduced the level of cytokines. The level of IL-6 decreased in the following order : 70% methanol fraction > methanol fraction > ethyl acetate fraction > water fraction > dexamethasone. The results demonstrated that IL-6 level on pre-treatment with 70% methanol and methanol fractions were comparable with control. When compared with LPS treated group, the level of IL-6 decreased in 70% methanol fraction (77.76 ± 3.2%), methanol fraction (70.97 ± 3.2%), ethyl acetate fraction (42.43 ± 2.3%) and water fraction (49.92 ± 1.8%). The level of IL-6 was decreased in positive control, dexamethasone, treated cells as well (20 ± 0.8%) when compared with LPS treated cells.

The level of IL-β decreased in the following order : 70% methanol fraction > methanol fraction > dexamethasone > water fraction > ethyl acetate fraction. The results demonstrated that the level of IL-β on pre-treatment with 70% methanol fraction was comparable with control. When compared with LPS treated group, the level of IL-β decreased in 70% methanol fraction (79.97 ± 2.2%), methanol fraction (52.70 ± 1.2%), water fraction (40.65 ± 2.8%) and ethyl acetate fraction (17.97 ± 1.3%) treated cells. Dexamethasone treated cells also showed a decrease in the level of IL-β (50.42 ± 1.8%). The level of TNF-α decreased in the following order: 70% methanol fraction > dexamethasone > methanol fraction > ethyl acetate fraction > water fraction. When compared with LPS treated group, the level of TNF-α decreased in 70% methanol

fraction (58.25 ± 2.5%), methanol fraction (32.17 ± 1.1%), ethyl acetate fraction (26.03 ± 0.8%) and water fraction (10.69 ± 0.8%) treated cells. Dexamethasone (43.65 ± 1.1%), treated cells also exhibited a reduction in TNF-α when compared with LPS treated cells. The level of INF-γ decreased in the following order: Dexamethasone > 70% methanol fraction > methanol fraction > ethyl acetate fraction > water fraction. When compared with LPS treated group, the level of IL-β decreased in 70% methanol fraction (66.62 ± 1.2%), methanol fraction (63.86 ± 1.8%) , ethyl acetate fraction (57.52 ± 2.3%) and water fraction (51.14 ± 2.7%) treated cells. The level of INF-γ decreased in dexamethasone (78.26 ± 1.2%) treated cells as well when compared with LPS treated cells.




IL-β
con. (pg) per 100 mg protein
1200
1000
800
600
400
200
0
#
#*
#*
#*
*
#*
Control
LPS
DXM
EA
ME
70%ME
WE


TNF-α
con. (pg) per 100 mg protein
1600
1400
1200
1000
800
600
400
200
0
#
#*
#*
#*
#*
*
Control
LPS
DXM
EA
ME
70%ME
WE

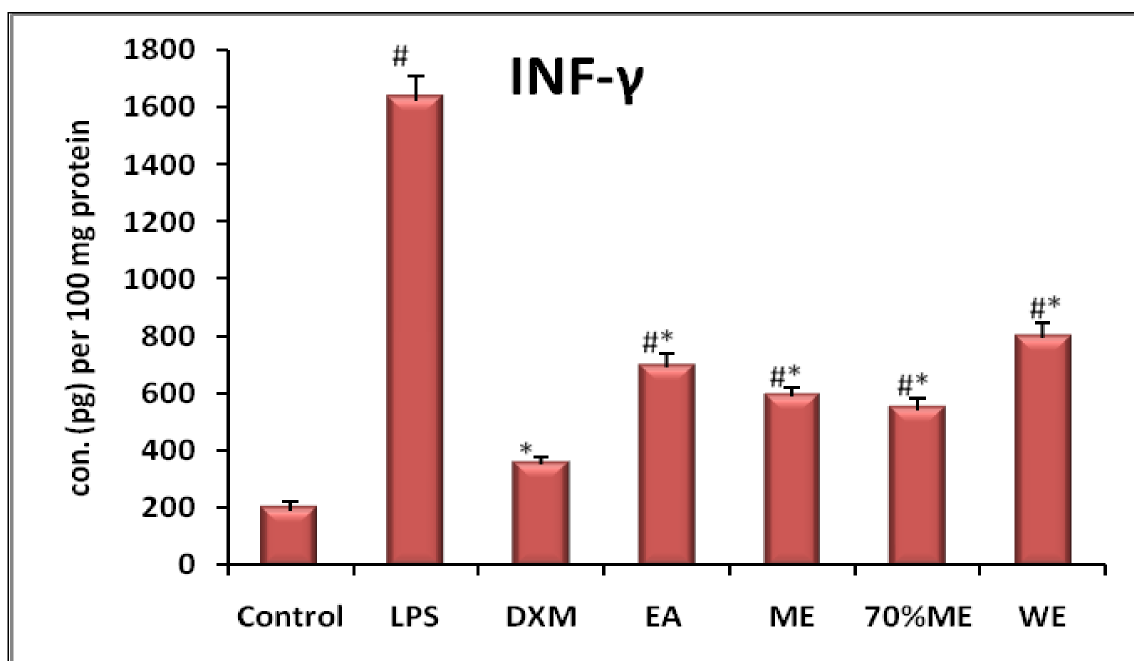


Fig 5.4: Effect of Syzygium cumini seed fractions on cytokine generation in RAW 264.7 macrophages.

Cells were pre-treated with ethyl acetate (EA), methanol (ME), 70% methanol (70% ME) and water fractions (WE) of *Syzygium cumini* seed (100 µg) along with LPS challenge (1 µg/mL). Positive control was dexamethasone (DXM). Each value represents mean ± SD (standard deviation) from triplicate measurements (n=3) of three different experiments. Significance test between different groups were determined by using one way ANOVA followed by Duncan's multiple range test the significance accepted at P≤0.05. #P≤0.05 verses control, * P≤0.05 verses LPS treated group.

5.5.4. Effect of *Syzygium cumini* seeds fractions on lipoxygenase activity

Lipoxygenases and its metabolite leukotrienes are known to be important modulators in inflammation and other vascular diseases. As it is evident from Fig.5.5, in the present study, the activity of lipoxygenase was upregulated following LPS induction. Pretreatment with dexamethasone and *Syzygium cumini* seed fractions (70% methanol, methanol and ethyl acetate) significantly reduced lipoxygenase activity in RAW 264.7 cell lines. The percentage inhibition of lipoxygenase activity of 70% methanol (51.03±1.9%), methanol (39.82±2.9%) and ethyl acetate fractions (34.5±2.4%) were significantly higher compared with LPS treated group.

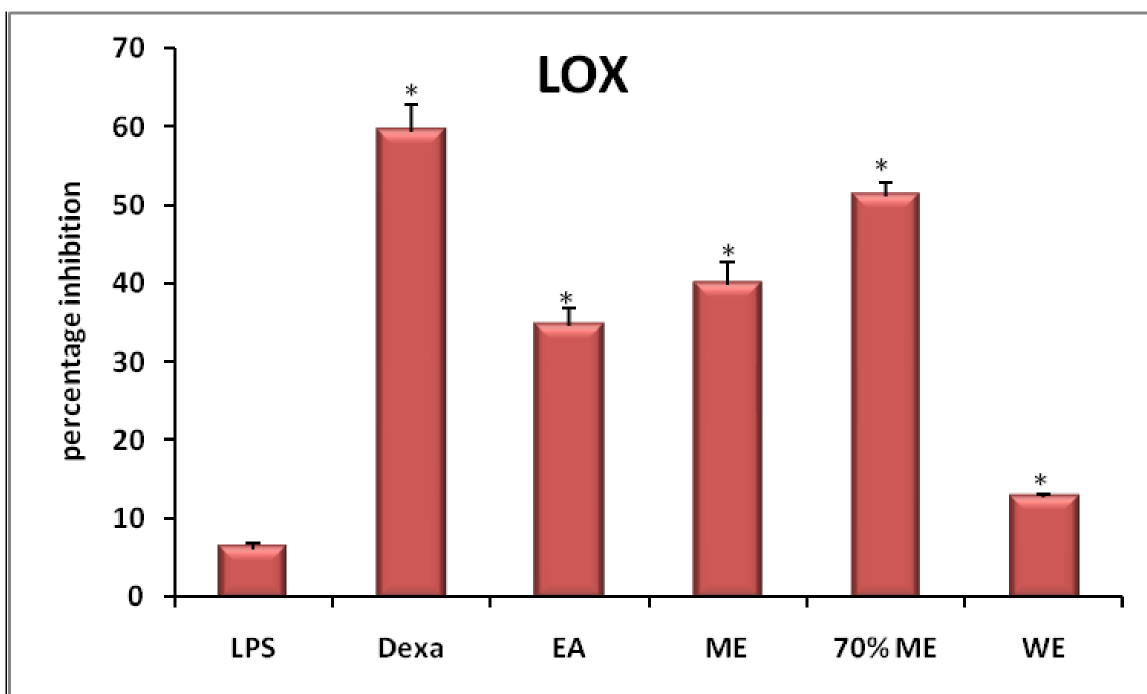


Fig 5.5: Effect of Syzygium cumini seed fractions on lipoxygenase level in RAW 264.7 macrophages.
Cells were pre-treated with different fractions of *Syzygium cumini* seed (100 µg) along with LPS challenge (1 µg/mL). Each value represents mean ± SD (standard deviation) from triplicate measurements (n=3) of three different experiments. Significance test between different groups were determined by using one way ANOVA followed by Duncan's multiple range test the significance accepted at P≤0.05. $^{\#}$P≤0.05 verses control, * P≤0.05 verses LPS treated group.

5.5.5. Effect of *Syzygium cumini* seeds fractions on LPS induced nuclear translocation of NF-κB (p65)

NF-κB (p65) is one of the crucial transcription factors in inflammatory pathways (Tak *et al*., 2001). LPS binds to toll-like receptor 4 (TLR4) and activates NF-κB pathway which in turn activates a series of factors. The effect of *Syzygium cumini* seeds in NF-κB translocation was studied by pretreating RAW 264.7 cell lines with fractions at 100 µg concentration. Pretreatment with LPS increased the level of NF-κB (p65) nuclear fraction compared with control group. *Syzygium cumini* seed fractions inhibited the LPS induced nuclear translocation of NF-κB (p65) and increased the level of p65 in the cytoplasmic fraction. 70% methanol fraction was found to be significantly (P≤0.05) effective in reducing the NF-κB (p65) level compared to dexamethasone treated group (Fig. 5.6).

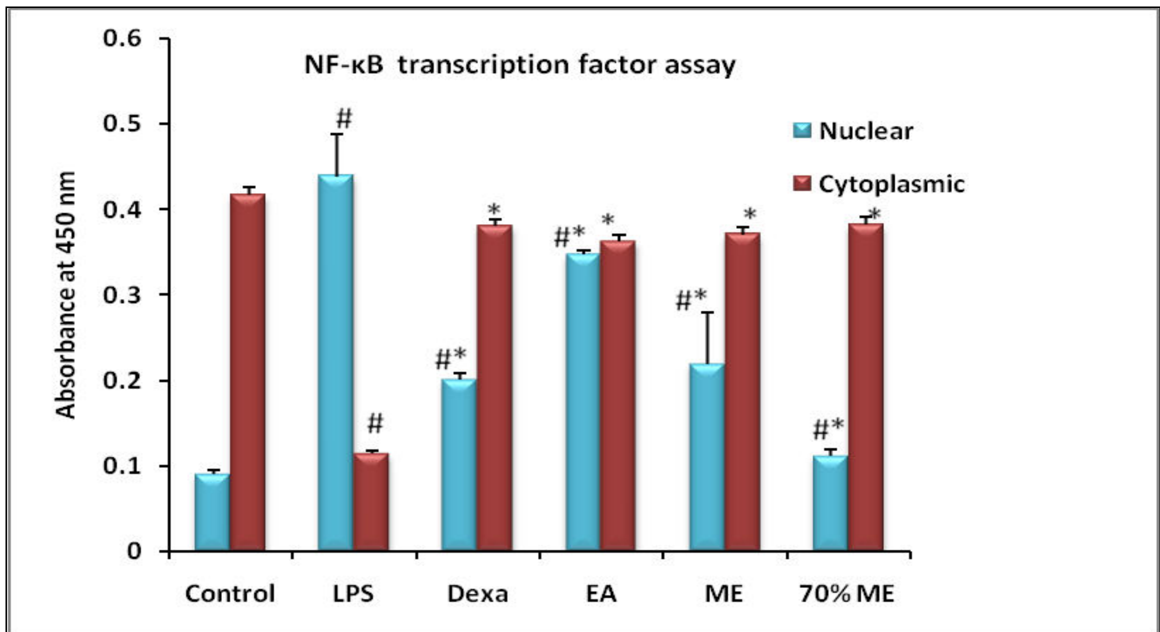


Fig 5.6: Effect of Syzygium cumini seed fractions on NF-κB translocation in RAW 264.7 macrophages.

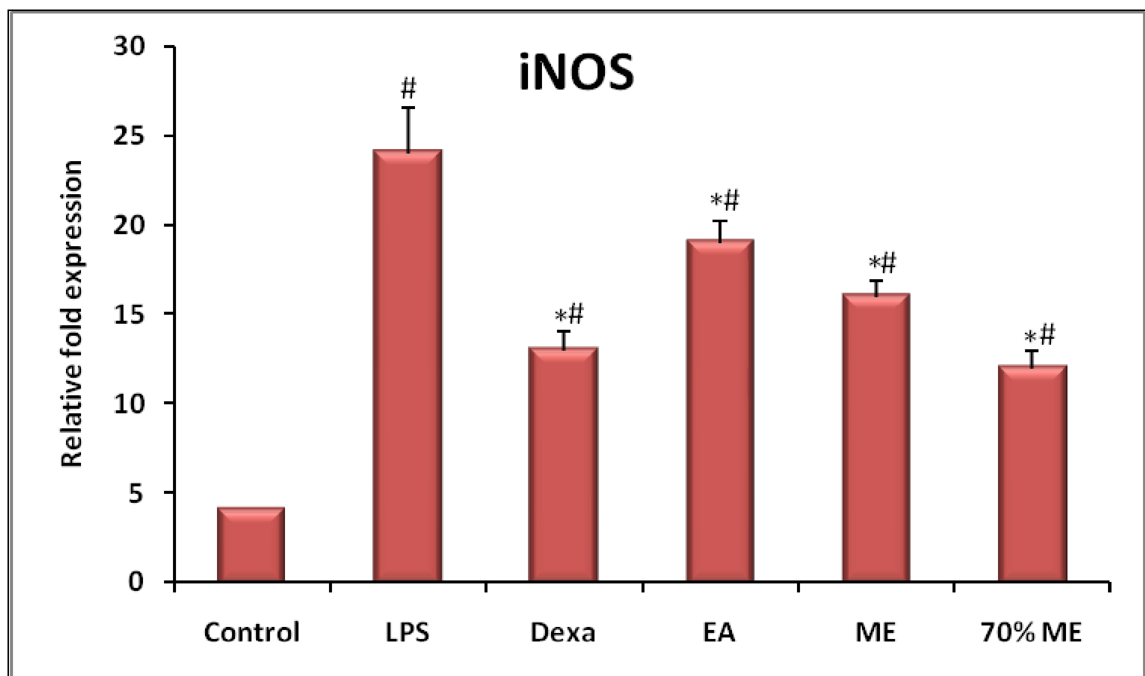
Cells were pre-treated with different fractions of *Syzygium cumini* seed (100 µg) along with LPS challenge (1 µg/mL). Each value represents mean ± SD (standard deviation) from triplicate measurements (n=3) of three different experiments. Significance test between different groups were determined by using one way ANOVA followed by Duncan's multiple range test the significance accepted at P≤0.05. #P≤0.05 verses control, * P≤0.05 verses LPS treated group.

This results proved that *Syzygium cumini* seed fractions exhibited anti-inflammatory effect by blocking NF-κB activation.

5.5.6. Effect of *Syzygium cumini* seeds fractions on iNOS and COX-2 gene expression

The inducible isoform, iNOS is involved in immune response and produces NO which is an important cell signaling molecule. Cyclooxygenase (COX), known as prostaglandin-endoperoxide synthase (PTGS), is an enzyme responsible for the formation of prostanoids. COX-2 is an enzyme responsible for inflammation and pain. To elucidate the role of iNOS and COX-2 in inflammatory pathway, iNOS and COX-2 mRNA levels were examined. The mRNA level of iNOS and COX-2 were significantly increased in LPS treated cells compared to control. The pretreatment with *Syzygium cumini* seed

fractions at a concentration of 100 µg significantly downregulated the expression of iNOS and COX-2 induced by LPS challenge. The results demonstrated that the mRNA level of iNOS and COX-2 in 70% methanol treated cells were comparable with that of positive control, dexamethasone (Fig. 5.7). The mRNA level of iNOS decreased in the following order after pre-treatment when compared with LPS induced group: 70% methanol (2 fold reduction) > dexamethasone (1.8 fold) > methanol (1.5 fold) > ethyl acetate fractions (1.2 fold). The mRNA level of COX-2 decreased in the following order after pre-treatment when compared with LPS induced group: Dexamethasone (1.8 fold) > 70% methanol (1.6 fold) > ethyl acetate fractions (1.4 fold) > methanol (1.1 fold). This real-time PCR data proved that *Syzygium cumini* seeds fractions could act as an anti inflammatory agent by down regulating LPS induced iNOS and COX-2 expression at the transcription level.



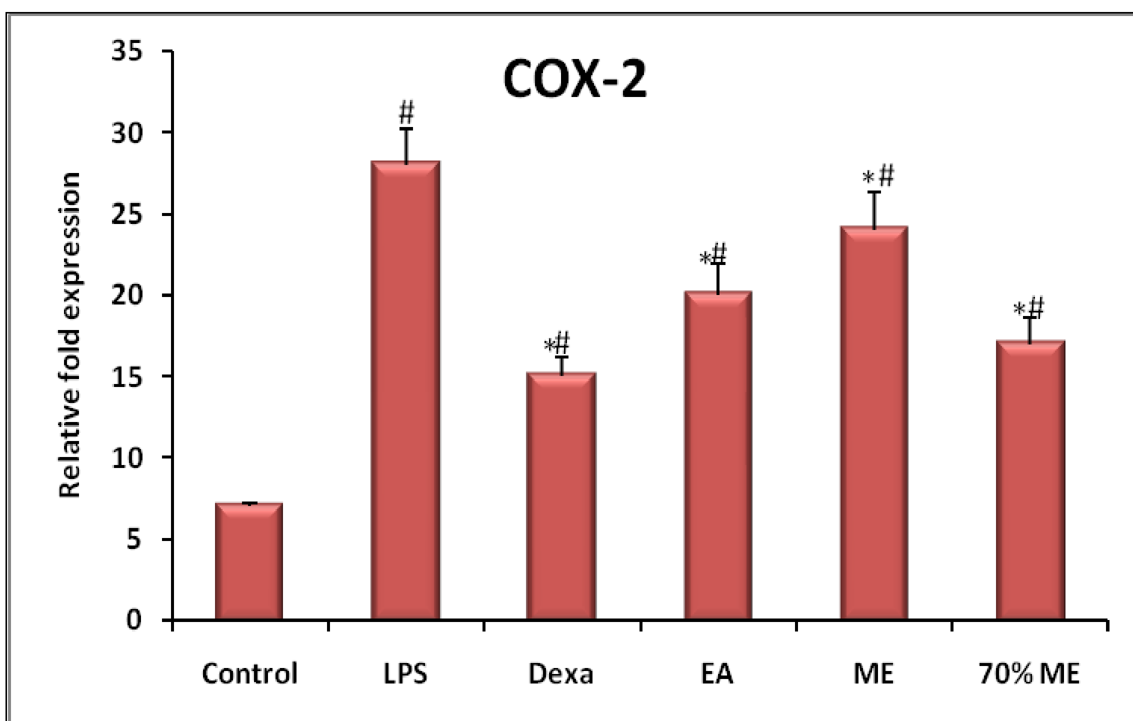


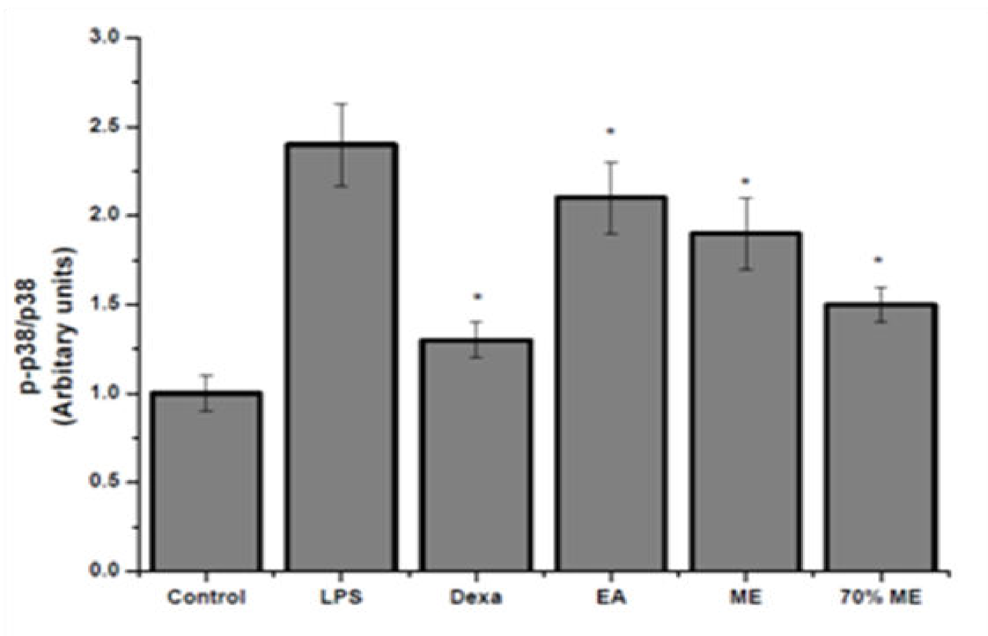
Fig 5.7: iNOS and COX-2 mRNA level expression of Syzygium cumini seed fractions on LPS induced RAW 264.7 macrophages.

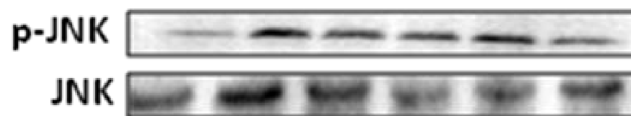
Cells were pre-treated with different fractions of *Syzygium cumini* seed (100 µg) along with LPS challenge (1 µg/mL). Each value represents mean ± SD (standard deviation) from triplicate measurements (n=3) of three different experiments. Significance test between different groups were determined by using one way ANOVA followed by Duncan's multiple range test the significance accepted at P≤0.05. #P≤0.05 verses control, * P≤0.05 verses LPS treated group.

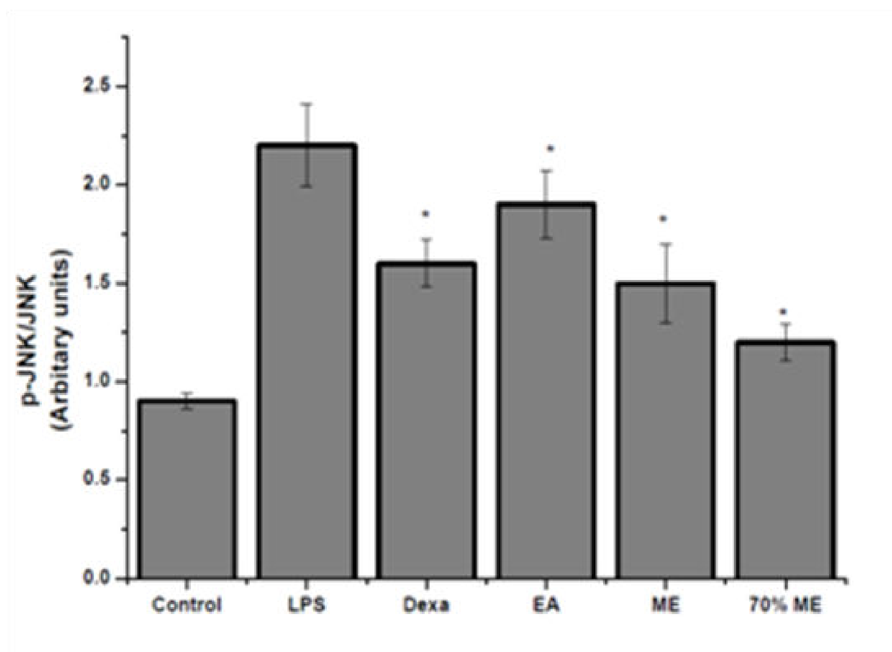
5.5.7. Effect of *Syzygium cumini seed* on phosphorylation of MAPK signaling pathway

To confirm the anti-inflammatory pathway, an immunoblotting study was done to evaluate the expressions of p38, p-p38, JNK, p-JNK, ERK and p-ERK. Protein expression studies also confirmed that on pre-treatment with LPS, the level of p- p38, p-JNK and p-ERK were upregulated. As shown in Fig. 5.8, *Syzygium cumini* seed fractions, especially 70% methanol, and methanol fractions down regulated LPS induced phosphorylation of p38, JNK and ERK. The order of down regulation was similar in all the three studied proteins, ie. 70% methanol > methanol > ethyl acetate fractions.

p-p38
p38


p-p38/p38
(Arbitary units)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control
LPS
Dexa
EA
ME
70% ME


p-JNK
JNK


p-JNK/JNK
(Arbitary units)
2.5
2.0
1.5
1.0
0.5
0.0
Control
LPS
Dexa
EA
ME
70% ME

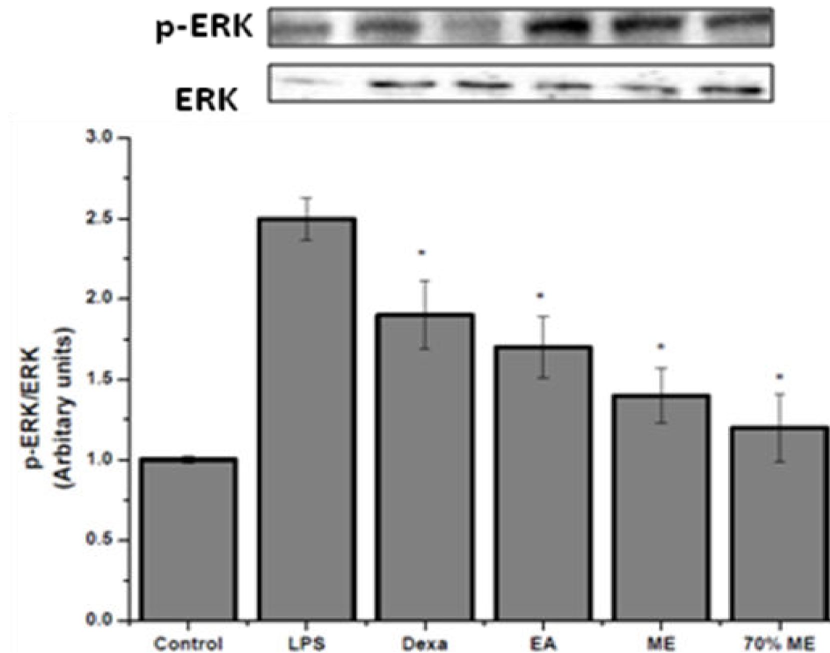


Fig 5.8: Bar graph showing the expression of p-p38, p-ERK and p-JNK of Syzygium cumini seed fractions on LPS induced RAW 264.7 macrophages.
Cells were pre-treated with different fractions of *Syzygium cumini* seed (100 µg) along with LPS challenge (1 µg/mL). Each value represents mean ± SD (standard deviation) from triplicate measurements (n=3) of three different experiments. Significance test between different groups were determined by using one way ANOVA followed by Duncan's multiple range test the significance accepted at $P \leq 0.05$. * $P \leq 0.05$ verses LPS treated group.

5.4. DISCUSSION

Inflammation and oxidative stress are key factors in the onset and development of diabetes (and its complications) and cardio vascular diseases (CVD) (Ceriello *et al*., 2004). Though many synthetic drugs are available to reduce and prevent the onset of inflammation, long term use of these drugs are associated with side effects. Therefore, the present study investigated the role of natural product, *Syzygium cumini* seeds, in alleviating the progress of inflamamtion. Earlier, various parts of *Syzygium cumini* (seeds, bark and leaves) were studied for anti-inflammatory activity in animal models (Muruganandan *et al*., 2001 ; Siani *et al*., 2013). Apart from these studies, there were no other reports showing the mechanism of action of crude extracts or isolated fractions

from *Syzygium cumini*. Hence, our study focuses to explore the anti-inflammatory property of *Syzygium cumini* (seed) and its isolated fractions.

NO is generated when LPS binds to its receptor (TLR-4) and it is also an important toxic defensive molecule against foreign infiltration. However, the uncontrolled production of NO will leads to various chronic inflammatory diseases (Bengmark *et al*., 2006). Therefore the level of NO should be kept in control using various anti-inflammatory agents. In this study ethyl acetate, methanol and 70% methanol fractions (100 µg) of *Syzygium cumini* seeds showed a reduction in the NO level, even more effectively than positive control, dexamethasone. Hence, our study showed a strong anti-inflammatory potential when compared with various compounds (gallic acid, ellagic acid, myricetin, ferulic acid, phenolic acids) reported earlier (Kassim *et al*., 2010). Due to the presence of polyphenols in ethyl acetate, methanol and 70% methanol frcations might be responsible for highest anti-inflammatory activity.

Proinflammatory cytokines (IL-1β, IL-6, TNF-α, and INF-γ) are produced predominantly by activated macrophages in response to foreign pathogens. These cytokines play an important role in generating immune response. The release of proinflammatory cytokines is beneficial only to some extent and similar to NO release, if they were not kept in control, they can eventually lead to serious inflammatory diseases. In our study, *Syzygium cumini* seed fractions, especially, methanol and 70% methanol fractions inhibited the level of IL-1β, IL-6, TNF-α and INF-γ in RAW 264.7 cell lines which were significantly stimulated by LPS. These results indicate the anti-inflammatory potential of *Syzygium cumini* seed fractions. Polyphenols have been reported to demonstrate a significant role in regulation of cytokine production (Santangelo *et al*., 2007) which might attribute to the inhibition of cytokine production in *Syzygium cumini* seed fractions.

LOX enzymes have a significant role in inflammatory responses, which helps in the conversion of arachidonic acid to leukotrienes. Over production of this leukotrienes are one of the major causes of inflammation in asthma and allergic rhinitis. Drugs and agents that can reduce inflammation by suppressing the pathway of production/activation of these inflammatory enzymes which inturn reduce the progress of inflammation. Polyphenols have been reported to play a crucial role in inhibiting lipoxygenases (Welton *et al*., 1986). In the present study, when compared with positive control dexamethasone, ethyl acetate, methanol, and 70% methanol fractions significantly acted on the lipoxygenase pathway of inflammation and proved the anti-inflammatory activity of *Syzygium cumini* seed fractions.

NF-κB is known to play a pivotal role in the expression of inflammatory enzymes and cytokines. NF-κB p65 is a key member of NF-κB transcription factor family which is located in the cytosol as inactive form when bound with inhibitor protein IκBα. Once it's activated by LPS and other extra cellular signals, IκB kinase got activated which inturn leads to the dissociation of IκBα from NF-κB. The activated NF-κB is then translocated into the nucleus. Therefore, by inhibiting the activation of NF-κB, the inflammatory responses can be reduced. This inhibitory pathway is considered as the mainstay for any compound or extract that has anti-inflammatory potential. In the present study, we found that *Syzygium cumini* seed fractions prevented the nuclear translocation of NF-κB p65 subunit which might be the reason for suppressing the level of pro-inflammatory cytokines. Also, the results showed that *Syzygium cumini* seed fractions acted at the transcriptional level of the inflammatory signaling pathway.

MAP kinases constitute major inflammatory signaling highways from the cell surface to the nucleus (Dong *et al*., 2002). LPS induced the release of cytokines from the cells and activated this pathway. ERK, JNK, and p38 are the major components in

MAPK pathway. Earlier studies have shown that MAPKs are the upstream enzymes and signaling molecules for NK-κB (Lopez *et al*., 2015; Sun *et al*., 2016). Our results showed that 70% methanolic fraction of *Syzygium cumini* seeds significantly inhibited the phosphorylation of ERK, JNK but had minimal effect on the phosphorylation of p38.

Macrophages play an important role in inflammation, and they are activated by pathogens or host derived molecules such as LPS. Activated macrophages secrete excessive amount of inflammatory mediators like inducible NO synthase (iNOS) and cycloxygenase (COX-2). iNOS catalyzes the oxidative deamination of L-arginine and produces a large amount of nitric oxide. COX-2 is an important enzyme involved in the biosynthesis of prostaglandin E2 (PGE2). Long term dysfunction of various pro-inflammatory genes, such as iNOS and COX-2 leads to chronic inflammation which will eventually lead to various pathological diseases including cancer (Chung *et al*., 2009). Thus reducing the level of these pro-inflammatory mediators would be an effective strategy for developing anti-inflammatory therapeutic compounds. In order to explain the mechanism responsible for the inhibitory effect of *Syzygium cumini* seeds on LPS induced RAW 264.7 cell lines, iNOS, and COX-2 mRNA gene expression was analyzed. The results showed that 70% methanol fractions of *Syzygium cumini* (100 µg) seed effectively inhibited iNOS and COX-2 mRNA expression in LPS induced cells. Studies had already reported on molecular targets of dietary polyphenols with anti-inflammatory property (Yoon *et al*., 2005). This supports that the presence of polyphenols in *Syzygium cumini* seed fraction may attribute to their anti-inflammatory properties.

In summary, the present study provides evidence that *Syzygium cumini* seed fractions especially 70% methanolic fraction exert significant anti-inflammatory potential in LPS induced RAW 264.7 cell lines by inhibiting the production of NO, LOX, and other pro-inflammatory cytokines. The activity is mediated by down regulating the

expression of iNOS and COX-2 via inhibiting NF-κB activation, ERK, JNK, and p38 phosphorylation. However, further studies are needed to characterize the individual compounds responsible for the anti-inflammatory activity of *Syzygium cumini* seed fractions. The anti-inflammatory activity demonstrated by *Syzygium cumini* seeds could be highly beneficial against the pathogenesis or development of complications of diabetes and CVD.

5.5. SUMMARY

Our research for the first time provided scientific evidence for the molecular mechanism of action of *Syzygium cumini* seeds in macrophage cell lines. The results from the present study showed the importance of ethyl acetate, methanol and 70% methanol fractions in controlling inflammation and its markers. The study demonstrated the role of *Syzygium cumini* seed fractions in managing the level of NO, cytokines, NF-κB, LOX and various proteins involved in inflammatory pathway. Thus the present study showed that *Syzygium cumini* seed fractions possess significant anti-inflammatory potential.

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CHAPTER 6

Summary & Conclusion

SUMMARY AND CONCLUSION

Life style diseases are diseases linked with the way people live their life. Diet, use of alcohol and tobacco, lack of physical exercise, heredity and environmental factors all these can lead to life style diseases. The major life style diseases in India are cardio vascular disease, diabetes, cancer and chronic respiratory disease. Today, more Indians are dying of life style diseases than infections- a reverse of the situation happened in some 20 years ago. In India, 25% of people die of life style diseases before they are 70. The synthetic drugs used to treat these life style diseases are often associated with detrimental side effects. Thus there is a growing demand for natural products for the prevention and treatment of these life style diseases.

Syzygium cumini, abundantly found in India and Indonesia are extensively used in various traditional systems of medicine like Ayurveda, Unani, Siddha, and Homeopathy. Literature survey had shown that almost all parts of *Syzygium cumini* have medicinal importance. But a detailed study demonstrating the mechanism of action of *Syzygium cumini* seeds or its biochemical targets relevant to cardiovascular disease, diabetes, and inflammation is not yet available in literature. Keeping this in mind, the main objectives of the present study was to determine the effect of *Syzygium cumini* seed and its fractions in relevant biochemical and molecular targets responsible for cardiovascular disease, diabetes, and inflammation.

Hence in the present study, *Syzygium cumini* fruits were collected from three different geographical locations namely Trivandrum (TVM), Trichy (TCH) and from Malampuzha (MPA). Seeds were separated from fruits and were dried, powdered and sequentially extracted using various solvents like hexane, ethyl acetate, methanol, 70% methanol and water fractions. TPC and TFC of all fractions were determined, and it was found that phenolic and flavonoid content increases in the following order for all the

geographical variants of *Syzygium cumini* - hexane fraction < water fraction < ethyl acetate fraction < methanol fraction < 70% methanol fraction. Antioxidant activity was checked using different assays like DPPH, nitric oxide scavenging, superoxide scavenging and ABTS scavenging activity. Antioxidant analysis also showed that 70% methanol fraction showed the highest antioxidant activity. Also, among all geographical variants, TVM variant was found to be the most active. Hence, HPLC profiling of all fractions of *Syzygium cumini* seeds were conducted, and each fractions were individually spiked with 13 different polyphenolic standards. Based on the retention time, the presence of polyphenols in each fractions were determined and were quantified using peak area. The presence of these polyphenols were also confirmed by LC-MS/MS analysis. The most active fraction was found to be 70% methanol fraction, and so it was further taken for compound isolation and identification. The compound β-sitosterol glucoside was isolated from 70% methanol fraction and was confirmed by NMR and HRMS data.

Then, we analysed the cardio protective effect of *Syzygium cumini* seed fractions using various *in vitro* cell free and cell based assays. It was found that methanol and 70% methanol fractions exhibited significant LDL oxidation, HMG-Co A reductase and ACE inhibition potential when compared with the positive controls ascorbic acid, pravastatin, and captopril respectively. Docking studies were also conducted to check the binding affinity of the major phenolics present in these fractions to the active site of these enzymes. The results of docking studies showed that these phenolics have more affinity towards the binding site of these enzymes thus blocking the active site from binding to the substrate. The cytotoxicity of *Syzygium cumini* seed fractions were checked in H9c2 rat cardiomyoblast cell lines. From the results, 100 μg concentration was taken for further cell line studies. TBHP (100 μM) was used for inducing oxidative stress.

Intracellular reactive oxygen species was determined by DCFH-DA by confocal microscopy and the results demonstrated that methanol and 70% methanol fractions exhibited significant reduction in ROS level. The results were confirmed by flow cytometry. The level of different antioxidant enzymes were determined and it was found that antioxidant level was decreased drastically on treatment with TBHP, but on further treatment with *Syzygium cumini* seed fractions especially 70% methanol fraction remarkedly improved the level of antioxidant enzymes.

The antidiabetic activity of *Syzygium cumini* seeds were explored using different targets relevant to diabetes. α- amylase, α-glucosidase, antiglycation and DPP-IV inhibition assays showed that 70 % methanol and methanol fractions possess significant antidiabetic potential when compared with positive controls. Their activity were further confirmed by docking the major phenolics with repective enzymes. Cytotoxicity of these fractions were checked in L6 mouse myoblast cell lines and 10 µg concentration was selected for further studies. Glucose uptake study in L6 cells using confocal microscopy showed that 70% methanol fraction exhibited significant increase in glucose uptake ability. This results were confirmed by flow cytometry. Results also showed that these fractions possess ability to inhibit PTP-1B enzyme activity that plays a crucial role in insulin signaling mechanism. Western blot analysis and PCR data of key signaling molecules involved in insulin signaling pathway exhibited that *Syzygium cumini* seed fractions enhance the insulin signaling by upregulating the level of IRS-1, PDL, PI3K, p-AKT and GLUT-4.

The anti-inflammatory effects of *Syzygium cumini* seed fractions were studied in RAW 264.7 mouse macrophage cell lines. From cytotoxicity assay, 100 µg concentartion was selected for further anti-inflammatory studies. Lipopolysaccharide (1 µg/mL) was used for inducing inflammation. Dexamethasone 1 µM was used as positive control in

inflamamtion studies. The level of nitric oxide and lipoxygenase were incraesed on tratment with LPS. But on further treatment with *Syzygium cumini* seed fractions especially 70% methanol and methanol fractions significantly reduced the level of nitric oxide and inhibited lipoxygenase. These fractions also significantly reduced the level of inflammatory cytokines like IL-6, TNF-α, INF-γ, IL-1β. NF-κB translocation assay also showed that 70% methanol possess significant ability to reduce the translocation of NF-κB from cytoplasm to nucleus. Protein expression analysis of key signaling molecules involved in inflammation pathway showed that these fractions of *Syzygium cumini* can down regulate the expression of proteins like p-p38, p-JNK, p-ERK and also down regulate the mRNA level expression of key molecules in inflammation pathway ie. iNOS and COX-2 gene.

The detailed investigation of *Syzygium cumini* seed fractions in various biochemical targets relevant to diabetes, inflammation and cardioprotective action showed that this seed fractions especially 70% methanol and methanol fractions possess significant antioxidant, cardio protective, anti-diabetic and anti-inflammatory activity. Thus this under-utilized seed can be used in the treatment of various life style diseases. More active compounds can be isolated from 70% methanol fraction and the activity of these fractions can also be can checked in *in vitro* and *in vivo* models in order to further substantiate these findings and to generate new chemicals as future drug.

Abbreviations

2-NBDG	2- (7-Nitrobenz-2-oxa-1,3-diazol-4-yl) amino-2-deoxy-D-glucose
70% ME	70% methanol
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid
ACE	Angiotensin converting enzyme
AGEs	Advanced glycated end products
ANOVA	One way analysis of variance
Apn	Apigenin
ARB	Angiotensin II receptor blockers
ARNIs	Angiotensin receptor neprilysin inhibitors
BSA	Bovine serum albumin
CA	Cinnamic acid
CAGR	Compound annual growth rate
CfA	Caffeic acid
ClA	Chlorogenic acid
CoA	Coumaric acid
COX	Cyclooxygenase
Ctl	Catechol
CVD	Cardiovascular disease
DCFH-DA	2,7-dichlorodihydrofluorescein diacetate
DM	Diabetes mellitus
DMEM	Dulbecco's modified Eagle's media
DMSO	Dimethyl Sulphoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
DPP-IV	Dipeptidyl peptidase-IV
EA	Elagic acid
EDTA	Ethylene diamine tetra acetic acid

EGCG	Epigallocatechin-3-gallate
ELISA	Enzyme linked immuno sorbent assay
ERK	Extra cellular signal-regulated kinase
FA	Ferulic acid
FACS	Fluorescent activated cell sorting
FAPGG	Furylacryloyl-Phenylalanyl-Glycyl-Glycine
FBS	Foetal bovine serum
FRAP	Ferric reducing antioxidant potential
GA	Gallic acid
GAE	Gallic acid equivalents
GSH	Glutathione
HIV	Human immuno deficiency virus
HLA	Human leukocyte antigen
HMGR	HMG-CoA reductase
HPLC	High Performance Liquid Chromatography
HRP	Horse radish peroxidase
IDF	International Diabetic Federation
IL-1ß	Interleukin-1ß
IL-6	Interleukin-6
iNOS	nitric oxide synthase
IV	Intra-venous
JNK	Jun N-terminus kinase
Kmp	Kaempferol
LDL	Low density lipoprotein
LOX	Lipoxygenase
LPS	Lipopolysaccharide
MAPKs	Mitogen-activated protein kinases

Mcn	Myricetin
MDA	Malondialdehyde
ME	Methanol
MPA	Malampuzha
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NADH	Nicotinamide adenine dinucleotide
NF- κB	Nuclear factor- κB
NMR	Nuclear Magnetic Resonance
NO	Nitric oxide
NSAIDs	Non-steroidal anti-inflammatory drugs
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
PGE2	Prostaglandin E2
PMS	Phenazine methosulfate
PNPP	p-nitrophenyl phosphate
ppm	parts per million
PTGS	Prostaglandin-endoperoxide synthase
PTP-1B	Protein Tyrosine Phosphatase 1B
PVDF	Poly vinylidene di fluoride
QE	Quercetin equivalents
Qtn	Quercetin
Q-TOF	Quadrapole – Time-of-Flight
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SA	Syringic acid
SD	Standard deviation

SNP	Sodium nitroprusside
SOD	Super oxide dismutase
SPSS	Statistical Program for Social Sciences
STZ	Streptozotocin
TBHP	ter-butyl hydrogen peroxide
TCH	Trichy
TEAC	Trolox equivalent antioxidant capacity
TFC	Total flavonoid content
TLR-4	Toll-like receptor 4
TM	Traditional Medicine
TNF-α	Tumor necrosis factor-α
TPC	Total phenolic content
TVM	Trivandrum
WE	Water
WHO	World Health Organisation

List of Publications

- **H P Syama**, S Asha, R Dhanya, P Nisha, Syed G Dastagar and P Jayamurthy (2014). Evaluation of underutilized vegetable leaves as a potent source of dietary antioxidant and antimicrobial agent. International Journal of Food and Nutritional Sciences, 3.
- **H P Syama**, P Nisha, P Jayamurthy (2016). Antioxidant activity, phenolic, flavonoid content, and high performance liquid chromatography profiling of three different variants of Syzygium cumini seeds: A comparative study. Journal of Intercultural Ethnopharmacology.
- **H P Syama**, A D Arya, R Dhanya, P Nisha, A Sundaresan, J Elizabeth, P Jayamurthy. Cardioprotective effect of polyphenol rich Syzygium cumini seed against tertiary butyl hydrogen peroxide induced oxidative stress in H9c2 cell lines and modulation of key enzymes involved in hypertension and cholesterol biosynthesis. Journal of Food Science and Technology (under revision).
- R. Dhanya, K B Arun, **H P Syama**, P Nisha, A Sundaresan, T R Santhosh Kumar, P Jayamurthy (2014). Rutin and quercetin enhance glucose uptake in L6 myotubes under oxidative stress induced by tertiary butyl hydrogen peroxide. Food Chemistry 158 (2014) 546–554.
- R Dhanya, K B Arun, V M Nisha, **H P Syama**, P Nisha, P Jayamurthy (2015). Preconditioning of L6 muscle cells with naringin ameliorates oxidative stress and glucose uptake. Plos one, doi:10.1371/journal.pone.0132429.
- **H P Syama**, K B Arun, G. Sinumol, R. Dhanya, S S Anusree, P. Nisha, L. Ravi Shankar, A. Sundaresan. Syzygium cumini seed exhibits antidiabetic potential via. multiple pathways involving inhibition of α-glucosidase, DPP-IV, glycation and ameliorating glucose uptake in L6 cell lines. Food Processing and Preservation (under review).
- **H P Syama**, T Sithara, K S Lekshmi, P Jayamurthy. Anti-inflammation effect of Syzygium cumini seeds on LPS treated murine macrophages RAW 264.7 cells through inhibiting TLR4/ NF-κB pathway activation (communicated).

- Sithara Thomas, K B Arun, **H P Syama**, T R Reshmitha, P Nisha (2017). Morin inhibits proliferation of SW480 colorectal cancer cells by inducing apoptosis mediated by reactive oxygen species formation and uncoupling of Warburg effect. Free Radical Biology and Medicine (under review).
- J Jacob, U R Reshma, **H P Syama**, P Jayamurthy, B S Dileep Kumar (2017). Enhanced antibacterial metabolite production through the application of statistical methodologies by a *Streptomyces nogalater* NIIST A30 isolated from Western Ghats forest soil. PLoS ONE (under revision).

Conference papers/posters

- *Evaluation of Underutilized vegetable leaves as a potent source of antioxidant and antimicrobial agent. International Conference on Advances in Food Technology and Health Sciences (ICFTHS-2014) on 15-16th October, 2014 at Jawaharlal Nehru University, New Delhi organized by International Institute of Food and Nutritional Sciences (IIFANS), New Delhi.* ***(Best Paper award).***
- *Phenolic profiling and anti diabetic activity of Syzygium cumini seed extracts from different geographical locations. International symposium on Phytochemicals, Trivandrum, 2015.*
- *Cardioprotective effects of polyphenol rich Syzygium cumini seeds. National Conference on 'Phytochemicals as Biotherapeutics – Unraveling the Mystery of Natural Products (HERBESCON 2016)' at Sri Ramachandra University, Porur, Chennai .*