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Antidiabetic Activity of *Cissus rotundifolia* Plant Growing in Saudi Arabia

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ANTIDIABETIC ACTIVITY OF *CISSUS ROTUNDIFOLIA* PLANT GROWING IN SAUDI ARABIA

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DISSERTATION ACCEPTANCE PAGE

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This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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ABBREVIATIONS

DM	Diabetes mellitus
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
ADA	American Diabetes Association
GDM	Gestational diabetes mellitus
PG	Plasma glucose levels
FPG	Fasting plasma glucose level
HbA1C	Glycated hemoglobin
TZDs	Thiazolidinediones
AGE	Alpha-glucosidase enzyme
GLP-1	Glucagon-like peptide 1
TCA	Tricarboxylic acid cycle (Krebs cycle)
AGIs	Alpha glucosidase inhibitors
PNPG	P-nitrophenyl- α-d-glucopyranoside
HepG2	Human liver cancer cell line
GLUTs	Glucose transporters
NIDDM	Non-insulin-dependent diabetes mellitus
PEPCK	Phosphoenolpyruvate protein kinase
CADD	Computer Aided Drug Design
FRED	Fast Rigid Exhaustive Docking

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ABSTRACT

ANTIDIABETIC ACTIVITY OF *CISSUS ROTUNDIFOLIA* PLANT GROWING IN SAUDI ARABIA

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Diabetes mellitus (DM) is a metabolic disease characterized by high levels of blood glucose resulting from defects in insulin production or action. A world-wide increase in the diabetes rate is projected to reach pandemic levels over the next 10-20 years. Current data shows that high numbers of the population in Saudi Arabia suffer from diabetes and prediabetes. Non-traditional treatments of diabetes from medicinal plants has shown potential antidiabetic activity. A considerable percentage of the population still prefer or use medicinal plants as a treatment or supplement to traditional medicine. *Cissus rotundifolia* (family Vitaceae) is a known plant in southwestern Saudi Arabia used by people in the region to treat skin diseases, burns and diabetes. The chemical and biological characteristics of *Cissus rotundifolia's* active compounds are unknown. The aim of this study to investigate the chemical and biological properties of this medicinal plant using bio-assay guided separation. Aqueous methanol extract of *Cissus rotundifolia* was fractionated by partitioning against hexane and ethyl acetate. Methanol, ethyl acetate and hexane extracts were then screened for antidiabetic activity using an alpha-glycosidase assay at a concentration of 12.5-50 mg/ml. Methanol extract showed a significant alpha-glucosidase inhibition percent of 58-95%. Further, LC separation

afforded six compounds isolated and characterized using 1H-NMR, 13C-NMR and 2DNMR. Two of the isolated compounds: 3 and 4 (1,4-dimethyl 2-hydroxybutanedioate and 3-hydroxy-4-methoxy-4-oxobutanoate, respectively) showed a significant inhibition of alpha-glycosidase enzyme in the range of 25% to 50% at a concentration range from 1.00 – 0.25 mg/ml. Also, since glucose uptake is considered one of the main pathways to control blood sugar levels, the fractions and the isolated compounds were investigated for their glucose uptake activity. Glucose uptake assay results showed that the ethyl acetate fraction has a significant uptake activity. The isolated compounds 3 and 4 showed increased of glucose uptake activity reach of 19 % and 25%, respectively, with Insulin used as the positive control. Structural similarities of isolated compounds to malate which is a main substrate of citric acid cycle, prompted us to build hypothesis that these compounds might interfere with gluconeogenesis process through inhibition of the citric acid cycle. Molecular modeling study of isolated compounds and their analogs targeting all enzymes involve in citric acid cycle was conducted to examine potential binding affinity of these compounds toward proteins in citric acid cycle. Docking study showed analogs S-2-aminosuccininamide (AN21), levulinic acid and isolated compound SAA4 exhibiting high affinity binding to the fumarase enzyme that plays an important role in citric acid cycle. Docking study clearly suggest compound SAA4 has potential inhibition of gluconeogenesis. In conclusion, this study authenticated that *C. rotundifolia* potential antidiabetic activity through inhibition of intestinal α-glucosidase, induction of glucose uptake activity and possible inhibition of gluconeogenesis.

1 CHAPTER ONE: GENERAL INTRODUCTION AND BACKGROUND

1.1 Natural products

1.1.1 Natural product overview

Plants produce different types of metabolites (primary and secondary) which regulate multiple functions.[1] Primary metabolites are synthesized by plants to maintain biochemical processes such as growth and development. Primary metabolites include amino acids, simple sugars, nucleic acids, and lipids. On the other hand, secondary metabolites are chemicals synthesized by plants to perform specific functions that are not necessary for biochemical processes. Alkaloid, terpenoid and phenolics are the major groups of compounds in plants (figure 1). Secondary metabolites are chemicals synthesized by living organisms to perform certain functions that are not specifically for biochemical processes; for example, defenses against herbivory or pollinator attraction. [2] Natural products are defined as organic compounds originating from living organisms such as animals, plants and microorganisms.[3] However, the term "natural products" is established from secondary metabolites.[3] According to the definition mentioned previously, natural products refers to whole organisms such as plants; animals; parts of organisms like leaves, roots or flowers; extracts from organisms; isolated animal organs; or pure compounds isolated from organism extracts such as flavonoid, terpenoid, alkaloid or steroid.[3]

1.1.2 Natural product history as source of novel medicine

Plants are always very rich sources of drugs, and the majority of currently available drugs on the market have been derived—directly or indirectly—from plants.[4, 5]

In fact, plants have actually provided a starting point for the synthesis of over 50% of available used pharmaceutical drugs.[6] For example, Glucophage® (metformin), one of the cheapest and most popular oral hypoglycemic drugs available nowadays on the market, is derived from *Galega Officinalis* (Fabaceae).[3] Based on ethno-botanical data, about 800 plant species show hypoglycemic activity and more than 1,000 plants worldwide have been used as folk medicine to control of type-II diabetes.[7] *Momordica charantia*, commonly known as bitter melon, is one of the most common plants used to treat diabetes in the world, particularly in Asia, South America and India. Folk medicine of various native communities has long used medicinal plants to treat diabetes. Examining this practice provides a new area of research on the antidiabetic effect of medicinal plants.[8] Natural products, especially medicinal plants, have played a very important role in treating human disease, and the use of medicinal plants has been dated back to ancient times.[3] Natural products are used in the treatment of numerous health conditions. Further, the use of natural products for medicinal purposes by countries such as India, China and South Africa has been practiced for thousands of years.[3] According to the World Health Organization (WHO), 65% of the global population is dependent on natural products for healthcare.[3] In China, for example, more than 7,295 plant species are used as sources of medicinal agents.[3] Today, in developed countries, 70% to 80% of the population uses some form of alternative or complementary medicine.[3] Moreover, herbal medicine is one of the most popular forms of traditional medicine in addition to being ranked as the most lucrative industry in the international marketplace.[3] Various cultures worldwide still rely on herbal medicines with approximately 38% of American adults using alternative medicines.[9] People depend on alternative or complementary medicine

primarily to manage side effects of pharmaceutical drugs or turn to it as an alternative when pharmaceutical options do not provide relief from disease. The drug discovery field depends on natural products for new medicines especially because existing therapies have many side effects that result in the recall of drugs.[10] Therefore, there is a need to study these medicinal plants in order to give scientific authentication to the proper use of these plants and remove any medications that may cause harm to patients. It is encouraging to see that, recently, many researchers became interested in giving scientific authentication to the activity of plants used in traditional medicine around the world.

1.1.3 Sources of natural products

Sources of natural products are numerous and may include plants, marines, animals and microbes. Plants are the richest source of natural products, and the use of certain plants can be seen in herbal medicine throughout human history.[6] The current predicted number of plant species worldwide is around 250,0000.[11] However, a limited number of these plant species have been screened for medicinal properties. In fact, less than 10% of medicinal plant species have been investigated and validated for their medical properties.[12] Furthermore, un-investigated plants could be a major source in drug discovery. Therefore, the search for effective, safe, affordable and convenient agents from medicinal plants has continued to be an important area of investigation. Numerous drugs currently available on the market have been derived from plants either directly or indirectly.[13, 14] Examples include the narcotic analgesic morphine from Papaver somniferum, the anticancer drug vincristine from Vinca rosea, the antimalarial drug artemisinin from Artemisia annua, the anticancer drug Taxol from Taxus brevifolia, the

antibiotic penicillin from Penicillium ssp and the most common antidiabetic available drug Metformin derived from Galega officinalis plant.[3, 14] (Figure 1-1)

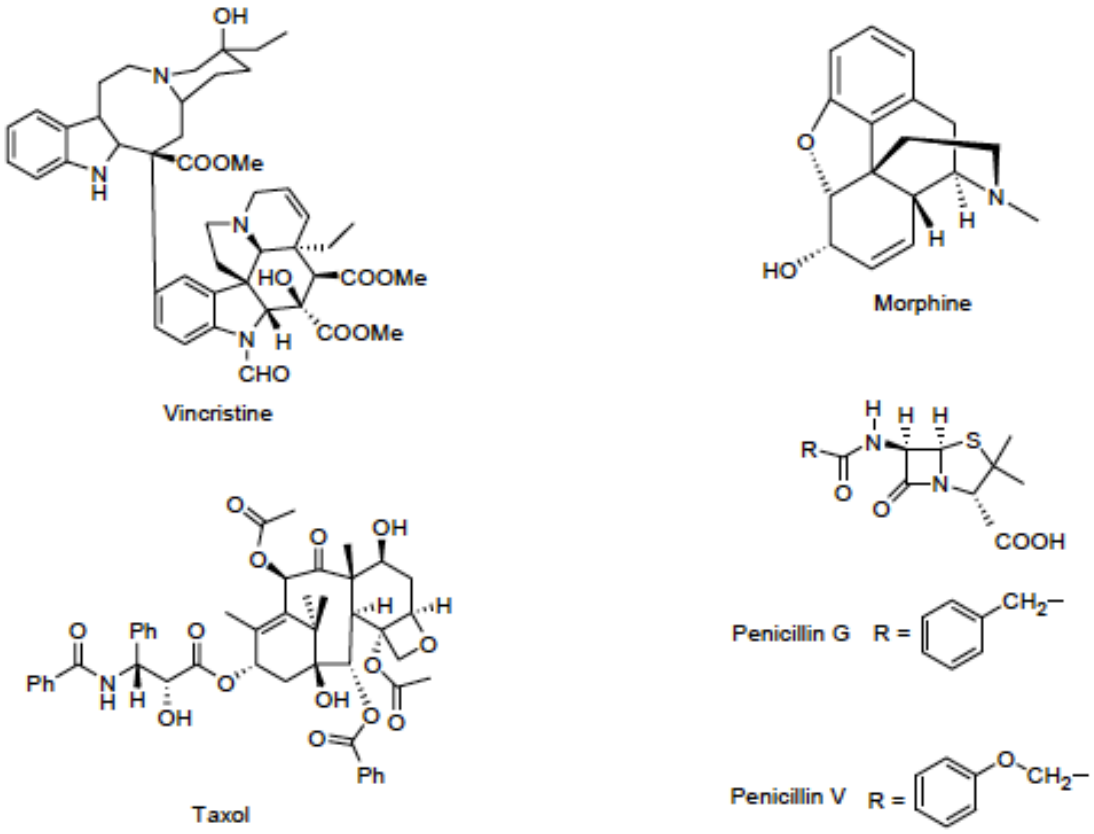

Figure 1-1: Structure of some important plant-derived drugs.

1.1.4 Drug discovery from natural product

Compounds isolated from natural products have a significant role in the discovery and developments of new medicines.[7] In spite of combinatorial chemistry's proposals of new drugs, natural products still remain the main sources of drug discovery and are involved in the most used clinical drug available today.[8] According to a study of all new chemical entities (1,355 total) accepted as new medicines in the period time between January 1981 and December 2010, 26% are natural products or their derivatives, and another 24% are synthetic natural product mimics or have natural product pharmacophores. Also, 15% are biological macromolecules such as peptides, 6% are vaccines and 29% are totally synthetic (Figure 1-2). [15] These data obviously prove that, as a minimum, 50% of all these new drugs are related to natural products. In 1999, one of the top twenty best-selling, non-protein medicines (simvastatin, pravastatin, lovastatin, augmentin, enalapril, atorvastatin, ciprofloxacin, cyclosporine clarithromycin and) were originally natural products or their synthetic derivatives, with expected sales exceeding $16 billion annually.[16] In addition to the important of natural products as sources of drug leads and the significant role of natural products in drug discoveries, natural products have greater chemical novelty and large-scale structural diversity than any other drug sources.[16]

About 40% of the natural products recorded in the natural products database (Dictionary of Natural Products, Chapman & Hall) are not derived from synthetic chemistry.[16] On the other hand, a variety of tools have been established to accelerate the drug discovery process. Among the most popular and frequently used tools in drug discovery are bioassay-guided fractionation and computer-aided drug design.

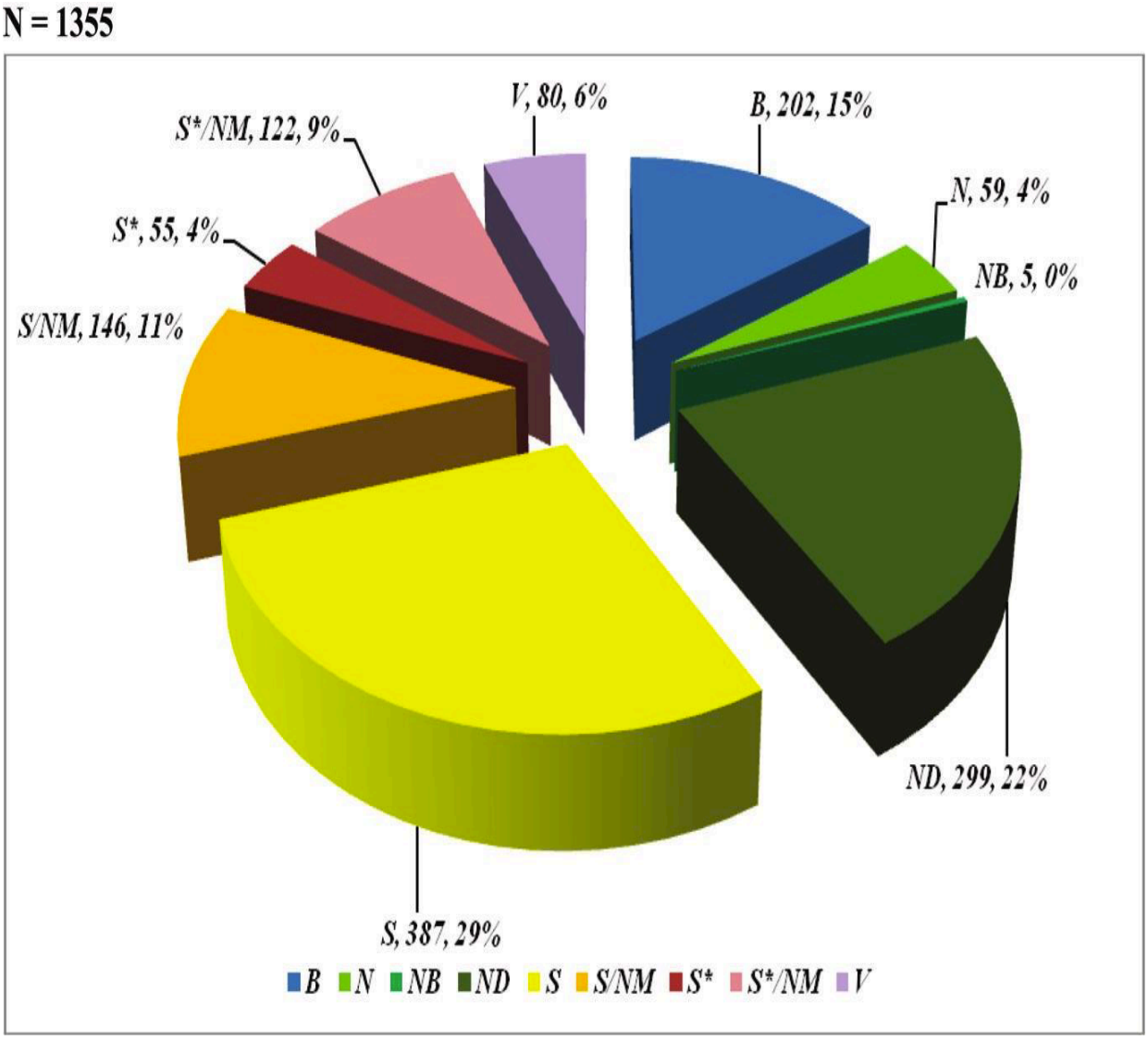

Figure 1-2: Natural products as sources of new drugs over 30 years (1981 to 2010) Reprinted from [17].

1.1.4.1 Bioassay-guided fractionation

The bioassay-guided fractionation method is frequently used in drug discovery involving natural products because of its ability to connect the chemical profile of extracts and fractions to specific biological tests.[18] Bioassay-guided fractionation is a process of generating a fractionated and refractionated extract using several analytical methods until a pure biologically active compound is isolated.[19] This technique begins

with fractionating the crude mixture into several fractions, then each fraction is estimated using different bioassay systems. Only biologically active fractions are exposed to a further fractionation process. The process of fractionation and biological assessment continues until a pure biologically active compound is isolated.[3] The general steps of bioassay-guided fractionation are presented in Figure 1-3.

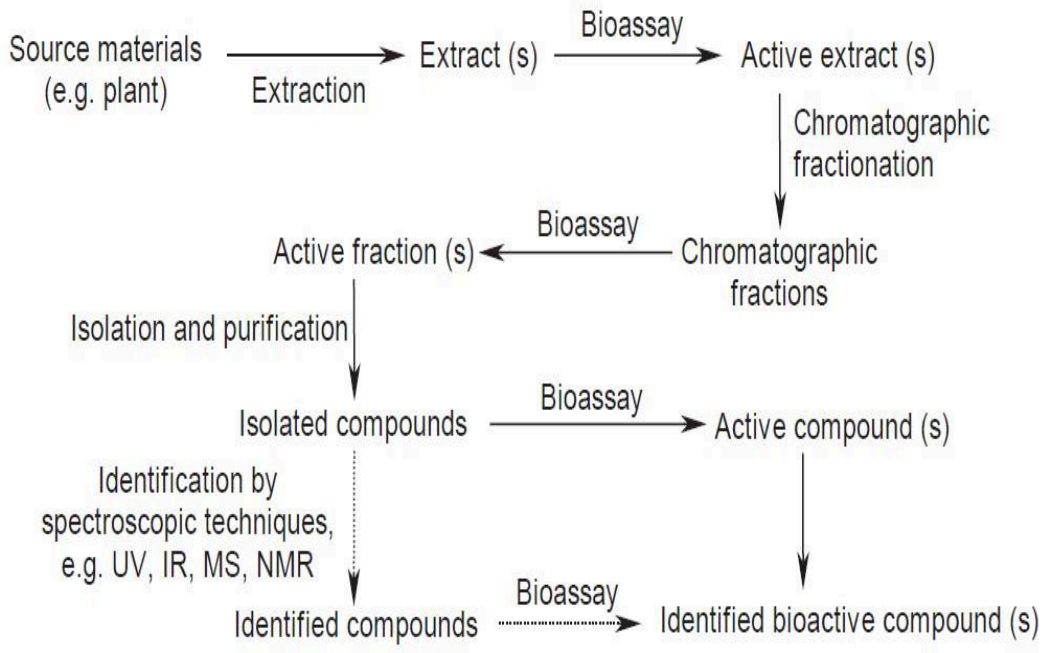

Figure 1-3: The general steps of bioassay-guided fraction.[3]

1.1.4.2 Computer-aided drug design

Connecting computational power and progressive technologies to combined chemical and biological fields has extremely accelerated drug discovery, design, development and optimization.[20] Computer Aided Drug Design (CADD) is an approach that plays an essential role in drug discovery and has increasingly become widespread in

the pharmaceutical industry.[20] Practically, Computer Aided Drug Design (CADD) refers to diverse terms like computational drug design, computer–aided molecular design (CAMD), rational drug design, computer–aided rational drug design, computer–aided molecular modeling (CAMM) and in silico drug design.[20] Computer Aided Drug Design (CADD) is generally expressed using computational tools and resources for the storage, management, analysis and modeling of compounds.[20] The fundamental goal of CADD "is to discover hits (active drug molecules), determine leads (the most likely candidates for further evaluation), and modify leads i.e. transform biologically active molecules into suitable drugs by improving their properties such as physicochemical, pharmaceutical, ADMET/PK (pharmacokinetic) properties."[20] Several computational tools are used in the computer-aided drug design process; these include ligand and structure-based pharmacophore modeling, homology modeling, docking and scoring, ligand-based quantitative structural activity relationship development, molecular dynamics simulation, similarity-diversity analyses, designing and enumerating virtual libraries, virtual high-throughput screening and computational prediction of absorption, distribution, metabolism, elimination (ADME) properties.[21] There are two types of CADD approaches based on the availability of the experimental 3D structures of the target proteins. The ligand-based drug design approach, such as pharmacophore analysis and Quantitative Structure Activity Relationship (QSAR), can be used when protein structures are unknown. Otherwise, the structure-based approaches, for example molecular docking, can be utilized when protein structures are known.[17]

1.1.4.2.1 Molecular docking

Molecular docking is the most commonly used strategy in the structure-based drug design approach. It is also used in the drug discovery method to find the highest matches between a receptor and a ligand.[17] The molecular docking process involves predicting the conformations and orientation of a compound in the active site of target macromolecules and detecting the preferred configurations and conformations of molecules interacting therein.[17] The major objectives of molecular docking are to perfect structural modeling and make a suitable prediction of activity.[3] Furthermore, molecular docking has been used in different stages of the drug discovery pipeline, such as quantitative structure activity relationship, virtual combinatorial library generation, lead optimization and the discovery of a potential lead through virtual screening.[22] In addition, the application of molecular molding has been used to study of the physico-chemical parameters of the ligand, involving absorption, distribution, metabolism and elimination/toxicity using different applications and programs.[20] In molecular modeling, the scoring function is commonly used to determine the interaction between the ligands and the binding sites of target macromolecules. For example, using one of the scoring methods employed in virtual high throughput screening, a virtual library of a significant number of ligands docked into the binding sites of target macromolecules is created, which are then scored based on their affinity to the binding sites of the target macromolecules.[21] There are large numbers of docking programs that have been developed and are used in the drug discovery process, including AutoDock, DOCK, ArgusDock and FTDock.[21] OpenEye Software sets (Fast Rigid Exhaustive Docking

(FRED), make Receptor, Omega, FRED and VIDA) were used to run the molecular docking studies.

1.2 Saudi flora

Saudi Arabia is a Middle Eastern country with a population of more than 25 million people. In Saudi Arabia, people live in both urban and rural areas and quit sectors are still use traditional natural medicine for health care and treatment of diseases.[23] Saudi Arabia has biodiversity, which consists of an admixture of elements from a variety regions like Asia, Africa and the Mediterranean. The mountainous area of southwestern Saudi Arabia is characterized by the richness of its flora and its species diversity.[24] This region, from Taif to the Yemen border, contains about 70% of Saudi flora. The southwestern part of Saudi Arabia has the largest number of these medicinal plants spread in area named Alsawdah mountain near the city of Abha, the capital of Asir province.[4] The climate in this region differs from all other regions of Saudi Arabia due to the heavy rainfall and high altitude.[4] The climate around the Abha area is characterized by differences in temperature and relatively high rainfall throughout the year coupled with complex topography, resulting in the construction of unique and diverse plant communities.[21]

Saudi flora contains 2,250 species arranged in 142 families; among these, more than 1,200 species are expected to have medicinal uses.[25] However, a limited number of these plant species in this rich flora area have been screened for medicinal properties.

1.3 *Vitaceae* family

Plants of the grape family, the Vitaceae family, are identified by their climbing vines and tendrils and the distinctive clusters of berries.[26] The leaves are represented

alternate, forming opposite from the tendrils and flowers. Flowers are regular with 4 or 5 small sepals.[27] There are 4 or 5 petals, which may be united at the tips, falling away as the flower opens with4 or 5 stamens. The ovary is positioned superior and consists of 2 (rarely 3 to 6) united carpels (syncarpous) with the partition walls present to form an equal number of chambers.[26, 27] It matures as a berry with 1 to 2 seeds per cell. Most members of the Grape family have edible leaves, stems, sap, and berries. The vegetation is often mildly astringent.[27]

1.3.1 Genus

The *Cissus* genus contains about 350 species; some of these species are used in herbal medicine as treatment or supplement for different ailments. For example, Bush Medicine Practitioners in Australia use *C. hypoglauca* as medicine for sore throat.[28] In Asia, many cultures use local species of *Cissus* to treat different aliments. In China, *C. assamica*, is often used as an anti-snake venom as a result of decreasing endothelin-1 and sarafotoxin 6B,[29] while in India and Sir Lanka, a species called *Cissus quadrangularis* is used for wound healing[30] and as agent with anti-obesity activity.[31] *C. hamaderohensis* is used in West Asia, it is reported to inhibit the angiotensin converting enzyme (ACE), aminopeptidase N (APN) and neutral endopeptidase (NGP).[32] In Africa, several countries use species of *Cissus* in their herbal medicine; for example, in Cameroon, they use *C. aralioides* as an antimicrobial agent.[33] *Cissus rotundifolia* is one of the known species in Africa and Asia. It shows anti-bacterial properties as a crude methanolic extract; however, it has not been investigated to identify the active constituent compounds. The methanolic extracts of cissus rotundifolia were screened for their antibacterial activity against different kind of bacteria such as against Bacillus cereus, Staphylococcus aureus,

Listeria monocytogenes, Escherichia coli.[34,35] *C. rotundifolia* is one of the common plant species growing in southwestern Saudi Arabia and is used locally to treat different aliments in traditional medicine such as skin disease , burns and to control the high blood glucose in diabetic patient. Based on the efficiency of cissus rotundifolia in traditionally uses, Investigation of the antidiabetic activity of C. rotundifolia might lead to a new effective and safe antidiabetic agent so I focused this study on *C. rotundifolia* to determine the plant compounds that have potential antidiabetic activity.

1.3.2 Cissus rotundifolia

Cissus rotundifolia is a perennial, climber, evergreen wild plant belonging to the family of Vitaceae (grape family).[36] It is known as a common Arabian wax cissus, Peruvian Grape Ivy, Venezuelan tree bine and, locally (in south of Saudi Arabia), as Algalaf.[37] It is commonly used as a food thickener. Moreover, it was found to have many therapeutic effects such as hypoglycemic and hypolipidemic as an extract however it has not been investigated for specific active compound.[34, 38] In addition, its extract shows antibacterial activity.[35] *Cissus rotundifolia* grows extensively in the southern part of Saudi Arabia, and its leaves are widely consumed after cooking as leafy vegetables. It is commonly used to prepare various dishes according to the traditional dietary culture of locals and used as alternative medicine to reduce the blood glucose for diabetic patients and skin burns.[37] However, it has not been studied for its antidiabetic activity. Therefore, this study aimed to evaluate the antidiabetic components of *Cissus rotundifolia* leaves.

1.3.3 Morphology

Figure 1-3 : Cissus rotundifolia (Arabian wax cissus, grape ivy)

The *Cissu rotundifolia* plant is a strong climber up to 30 ft long. Stems are often 4-5 feet sloping and soft of hairless.[27] Tendrils are 2-fid. *C. Rotundifolia* leaves are simple and sometimes lobed. Leaf-stalks are soft, up to 1 cm long and up to 8 x 8 cm in a circular to ovate shape, blunt at the tip, heart-shaped at the base with a toothed margin, velvety to hairless on both sides, thick and fleshy.[27] Stipules are up to 4 mm long, semicircular, and hairless. The flowers are borne in lax cymes, leaf-alternated and at branch ends.[36] Cymes are carried on 3 cm long stalks and flower-stalks are 4-5 mm and flower-buds are 3.5 x 1.5 mm.[36] Petals are green and the ovary hairless with a length of 0.5 mm. Fruit is 1.5 x 1.3 cm and red when ripe. Seeds are 1-2 per fruit, 9 mm. long, and smooth with a single crest.[27, 36] *Cissus rotundifolia* is native to Africa and the Arabian Peninsula.[37]

1.3.4 Taxonomy

The generic name originated from the Greek word kissos, meaning "ivy."[39] In the 1980s, the genus was divided according to certain details of the flower. The large caudiciform species were changed to the new genus Cyphostemma. The genus name was started by Car Linnaeus who used species epithets that are adjectives with the feminine grammatical gender in Latin (e.g., C. trifoliata L.). This matches the form of tree names ending in -us in Latin and having feminine gender.[40] The *Cissus rotundifolia* plant belongs to one of the biggest kingdoms of plant called Plantae. The general taxonomy of *Cissu rotundifolia* as shown in table 1.

Kingdom	Plantae– plantes, Planta, Vegetal, plants
Subkingdom	Viridiplantae – green plants
Infrakingdom	Streptophyta – land plants
Super division	Embryophyta
Division	Tracheophyta – vascular plants, tracheophytes
Subdivision	Spermatophytina – spermatophytes, seed plants, phanérogames
Class	Magnoliopsida
Superorder	Rosanae
Order	Vitales
Family	Vitaceae – grapes
Genus	Cissus L. – treebind, treebine
Species	Cissus rotundifolia (Forssk.) Vahl – Venezuelan treebine

Table 1-1: General taxonomy of Cissu rotundifolia.

The species of *Cissus* genus are frequently used as medicinal plants as they contain vitamins, proteins, polyphenols and carbohydrates. Leaves of Cissus *genus* contain Sterols, Quinones, and Phenolic compounds. In addition to, Anthocyanins, Saponins and flavonoids are found in the plants leaves and fruits.[41] Phytochemical studies on methanol extract has been reported the presence of triterpenes including α- and β-amyrins, β-sitosterol, ketosteroids, phenols, tannins, carotene and vitamin C. [41]

1.3.5 Occurrences of *Cissus rotundifolia*

Cissus rotundifolia is one of the most common plants that grow freely in the wild with no agricultural treatments that allow this plant to be consumed as a food.[37] This species of plant is consumed worldwide in both developing and developed nations and provides nutrition and food security for poor rural communities in several regions.[42, 43] For example, *C. rotundifolia* serves as a diet supplement in Japan, Europe and North American.[44-46] The known plants of these species are rich in minerals, dietary fiber, vitamins, amino acids and fatty acids.[47, 48] Previous studies showed that the corresponding domesticated types of these plants have some therapeutic potential; for example, phytic acid has been shown to have anticancer and antioxidant activity.[49, 50] Therefore, there are indeed reasons to investigate the nutritional impacts of this plant. Moreover, it is necessary to understand its impacts on consumer's health[51].

1.3.6 Medical uses of *Cissus Rotundifolia*

Cissus rotundifolia (fam, Vitaceae) spread from the African Mediterranean region and are currently cultivated in many countries worldwide.[37] It was consumed by the ancient Greeks, Romans and Persians and reached the Far East being used either as a food or alternative medicinal as anti-inflammatory.[26] In the southwestern part of Saudi

Arabia, people use the leaves of *cissus rotundifolia* to control a variety of medical issues like skin disease, burns, cold symptoms and, in traditional medicine for the patient who suffers from diabetes to control the high level of glucose in the blood.[37]

1.4 Diabetes mellitus overview

Diabetes mellitus (DM) is a metabolic disease characterized by high levels of blood glucose resulting from defects in insulin production or insulin action. The increase of the diabetes rate world-wide is projected to reach pandemic levels over the next 10-20 years.

1.5 Classification of diabetes mellitus

In 1997, the American Diabetes Association (ADA) established new classification criteria for diabetes mellitus disease.[52] The ADA classified Diabetes in four clinical classes: the first clinical class of diabetes is Type 1 diabetes (T1DM) based on β-cell destruction leading to absolute insulin deficiency. The second class is the most common type of diabetes; type 2 diabetes mellitus (T2DM) diagnosed due to a progressive insulin secretory weakness on the background of insulin resistance. The third clinical class of diabetes includes other specific types of diabetes resulting from other causes such as genetic defects in insulin action, genetic defects in β-cell function and diseases of the exocrine pancreas. The fourth class is Gestational diabetes mellitus (GDM) that occurs under specific health conditions and is diagnosed during pregnancy.[53]

1.6 Clinical diagnosis of diabetes

Diabetes can be diagnosed through direct blood glucose level testing during individuals primary clinical care with low-risk and diabetes risk assessment. Early diagnosis of type 2 diabetes mellitus (T2DM) is recognized by blood tests that measure plasma glucose levels (PG).[52] The fasting plasma glucose level (FPG) is the most

preferred test to detect the blood glucose level in the body with a level of ≥126 mg/dL or 7.0 mmol/L verified by doing the test again on another clinic visit. This test needs fasting for at least 8 h. Another test is the 2-hour plasma glucose (PG) of ≥200 mg/dL or 11.1 mmol/L in a patient with frank symptoms of diabetes like polyuria, unexplained weight loss and polydipsia.[54] A positive result of the oral glucose tolerance test (OGTT) will demonstrate a PG level of ≥200 mg/dL or 11.1 mmol/L after a glucose load containing 75 g of glucose solution in water. Although the two-hour PG OGTT test is more accurate, it is not commonly used in the clinic because of less convenience and more expense for patients.

1.7 Clinical management of diabetes

The initial evaluation required for a patient suffering from a high blood glucose level is specific to the patient's risk factors and symptoms. Comprehensive care for a patient to help to optimally manage the diabetic case or even diagnose prediabetes is necessary. The main way to control diabetes proceeds from Non-pharmacological therapy (e.g. lifestyle change) to using antidiabetic therapy followed by blood glucose monitoring.

1.7.1 Non-pharmacological Management

1.7.1.1 Lifestyle Modification

Lifestyle modification is the most effective way to prevent one of the most common type of diabetes (T2D) and leads to about a 58% reduction in risks over 3 years .[55] The patient suffering from one or two of high risk factors is highly recommended to implement lifestyle changes such as healthy diet and more time exercising. Later, the diabetic patient already suffering from diabetes, needs to follow up with nutrition

specialists to follow the right diet that will help to avoid sugar sources that increase the level of blood glucose. On the other hand, the patient diagnosed with diabetes needs to moderate his/her weight loss (~ 7% of body weight) as one of the important tactics to control diabetes and impact the cholesterol level in addition to positively controlling blood pressure.[55] Diabetic patient can manage weight loss with a balanced diet, avoiding carbohydrates and control of the calories daily consumed.[56, 57]

Also, other studies show that exercise plays an important role in preventing and controlling hyperglycemia (lower HbA1C level by 0.66%).[57] Exercise is considered an essential part in order to prevent and control both prediabetes and diabetes. The U.S. Department of Health and Human Services recommends that adults ≥18 years of age need to do at least 150 min/week of moderate exercise, such as walking for 15 to 20 min, or 75 min/week of intensity physical activity like running and aerobics over at least 3 days per week to achieve the benefits.[52, 54] For patients younger than 18 years old, the U.S. Department of Health and Human services recommends 60 min of physical activity daily.[52]

In addition to the lifestyle modifications mentioned previously, the diabetes patient needs to moderate alcohol consumption, especially the patient suffering from any another cardiovascular disease, such as hypertension, with diabetes. Also, consumption of alcohol in a fasted state is considered life-threatening in that it can cause hypoglycemia and coma. In general, diabetic patients need counseling and psychosocial support to successfully fight the complication effects of diabetes.[58]

1.7.2 Pharmacological Management

Diabetes mellitus is a chronic disease difficult to control but the management of this disease emphasizes keeping the plasma glucose levels in the blood as close as possible to a normal range with the support of glucose lowering medications, healthy nutrition and exercise.[52] Target goals depend on each patient and the optimal way to control the diabetes is determined after consultation with a medical practitioner. The treatment goal differs from young, adult and elderly to reach normoglycemia without significant hypoglycemia. Hemoglobin A1c (**HbA1c**) is the shape of hemoglobin (a blood pigment that carries oxygen) that binds to glucose. The HbA1c test is used to screen patient glycaemia and the key element is patient education and understanding of the disease and complications related to diabetes, such as cardiovascular disease. Management of diabetes depends on the type of diabetes. In general, Type 1 diabetes mellitus is produced by absence of insulin; therefore, insulin should be controlled in a patient suffering with Type 1 diabetes. Type 2 diabetes is described by insulin resistance and treatments include oral anti-diabetic medications.[59, 60]

1.7.2.1 Insulin Therapy

Insulin is used for treatment of diabetes mellitus Type 1 and includes management of combined split-mixed injections. Moreover, insulin is used in acute diabetes emergencies, for example diabetic ketoacidosis (DKA) and pregnancy. Also, insulin can be used as a supplement in diabetes Type 2 in case oral therapy fails to control blood glucose concentrations. The main adverse effect observed with insulin therapy is hypoglycemia, which might lead to brain damage. Mostly, patients with chronic renal

impairment needed less insulin, so regular control of blood glucose levels must be made to minimize the risk of hypoglycemia.[61]

1.7.2.2 Oral antidiabetic

There are major classes of oral antidiabetic medications, including biguanides, sulfonylureas, meglitinide, thiazolidinedione (TZD), dipeptidyl peptidase 4 (DPP-4) inhibitors and α-glucosidase inhibitors. Diabetes diagnosis depends on the HbA1C test result, if HbA1C level rises to 7.5% while on medication or if the initial HbA1C is ≥9%, combination therapy with two oral agents, or with insulin, may be considered.[61, 62] However, these medications have limitations regarding efficiency, side effects and patient compliance.[61]

1.7.2.2.1 Biguanide

Biguanide and its derivatives are the most common and available treatment for diabetes. The discovery of biguanide started from extraction of a plant called Galega officinalis; this plant was found to have different components, such as guanidine, galegine and biguanide, which showed potential antidiabetic activity through decreasing blood glucose levels.[63] Metformin is the most common, cheapest available and the first-line oral medicine for type 2 diabetes mellitus (T2DM). Metformin is a biguanide that activates adenosine monophosphate-activated protein kinase in the liver, causing hepatic uptake of glucose and inhibiting gluconeogenesis through complex effects on the mitochondrial enzymes.[63] Metformin is a small molecule synthesized based on the pharmacophore of the natural product compound isolated called galegine from *Galega officinalis*. Metformin is the first line drug of T2DM, and it reduces the risk of complications and mortality rates in patients through decreasing hepatic glucose synthesis

(gluconeogenesis) and sensitizing peripheral tissues to insulin.[63] However, metformin has side effects: a risk of hypoglycemia and low chances of weight gain. Metformin might also cause vitamin B12 and folic acid deficiency.[64] This needs to be monitored, especially in elderly patients. Another potential problem resulting from the use of metformin is a decrease in the drug's efficiency as diabetes progresses. Metformin is highly effective when there is enough insulin production; however, when diabetes reaches the state of breakdown of β-cells and results in a type 1 phenotype, metformin loses its efficacy.[61]

1.7.2.2.2 Sulphonylureas

Sulphonylureas is a class of organic compounds used as oral antihyperglycemic drugs. The mechanism of action of sulphonylurease occurs through binding to the sulfonylurea receptor on the beta cell surface to indicate secretion of insulin from the pancreatic beta cells. Sulphonylureas are classified into two main groups: first generation and second generation. Second generation sulphonylureas are characterized by fewer side effects and more effective action than the first generation. The duration of action is shorter in the first generation compared to the second generation. Universally used first generation drugs are tolazamide, acetohexamide, tolbutamide and chlorpropamide. Examples of the most commonly used second generation drugs are glibenclamide, glipizide, glimepiride and gliclazide. Sulphonylurease is used either as monotherapy or in combination with other antihyperglycemic drugs. The major side effects of this class of antidiabetic drugs are hypoglycemia and weight gain.[54, 65]

1.7.2.2.3 Thiazolidinediones (TZDs)

Thiazolidinediones (TZDs) are a class of antidiabetic drugs use by patients suffering from type 2 diabetes (T2DM). Rosiglitazone and Pioglitazone are the most

common TDZ drugs currently available. The mechanism of action of TDZs depends on binding to the peroxisome proliferator activated receptor gamma (PPAR-γ), a kind of nuclear regulatory protein located in fat, muscle and liver. TZDs are expected to increase insulin sensitivity, decrease cellular resistance and improve glycaemic control based on enough production of insulin by pancreatic beta cells. TZDs might be used either as monotherapy or in arrangement with other oral antidiabetic medications. In fact, use of this class of drugs (TZDs) has been reduced due to side effects, such as myocardial infarction, angina fluid retention, heart failure, hypoglycemia and liver injury as indicated by multiple retrospective studies.[65, 66]

1.7.2.2.4 α-glucosidase inhibitors

Alpha glucosidase inhibitors (AGIs) are one of the first line therapy classes of anti-diabetic drugs. These oral antidiabetic medicines are enzyme inhibitors that do not affect the pancreas during the mechanism of action. The mechanism of action of AGIs depends on the delay of carbohydrate absorption in the gastrointestinal tract. In addition, AGIs control postprandial hyperglycemia and provide cardiovascular benefit. Carbohydrates are present as oligo — or poly — saccharides. Polysaccharides need to be broken down to monosaccharides (glucose, fructose, galactose) for digestion. For example, starch is one of the most common polysaccharides digested through the alpha glucosidase enzyme that breaks starch (polysaccharides) down to monosaccharides. Acarbose inhibits alpha-glucosidase enzyme, thus preventing absorption of starch from the brush border of the intestine. Therefore, AGIs class of antidiabetic medicine, which delays intestinal carbohydrate absorption, reduces postprandial glycaemia and helps manage diabetes.[67]

1.8 Diabetic case statistics in Saudi Arabia

425 million people suffer from diabetes worldwide according to the World Health Organization and more than 39 million people in the MENA region. Saudi Arabia is one of the MENA region countries that has a high prevalence rate of diabetes disease. By 2045, the expected number of diabetic patients around the world will rise to 67 million. There were 3.852.000 people suffering from diabetes in Saudi Arabia in 2017 while the total adult population is about 20,770,000 people, so the prevalence of diabetes in adults is approximately 18.5%.[61]

In Saudi Arabia, diabetic disease occurs as one of the major medical issues and cardiovascular disease is considered the first leading cause of death. Saudi Arabia has the highest prevalence rates of diabetics with the increase of diabetes cases in Saudi Arabia affecting more than one fourth of the adult Saudi population.[68] The dramatic increase in the prevalence rate of diabetes is a result of rapid socioeconomic development, lifestyle changes and a change in dietary patterns. Etiological factors that cause high blood glucose level like obesity, dietary habits and lack of exercise also contribute.[69]

1.9 *Cissus rotundifolia* as potential therapy for different medical issues

Cissus Rotundifolia as a crude extract has been reported to possess biological activities.[70, 71 72] This section provides a summary of the potential antimalarial and antidiabetic effects of *cissus rotundifolia as* an extract.

1.9.1 Antimalarial activity of *cissus rotundifolia* as a crude extract

Malaria is an infectious disease that remains associated with considerable morbidity and mortality and economic impact on developing societies.[70] According to the World Health Organization (WHO), malaria is widespread in 91 countries, mostly in

Africa, Asia and Latin America. Approximately 40% of the world's population is at risk and it will continue to be distributed widely, essentially due to the drug resistance developed by Plasmodium falciparum.[72] A study of medicinal plant assessment showed the antimalarial activity of crude extracts of *Cissus rotundifolia*.[70] The results confirm that this plant, consumed in traditional medicine, has significant antimalaria activity in vitro and verified its use in traditional medicine.[72, 73] However, in vivo studies of this medicinal plant need to be completed to determine toxicity of the active constituents, pharmacokinetic properties and diffusion in different body sites in addition to side effects.[73]

1.9.2 Antidiabetic activity of *cissus rotundifolia* as a crude extract

Cissus rotundifolia is a climbing or prostrate shrub found throughout Africa and the Arabian Peninsula being used as food. *C. rotundifolia* leaves extract has shown anti-diabetic as well as antiparasitic properties.[34, 74] In Saudi Arabia, the boiled leaves of *C. rotundifolia* are prepared with meals as an appetizer and also used as an antipyretic in the treatment of malaria and dengue fever.[71] The present study was designed to evaluate the hypoglycemic activity of *C. rotundifolia* components using different pathways to control glucose blood levels compared to the most common antidiabetic drugs like acarbose and metformin.

1.10 Molecular target of diabetes mellitus

Diabetes mellitus known as high level of glucose in the blood. The two main sources of glucose in the body comes from either degradation of complex carbohydrates or the producing of the glucose by liver as result of gluconeogenesis process. In order to control of blood glucose level in early stage need to delay the absorption of glucose to the

blood through inhibition Alpha-glucosidase enzyme as a molecular target that used to breakdown the carbohydrate consumed to glucose. Moreover, through reduce the production of glucose in the body that result from gluconeogenesis process.

1.10.1 Alpha-glucosidase (AG) enzyme

Alpha-glucosidase enzyme is located in the brush border in the small intestine, which is the key enzyme in carbohydrate synthesis and breakdown.[75] Alpha-glucosidase is an exo-type carbohydrase, breaking glycosidic bonds in complex polysaccharides to release absorbable monosaccharides (Fig. 1-4).[76] The degradation of dietary carbohydrates is considered the principal source of increased levels of glucose in the blood. After hydrolysis of the complex dietary carbohydrate, the consequent absorption in the intestine is made by alpha-glucosidases.[77] One of the main strategies to control type 2 Diabetes mellitus (T2D) is to decrease postprandial high blood glucose levels by delaying the absorption of glucose through inhibition of carbohydrate-hydrolyzing enzymes, alpha-glucosidase, in the digestive processes.[78] Furthermore, one interesting property of the alpha-glucosidase inhibitor is its ability to both improve and extend glucagon-like peptide 1 (GLP-1) secretion in normal individuals and patients with Type 2 diabetes mellitus disease.[79, 80]

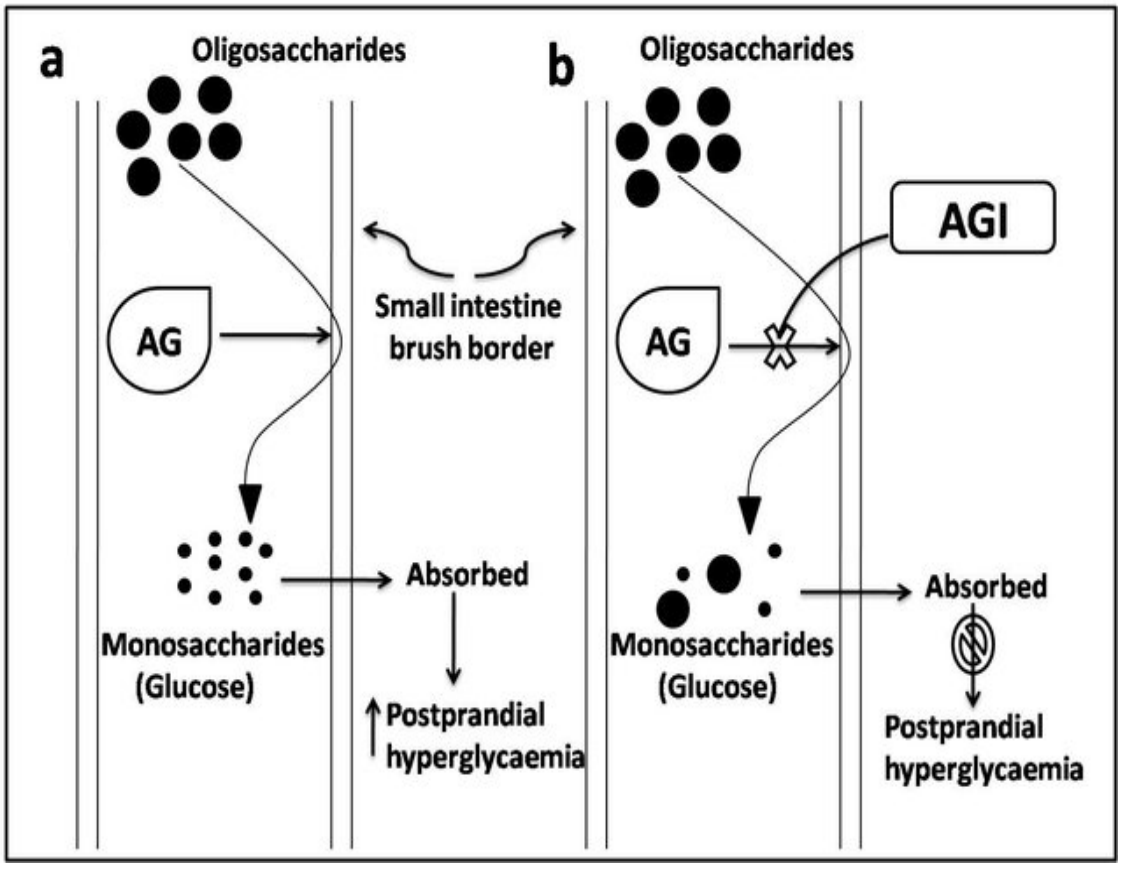

Figure 1-4: Mechanism of action for alpha-glucosidase enzyme.[81]

1.10.2 Gluconeogenesis Inhibition

Gluconeogenesis is the main pathway to produce glucose in human body. In diabetic patients, gluconeogenesis is high so the current target to control diabetic disease is to inhibit gluconeogenesis in order to manage hyperglycemia. Gluconeogenesis is the reversal of glycolysis, with some workarounds for the irreversible reactions in this pathway.[82] The reactions that interact for glycolysis and gluconeogenesis are presented in blue in the figure 2-1, whereas gluconeogenesis's reactions are shown in red.[83] It is realized that both pyruvate and oxaloacetate as substrates from the citric acid cycle are starting points for gluconeogenesis. These pathways are presented here by green arrows. The major substrate source for gluconeogenesis is protein, both dietary and endogenous.

Moreover, any of the TCA cycle intermediates can serve as substrates for gluconeogenesis.

Gluconeogenesis occurs only in the liver and the kidneys, and the liver is synthesizing most of the glucose. Gluconeogenesis provides stable blood glucose levels between meals; however, it is found to be high in diabetic patients. Gluconeogenesis also helps us to maintain glucose levels when on a diet that is low in carbohydrates.[83] Therefore, based on the similarity in the chemical structure between the isolated compound from *cissus rotundifolia* and citric acid cycle substrates, our hypothesis builds on the investigation of the binding affinity of isolated compound and citric acid cycle enzymes in order to screen the inhibition activity for gluconeogenesis to control the hyperglycemia.

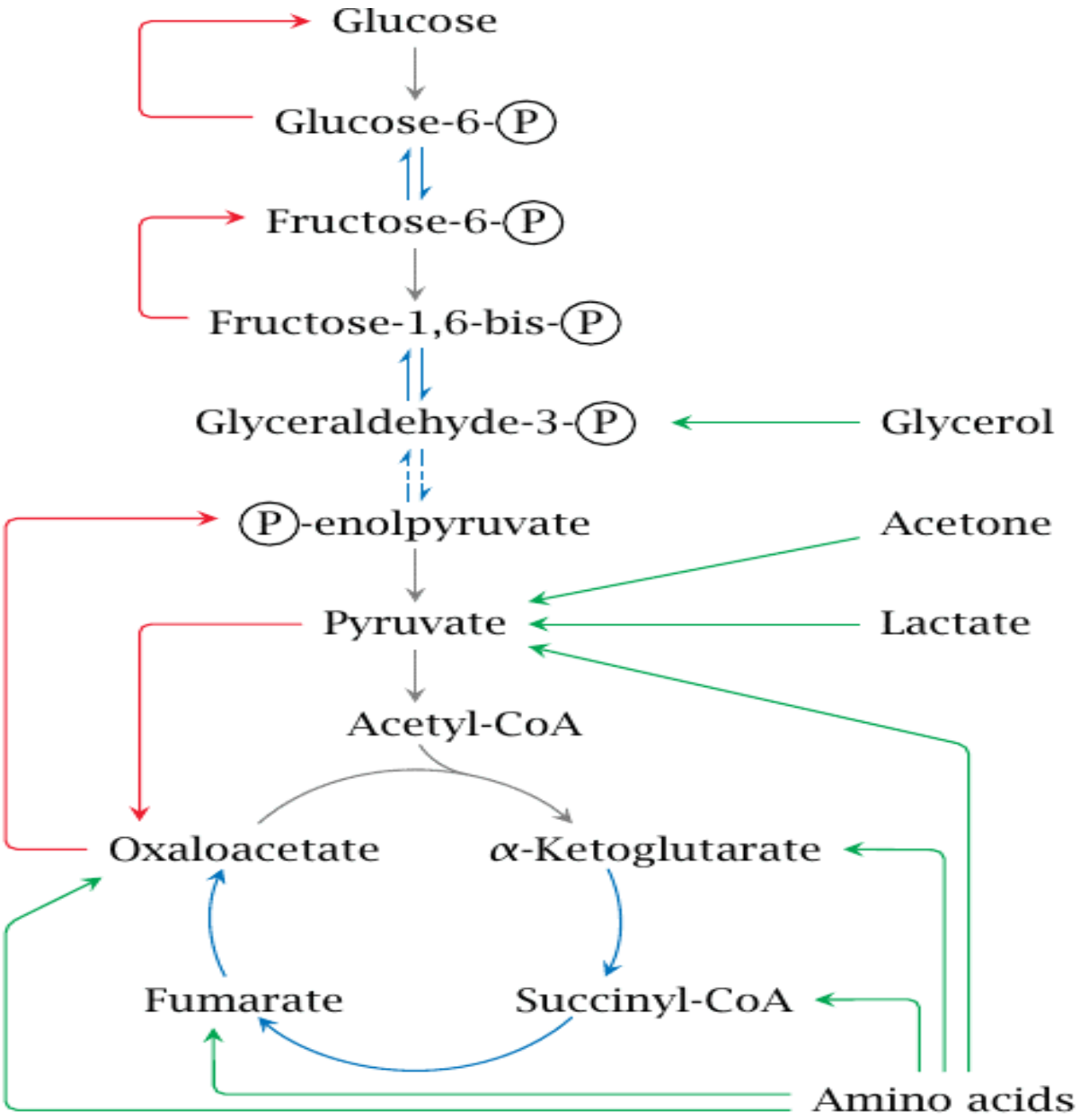

Figure 1-5: Gluconeogenesis process (in red) starts by oxaloacetate substrate from citric acid cycle reactions.

The citric acid cycle, or tricarboxylic acid cycle, has a fundamental role in the gluconeogenesis process that is considered the main pathway of the hepatic production of glucose. The citric acid cycle is a series of reactions in mitochondria that result from

oxidization of the acetyl moiety of acetyl-CoA to CO_2 and decreases coenzymes that are reoxidized through the electron transport chain related to the formation of ATP. The enzymes involving in citric acid cycle play important role in regulation of gluconeogenesis through catalyzes each step of the cycle to form the substrates. Oxaloacetate is a substrate of citric acid cycle and it is the starting material for gluconeogenesis. Although the properties of citric acid cycle enzymes have been studied, the reports of targeted small molecule modulators of the activity have been limited. Therefore, in this study the isolated compound and small analogs investigated for their binding affinity to citric acid cycle enzymes that might lead to inhibition activity of gluconeogenesis.

1.11 Project objectives

1. **Extraction, isolation, and structural elucidation of the active constituents from *Cissus Rotundifolia* with potential anti-diabetic activity using bioassay-guided fractionation.**

   Cissus Rotundifolia (Vitaceae), which is locally called Algalaf, is commonly used in Saudi folk medicine to control high blood glucose level (Diabetes Mellitus). The overall objective of this study was the extraction, isolation and structural elucidation of the active constituents with potential antidiabetic activity from *Cissus Rotundifolia* using bioassay-guided fractionation. The antidiabetic activities of the extracts, fractions and pure isolated compounds obtained from the bioassay-guided fractionation were evaluated in vitro using alpha glucosidase enzyme inhibition bioassay and glucose uptake induction to the

cell using liver cell line (HepG2). The chemical structures of the pure isolated compounds were elucidated using H NMR, C^{15} NMR and 2D NMR. To the best of our knowledge, this is the first chemical profile investigation of the antidiabetic activities in *Cissus Rotundifolia*.

2. **In-vitro evaluation of anti-diabetic activity of isolated compounds.**

One of the current targets of novel antidiabetic agents to control high blood glucose in the blood is glucose uptake induction. In fact, the most important effects of insulin on glucose metabolism are the regulation of GLUT4 trafficking and, consequently, glucose uptake.[84] Moreover, glucose transport plays the essential role in insulin-regulated glucose metabolism. The isolated compounds with potential antidiabetic activity from *cissus rotundifolia* extract conduct an in vitro evaluation of glucose uptake induction through glucose uptake assay.

3. **In-silico molecular modeling study of isolated compounds targeting citric acid cycle enzymes.**

Gluconeogenesis provides stable blood glucose levels between meals; however, it is found high in diabetic patients. Gluconeogenesis also helps us to maintain appropriate glucose levels when on a diet low in carbohydrates. Therefore, based on the similarity in the chemical structure between the isolated compound from *cissus rotundifolia* and citric acid cycle substrates, our hypothesis builds on investigations of the binding affinity of the isolated compound and citric acid cycle enzymes in order to screen the inhibition activity for gluconeogenesis to control the hyperglycemia. Gluconeogenesis steps start by oxaloacetate, which

is one of the citric acid cycle substrates. Therefore, gluconeogenesis could be reduced through inhibition of citric acid cycle enzymes.

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2 CHAPTER TWO: ISOLATION OF ANTIDIABETIC CONSTITUENTS FROM *CISSUS ROTUNDIFOLIA* THROUGH BIOASSAY – GUIDED FRACTIONATION.

2.1 Introduction

Nature has long been the source of the basic elements of life such as food, medicine, clothing and shelter.[1] Natural products performed a significant role in human disease therapy and compounds derived from natural products have continuously been noted as a valued sources for drug discovery.[1, 2]

Plants are the richest source of remedies and the use of some plants is known in herbal medicine throughout human history.[3-5] The current predicted number of plant species worldwide is around 250,000 species.[6, 7] However, a limited number of these plant species have been screened for medicinal properties. In fact, less than 10% of medicinal plants species have been investigated and validated for their medical activities.[6] Plants have always been a very rich source for drug discovery, and the majority of currently available drugs on the market have been derived directly or indirectly from plants.[6, 8] For example, several available drugs derived from plants for different diseases therapy exist, such as the anticancer drug vincristine from *Vinca rosea*, the narcotic analgesic morphine from *Papaver somniferum,* the antimalarial drug artemisinin from *Artemisia annua*, the anticancer drug Taxol from *Taxus brevifolia*, the antibiotic penicillin from *Penicillium ssp* [1] and the most common antidiabetic available drug, Metformin, derived from *Galega officinalis*.[9] [10]

The *Vitaceae* is a commonly known plant family that forms one of the largest and most diverse plant families worldwide, growing in a variety of environments. This family contains more than 900 known species, occurs in 14 genera, and is mainly spread in tropical and subtropical areas of the world.[11] The largest genus of this family is *Cissus,* including about 350 species. Some of the important genera belonging to the Vitaceae family include *Acareosperma, Ampelocissu, Cyphostemma, Amplopsis, Psedra, Leea*, *Vitis* and *Cissus*.[4] Most of the plants in this family are identified by their climbing vines, tendrils and distinctive clusters of berries. Their leaves are alternate, forming opposite from the tendrils and flowers. Flowers are regular with 4 or 5 small sepals.[12] Vitaceae is also considered one of the most economically important plant families due to the cultivation of many edible Vitaceae species, including the European wine grape (*V. vinifera*) and the North American fox grape (*V. labrusca*) which are the parent species of most of the cultivated slipskin American grapes. The Boston ivy (*q.v.; Parthenocissus tricuspidata*) and the Virginia creeper (*q.v.; P. quinquefolia*) are the most common woody vines in the eastern United States.[4, 13] The Vitaceae family has drawn the attention of many researchers since their fruits, leaves and vegetables are traditionally consumed as food and medicine to treat some skin burns in addition to uses as a diet supplement to reduce the blood glucose level in diabetic patients .[14] A number of plant species from the Vitaceae family have been extensively reviewed in the literature for their medicinal properties, such as *Cissus quadrangularis, C. araloides, C. assamica*, *C. hamaderohensis, C. hypoglauca, C. sicyoides, C. debilis, C. ibuensis, C. populne, C. verticillata* and *C. rotundifolia screened as a crude extracts and showed that they have medicinal activity as anti-inflammatory and anti-malaria, in addition to hypoglycemic*

activity as a methanolic extract however, it is not significantly investigate for the active constituents compounds.[11] Several parts of the plants, including the seeds, fruits and leaves, of the Vitaceae family have been consumed by humans and reported to have medicinal properties.[11, 15]

Cissus rotundifolia is a perennial, climber, evergreen, wild plant belonging to the family of Vitaceae (grape family). It is known as a common Arabian wax cissus, Peruvian Grape Ivy, Venezuelan tree bine and locally (in the south of Saudi Arabia) as Algalaf. It is commonly used as a food thickener. Moreover, it was found to have many therapeutic effects as a hypoglycemic and hypolipidemic , based on the literature study, the hypoglycemic activities of plant extracts of C. rotundifolia still not fully investigated to specific mechanism of action however the study mentioned that the antidiabetic activity of C. rotundifolia extract could be due to the stimulation of insulin secretion from the remaining β-cells which promotes tissue glucose utilization in diabetic rats either by enhancing its uptake and metabolism or by inhibiting hepatic gluconeogenesis. Moreover, C. *rotundifolia* extracts significantly corrected the levels of cholesterol and triglycerides towards normal. This improvement may be partly attributed to the increase in insulin secretion that affects lipid metabolism and to the regeneration of β-cell as a result of the decrease in production of free radicals by lipid peroxidation.[16] In addition, the methanolic extract of *cissus rotundifolia* has been reported to possess antibacterial activity when tested against E. coli, S. infantis, S. aureus and L. monocytogenes in study where only the extracts of edible plants were screened for their antibacterial activity however the investigation of specific active compound need future studies.[17]

.[18] *Cissus rotundifolia* grows extensively in the southern part of Saudi Arabia, and its leaves are widely consumed after cooking as leafy vegetables. *Cissus rotundifolia* extract has been reported to contain components such as flavonoid, hydrolysable tannin and vitamin C. *In addition to,* an appreciable amount of protein, fat, crude fiber and minerals. According to nutritional evaluation of *cissus rotundifolia*, the protein fraction includes a relatively high level of essential amino acids; fat contains a high concentration of unsaturated fatty acids; Macro elements (Magnesium, Sodium, Potassium; Microelements (Iron, Zinc, Manganese, Copper and Chromium). However, up to present, no phytochemical studies have been reported regarding the isolation and identification of the phytoconstituents of *C. rotundifolia.* It is commonly prepared in various dishes according to the traditional dietary culture of locals and is also used as an alternative medicine. However, it has not been studied for its antidiabetic activity. Therefore, this study aims to evaluate the antidiabetic components of *Cissus rotundifolia* leaves.

2.2 Distribution and uses of *cissus rotundifolia* plant in Saudi Arabia

Saudi Arabia is one of the Middle East countries with a population of more than 25 million people. In Saudi Arabia, people live in both urban and rural areas and sectors still use traditional, natural medicine for health care and treatment of diseases.[19] Saudi Arabia has plant biodiversity, which consists of an admixture of elements from variety regions like Asia, Africa, and the Mediterranean. The mountainous area of southwestern Saudi Arabia is characterized by the richness of its flora and species diversity.[20] *Cissus rotundifolia* is a perennial, climber, evergreen wild plant belonging to the family of Vitaceae (grape family).[21] It is known as a common Arabian wax cissus, Peru-vian Grape

Ivy, Venezuelan tree bine and, locally (in south of Saudi Arabia), as Algalaf.[22] It is commonly used as a food thickener. *Cissus rotundifolia* grows extensively in the southern part of Saudi Arabia, and its leaves are widely consumed after cooking as leafy vegetables. It is commonly used to prepare various dishes according to the traditional dietary culture of locals and used as alternative medicine to treat some skin burns and also uses to reduce the blood glucose for diabetic patients. However, it has not been investigated for its antidiabetic activity. Therefore, this study aimed to evaluate the antidiabetic components of *Cissus rotundifolia* leaves using the bioassay guided separation of methanolic extract of *C. rotundifolia* leaves.

2.3 Aim of the study

Identification of the chemical constituents of the plants used in traditional medicine is very important since several studies have found them to have potential biological activities, such as potential antidiabetic activity.[23] All established information will expand our current knowledge of the ethnopharmacology of the plants that have medicinal properties and are used in traditional medicine and might aid in identifying new chemical entities with antidiabetic activities.[23] The aim of the present study was the extraction, isolation and structural elucidation of the active constituents with potential anti-diabetic activity from *Cissus rotundifolia* using bioassay-guided fractionation. *Cissus rotundifolia* (Figure 2-13) (Vitaceae), which is locally called Algalaf, is used in Saudi traditional medicine for the treatment of skin burn, malaria and control of diabetes

disease. *Cissus rotundifolia* grows widely in different regions close to Abha city, Saudi Arabia.

2.4 Molecular target of diabetes mellitus

Diabetes mellitus known as high level of glucose in the blood. The two main sources of glucose in the body comes from either degradation of complex carbohydrates or the producing of the glucose by liver as result of gluconeogenesis process. In order to control of blood glucose level in early stage need to delay the absorption of glucose to the blood through inhibition Alpha-glucosidase enzyme which play an important role on regulation of blood glucose level in the blood.

2.4.1 Alpha-glucosidase (AG) enzyme

Alpha-glucosidase is a carbohydrase enzyme, breaking glycosidic bonds in complex polysaccharides to release absorbable monosaccharides (fig. 2-1).[24] The degradation of dietary carbohydrates is considered the principal source of increased glucose in the blood. After the hydrolysis of the complex dietary carbohydrate, the consequent absorption in the intestine is made by alpha-glucosidases.[25] One of the main strategies to control type 2 Diabetes mellitus (T2D) is to decrease postprandial high blood glucose level by delaying the absorption of glucose through inhibition of carbohydrate-hydrolyzing enzymes, alpha-glucosidase, in the digestive processes.[26]

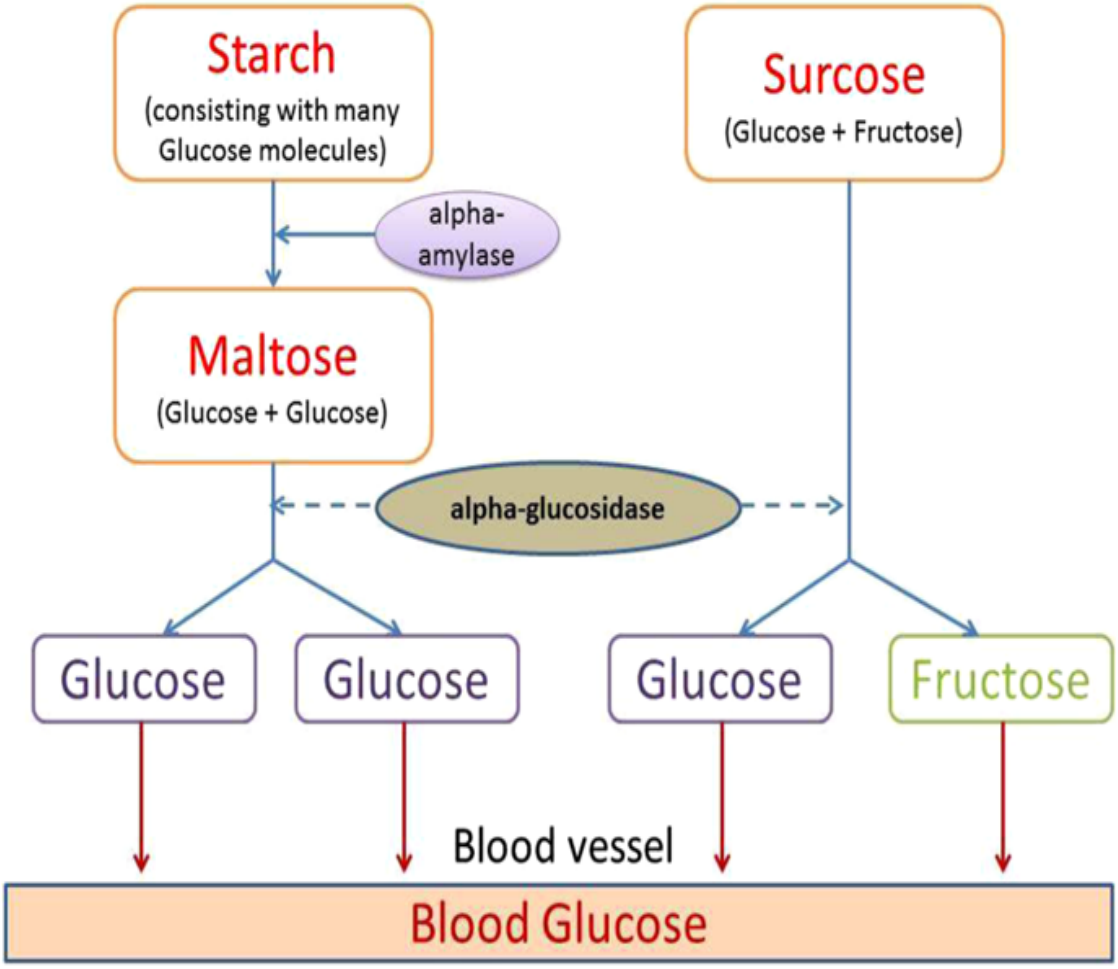


Figure 2-1: Hydrolysis of Polysaccharides to glucose as catalyzed by alpha-glucosidase.

2.5 Materials and methods

2.5.1 General procedures and reagents

^{1}H-NMR, ^{13}C-NMR and 2D-NMR were conducted using Bruker AVANCE-400 MHz and 600 MHz NMR spectrometer in deuterated chloroform ($CDCl_3$) using tetramethylsilane (TMS) as the internal standard; chemical shifts are given in ä (ppm) values. Column chromatography was carried out using silica gel (230–400 mesh) purchased from Sorbent Technologies (Norcross, GA, USA). TLC was performed using pre-coated silica gel PE Sheets purchased from Sorbent Technologies (Norcross, GA, USA), visualized under ultraviolet at 254 nm, stained with Ceric Ammonium Molybdate (CAM) stain and followed by heating. All solvents were obtained from commercial suppliers and used as received.

α-Glucosidase enzyme (Cat. No. G 5003, Sigma Aldrich Chemical Co, USA). Bovine serum albumin (Sigma Aldrich Chemical Co, USA), Sodium azide (Sigma Aldrich Chemical Co, USA). Para nitrophenyl-α-d-glucopyranoside (Cat No: N 1377, Sigma Aldrich Chemical Co, USA). Acarbose (Sigma Aldrich, PHR1253, USA).

2.5.2 Plant materials

Fresh leaves of *Cissus rotundifolia* were collected in June 2016 from the area near to Abha, Saudi Arabia. Al-Sawdah Mountain, the site of *Cissus rotundifolia* plant collection, is located east of Abha, in area between latitude 17°30’ north and 21°00’ north

and longitude 41°30’ east and 44°30’. The plant was botanically authenticated, and a voucher specimen was deposited in Pharmacognosy Department Herbarium, College of Pharmacy, King Khaled University, Abha, Saudi Arabia.

2.5.3 Preparation of plant extracts and fractions

The leaves of *Cissus rotundifolia* (7 kg) were cut into small pieces and homogenized in methanol (a blender was filled to third (1/3) volume with leaves, 1.5 L of methanol was added, the mixture was homogenized for 5 min). Then, the mixture was macerated in methanol for 72 hours. The methanol extract was filtered, concentrated under reduced pressure at 40°C using a rotary evaporator and lyophilized to afford a residue. The dried methanol extract (210 g) was dispersed in deionized water (500 ml) and partitioned sequentially with n-hexane (500 ml×3) and ethyl acetate (500 ml×3).The combined solvent of each partitioned extract was concentrated under reduced pressure at 40°C using the rotary evaporator and freeze dried for 72 hours to yield an n-hexane fraction (32 g), an ethylacetate fraction (20 g) and the remainder of the water fraction (158 g). All fractions were tested for their antidiabetic activity through evaluation of their inhibition activity of α -glucosidase enzyme using α -glucosidase inhibition bioassay.

2.5.4 Isolation

According to the bioassay-guided fractionation, the ethyl acetate fraction showed the greatest potential antidiabetic activity, thus this fraction was selected for further study. The EtOAc fraction was subjected to column chromatography on silica gel (300 g) and eluted with stepwise gradients of n-hexane/EtOAc (100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 45:55, 40:60, 30:70, 20:80, 10:90, 0:100 v/v) and finally with 2 L methanol.

A total number of 532 fractions (20mL each) were collected and combined on the basis of their TLC profiles into five main fractions as follows: fraction I (70-136) (941 mg), Fraction II (136-237) (633 mg), Fraction III (238-298) (460g), Fraction IV (299-363) (302g) and Fraction V (362-534) (720 mg). All these sub-fractions are examined for α -glucosidase inhibition bioassay in order to evaluate their antidiabetic activity through inhibition activity of α -glucosidase enzyme.

2.5.5 Alpha-glucosidase inhibition assay

α-Glucosidase inhibitors act as competitive inhibitors of intestinal α-glucosidase enzyme, which results in delay of the digestion and subsequent absorption of elevated blood glucose levels.[27] The extracts were pre-incubated with the α-glucosidase enzyme before adding the substrate p-nitrophenyl- α-d-glucopyranoside (PNPG). The α-glucosidase enzyme (Cat. No. G 5003, Sigma Aldrich Chemical Co, USA) was dissolved at a concentration of 0.1 U/ml in 100 mM phosphate buffer, pH 7.0, containing bovine serum albumin 2000 mg/ml (Sigma Aldrich Chemical Co, USA), and sodium azide 200 mg/ml (Sigma Aldrich Chemical Co, USA), which was used as enzyme source. Para nitrophenyl-α-d-glucopyranoside (Cat No: N 1377, Sigma Aldrich Chemical Co, USA) was used as substrate. *Cissus rotundifolia* extract was weighed and serial dilutions of 50, 25, and 12.50 mg/ml were made up with equal volumes of dimethylsulfoxide and distilled water. Ten microliters of *C. rotundifolia* extract dilutions were incubated for 5 min with 50 µl of the α-glucosidase enzyme source. Then, 50 µl of substrate was added and incubated for 5 min at room temperature. The pre-substrate and post substrate addition absorbance was measured at 405 nm on a microplate reader (Biotek Instruments Inc, USA). Percent α-glucosidase inhibition was calculated as follows: (1–B/A) Å~ 100,

where (A) is the absorbance of control and, (B) is the absorbance of samples containing extracts.

The activity of the α-glucosidase enzyme was measured by determining the color developed by the release of p-nitrophenol resulting from the hydrolysis of substrate PNPG by α – glucosidase using a spectrophotometric method. Experiments were done in triplicate. Acarbose (Sigma Aldrich, PHR1253, USA) was dissolved in distilled water and serial dilutions of 10, 5 and 2.5 mg/ml were made and used as positive controls.

2.6 Results and Discussion

2.6.1 Isolation and structure elucidation

The methanolic extract of the leaves of *Cissus rotundifolia* was dispersed in deionized water and partitioned sequentially with n-hexane and ethyl acetate. Based on the bioassay-guided fractionation, the ethyl acetate fraction showed the highest potential antidiabetic activities and thus it was subjected to a series of chromatography techniques to yield 6 compounds (Figure 2-12).

Compound **1** was isolated as a white powder, then identified using ^{1}H, ^{13}C and 2D NMR spectra. ^{1}H NMR spectrum showed signals mainly in the upfield region. The spectra showed only two signals with sharp chemical shifts values; the first one resonated in the olefinic region and the other was observed a little up field region. The ^{1}H-NMR spectra of compound 1 also exhibited a signal corresponding to the proton connected to C-3 hydroxyl group, which appeared as a multiplet at δ 3.46 (1H, m). Six other proton signals were marked, including four secondary methyl groups (δ_H 0.91, 0.82, 0.81 and 0.79 all doublets with J = 6.6, 7.2, 6.4 and 6.4 Hz respectively) and two tertiary methyl groups (δ_H 0.66 and 0.99. Since *Cissus rotundifolia* extract has been

reported to contain components such as sterols. The olefinic signal at δ 5.28 (1H, J = 4.8 Hz) appeared to be characteristic of the sterols, and it was assigned to the H-6 proton in the β–sitosterol (1) chemical skeleton. The ^{13}C NMR spectra exhibited 29 carbon signals characteristic of phytosterols: ^{13}C NMR (151 MHz, $CDCl_3$) δ 140.77, 121.74, 71.82, 56.78, 56.06, 50.14, 45.84, 42.34, 42.32, 39.79, 37.27, 36.52, 36.16, 33.95, 31.93, 31.92, 31.68, 29.72, 29.15, 28.27, 26.07, 24.32, 23.08, 21.10, 19.84, 19.04, 18.79, 12.00, 11.88. In addition to 2D, DEPT135 showed that 11 carbon signal inverted as represent of CH2 group: ^{13}C NMR (151 MHz, CDCl3) δ 121.75, 71.83, 56.78, 56.06, 50.13, 45.83, 42.31 (inverted), 39.78 (inverted), 37.26 (inverted), 36.16, 33.95 (inverted), 31.91, 31.67 (inverted), 29.73 (inverted), 29.14, 28.27 (inverted), 26.06 (inverted), 24.32 (inverted), 23.07 (inverted), 21.98, 21.10 (inverted), 19.82, 19.42, 18.79, 12.00, 11.88.. These data corresponded with the structure of the β-sitosterol compound (Figure 2-2). The NMR data of compound 1 (β-sitosterol) is in agreement with the published values.[28]

Compound **2** was isolated as white powder. ^{1}H NMR spectra of **2** showed one methyl group signal at δ 3.72 (s, 3H) and a doublet signal at δ 2.84 (m, 1H) and 2.71 (d, J=*1.2Hz*, 1H). Further, it showed a proton signal at δ 4.54- 4.62 (d, *J* = 2.5 Hz, 3H) with a secondary alcoholic proton signal δ 2.09 (d, *J* = 49, 2.8 Hz, 1H). The ^{13}C-NMR data of compound **2** showed the presence of seven carbon signals in addition to two carbon signals for acetone solvent at δ 30.03 and 202.07. The ^{13}C-NMR data present two carbonyl signal at δ 174.25, 171.98 and two peaks showed at the olefinic region at δ 110.23 and 145.33. One carboxylate methyl ester at δ 52.28.19 and C2 hydroxyl at 68.19 ppm respectively. One carbon signal at δ 39.36 which was assigned to methylene CH2 based on calculated NMR data and 2D NMR Dept 135 spectrum that showed signal at δ

39.36 (inverted). Stereochemistry at C-2 could not be established at this point, further X-ray analysis to establish the chirality at C-2 at this point could not be conducted. The above data indicated that structure **2** was characterized as 1-methyl 4-vinyl 2-hydroxysuccinate (Figure 2-2). The NMR data of compound **2** corresponds with the published values. These dicarboxylic acids derivatives are widespread in nature and often appear with other functional groups. In addition, several synthesized dicarboxylate have wide spread applications in food industry and supplement preparations.[29]

Compound **3** was isolated as yellow powder. ^{1}H NMR (600 MHz, $CDCl_3$) spectra of **3** showed two methyl group signals at δ 3.81 (s, 3H) and 3.71 (s, 3H) and a hydroxyl group proton at δ 3.39 (s, 1H) with a doublet signal at δ 2.83 (ddd, J = 22.7, 16.4, 5.3 Hz, 2H) and secondary alcoholic proton signal at δ 4.53 – 4.50 (m, 1H). The ^{13}C-NMR data of compound **3** showed the presence of six carbon signals in addition to a carbon signal for chloroform $CDCl_3$. The ^{13}C-NMR data presented two carbonyl signals at δ 173.74 and 171.03, and three oxy carbons signals at δ 67.24, 52.82 and 52.03 ppm, which correspond to the dimethyl ester and C-2. One carbon signal, at δ 38.44, was assigned as C2 as result of 2D NMR Dept 135 spectrum that showed signal at δ 38.44 (inverted). Stereochemistry at C-2 could not be established at this point, further X-ray analysis to establish the chirality at C-2 at this point could not be conducted. The above data indicated that structure **3** was characterized as 1,4-dimethyl 2-hydroxybutanedioate (Figure 2-2). The NMR data of compound 3 is in agreement with a cited literature value.[30]

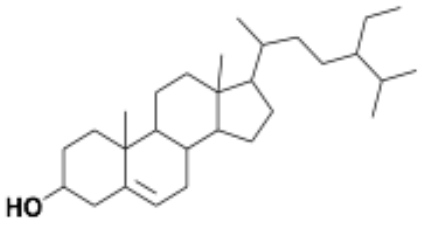
Compound **4** was isolated as white crystal. ^{1}H NMR (600 MHz, Acetone) spectra of **4** showed carboxylic acid proton at δ 8.42 (s, 1H), and one methyl group signal at δ 3.71 (s, 3H) with a doublet signal at δ 2.76 (ddd, J = 23.4, 16.2, 5.9 Hz, 2H) and

secondary alcoholic proton signal at δ 4.54 (dd, J = 7.2, 4.7 Hz, 1H). The ^{13}C-NMR data of compound **4** showed the presence of five carbon signals, in addition to two carbon signals for acetone solvent. The ^{13}C-NMR data presented two carbonyl signals at δ 174.39 and 172.68, and two carboxylate methyl signals showed at δ 68.29 and 52.58 ppm. One oxy-carbon signal at δ 39.36 which was assigned to C2 hydroxyl based on calculated NMR data and 2D NMR Dept 135 spectrum that showed signal at δ 39.36 (inverted). Stereochemistry at C-2 could not be established at this point, further X-ray analysis to establish the chirality at C-2 at this point could not be conducted. The above data indicated that structure **4** was characterized as 3-hydroxy-4-methoxy-4-oxobutanoate (Figure 2-2). The NMR data of compound **4** is in agreement with the published values.[31]

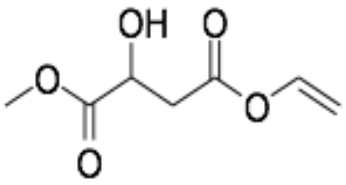
Compound **5** was isolated as a white powder, then identified using ^{1}H, ^{13}C and 2D NMR. ^{1}H NMR (600 MHz, $CDCl_3$) spectra of **5** showed two proton signals at δ 7.88 (s, 1H) and 6.82 (s, 1H), assigned for C-1 and C-2. Then, a proton signal at δ 4.50 (d, 1H) assigned for CHOH carbon proton and at δ 2.84 (m, 1H) proton assigned for hydroxyl group proton. In addition, a signal proton showed at δ 3.74 (t, 3H) and δ 3.65 (m, 3H) assigned for C-3 and C-5 as two methyl groups. Two signal peaks showed at δ 2.78 (m, 2H) and δ 2.70 (m, 2H) assigned for CH2 at C-6 and C-7. The ^{13}C-NMR data of Compound **5** presented twelve carbon signals. Two carbonyl signals at δ 173.78 and 171.15, and three methoxy carbons signal showed at δ 67.22, 52.42 and 52.12 ppm. One oxy-carbon signal at δ 38.44 which was assigned to C2 hydroxyl based on calculated NMR data and 2D NMR Dept 135 spectrum that showed signal at δ 39.36 (inverted). Stereochemistry at C-2 could not be established at this point, further X-ray analysis to establish the chirality at C-2 at this point could not be conducted. Two carbon peaks of **5**

spectra showed at δ 132.34 and δ 115.29 assigned for C-3 and C-4. The above data indicated that structure **5** was characterized as Dimethyl-2-hydroxy-5-methylenehexanedioate (Figure 2-2). The NMR data of compound **5** is in agreement with a cited literature value.[32]

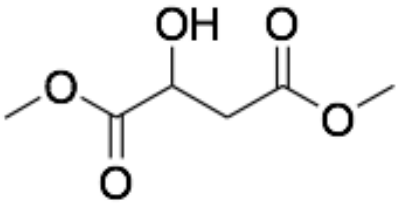
Compound **6** was isolated as a white powder, then identified using ^{1}H, ^{13}C and 2D NMR. ^{1}H NMR (600 MHz, $CDCl_3$) spectra of **6** showed proton signals at δ 4.72 (s, 1H) assigned for C-2 as and proton signal at δ 2.64 (m, 1H) assigned for hydroxyl group at C-2. The ^{1}H-NMR spectra of compound **6** also exhibited a signal corresponding to the proton connected to C-3, which appeared as a multiplet at δ 2.66-2.54 (m, 2H). The ^{13}C NMR spectra of compound **6** exhibited 4 carbon signals δ 178.26, 175.55, 67.287 and 39.36. At δ 178.26, AT δ 175.55 showed two carbon signals assigned for carbonyl groups at C-1 and C-4. Also, compound **6** spectra showed carbon peak at δ 67.287 and was assigned for methoxy group at C-2. Stereochemistry at C-2 could not be established at this point, further X-ray analysis to establish the chirality at C-2 at this point could not be conducted. Carbon peak signal showed up filed at δ 39.36 and was assigned to CH_2 at C-3. The above data indicated that structure **5** was characterized as 2-hydroxysuccinic acid (Figure 2-2). The NMR data of compound **6** is in agreement with the published values.[33]



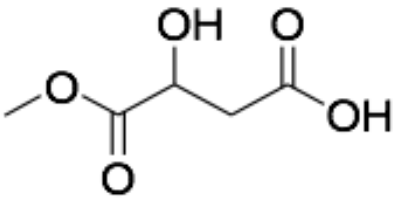
β-sitosterol
(1)



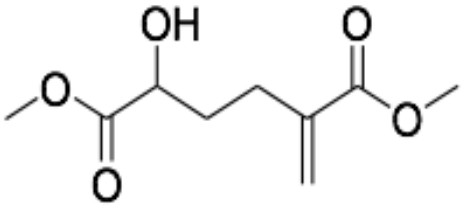
1-methyl 4-vinyl 2-hydroxysuccinate
(2)



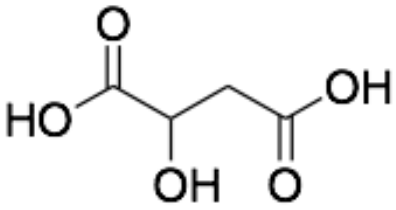
1,4-dimethyl 2-hydroxybutanedioate
(3)



3-hydroxy-4-methoxy-4-oxobutanoate
(4)



Dimethyl-2-hydroxy-5-methylenehexanedioate
(5)



2-hydroxysuccinic acid
(6)

Figure 2-2: Isolated compounds (1-6) from Cissus extract

2.6.2 Biological evaluation

α-Glucosidases are a series of enzymes located on the intestinal brush-border. The source of important carbohydrates in diet (e.g. starch and sucrose) are hydrolyzed to monosaccharide (glucose and fructose) by α-glucosidase, and then absorbed into the blood to increase blood glucose level. Normally, these processes happen in the upper portion of the small intestine and greatly increase blood glucose concentration, especially in diabetic patients. [34] According to literature study proposed that α-Glucosidase inhibitors (e.g. acarbose) increases the duration time of carbohydrate absorption and flatten the blood-glucose concentrations. Therefore, acarbose have been used as the first-line drugs in treatment of diabetes.[34] In this study, the aims were to explore the active compound compared with acarbose and evaluate how this active compound inhibited α-glucosidase activity. Hexane, ethyl acetate and aqueous extracts, *cissus rotundifolia* showed variable inhibition of alpha-glucosidase enzyme based on alpha-glucosidase inhibition assay. Alpha-glucosidase inhibition was measured in the concentration range of 12.5 mg/mL to 50 mg/mL for each fraction and 12.5 uM to 100 uM for each pure compound. As shown in figure 2-2, the ethyl acetate fraction exhibits significant inhibition effects on alpha-glucosidase enzyme at different concentrations of 12.50, 25, and 50 mg/mL, respectively, while the n- hexane fraction was found to have lowest inhibition activity. The aqueous fraction showed slight inhibition activity against alpha-glucosidase enzyme. In addition, the ethyl acetate fraction showed a concentration-dependent inhibitory effect on the alpha-glucosidase enzyme. These results obviously suggest that the ethyl acetate fraction possesses the highest inhibition activity and led us

to carry out a study to identify the active constituents that may contain potential antidiabetic compounds.

The ethyl acetate fraction subjected to column chromatography on silica gel to give five main fractions (F1- F5). Fraction 1 afforded pure sitosterol compound **1** (Figure 2-12). Both fractions 3 and 4 demonstrated the highest inhibition activity in a concentration-dependent manner. The bioassay guided purification of subfractions resulted in the isolation and identification of six compounds: SAA1 (**1**), SAA2 (**2**), SAA3 **(3),** SAA4 **(4**), SAA5 **(5**) and SAA6 **(6**) (Figure 2-12). Compound **3** showed the most promise with significant inhibition 51 % of alpha-glucosidase enzyme activity. The dose-dependent manner present at four different concentration 100, 50, 25 and12.5 uM of the promising compounds and acarbose was used as a positive control (figure 2-8).

In most reports, the mechanism of the inhibition against α-glucosidase was not yet clear. [26] In our experiments, six compounds isolated from *Cissus rotundifolia* showed different degree of activity against α-glucosidase.1,4-Dimethyl 2-hydroxybutanedioate **(3)** and 3-hydroxy-4-methoxy-4-oxobutanoate **(4)** had promising inhibition activity, and that might be because of C-2 hydroxyl group and dimethyl carboxylate at C-1 and C-4 in compound **3** that also strengthen α-glucosidase inhibition activity. At the same time, from compounds **2**, **5** and **6**'s structures, we inferred that if dimethyl group at C-1 and C-4 were changed, it might cut down α- glucosidase inhibition activity. The difference inhibition activity between compound **2** and compound **3** could be related to the structural difference at C-4 the vinyl ester). However, the increase of compound **3** inhibition activity could be attributed to the dicarboxylate methyl ester at C-1 and C-4. It is worth mentioning that dimethyl ester is relatively non-polar and therefore the polarity

difference between active and nonactive compounds could be the reasons of this differences in the inhibition activity. The data from this study also indicated that 1,4-dimethyl 2-hydroxybutanedioate **(3)**, as the most effective compound, displayed a significantly inhibitory activity against α-glucosidase. All the findings indicated that *cissus rotundifolia* has potential activity for treatment of diabetes, and 1,4-dimethyl 2-hydroxybutanedioate **(3)** as the most active compound showed inhibition of α-glucosidase activity.

2.7 Conclusion

Cissus rotundifolia (Vitaceae), called Algalaf in Arabic, is used in Saudi folk medicine for the treatment of skin, malaria and control of diabetes. The chemical constituents were examined to determine chemical profile and potential antidiabetic activity. In the present study, bioassay-guided fractionation and purification were used to isolate the antidiabetic compounds of an extract of *Cissus rotundifolia* leaves. All fractions, sub-fractions and pure compounds were screened for their antidiabetic activity against the alpha-glucosidase enzyme. The highest inhibition activity was found to be in the ethyl acetate fraction, resulting in the isolation of six compounds identified as sitosterol (1), SAA2 (2), SAA3 (3), SAA4 (4) and SAA5 (5). Among the compounds isolated and tested for the first time, SAA3 and SAA4 showed potent antidiabetic activities through inhibition of the alpha-glucosidase enzyme different concentrations. This finding may help us to identify new antidiabetic compounds as inhibitor of alpha-glucosidase enzyme.

Inhibitory activity for *cissus rotundifolia* fractions against alpha-glucosidase enzyme

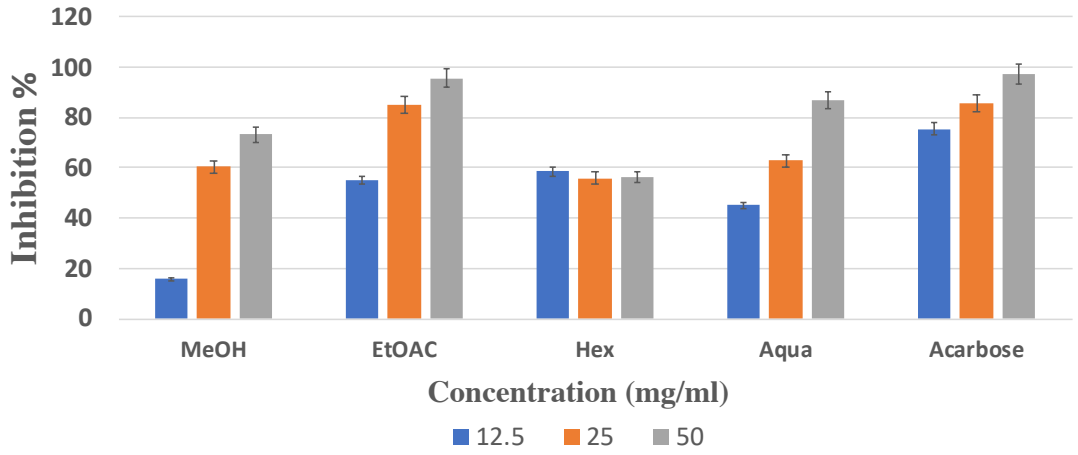


Figure 2-3 : Alpha-glucosidase enzyme inhibition of Cissus rotundifolia fractions

Inhibitory activity for ethyl acetate sub-fractions against alpha-glucosidase enzyme

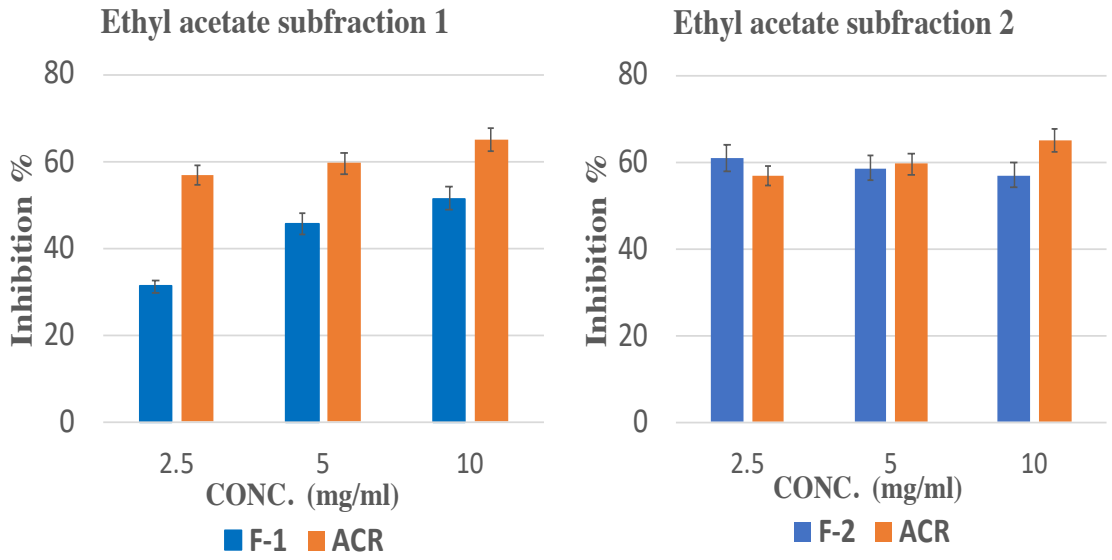


Figure 2-4: Ethyl acetate sub-fractions (1&2) at three different concentrations (10, 5, 2.5 mg/l), represented in blue,

compared with Acarbose as positive control, represented in orange, to show the inhibition of alpha-glucosidase enzyme activity.

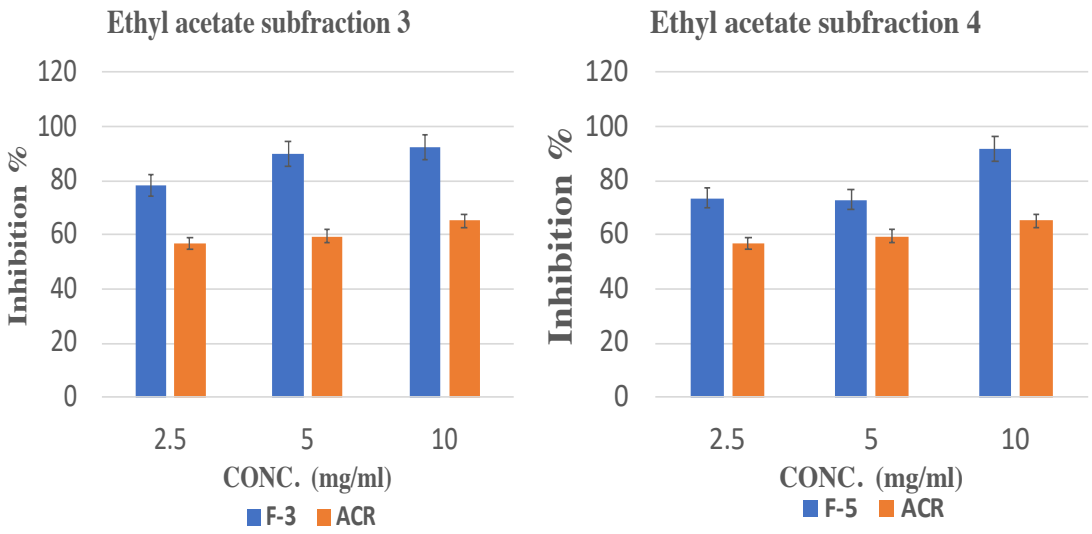


Figure 2-5 : Ethyl acetate sub-fractions (3 & 4) at three different concentrations (10, 5, 2.5 mg/l), represented in blue, compared with Acarbose as positive control, represent in orange, to show the inhibition of alpha-glucosidase enzyme activity.

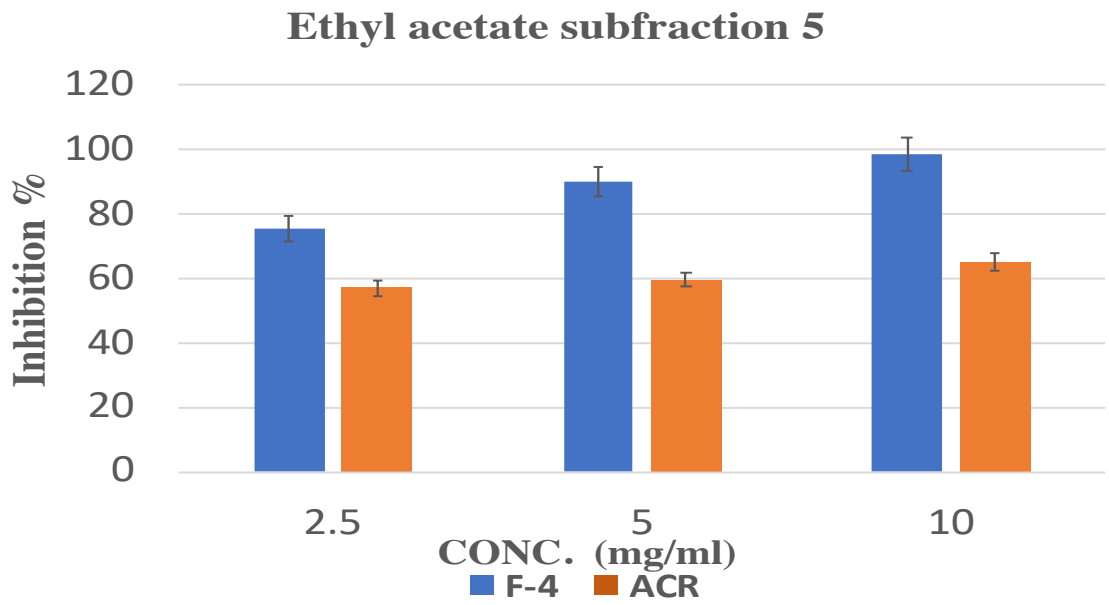


Figure 2-6 : Ethyl acetate sub-fraction (5) at three different concentrations (10, 5, 2.5 mg/l), represented in blue, compared with Acarbose as positive control, represented in orange, to show the inhibition of alpha-glucosidase enzyme activity.

Inhibitory activity for isolated compounds against alpha-glucosidase enzyme.

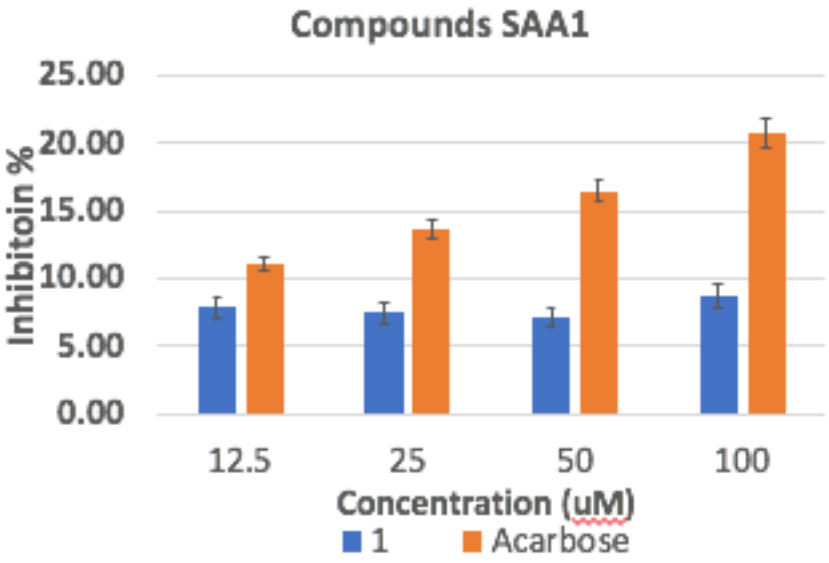


Figure 2-7 : Isolated compound (1) at four different concentrations (100, 50, 25, 12.5 µM), represented in blue, compared with Acarbose as positive control, represented in orange, to show the inhibition of alpha-glucosidase enzyme activity.

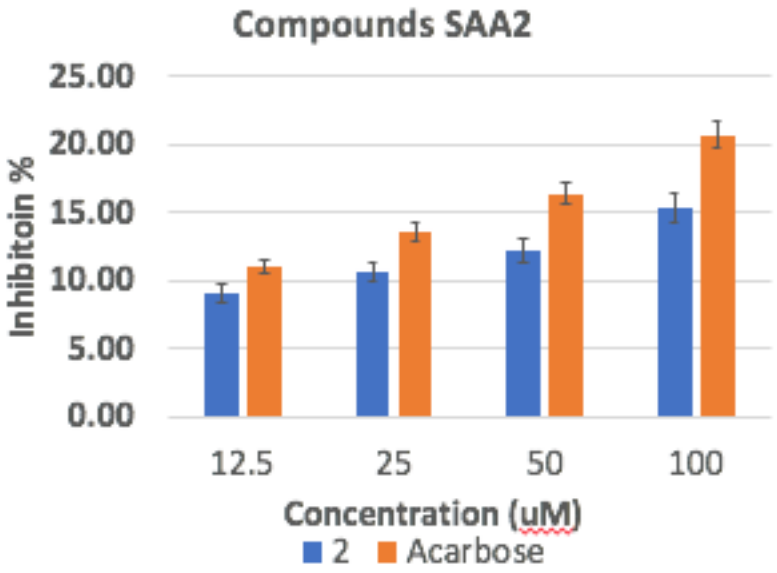


Figure 2-8 : Isolated compound (2) at four different concentrations (100, 50, 25, 12.5 µM), represented in blue, compared with Acarbose as positive control, represented in orange, to show the inhibition of alpha-glucosidase enzyme activity.

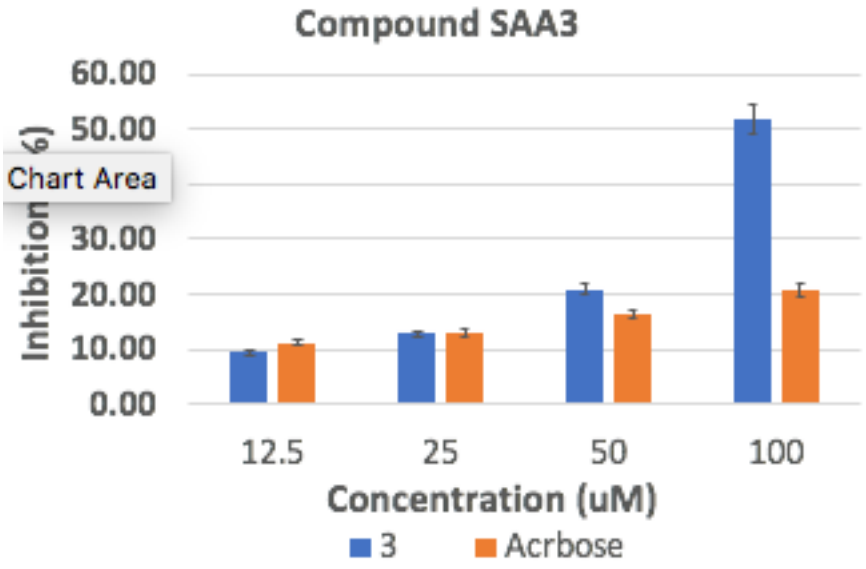


Figure 2-9 : Isolated compound (3) at four different concentrations (100, 50, 25, 12.5 µM), represented in blue, compared with Acarbose as positive control, represented in orange, to show the inhibition of alpha-glucosidase enzyme activity.

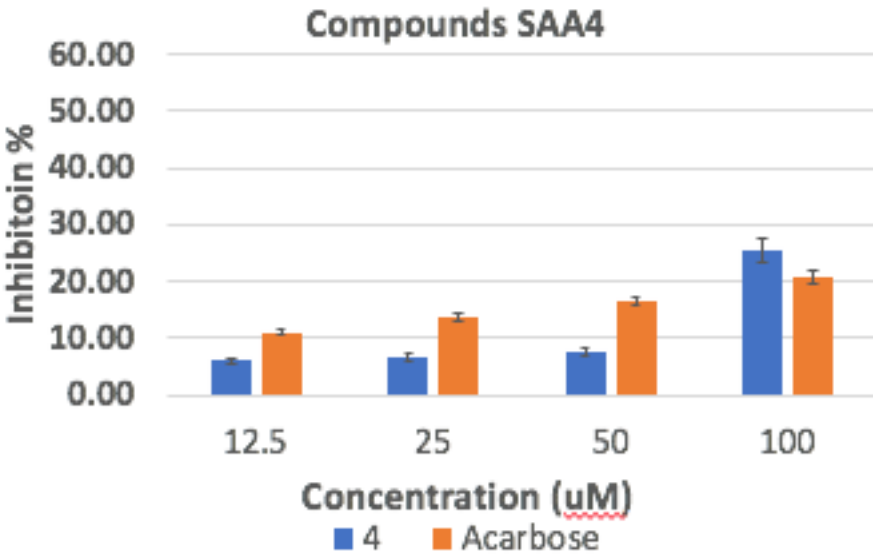


Figure 2-10 : Isolated compound (4) at four different concentrations (100, 50, 25, 12.5 µM), represented in blue, compared with Acarbose as positive control, represented in orange, to show the inhibition of alpha-glucosidase enzyme activity.

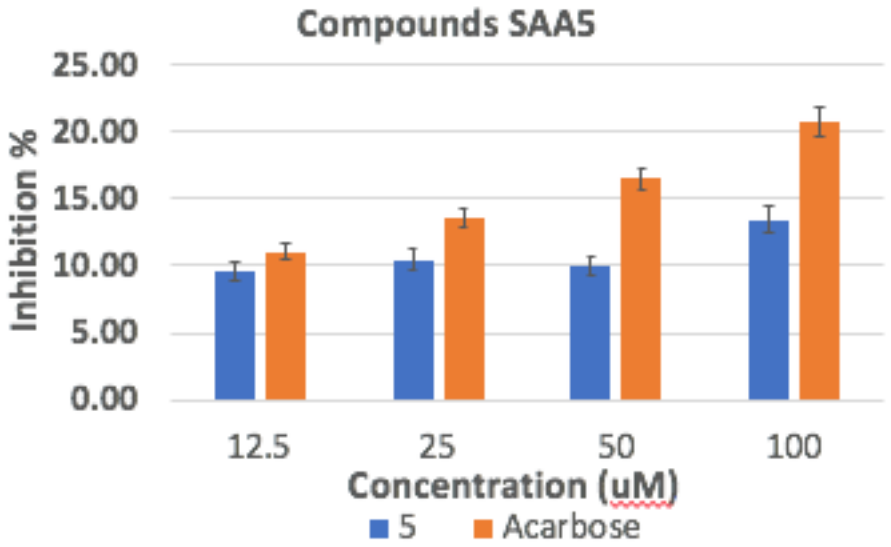


Figure 2-11 : Isolated compound (5) at four different concentrations (100, 50, 25, 12.5 µM), represented in blue, compared with Acarbose as positive control, represented in orange, to show the inhibition of alpha-glucosidase enzyme activity.

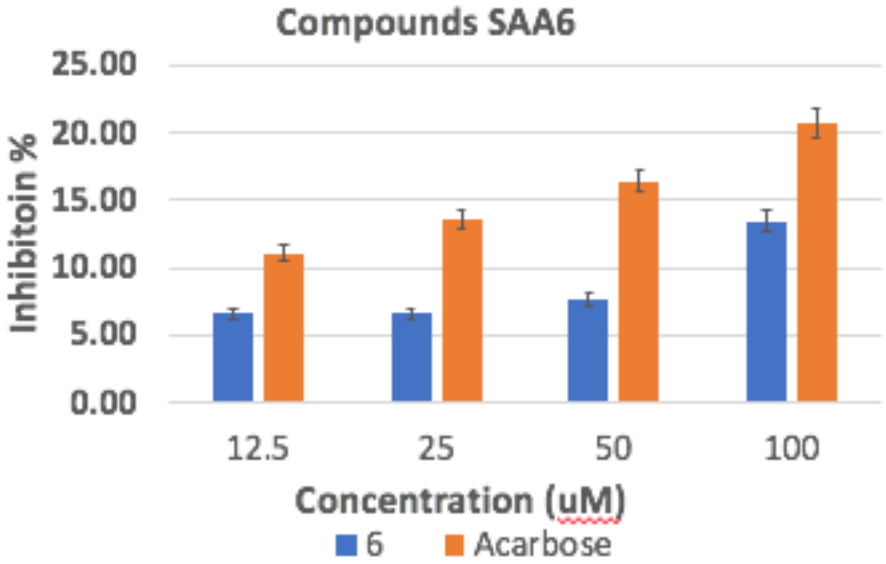


Figure 2-12 : Isolated compound (6) at four different concentrations (100, 50, 25, 12.5 µM), represented in blue, compared with Acarbose as positive control, represented in orange, to show the inhibition of alpha-glucosidase enzyme activity.

Figure 2-13 : Photo of: Cissus Rotundifolia plant. (Vitaceae)

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3 CHAPTER THREE: IN-VITRO EVALUATION OF ANTI-DIABETIC ACTIVITY OF ISOLATED COMPOUNDS.

3.1 Introduction

Diabetes mellitus is a metabolic disease characterized by high blood glucose level. The major risk of diabetes is obvious based on the dramatically high distribution rate of this disease. Recently, current estimations showed that the number of diabetics will increase up to 439 million in 2030.[1, 2]The majority of diabetics, more than 90%, are type 2 diabetics.[3] Type 2 diabetes mellitus (T2DM) is characterized by insulin resistance in the hepatic and peripheral tissues. Glucose transporter 4 (GLUT4) plays a main role in the pathophysiology of T2DM[4]. Generally, it is defective expression or translocation to the peripheral cell plasma membrane in type 2 diabetes mellitus patients that delays the entrance of glucose into the cell for energy production.[5] Type 2 diabetes (T2DM) is identified by insulin resistance and a progressive weakening in β-cell function.[6, 7] Recently, it has been reported that insulin resistance is one of the most important factors in the pathogenesis of type 2 diabetes, which is known not only by reduced responsiveness of the peripheral target tissues like liver, skeletal muscle and adipose tissue to insulin, but also by a remarkable decrease in glucose uptake and utilization.[8, 9] Therefore, inducing glucose uptake in the above three key tissues is one of the main effective therapeutic approaches for treating Type 2 diabetes.[10] Meanwhile, natural products are the main source of drug discovery.[11]

Cissus rotundifolia (fam. Vitaceae) is one of the traditional medicinal plants widely distributed in south western part of Saudi Arabia. *C. rotundifolia* leaves have been used as a folk herbal drug to treat skin, bacterial infections, inflammatory conditions and flu in alternative medicine.[12, 13] It has also been reported to possess antibacterial activity when tested against E. coli, S. infantis, S. aureus and L. monocytogenes in study where only the extract of edible plant were screened for their antibacterial activity however the investigation of specific active compound need future studies.[14].[15] *Cissus Rotundifolia* as a crude extract has been reported to possess biological activities.[16, 17, 18] For example, antimalarial and anti-inflammatory effects as an extract.[16, 19] However, there is no report on the antidiabetic activity of *C. Rotundifolia* leaves antidiabetic activity such as glucose uptake or metabolism. Therefore, this study was to investigate whether the leaves of *C. rotundifolia* fractions possessed antidiabetic activity or not through inducing glucose uptake.

3.2 Glucose uptake pathway

Through an intracellular pathway, insulin induces translocation of glucose transporters (GLUTs) into the cell membrane of the cells.[20-22] Actually, the most important effects of insulin on glucose metabolism are the regulation of GLUT4 trafficking and, consequently, glucose uptake.[23] Furthermore, glucose transport plays the essential role in insulin-regulated glucose metabolism, including glycogen synthesis, glycolysis and lipogenesis, where a dysfunction in this route in muscle and adipose tissue signifies an important defect in the insulin action.[24, 25] Insulin receptor is a heterotetramer, involving two extracellular α subunits (binding fraction) and two transmembrane β subunits (with intrinsic tyrosine kinase) linked by disulfide bonds.[26] In insulin-responsive

tissues, such as liver, skeletal muscle and adipose tissue, the action of insulin begins by binding to its specific receptor.[27] The activation of the insulin receptor makes a structural change in the α subunit, leading to autophosphorylation of a tyrosine kinase domain of β subunits and subsequent tyrosine phosphorylation of numerous protein intermediates, involving insulin receptor substrate (IRS -1, 2, 3 and 4).[28, 29] Both of these phosphorylated substrates identify and bind to domains with homology SH2, especially phosphatidylinositol-3-kinase (PI3-K).[28, 30] Then, upon attachment to the cell surface, protein kinase B (AKT) is activated, resulting in the AKT-dependent phosphorylation of many substrates. In addition to glucose uptake, virtually all of insulin's metabolic effects are regulated by AKT.[31] For example, AKT-dependent phosphorylation of glycogen synthase kinase 3 (GSK-3β) leads to the activation of glycogen synthase and enhances glucose storage as glycogen.[32, 33] Generally, the most important effects of insulin on glucose metabolism are the regulation of GLUT4 trafficking and, consequently, glucose uptake.[23] Moreover, glucose transport plays the essential role in insulin-regulated glucose metabolism. Therefore, the isolated compounds with potential antidiabetic activity from *cissus rotundifolia* extract conduct to an in vitro evaluation of glucose uptake induction through glucose uptake assay.

3.3 Aim of the study

Natural bioactive compounds have a long history of effective use in the treatment of diabetes mellitus. *Cissus rotundifolia* (Vitaceae) have been used locally (in southwestern part of Saudi Arabia), for the treatment of different ailments such as skin

disease, malaria and hypoglycemia.[34]. Although the hypoglycemic effect of *C. rotundifolia leaves* extract has been reported, the exact mechanism of this effect has not been fully discovered. [19] In fact, the most important effects of insulin on glucose metabolism are the regulation of GLUT4 trafficking and consequently glucose uptake.[23] Moreover, glucose transport plays the essential role in insulin-regulated glucose metabolism, including glycogen synthesis, glycolysis and lipogenesis. The present work aims to study the effect of fractions and the isolated bioactive compounds of *Cissus rotundifolia* extract on glucose uptake in liver cell lines (HepG2). This study evaluates the glucose uptake activity of *cissus rotundifolia* leaves methanolic extract fractions, sub fractions and the isolated compounds in HepG2 cell *in vitro.* The isolated compounds, cell viability and antidiabetic activity of the extract were also discussed.

3.4 Materials and methods

3.4.1 Reagents

Dimethyl sulfoxide (DMSO), 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium-bromide (MTT), sodium dodecyl sulphate (SDS). Fetal bovine serum (FBS) was purchased from HyClone (Logan, UT, USA). Dulbecco's modified Eagle medium (DMEM), phosphate buffered saline (PBS) 1X solution and trypsin were purchased from Gibco (Grand Island, NY, USA). Glucose (Oxidase) Liquid Reagents were purchased from Fisher Scientific (Turnberry Hanover, IL, USA). Insulin solution from bovine pancreas at 10 mg/mL insulin, pH 8.2, BioReagent, sterile-filtered, suitable for cell culture was used as positive control.

3.4.2 Extracts and Isolated Compounds

Fresh leaves of *Cissus rotundifolia* were collected in June 2016 from the area near Abha, Saudi Arabia. The plant was botanically authenticated, and a voucher specimen was deposited in Pharmacognosy Department Herbarium, College of Pharmacy, King Khaled University, Abha, Saudi Arabia.

3.4.3 Preparation of plant extracts and fractions

The leaves of *Cissus rotundifolia* (7 kg) were cut into small pieces and homogenized in methanol (a blender was filled to third (1/3) volume with leaves, 1.5 L of methanol was added, and the mixture was homogenized for 5 min). Then, the mixture was macerated in methanol for 72 hours. The methanol extract was filtered, concentrated under reduced pressure at 40°C using a rotary evaporator and lyophilized to afford a residue. The dried methanol extract (210 g) was dispersed in deionized water (500 ml) and partitioned sequentially with n-hexane (500 ml×3) and ethyl acetate (500 ml×3).The combined solvent of each partitioned extract was concentrated under reduced pressure at 40°C using the rotary evaporator and freeze dried for 72 hours to yield an n-hexane fraction (32 g), an ethylacetate fraction (20 g) and the remainder of the water fraction (158 g).

3.4.4 Cell Culture

A human cancer liver cell line (HepG2) was obtained from American Type Cell Culture (ATCC, Rockville, MD, USA). The HepG2 cell line was maintained at 37°C in a humidified atmosphere of 5% CO2 in a DMEM medium containing 10% fetal bovine serum and antibiotics (100 IU/mL penicillin and 100 μg/ml).

3.4.5 Cell viability assay

Cell viability was determined by the MTT method, as previously described, with a modification. Briefly, cells (1 ×104cells/well) were seeded into a 96-well plate and allowed to attach to the well overnight. Fractions were then added at different concentrations (100, 50, 25, 12, 50 mg/ml) and cells were incubated for a further 24 hours. After incubation, 10 µL of 5 mg/mL of MTT dye was added to the cells for 4 hours at 37°C, followed by the addition of 100 µL of 10% SDS in 0.01 N HCl as a solubilizing agent. The absorbance at 570 nm was recorded using an ELISA microplate reader. The results of viability were expressed as a percentage of the control.

3.4.6 Glucose uptake assay

Glucose uptake using human liver cancer cell line (HepG2) as liver tissue plays a significant role in controlling blood glucose level. Briefly, HepG2 cells were seeded in 96-well plate with some wells left blank. Then, when the cells gained confluence, the medium was exchanged for DMEM containing 0.2% BSA and incubated for 18 hours. After incubation, the cells were treated with DMEM containing 0.2% BSA with the selected concentrations of test samples for another 24 h. Then 10 uL of medium was removed from each well and placed into a new 96-well plate that contained 200 uL of glucose oxidase reagent (Glucose CII-Test, IL, USA). Finally, the plate was incubated at 37C for 15 min and the optical density (OD) was measured at 490 nm using a microplate reader. The percent of glucose uptake was estimated by subtracting the glucose concentrations of the blank wells from the remaining glucose in the cell plated wells. Insulin (10 mg/mL) was used as positive control.

3.5 Results and Discussion

3.5.1 Cell viability assay

The cell viability of the methanol extract and the fractions of ethylacetate and hexane from *Cissus rotundifolia* on HepG2 cells was assessed by MTT assay (Figure 3-1). At the concentration of 100 mg/mL, the cell viability of the extract was about 85% for HepG2 and the cell viability of the ethylacetate fraction was evaluated at concentration 100, 50, 25, 12, 50 mg/ml respectively as presented in figure 3-1. Cissus rotundifolia extracts toxicity was tested in vitro in HepG2 cell line using MTT assay. Extract concentrations that kept at least 80% cell viability were considered as safe. MeOH extract (Fig. 3-1) was found to be safe up to 100 mg/ml where's hexane (Fig. 3-1) and aqueous extract (Fig. 3-1) were found to be lower than 80% cell viability at 100 mg/ml. Ethyl acetate extract (Fig. 3-1) was found to be safe up to 100 mg/ml that showed 86% cell viability at 100 mg/ml. Accordingly, all the efficacy studies for the three extracts were performed at safe concentrations. Therefore, different doses of the methanol extract and fractions from *C. rotundifolia* leaves (12.5, 25, 50 and 100 mg/mL) were used in the following glucose uptake experiments.

Cell Viability effect of *Cissus rotundifolia* on HepG2 cell line

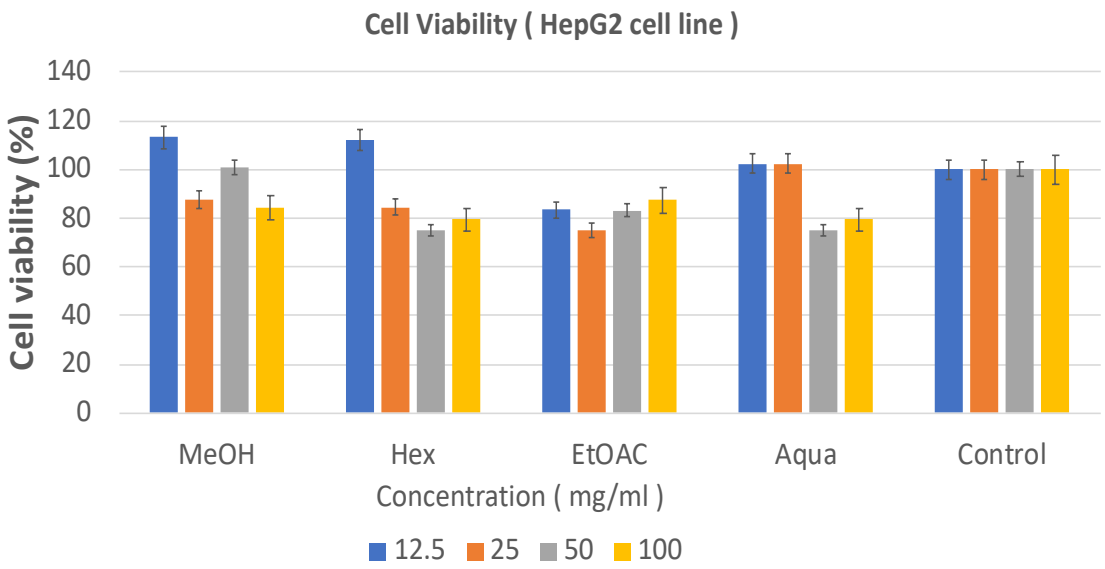

Figure 3-1 : Comparison of cell viability results obtained using MTT. Cells were treated with 12.5-100 mg/ml of each fraction represent in (12.5 (blue), 25 (orange), 50 (gray), and 100 (yellow)). MTT assay was used to measure the cell viability %..

3.5.2 Glucose uptake assay

The effect of methanol, ethylacetate and hexane extracts of *C. rotundifolia* leaves on glucose uptake in HepG2 cells are illustrated in Figure 3-2. The ethylacetate fraction showed a significant induction of glucose uptake in HepG2 cells with a value of 27% at the concentration of 50 mg/mL compared with insulin as positive control. Insulin increased glucose uptake in HepG2 cells of about 18%, while the hexane fraction showed 14 % inducing glucose uptake activity and the aqueous fraction showed 20 % induction of glucose uptake activity in HepG2 cells. So, the result indicates that the ethylacetate fraction has a promoting effect on HepG2 cells and the ethylacetate fraction has the most promising induction of glucose uptake, represented in figure 3-2. Moreover, the present study was conducted to evaluate the effect of six compounds were isolated from *cissus rotundifolia* on glucose uptake activity in HepG2 cell line, and insulin used as positive control. Furthermore, this study highlights potential anti-diabetic compounds in cissus

rotundifolia. The results indicate that the isolated compounds 3-hydroxy-4-methoxy-4-oxobutanoate (SAA4) and 1,4-dimethyl 2-hydroxybutanedioate (SAA3) have promising glucose uptake inducing activity (Figure 3-4). 3-Hydroxy-4-methoxy-4-oxobutanoate (SAA4) has the most significant induction of glucose uptake activity about 26% at 100 uM concentration. Additionally, isolated compound 1,4-dimethyl 2-hydroxybutanedioate (SAA3) showed the enhancement in glucose uptake by 24% at 100 uM concentration. While the rest compounds β-sitosterol (SAA1), dimethyl-2-hydroxy-5-methylenehexanedioate (SAA5), and 2-hydroxysuccinic acid (SAA6) showed no effect on glucose uptake inducing activity in comparison to control in HepG2 cell line (figure 3-7). Compound 4 was found to be most promising compound over the isolated compounds for inducing glucose uptake in liver cell line. Generally, liver is major site of glycogen storage and thus, glucose uptake activity is important to maintain normal blood glucose level[35]. Present study showed that the isolated bioactive compounds SAA4 and SAA3 enhance the glucose uptake in HepG2 at dose dependent manner which may be due to its effect on the receptors on the cell membrane in liver cell line (HepG2). Future in vivo study to confirm our data is important to support the novel glucose uptake induction of these compounds.

3.6 Conclusion

In the present study, bioassay-guided fractionation and purification were used to isolate the antidiabetic compounds of an extract of *Cissus rotundifolia* leaves as mentioned in Chapter2. All fractions, sub-fractions and pure compounds were screened for their antidiabetic activity through glucose uptake inducing activity on the cell. The highest inducing activity was found to be in the ethyl acetate fraction and two isolated c

compounds SAA3 (3), SAA4 (4). Compounds SAA4 and SAA3 showed potential antidiabetic activity through inhibition of glucose uptake in comparison to insulin. Future investigation of antidiabetic activities of isolated compounds in vivo to elucidate the mechanism could highlight the potential medicinal value of Cissus *rotundifolia and isolated compounds.*

Effect of Cissus rotundifolia fractions on glucose uptake

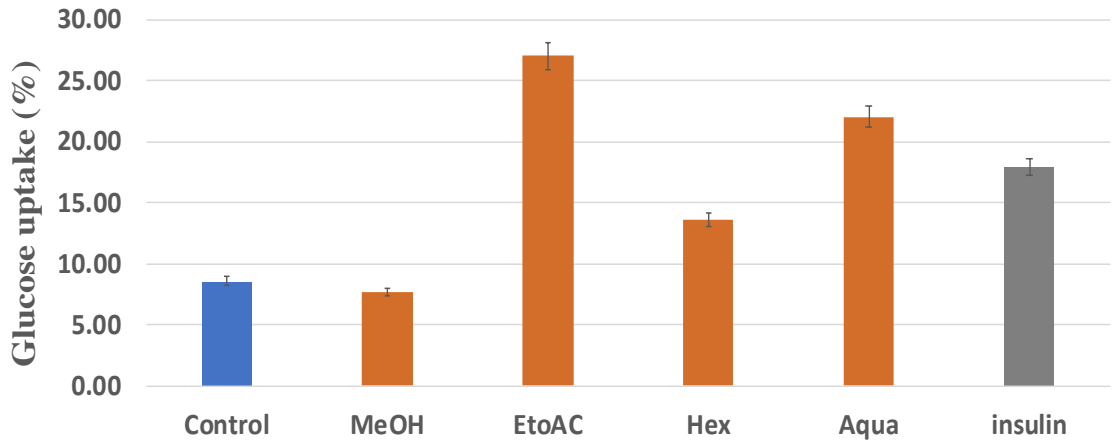

Figure 3-2 : Effect of Cissus rotundifolia fractions on glucose uptake. Fractions of Methanol, Ethylacetate, hexane and aqueous represent in orange, compared with insulin as positive control represent in gray to show the induction of glucose uptake activity.

Effect of isolated compounds from *Cissus rotundifolia* on glucose uptake

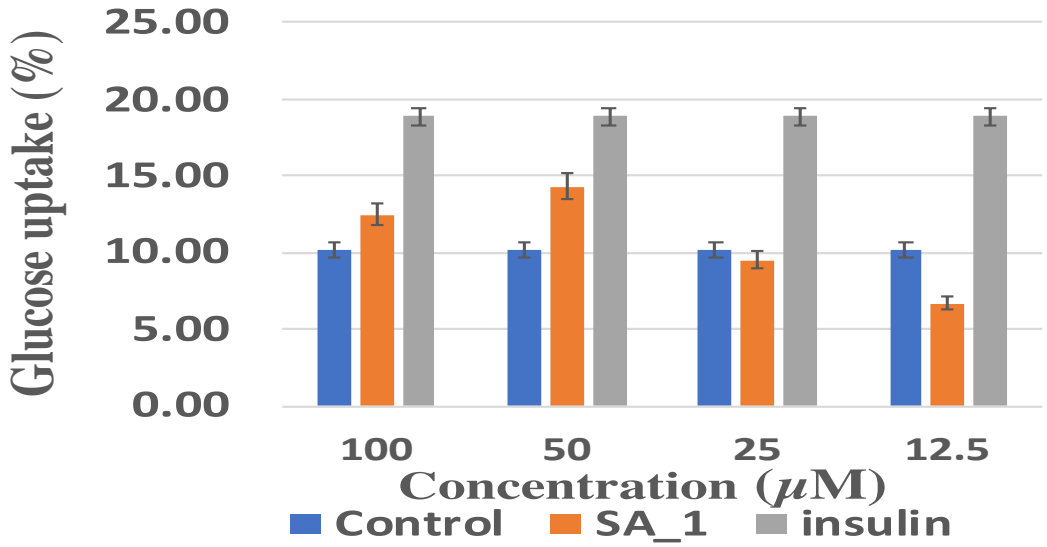

Figure 3-3 : Isolated compound (1) at four different concentrations (100, 50, 25, 12.5 µM), represented in orange, compared with insulin as positive control, represented in gray, to show the induction of glucose uptake activity.

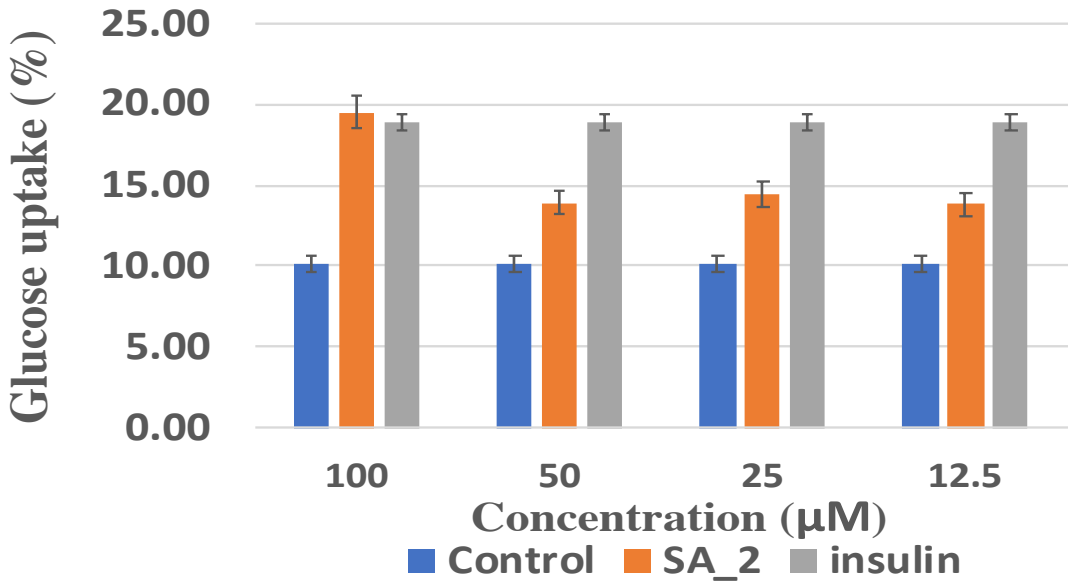

Figure 3-4 : Isolated compound (2) at four different concentrations (100, 50, 25, 12.5 µM), represented in orange, compared with insulin as positive control, represented in gray, to show the induction of glucose uptake activity.

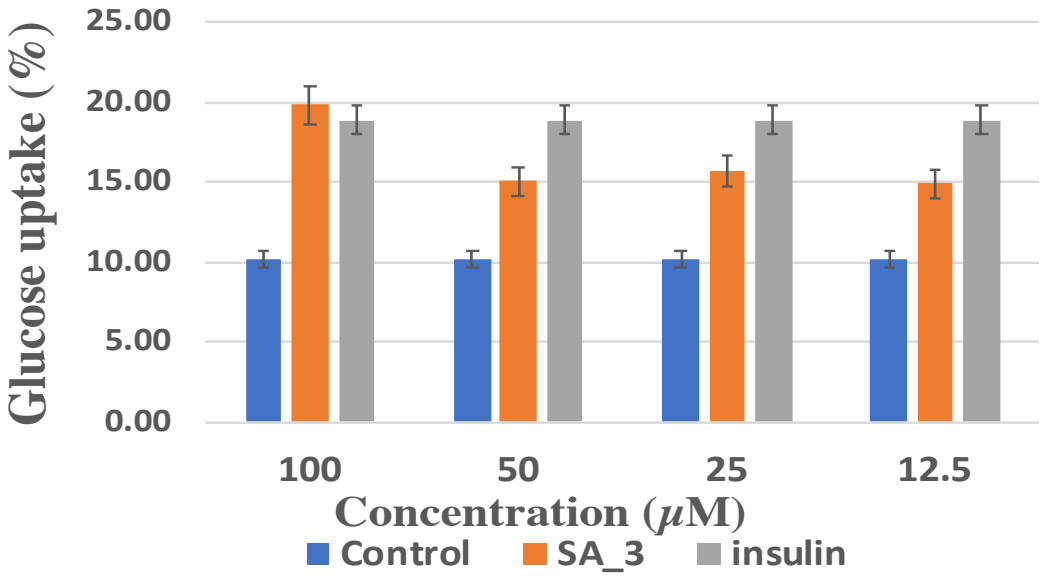

Figure 3-5 : *Isolated compound (3) at four different concentrations (100, 50, 25, 12.5 µM), represented in orange, compared with insulin as positive control, represented in gray, to show the induction of glucose uptake activi*

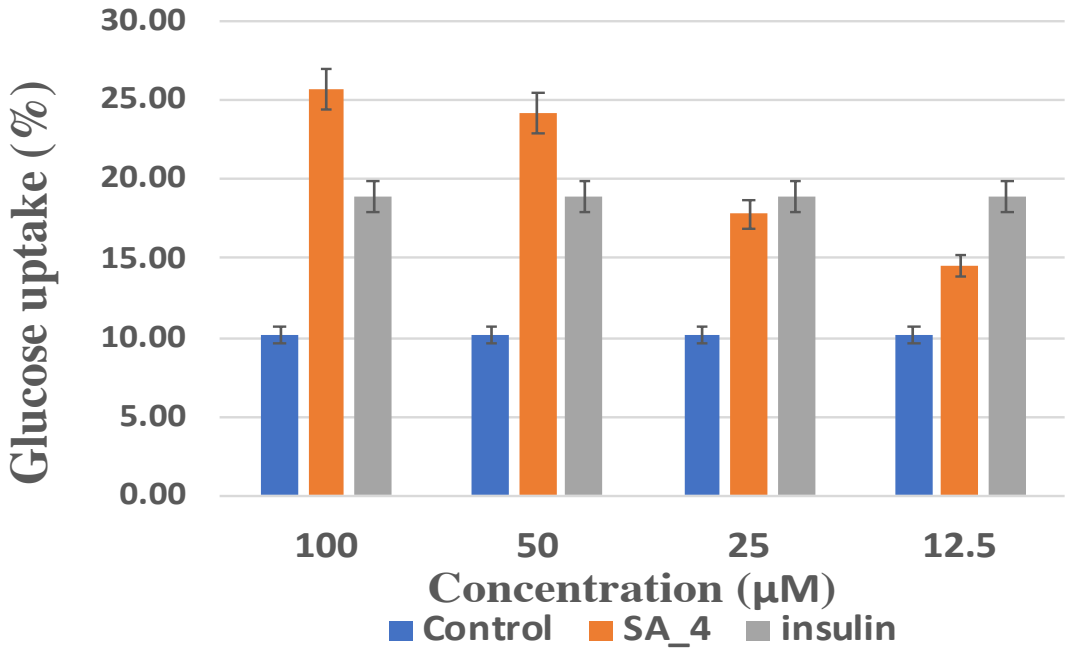

Figure 3-6 : Isolated compound (4) at four different concentrations (100, 50, 25, 12.5 µM), represented in orange,

compared with insulin as positive control, represented in gray, to show the induction of glucose uptake activity..

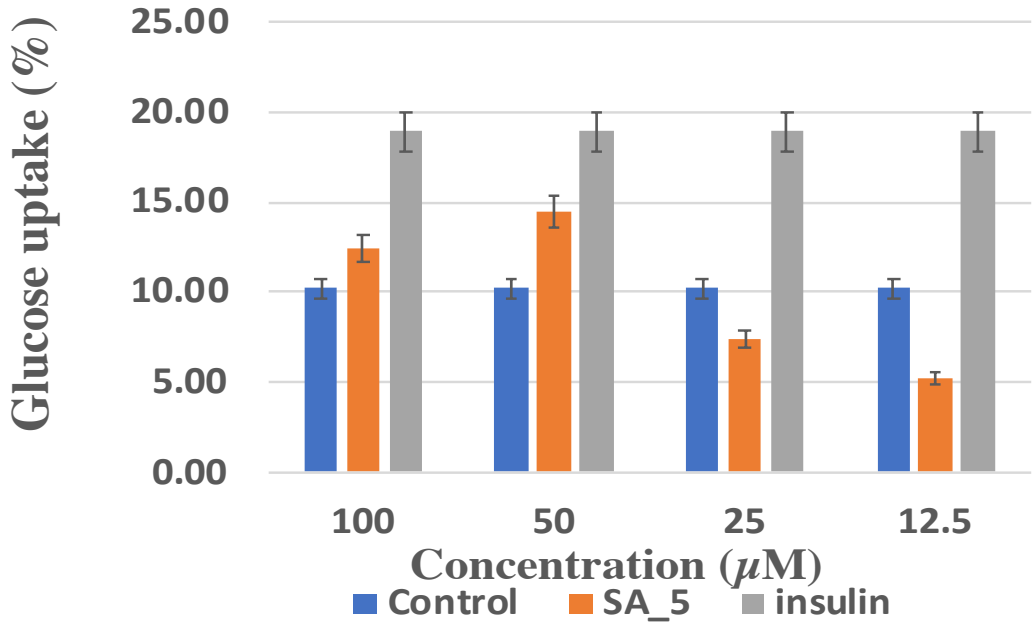

Figure 3-7 : Isolated compound (5) at four different concentrations (100, 50, 25, 12.5 µM), represented in orange, compared with insulin as positive control, represented in gray, to show the induction of glucose uptake activity..

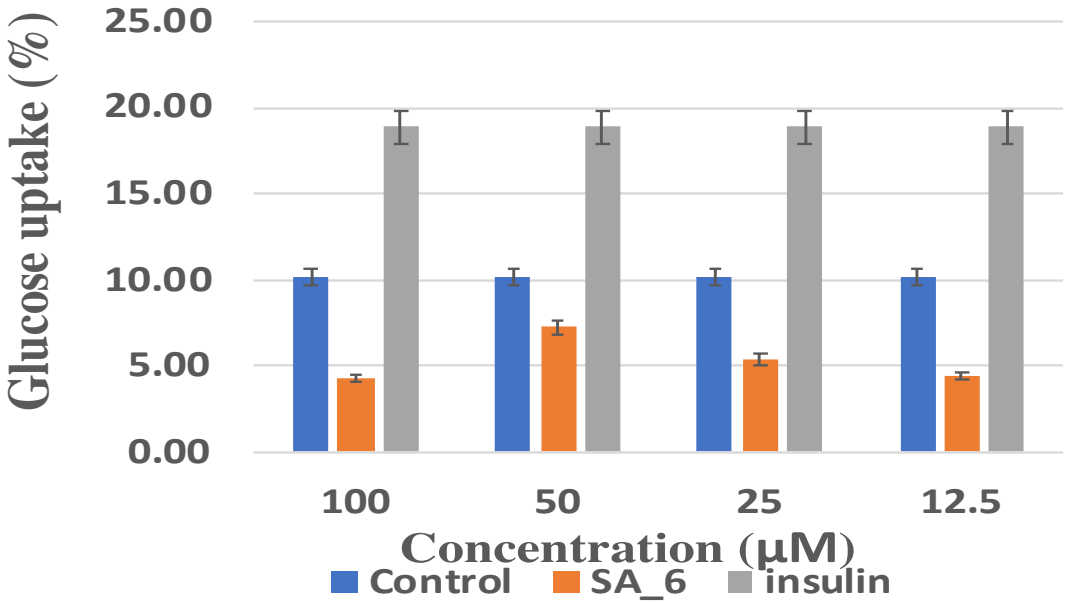

Figure 3-8 : Isolated compound (6) at four different concentrations (100, 50, 25, 12.5 µM), represented in orange, compared with insulin as positive control, represented in gray, to show the induction of glucose uptake activity.

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4 CHAPTER FOUR: IN-SILICO MOLECULAR MODELING STUDY OF ISOLATED COMPOUNDS TARGETING CITRIC ACID CYCLE ENZYMES

4.1 Introduction

Glucose homeostasis is physiologically maintained by balance between glucose production by liver and glucose consumption by the peripheral tissues.[1] Hepatic glucose production and glucose utilization is controlled by insulin, so, in non-insulin-dependent diabetes mellitus (NIDDM), hepatic glucose production is high and demonstrates a positive correlation with concentrations of glucose in plasma.[2] Moreover, increases in hepatic glucose production are considered the principal cause of fasting hyperglycemia in NIDDM.[3] In the liver, two processes produce glucose (gluconeogenesis and glycogenolysis); however, gluconeogenesis develops to be significantly increased in NIDDM.[3] When the process of gluconeogenesis increases, it will further cause increased hepatic glucose production and a positive correlation between the rates of gluconeogenesis and fasting plasma glucose concentration has been discovered in NIDDM subjects.[4, 5]Therefore, reduced hepatic glucose production also plays an important role in controlling blood glucose level and is considered a new target for antidiabetic research.[6] Gluconeogenesis begins in the mitochondria through citric acid cycle intermediates, starting throughout conversion to oxaloacetate, which can also function as substrates for gluconeogenesis.[7] Gluconeogenesis is a pathway involving a couple of enzyme-catalyzed reactions.[8] This pathway starts in the mitochondria or cytoplasm based on the substrate being used.[7] Gluconeogenesis begins with the formation of oxaloacetate from carboxylation of pyruvate. This reaction needs one molecule of ATP and it is catalyzed by an enzyme called pyruvate carboxylase.[9] Pyruvate carboxylase is stimulated by high levels of acetyl-CoA.[10] Over the reaction of the citric acid cycle,

Malate is the intermediate oxidized to oxaloacetate using NAD+ in the cytoplasm, where other steps of gluconeogenesis show.[11] Oxaloacetate is decarboxylated and phosphorylated to generate phosphoenolpyruvate by phosphoenolpyruvate carboxykinase.[12]

Gluconeogenesis provides stable blood glucose levels between meals; however, it is found to be high in diabetic patients. Gluconeogenesis also helps maintain glucose levels when on a diet low in carbohydrates.[13] Therefore, based on the similarity in the chemical structure between the isolated compounds from *cissus rotundifolia* and citric acid cycle substrates, our hypothesis that molecular modeling to study binding affinity of the isolated compound with citric acid cycle enzymes could reveal potential mechanism of compounds isolated in our study to inhibit gluconeogenesis and control hyperglycemia through inhibition of hepatic glucose production.

4.2 Citric acid cycle (Kreb's Cycle)

The citric acid cycle, also called the Krebs or tricarboxylic acid cycle, is a series of reactions in mitochondria that result from oxidization of the acetyl moiety of acetyl-CoA to CO_2 and decreases coenzymes that are reoxidized through the electron transport chain related to the formation of ATP.[14] The tricarboxylic acid cycle is a pathway for the oxidation of carbohydrates, proteins and lipids because glucose, most amino acids and fatty acids are metabolized to acetyl-CoA or intermediates of the cycle.[15] The citric acid cycle has a fundamental role in the gluconeogenesis process that is considered the main pathway of the hepatic production of glucose. These processes occur in the liver tissues,

the abnormally increased rate of **hepatic gluconeogenesis** contributes to hyperglycemia in diabetes.[13, 16]

4.3 Steps of the citric acid cycle

Several steps during the citric acid cycle occur through enzymatically catalyzed reaction in order to generate the molecules produced.[17] The molecules produced from the citric acid cycle, such as NADH, FADH, and ATP, are important as sources of energy, while the intermediate produce through the citric acid cycle are important in another process.[18] For example, oxaloacetate is one of intermediates of the citric acid cycle that plays an essential role and is the starting point of the gluconeogenesis process to produce glucose.[19] The steps of the citric acid cycle begin when acetyl CoA joins with oxaloacetate, four carbon molecules, to form a six-carbon molecule (citrate). This step is catalyzed by an enzyme called **citrate synthase**. The second step, which is a two-step process, occurs when citrate is converted into its isomer, isocitrate. This step is catalyzed by an **aconitase** enzyme and it involves the removal and then the addition of a water molecule, which is the reason that the citric acid cycle is sometimes defined as having nine steps rather than the eight. The third step, the enzyme **isocitrate dehydrogenase** catalyzes this step when the isomer of citrate, isocitrate, is oxidized and releases a molecule of carbon dioxide to form a five-carbon molecule called α-ketoglutarate. Next, the fourth step begins when α-ketoglutarate oxidizes and releases a molecule of carbon dioxide to form a four-carbon molecule. These four-carbon molecules pick up Coenzyme A, producing the unstable compound succinyl CoA. The enzyme, **α-ketoglutarate dehydrogenase**, is catalyzed in this forth step and also it is important in the regulation of the citric acid cycle. In step five in citric acid cycle, the CoA of succinyl CoA is

substituted by a phosphate group, then transferred to ADP to make ATP. The four-carbon molecule produced in this step is called succinate. The enzyme that catalyzes this step is the **succinyl CoA synthetase** enzyme. Then**,** in step six, succinate is oxidized, creating another four-carbon molecule called fumarate. An enzyme called **succinate dehydrogenase** catalyzes this step. The result of this reaction, two hydrogen atoms with their electrons, are transferred to FAD, forming FADH2. The enzyme involved in this step is implanted in the inner membrane of the mitochondrion, so FADH2 can transfer its electrons directly into the electron transport chain. Step seven in citric acid cycle involves the four-carbon molecule fumarate converting into another four-carbon molecule called malate. The **Fumarase** enzyme catalyzes this step. The last step of the citric acid cycle, step eight, creates oxaloacetate, a four-carbon compound formed by oxidation of the malate molecule. **Malate dehydrogenase** catalyzes this step.

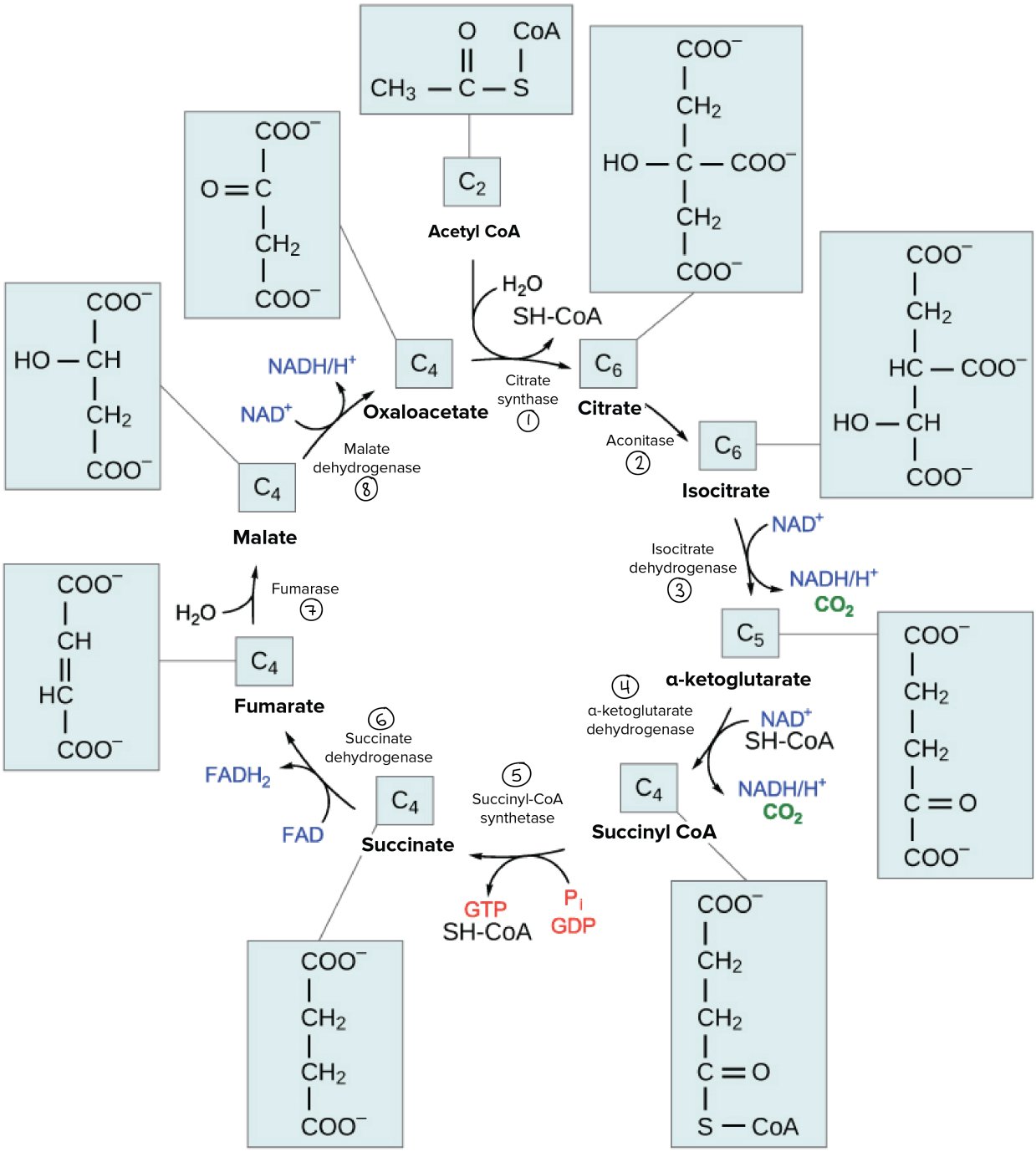

Figure 4-1 : Citric acid cycle steps.

4.4 Gluconeogenesis

Gluconeogenesis is a pathway to produce glucose in the human body.[20] In diabetic patients, gluconeogenesis is active, especially during fasting, so the current target to control diabetic disease is to inhibit gluconeogenesis in order to manage hyperglycemia.[21] Gluconeogenesis is a process to produce glucose from precursors such as lactate, glycerol, pyruvate and amino acids.[21, 22] A fasting condition requires synthesis of glucose from non-carbohydrate sources. Most precursors must enter the citric acid cycle at some point to be converted to oxaloacetate.[3] Oxaloacetate is an intermediate of the citric acid cycle that is the starting material for gluconeogenesis.[23]

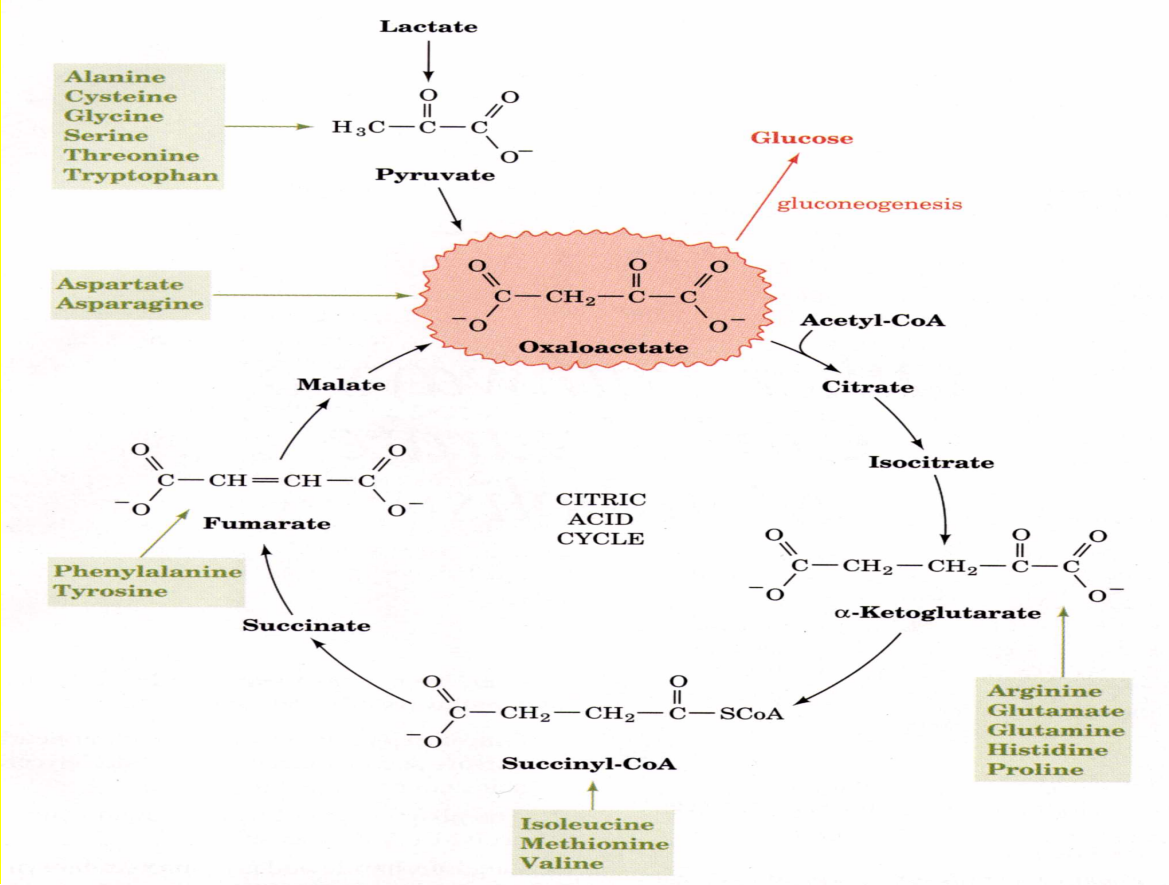

Figure 4-2 : Citric acid cycle intermediate, Oxaloacetate, as starting material of Gluconeogenesis process

The citric acid cycle, or tricarboxylic acid cycle, has a fundamental role in the gluconeogenesis process that is considered the main pathway of the hepatic production of glucose. The citric acid cycle is a series of reactions in mitochondria that result from oxidization of the acetyl moiety of acetyl-CoA to CO_2 and decreases coenzymes that are reoxidized through the electron transport chain related to the formation of ATP. The enzymes involving in citric acid cycle play important role in regulation of gluconeogenesis through catalyzes each step of the cycle to form the substrates. Oxaloacetate is a substrate of citric acid cycle and it is the starting material for gluconeogenesis. Although the properties of citric acid cycle enzymes have been studied, the reports of targeted small molecule modulators of the activity have been limited. Therefore, in this study the isolated compound and small analogs investigation of their binding affinity to citric acid cycle enzymes that might lead to a new discovery for inhibition activity of gluconeogenesis.

4.5 Molecular docking

Molecular docking is a structure-based drug design approach. It is frequently used in the drug discovery process to determine the best matches between a receptor/target protein and a ligand/drug.[24] The molecular docking method involves predicting the conformations and orientations of a molecule in the active site of target macromolecules and labeling the preferred configurations and conformations of molecules binding with the active site of target macromolecules.[24] The two major purposes of molecular docking are right structural modeling and correct prediction of activity.[25] Moreover, molecular docking has been used in different phases of the drug discovery pipeline, such as quantitative structure activity relationship, virtual combinatorial library generation, lead

optimization and discovery of a potential lead through virtual screening.[25] In addition, the application of molecular molding has extended to the analysis of physico-chemical parameters of the ligand, including absorption, distribution, metabolism and elimination/toxicity using a variety of applications and programs.[25, 26] Therefore, in this present study, we use computational molecular modeling tools to study and predict the interaction of isolated compounds from *cissus rotundifolia* extract analogs enriched with functionalities with citric acid cycle enzymes and gluconeogenesis enzyme PEPCK.

4.6 Aim of the study

The promising isolated compounds from *cissus rotundifolia* identified as the dicarboxylic aicds analogs showed similarity to malate structure (one of the main intermediates of citric acid cycle). Therefore, the similarity in the chemical structure prompt us to build a hypothesis for investigating the ability of the isolated compounds to inhibit the gluconeogenesis process through inhibition of the citric acid cycle, one of the main pathways to reduce the blood glucose level. The molecular docking process includes predicting the conformations and orientations of a molecule in the active site of target macromolecules and identifying the preferred configurations and conformations of molecules interacting with the active site of target macromolecules. In this study, we used molecular docking as one of the main strategies in the structure-based drug design approach, so we use computational molecular modeling tools to study and predict the interaction of the isolated compounds from *cissus rotundifolia* identified above.

4.7 Materials and methods

4.7.1 Compounds

Six compounds were isolated from *cissus rotundifolia* as previously mentioned in Chapter 2. In addition to, 2-aminosuccinicamide, 4-methoxy-2,4, dioxobutanic acid and levulinic acid compounds. For docking, a vertical library of thirty analogs docked including metformin as standard.

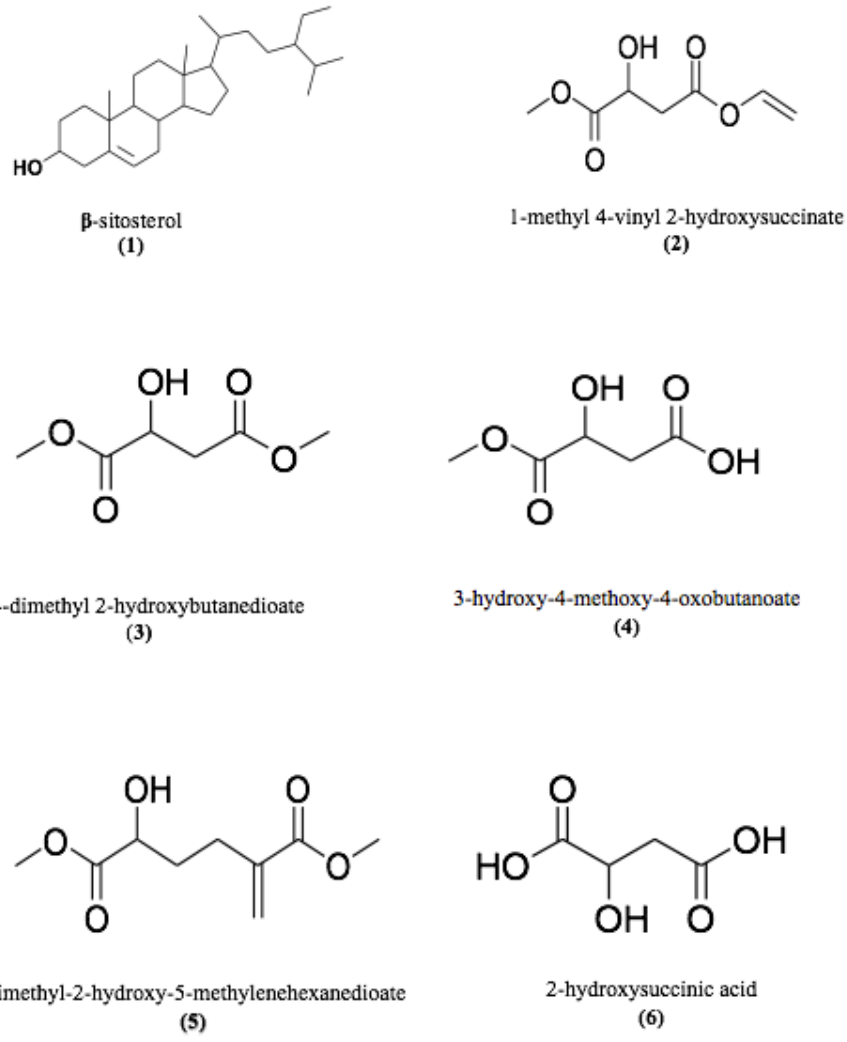

Figure 4-3: Chemical structure of Isolated compounds (1- 6) from Cissus rotundifolia extract.

4.7.2 Molecular modeling

Molecular modeling was completed using four application softwares (make receptor , Omega, Fred and Vida) using the method previously published by our group.[27]

4.7.3 2D and 3D Structures

A virtual library of thirty analogs and standard metformin compound, along with known antidiabetic therapies for the treatment of diabetic mellitus, were prepared using ChemOffice Ultra. The energies of the 3D structures were minimized using semi-empirical PM3 calculations. The energy-minimized structures were then converted into pdb files, maintaining all heavy atoms.

4.7.4 Generation of conformers

The energy-minimized structures were combined into a single continuous pdb file to be used as an input for Omega. The Omega utility uses the MMFF94 force field to form multiple conformations for each input ligand in the library in order to induce ligand flexibility in an otherwise rigid model.

4.7.5 Receptor preparation

All of the citric acid cycle enzymes and phosphonyl pyruvate kinase enzyme were prepared for the molecular docking process. Citrate synthase (PDB ID: 1cts), aconitase (PDB ID: 7acn), isocitrate dehydrogenase (PDB ID: 3blw), alpha-ketoglutarate dehydrogenase (PDB: 2jGD), succinyl CoA synthetase (PDB: 2FP4), succinate dehydrogenase (PDB: 1neck), fumarase (PDB: 1fuo), malate dehydrogenase (PDB: 1MLD) and phosphonyl pyruvate kinase enzyme (PECK) (PDB ID:) structures were downloaded from the RCSB Protein Data Bank and prepared for modeling using Fast Rigid Exhaustive Docking (FRED). The application allows for the creation of a grid box

by the mode selection pane and adjustment of its size using the mode controls. The box size would not exceed 50000–60000Å. Once the grid box size has been created, the receptor is ready for use in the docking calculations.

4.7.6 Docking

Multiple scoring functions were used in order to identify a consensus structure and score in the final output. The scoring functions involve Shapegauss, Chemgauss3, Oechemscore, Screen score and PLP. Snapshots and visualizations of the chemical interactions between the analogues and receptors were obtained using the VIDA application.

4.8 Results and discussion

4.8.1 Molecular modeling

OpenEye Software sets (Fast Rigid Exhaustive Docking (FRED), make Receptor, Omega, FRED and VIDA) were used to run the molecular docking studies.[27] The crystal structure of the citric acid cycle enzymes was used as a molecular target. A virtual library of compounds was docked into a ligand binding domain of all the eight enzymes involved in each step of the citric acid cycle in addition to phosphoenolpyruvate kinase protein (PECK). The molecular docking results showed that analog 2-aminosuccininamide (AN21), levulinic acid, and SAA4 compounds possess a high binding affinity on the fumarase enzyme. Levulinic acid formed a hydrogen bonding interaction between the carboxylic group at C-1 and the amino acid residue ASN135, in addition to another two hydrogen bonding interactions between the oxygen of carbonyl group at C-4 with the amino acid residues ASN141 and SER140 in fumarase (Figure 4.7). Meanwhile the isolated compound, SAA4, is one of the top compounds that showed high binding affinity

with the fumarase enzyme. The isolated compound SAA4 formed two hydrogen bonds between the oxygen on the groups at C-1and C-3 with SER139 and ASN141, respectively (Figure 4-8). Also, analog (AN21) exhibited a high binding affinity with no hydrogen bonding interaction (Figure 4.9). Meanwhile, SAA3 compound, which is an isolated compound from *cissus rotundifolia*, formed only one hydrogen bond interaction between the carboxylic group at C-1 and the amino acid residue SER140 in fumarase (Figure 4.10). Alternatively, SAA4 and SAA3 compounds isolated from *cissus rotundifolia* extract showed a binding affinity to three of the citric acid enzymes targeted: isocitrate dehydrogenase, succinate dehydrogenase and succinyl CoA. The SAA4 isolated compound showed the highest binding affinity to isocitrate dehydrogenase in comparison to other isolated compounds and analogs. SAA4 formed hydrogen bonding interaction between C-1 and the amino acid residue HIS301 (figure 4.12). SAA3 compound showed a high binding affinity to the succinyl CoA enzyme forming two hydrogen bonding between the hydroxy group C-2 and carboxylate oxygen C-1 with the amino acid residue LYS172 and TYR173, respectively (figure 4.11). Meanwhile, analogs AN21 and levulinic acid compounds showed a high binding affinity with the malate dehydrogenase enzyme. The AN21 analog formed three hydrogen bond interactions between the amide group at C-3 with the amino acid residue ASN118; in addition, two hydrogen bonds appeared between the oxygen of carbonyl group at C-1 with two amino acid residues, THR211 and ARG80, respectively (Figure 4.13). The isolated compound SAA3 showed a high binding affinity to malate dehydrogenase, which exhibited a hydrogen bond interaction to malate dehydrogenase enzyme between the hydroxyl group at C-2 with the amino acid reside ALA223 (Figure 4.14). On the other hand, compound SAA2 showed

less binding affinity on fumarase enzyme and show no hydrogen bond to malate dehydrogenase. Structural differences between SAA3 and SAA2 are obvious reason for different binding activities. SAA5 does not show binding affinity to fumarase enzyme. Higher binding affinity of SAA3 could be attributed to its structural difference from other compounds at C-1 and C-4. Furthermore, binding affinity study showed that C-2 hydroxy group hydrogen bond to malate dehydrogenase enzyme is the key point behind higher binding affinity to malate dehydrogenase enzyme. Phosphoenolpyruvate protein kinase (PEPK) plays an important role in the gluconeogenesis process. The results of docking of PEPK with the isolated compounds from *cissus rotundifolia* and analogs showed that SAA3 possesses a high binding affinity. SAA3 formed hydrogen bonding interaction between C-1 and the residue of the amino acids VAL335, SER286 and LYS290. (Figure 4.15). Generally, the result of the top twenty analogs showed a high binding affinity to citric acid cycle enzymes and the phosphoenolpyruvate protein kinase. Analogs (AN21), (AN4) and Levulinic acid, in addition to, the isolated compounds from *cissus rotundifolia* (SAA4 and SAA3) showed the most promising binding affinity to more than one target of enzymes that involved in citric acid cycle, so these molecules based on molecular docking result need for further studied using biological evaluation in order to investigate the antidiabetic activity through inhibition of gluconeogenesis using in vitro and in vivo assays.

4.9 Conclusion

In this study, isolated compounds from *cissus rotundifolia* and their analogs, including analogs with metformin like functionalities, were studied and their ability to

bind to the citric acid cycle enzymes were analyzed using molecular modeling. The molecular docking results showed that analog (AN21), levulinic acid and the isolated compound SAA4 compounds possess a high binding affinity on the fumarase enzyme, In addition to the isolated compound SAA3 and SAA4 showed high binding affinity on malate dehydrogenase enzyme, suggesting that they may have potential for treating diabetes mellitus through inhibition of citric acid cycle based on the high binding affinity of these molecule to fumarase and malate dehydrogenase enzymes. More interestingly, our molecular modeling and binding affinity results on the isolated compounds suggests they may have inhibition activity for gluconeogenesis due to the high binding affinity to citric acid cycle enzymes, in addition to the antidiabetic activity observation from chapter 2 and chapter 3, since the isolated compounds exhibit significant inhibition activity against the alpha glucosidase enzyme and inducing cellular glucose uptake activity.

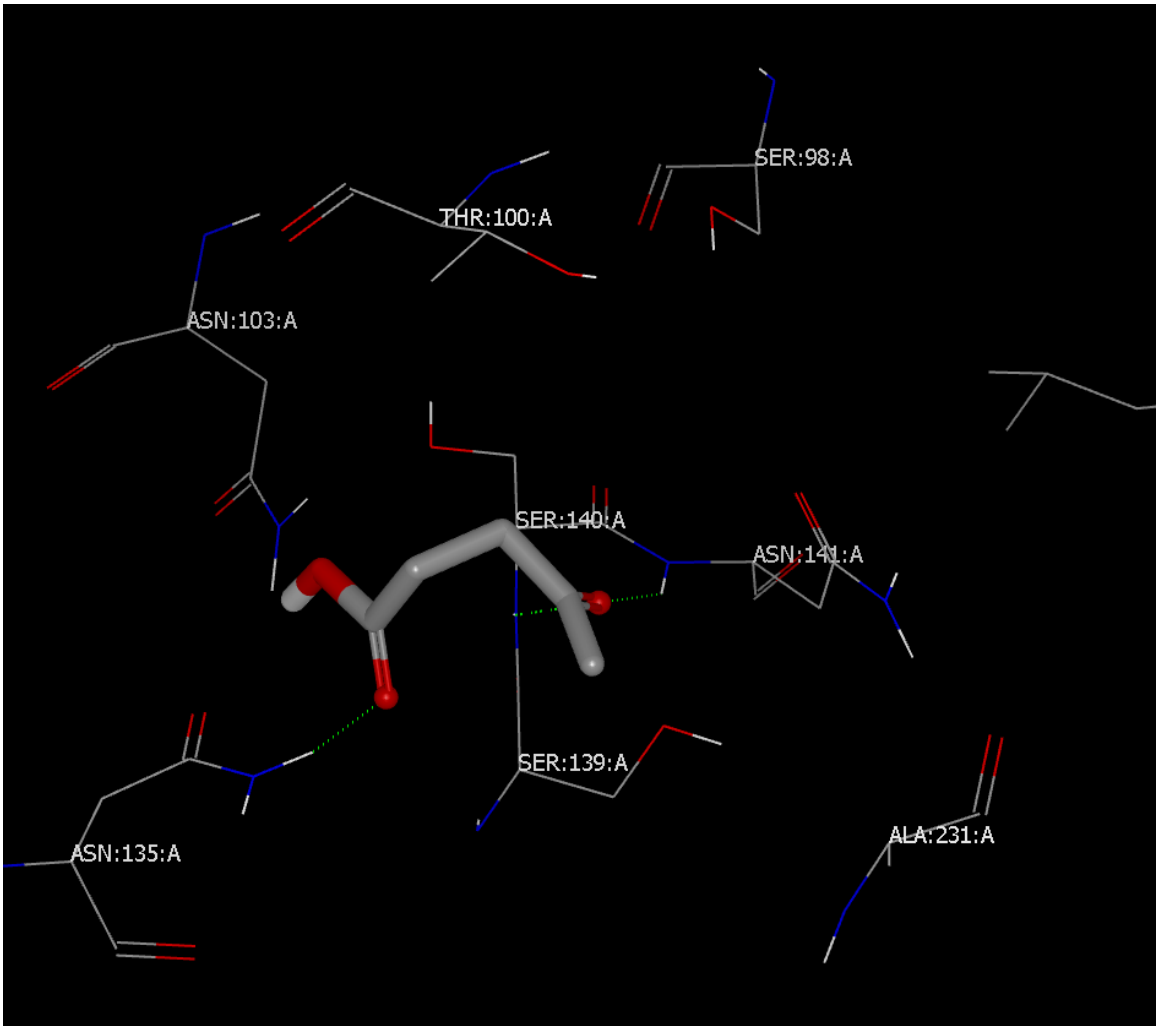

Figure 4-4: Visual representation of levulinic acid analog on fumarase enzyme, where the binding showed at the formation of a hydrogen bonds between the group at C-1 and C-4 with the amino acid residues ASN 141, SER 140 and ASN 135.

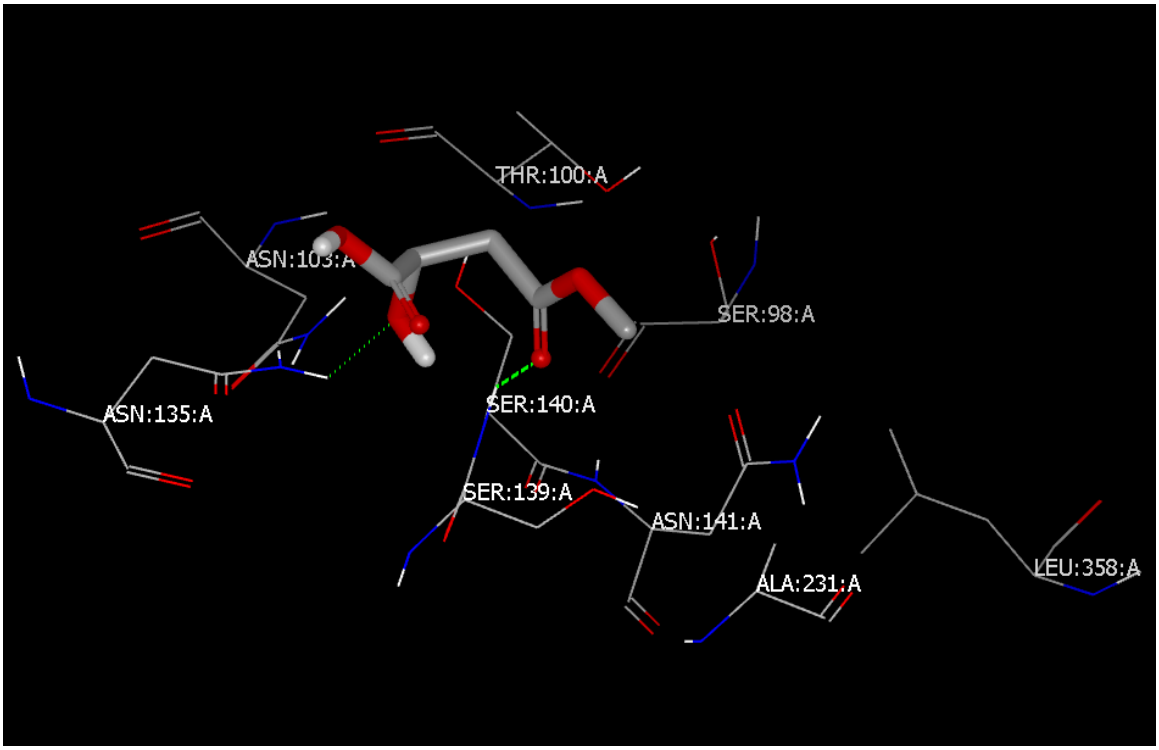

Figure 4-5:Visual representation of isolated compound SAA4 binding on the fumarase enzyme, where the binding showed at the formation of a hydrogen bond between the carboxylate group with the amino acid residues SER 140, and hydrogen bond between the hydroxyl group with the amino residue ASN 141.

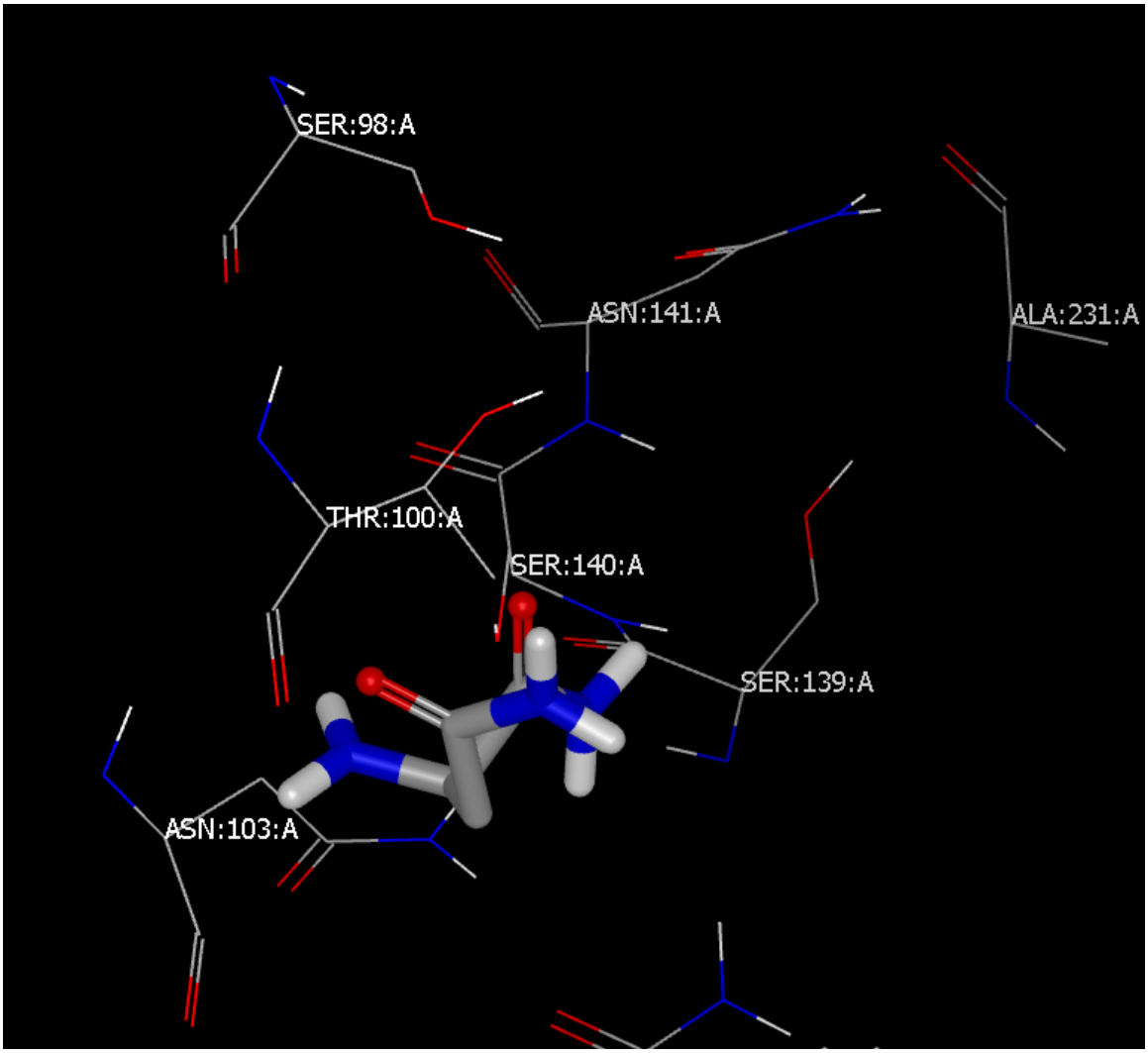

Figure 4-6: Visual representation of AN21 analog on the fumarase enzyme.

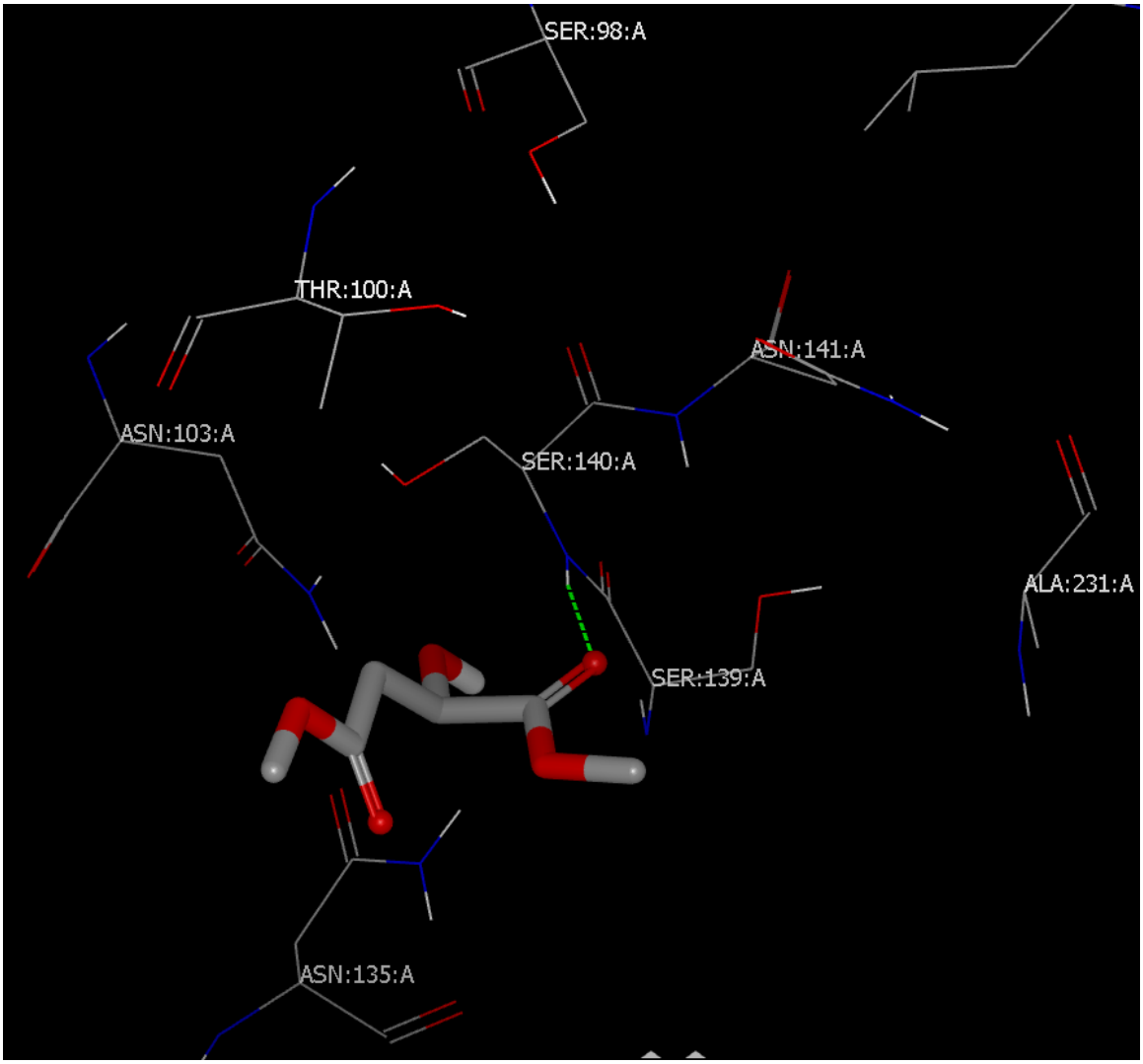

Figure 4-7: Visual representation of SAA3 analog on the fumarase enzyme, where the binding showed at the formation of a hydrogen bond between the oxygen of carbonyl group at C-1 with the amino acid residues SER 140.

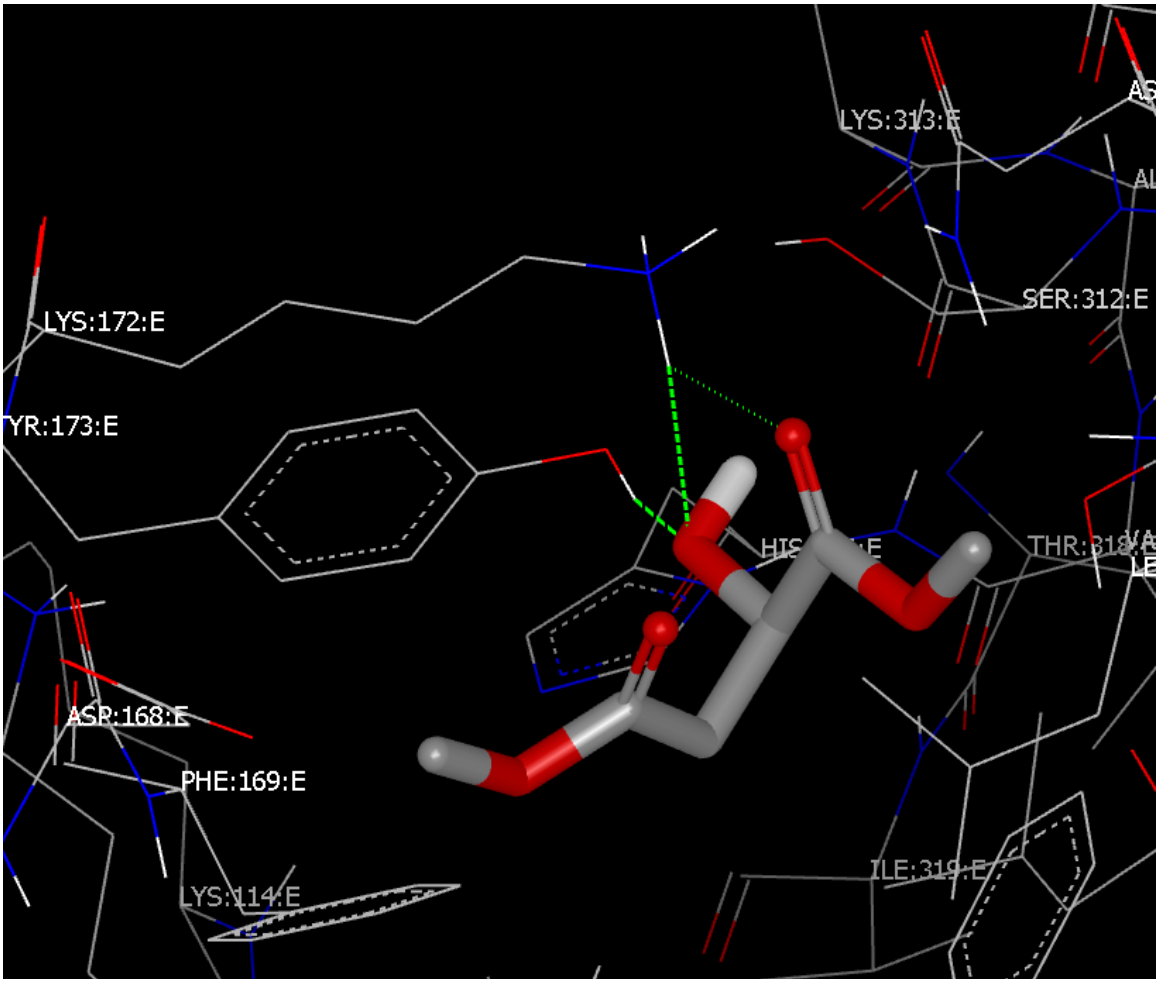

Figure 4-8: Visual representation of SAA3 isolated compound from cissus rotundifolia on isocitrate dehydrogenase enzyme, where the binding showed at the formation of a hydrogen bonds between the methoxy group with the amino acid residues LYS 171 and TYR 173.

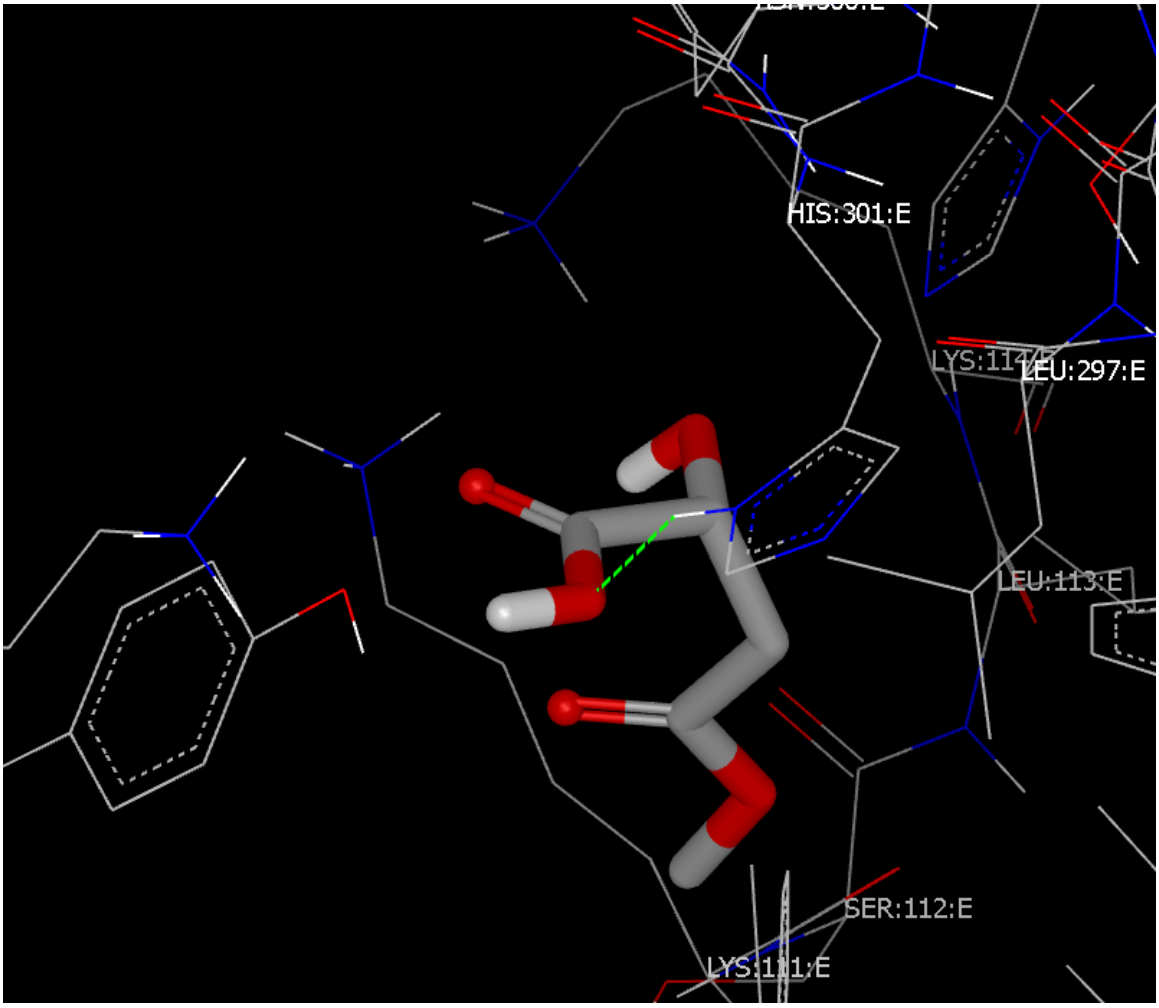

Figure 4-9: Visual representation of SAA4 isolated compound from cissus rotundifolia on isocitrate dehydrogenase enzyme, where the binding showed at the formation of a hydrogen bond between the methoxy group with the amino acid residues HIS 301.

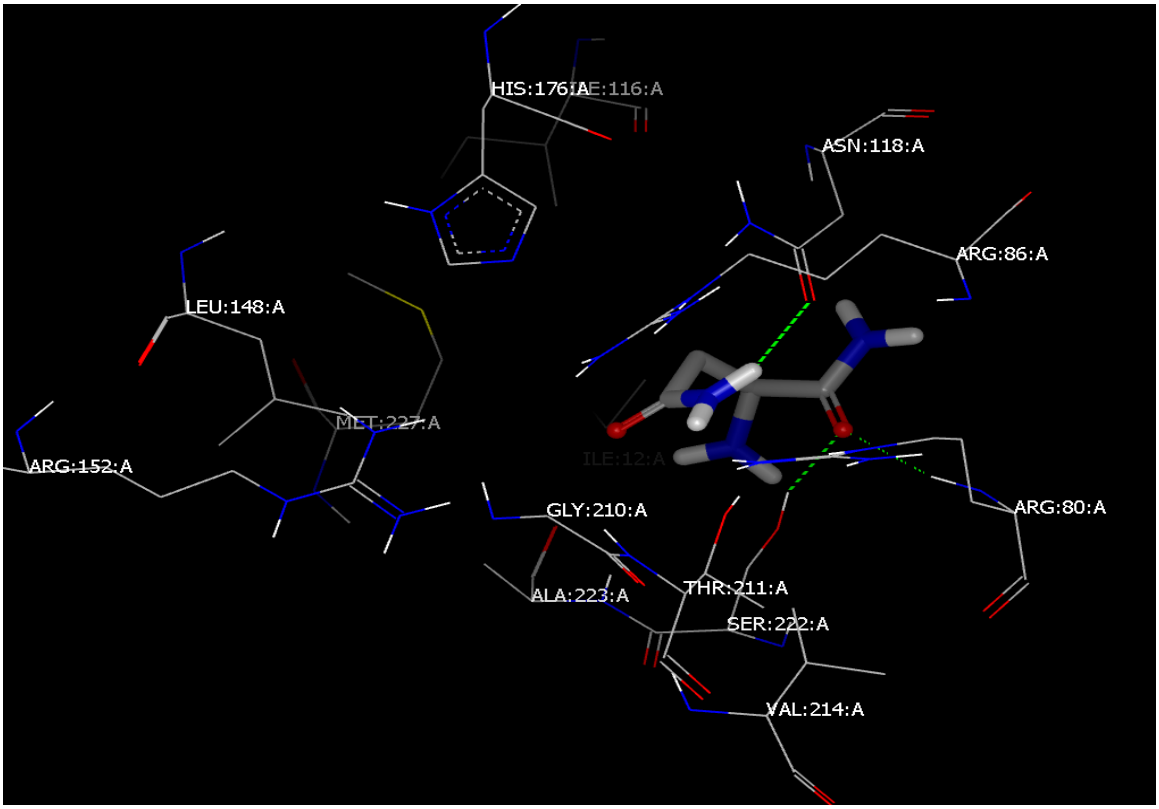

Figure 4-10: Visual representation of analog AN21 on malate dehydrogenase enzyme. AN21 analog formed three hydrogen bonds between the amide group at C-3 with the amino acid residue ASN118; in addition, two hydrogen bonds occurred between the oxygen of carbonyl group at C-1 with two amino acid residues: THR211 and ARG80, respectively.

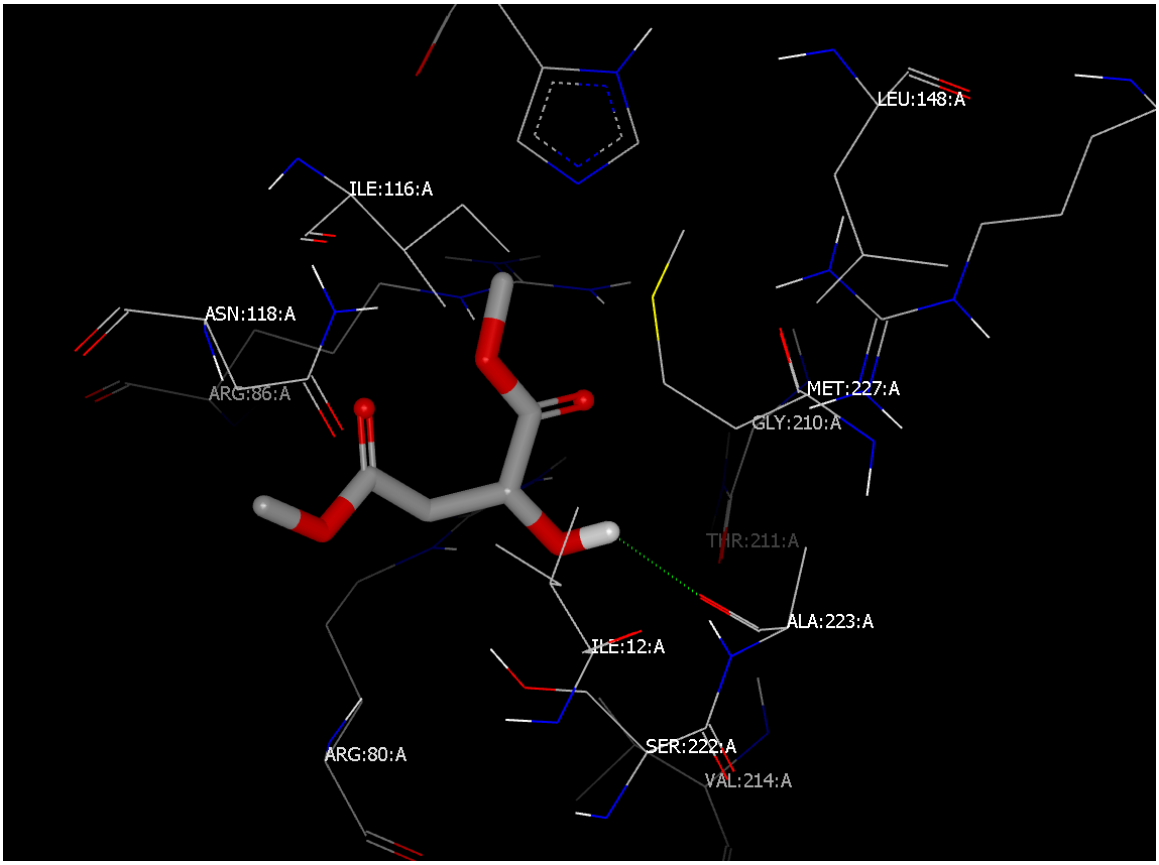

Figure 4-11: Visual representation of isolated compound SA19 on malate dehydrogenase enzyme. SA19 represents a hydrogen bond interaction on the malate dehydrogenase enzyme between the hydroxyl group at C-2 with the amino acid reside ALA223.

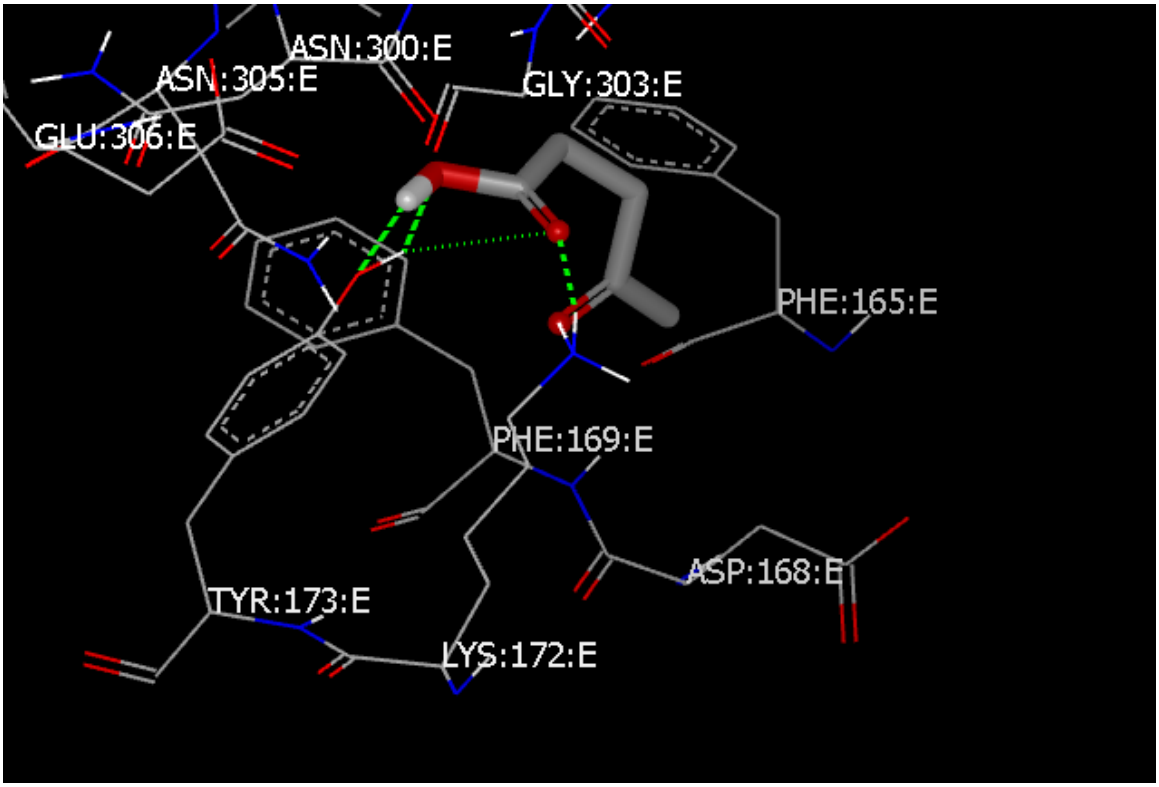

Figure 4-12: Visual representation of levulinic acid analog on isocitrate dehydrogenase enzyme, where the binding occurred at the formation of a hydrogen bonds between the group at C-1 and C-2 with the amino acid residues PHE169, GLU306 and TYR173.

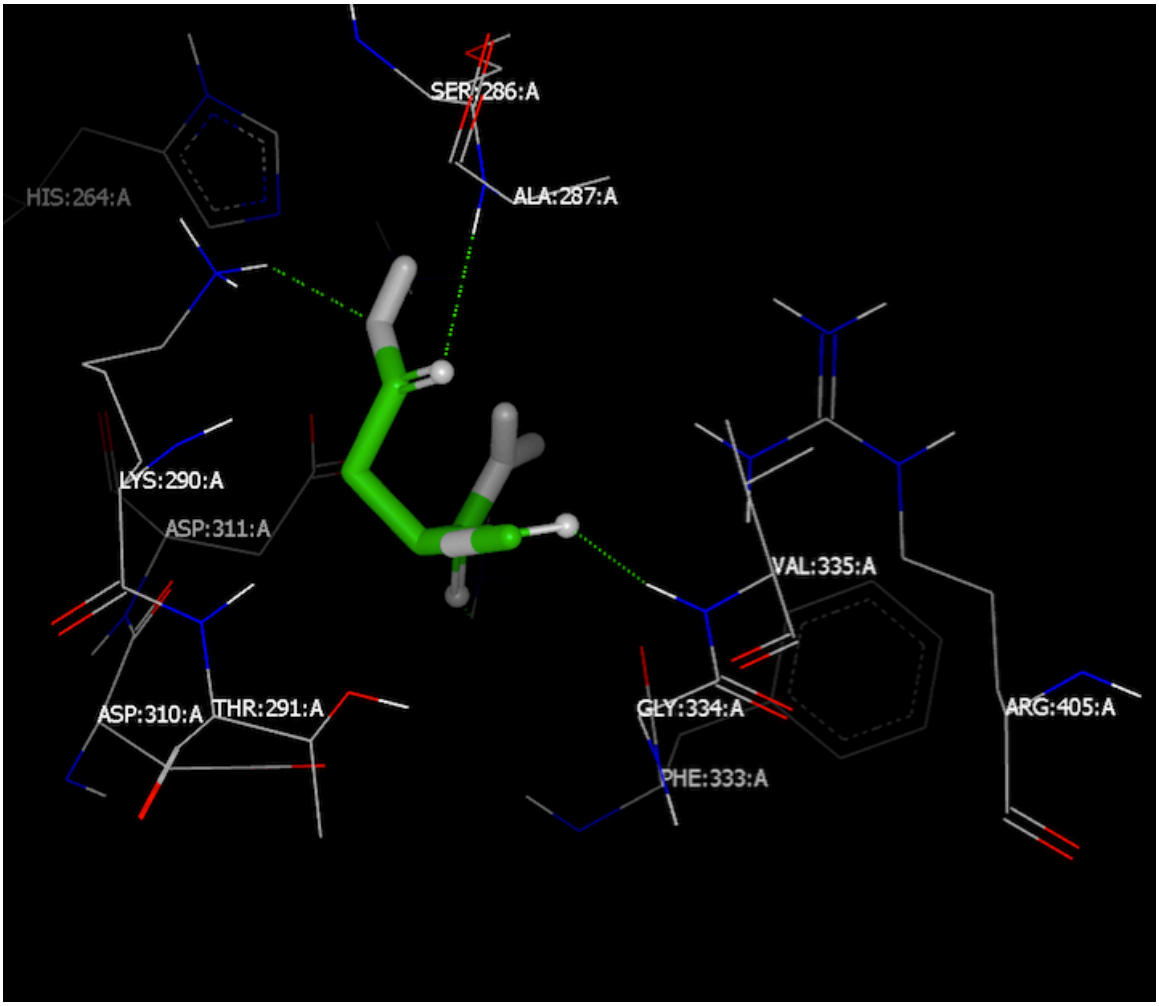

Figure 4-13: Visual representation of SAA3 analog on phosphoenolpyruvate protein kinase enzyme (PECK), where the binding showed at the formation of a hydrogen bonds between the group at C-1 and C-3 with the amino acid residues VAL335, SER286 and LYS290.

4.10 References

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5 Appendix

5.1 Structure elucidation NMR spectrum

^{1}H-NMR, ^{13}C-NMR and 2D-NMR were conducted using Bruker AVANCE-400 MHz and 600 MHz NMR spectrometer in deuterated chloroform ($CDCl_3$) using tetramethylsilane (TMS) as the internal standard; chemical shifts are given in ä (ppm) values.

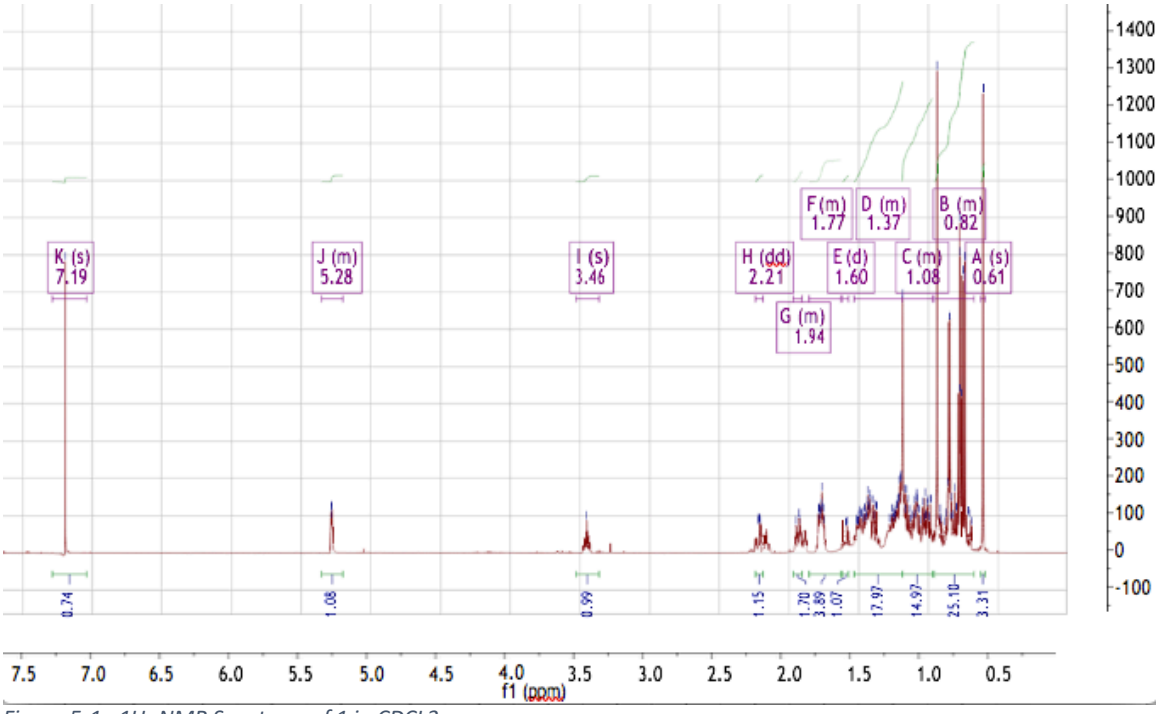

Figure 5-1 : 1H -NMR Spectrum of 1 in CDCL3

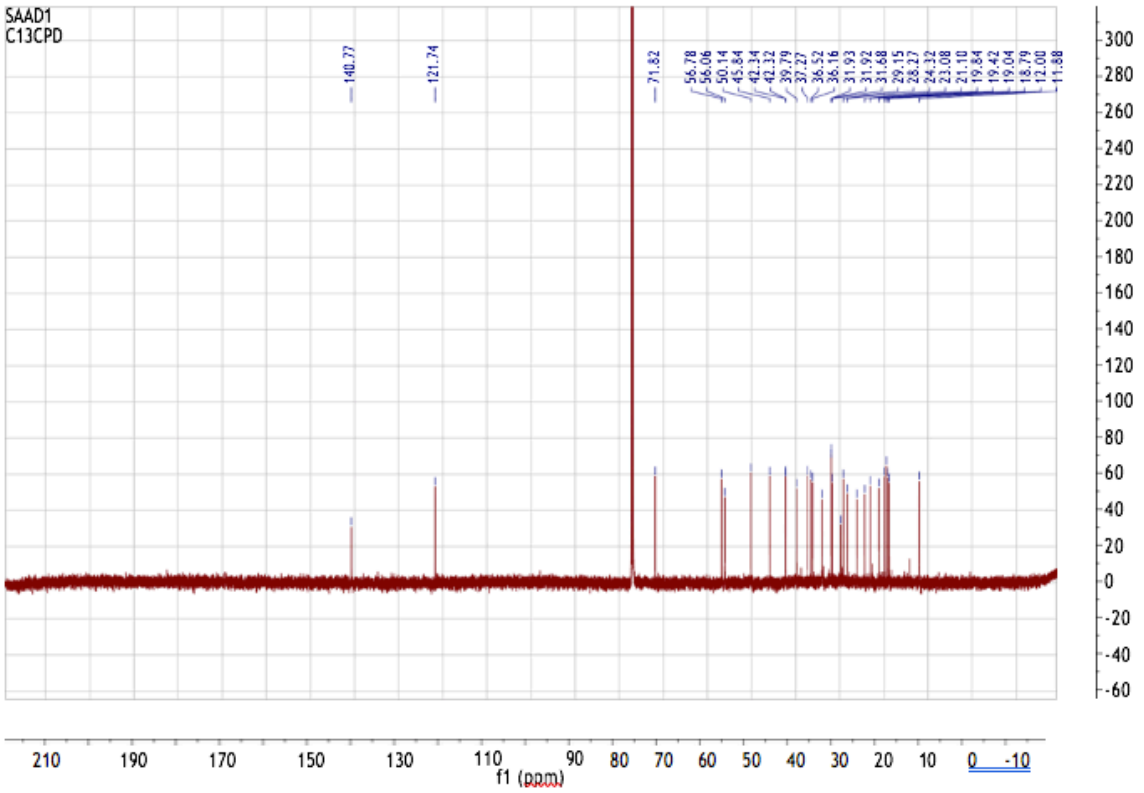

Figure 5-2 : 13C -NMR Spectrum of 1 in CDCL3

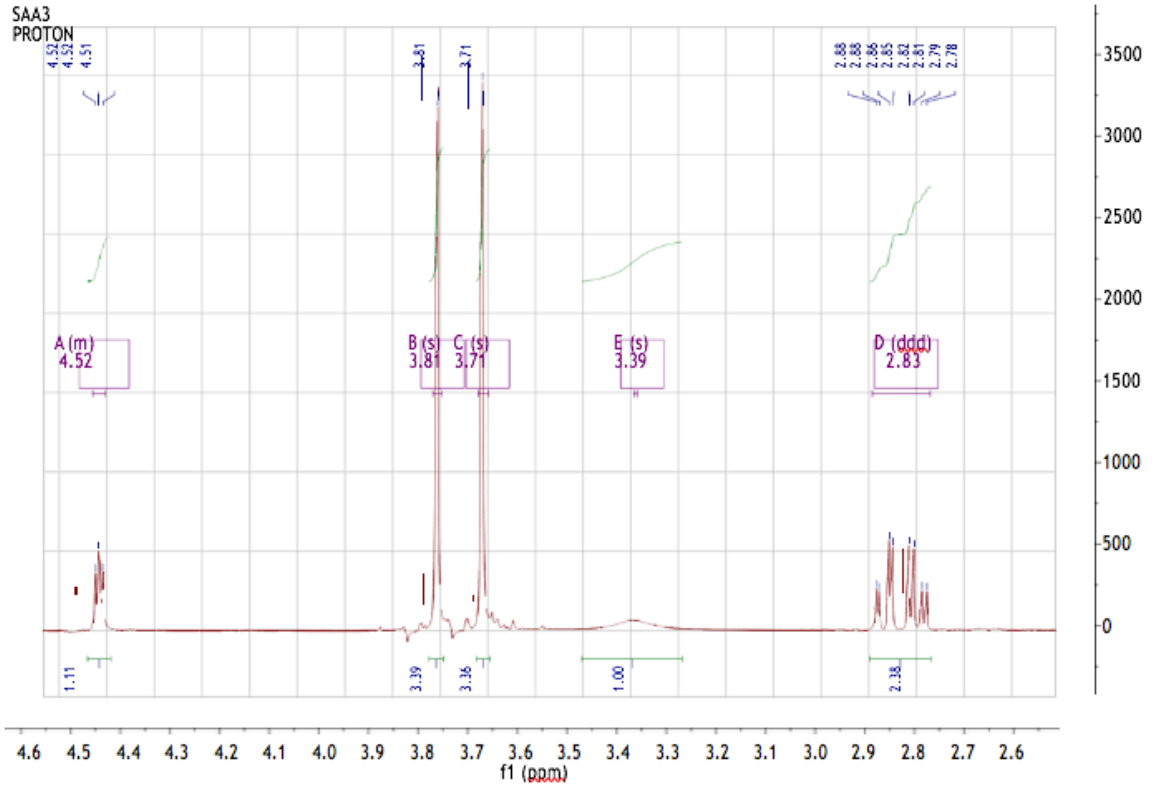

Figure 5-3 : 1H -NMR Spectrum of 3 in CDCL3

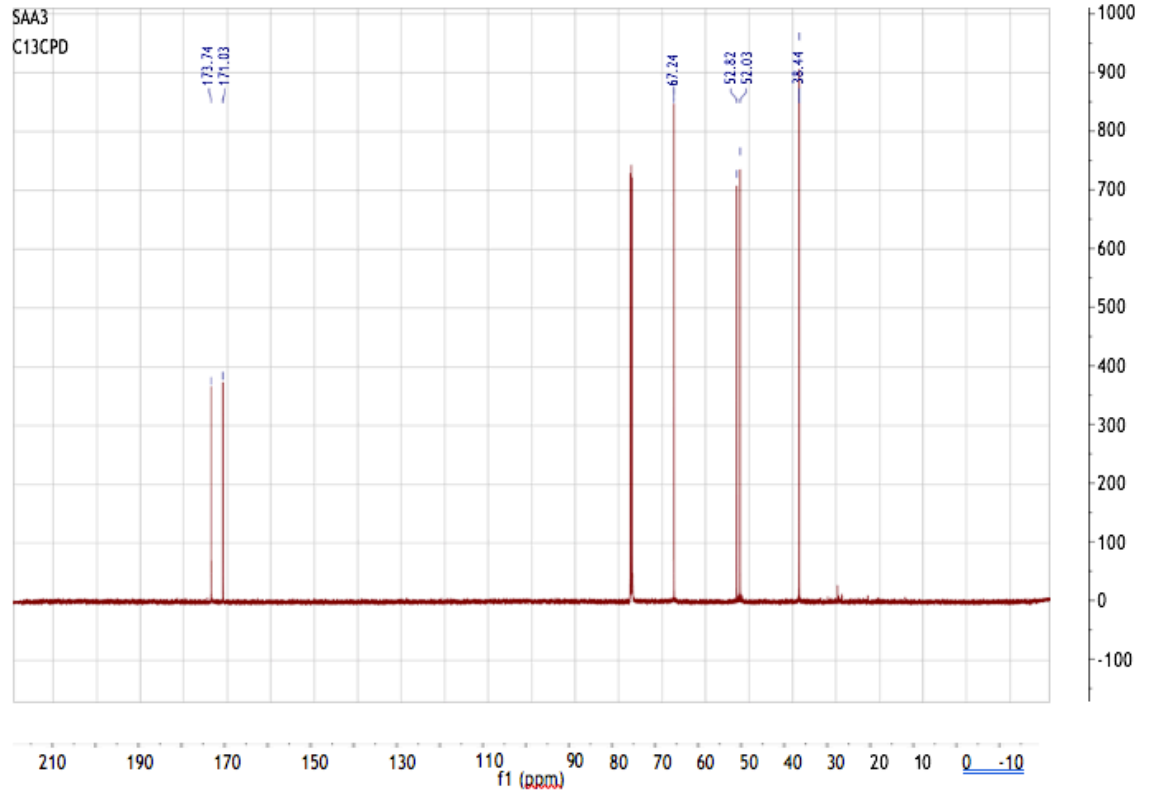

Figure 5-4 : 13C -NMR Spectrum of 3 in CDCL3

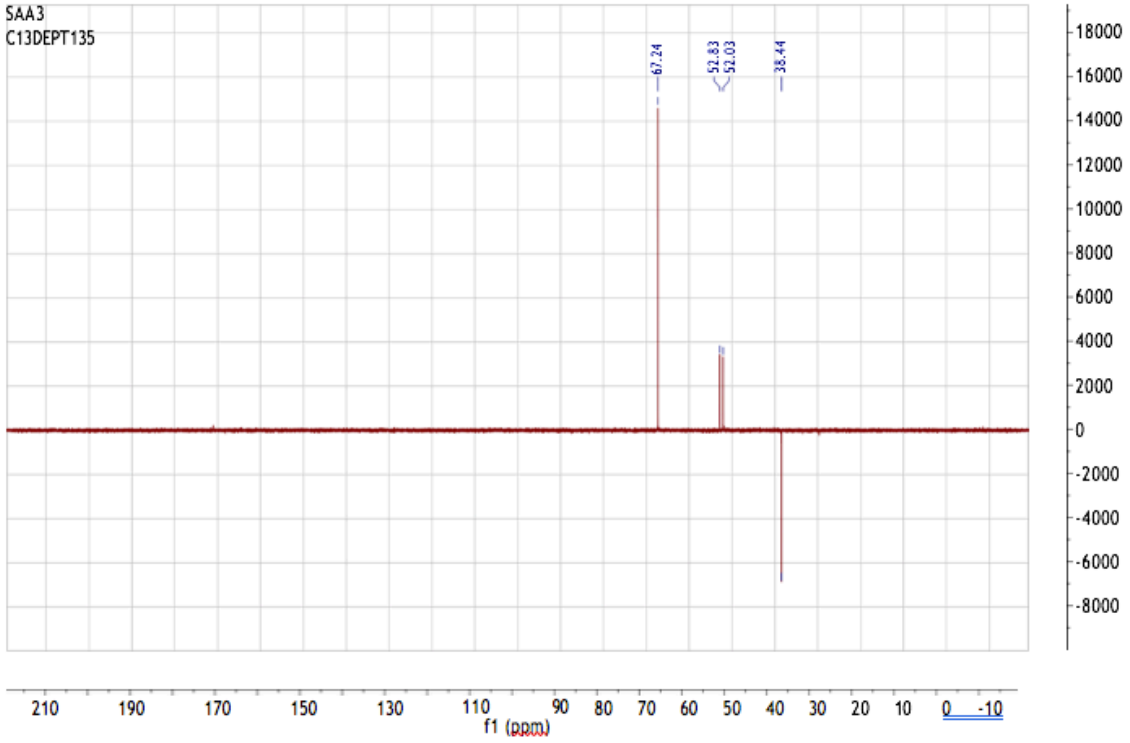

Figure 5-5 : DEPT135 -NMR Spectrum of 3 in CDCL3

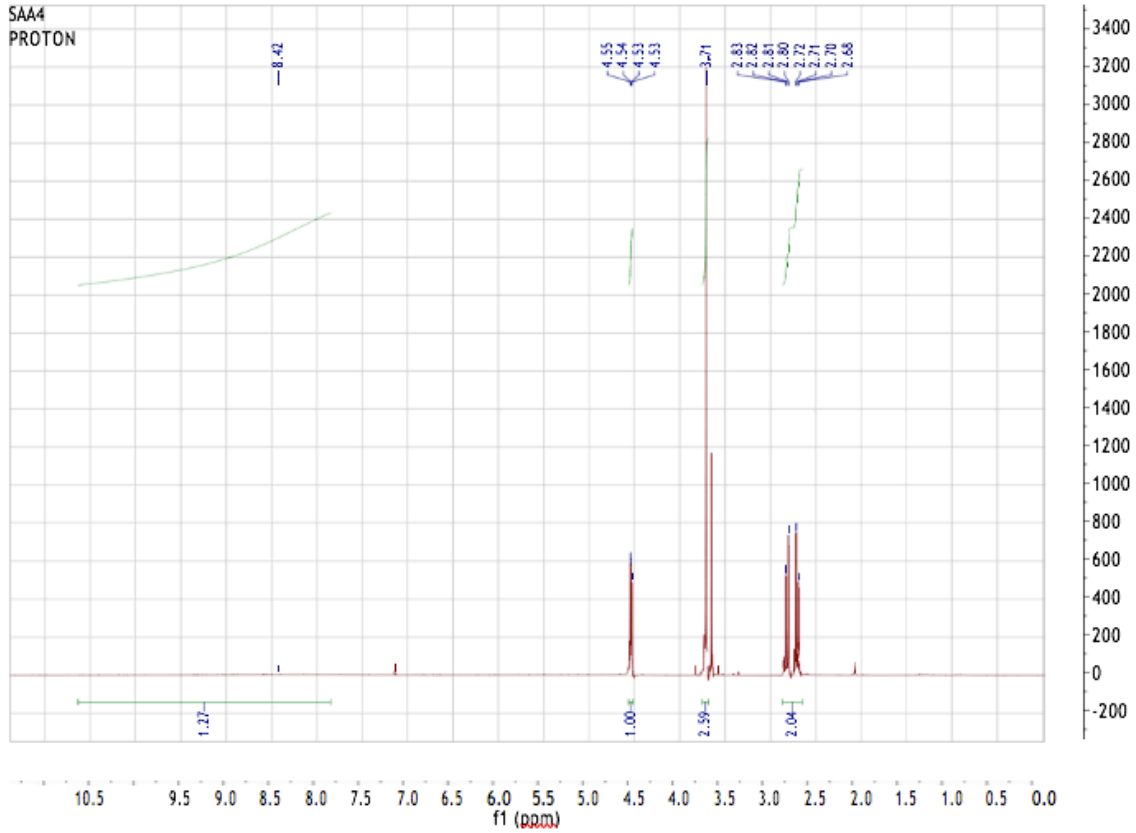

Figure 5-6 : 1H -NMR Spectrum of 4 in Acetone

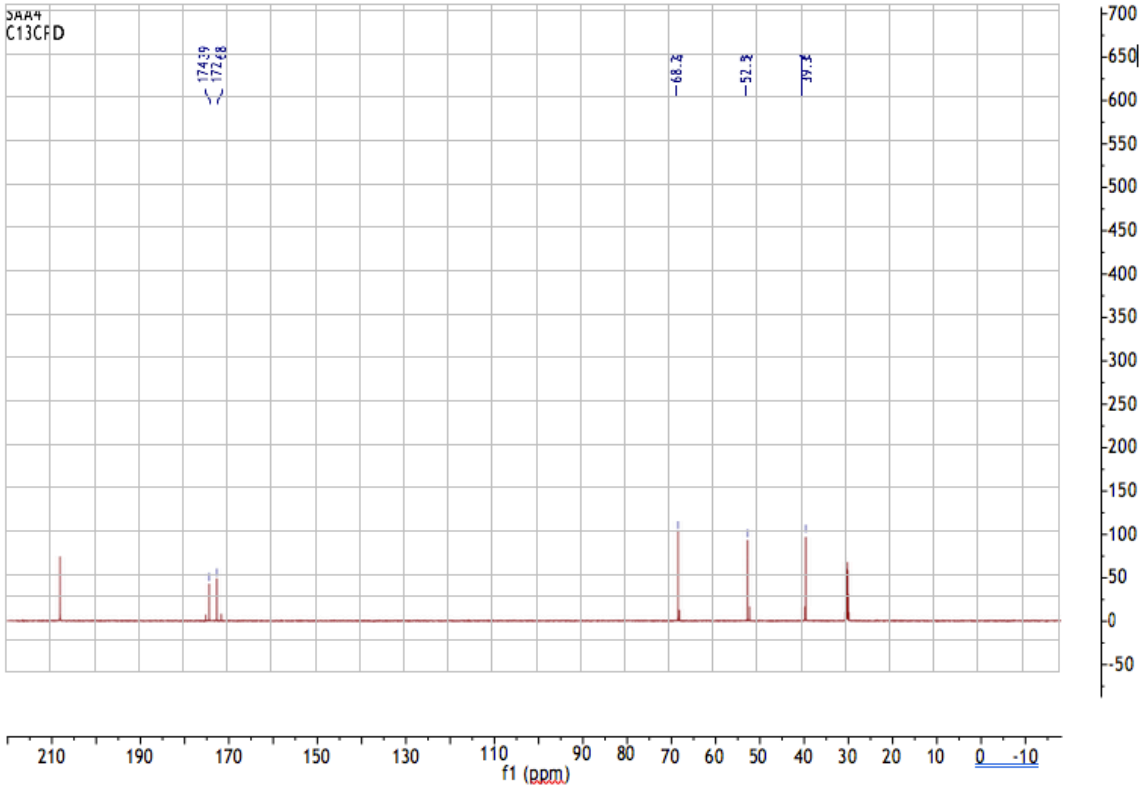

Figure 5-7 : 13C -NMR Spectrum of 4 in Acetone

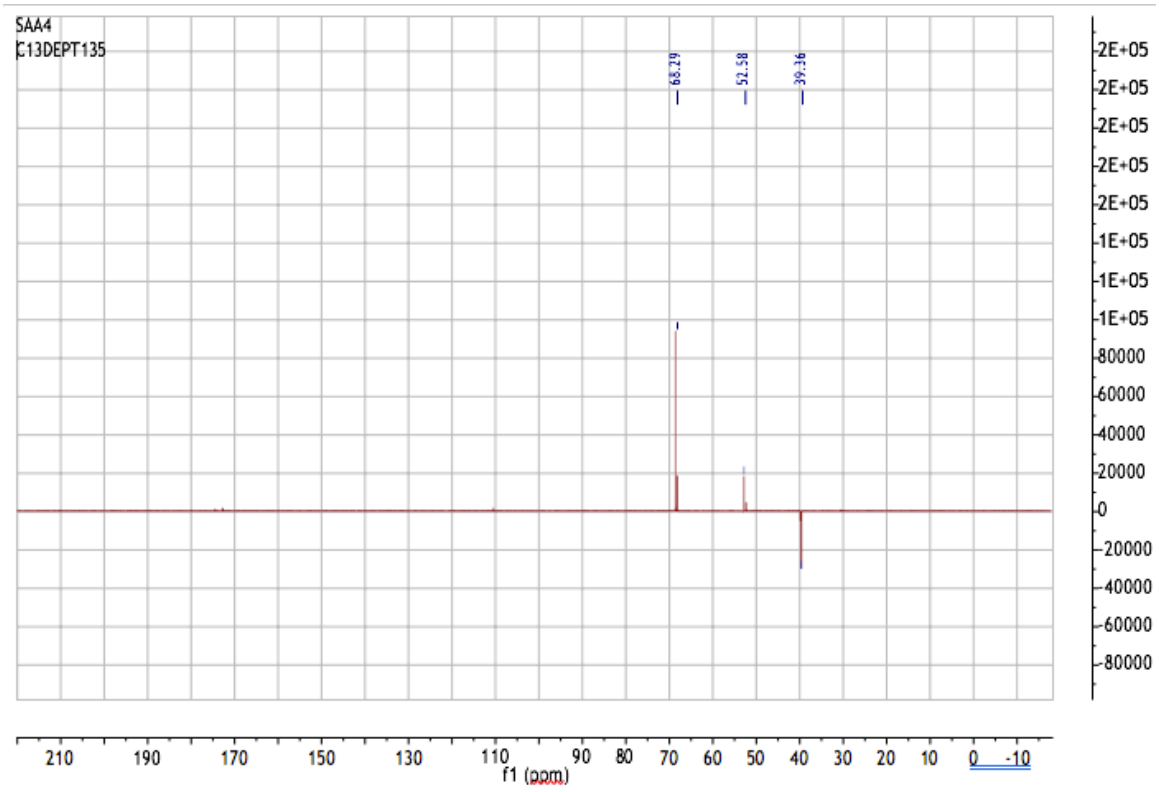

Figure 5-8 : DEPT90 -NMR Spectrum of 4 in Acetone