EVALUATION OF THE MODULATORY EFFECT OF METHANOLIC EXTRACT OF *CADABA FARINOSA* Forssk ON CARBONIC ANHYDRASE IN STREPTOZOTOCIN INDUCED DIABETIC RATS

BY

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DEPARTMENT OF BIOCHEMISTRY

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THESISSUBMITTED TO THE SCHOOL POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA-NIGERIA. IN PARTIAL FULFILLMENT FOR THE AWARD OF DOCTOR OF PHILOSOPHY DEGREE (Ph.D) IN BIOCHEMISTRY

DEPARTMENT OF BIOCHEMISTRY

AHMADU BELLO UNIVERSITY,

ZARIA-NIGERIA.

JULY, 2017

DECLARATION

I declare that the work in this Project Thesis entitled 'EVALUATION OF THE MODULATORY EFFECT OF METHANOLIC EXTRACT OF *CADABA FARINOSA* ForsskON CARBONIC ANHYDRASE ACTIVITY IN STREPTOZOTOCININDUCED DIABETIC RATS'has been carried out by me in the Department of Biochemistry, Ahmadu Bello University, Zaria.The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other Institution.

Salihu Ismail Ibrahim…… ……
PhD/SCIE/1254/2011-2012Signature Date

CERTIFICATION

This thesis titled 'EVALUATION OF THE MODULATORY EFFECT OF METHANOLIC EXTRACT OF *CADABA FARINOSA* ForsskON CARBONIC ANHYDRASE ACTIVITY IN STREPTOZOTOCININDUCED DIABETIC RATS' by Salihu Ismail IBRAHIM meets the regulations governing the award of the degree of Doctor of Philosophy (Ph.D) of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

Prof. D. A. Ameh

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Prof. S.Z. Abubakar

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Dean, School of Postgraduate Studies Signature Date

DEDICATION

I dedicate this work to my family for their abundant support, understanding and love

ACKNOWLEDGEMENTS

All praise is due to Allah for bestowing me with the knowledge and wisdom to carry out this research.

Firstly, I would like to acknowledge my supervisor(s) Prof. D.A Ameh, Prof. S.E. Atawodi and Prof. I.A. Umar for the opportunity they have provided me in putting this thesis together.I am appreciative of their encouragement, insight and their diligence towards the successful completion of this project. Without their guidance, this project would nothave gone forward or been as interesting.

I would like to express deep gratitude to the Head Department of Biochemistryfor his intellectual advice.

I would like to also thank Prof A.J. Nok, Dr Auwal Ibrahim and all the staffs of the Department of Biochemistry for their support and help.

I would like to recognize Bashir Musa of Multiuser Research Laboratories who was there in the beginning to show me the fundamentals of carrying out laboratory analysis. I would like to recognize Dr. James Habila of the Department of Chemistry, Mal Muazu, Mal. Abdullahi and Aliyu for theirassistance with the laboratory analysis and animal work.

I will not end without showing appreciation to my fellow PhD research students for providing an important support network.

Finally the biggest thanks go to my family, May Allah rewards them abundantly.

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LIST OF ABBREVIATION

FTIR	Fourier Transform infra-red spectroscopy
GC-MS	Gas Chromatography and Mass Spectra Analysis
CA	Carbonic anhydrase
NMR	Nuclear Magnetic Resonance
STZ	Streptozotocin
GSK3	Glycogen synthase kinase 3
PDH	Pyruvate dehydrogenase
CPT	Carnitine palmitoyl transferase
HGP	Hepatic glucose production
PC	Pyruvate carboxylase
HCO_3^-	Bicarbonate
PTP-1B	Protein tyrosine phosphatase-1B
MCT	Monocarboxylate transporter
DPP-IV	Dipeptidyl peptidase
hCA	Human carbonic anhydrase
HIV	Human immunodeficiency virus
GLP-1	Glucagon like peptide-1
RBC	Red blood cell
SOD	Superoxide dismutase
CAT	Catalase

ABSTRACT

Carbonic anhydrase (CA) is an ubiquitous enzyme catalyzing the reversible hydration of CO_2 to HCO_3^- and H^+. CA plays a crucial role in CO_2 transport, acid-base balance, and in linking intracellular acidosis to O_2 unloading from hemoglobin and also facilitates lactate shuttling across the monocarboxylate transporters (MCT). The study was aimed at investigating the influence of carbonic anhydrase on blood glucose concentration in Streptozotocin (STZ) induced type 1 diabetic rats, and to verify the potential antidiabetic activity of the leaves of *C. farinosa Forssk*. Carbonic anhydrase activity was determined by measuring the release of p-nitrophenol from p-nitrophenyl acetate. Glycated hemoglobin (HbA1c) was determined by ion exchange method. Glucose was determined based on glucose oxidase/ mediator reaction. Blood lactate was determined by lactate oxidase reaction. Superoxide dismutase (SOD) activity was determined by reacting with O_2^- substrate generated indirectly in the oxidation of epinephrine at alkaline pH.In the early phase of diabetes it was observed that carbonic anhydrase activity and blood lactate increases significantly ($P<0.05$) in diabetic when compared to normal control. However, as the course of diabetes progresses a significant ($P<0.05$) decrease of carbonic anhydrase activity was observed with a 2.2 fold increase in blood lactate level when compared with normal control. Treatment of diabetic rats with Acetazolamide (3.6mg/kg b.w.) resulted in 6.9 fold increase in blood lactate level and a significant($P<0.05$) reduction in carbonic anhydrase activity when compared with diabetic control. Similarly treatment with Metformin (14.2 mg/kg b.w.) increased blood lactate level and reduced carbonic anhydrase activity significantly ($P<0.05$) when compared with diabetic control. Metformin, Acetazolamide and Methanol leaf Extract of *C. farinosa* were all found to have significantly($P<0.05$) reduced blood glucose concentration when compared with control groups, *invivo.* Metformin and methanol leaf extract of *C. farinosa* were found to inhibit carbonic anhydrase enzyme activity *in vitro*against acetazolamide with an $IC_{50}$was found to be 13.57µg/100µL. The inhibition of carbonic anhydrase activity was found to be associated with increase in glycation of hemoglobin, increase in SOD activity, decrease in the activity of catalase and a reduction in the rate of lipid peroxidation (TBARS). Fourier transform infra-red spectroscopy analysis of the inhibitor fraction of the methanol leaf extract of *C. farinosa* revealed a characteristic band absorption spectrum similar to aminonitrile. GC-MS analysis revealed the presence of 4-methyl-3-(2-methylhydrazino)-phenol; 2,3,4,5-tetraacetate-D-ribonitrile; 5-ethyl-2-ethylpyridine 1-oxide derivatives of hydrazine, ribonitrile and pyridine. The ^{13}C NMR spectra revealed the presence of carboxylic acid moiety at dC 172.21 (C-1), OH-bonded methyl at dC 71.91 (C-3), and acetyloxy bonded to methylene adjacent to carbonyl dC 132.37 (C-6). On the basis of these findings, compound 1 was assigned as 6-acetyloxy-3-hydroxy-5-hexenoic acid.It can be concluded that inhibition of carbonic anhydrase activity reduced blood glucose concentration and may therefore be used as a possible therapeutic target for reduction of hyperglycemia in diabetes. The result indicated that *C. farinosa*was useful in reducing blood glucose concentration and can be used as antidiabetic medicine. The plant may also be a useful source in reducing oxidative stress.

CHAPTER ONE

1.0 INTRODUCTION

Diabetes mellitus is a metabolic disorder of impaired glucose regulation, characterized by persistent hyperglycemia. Hyperglycemia has been associated with increased morbidity and mortality in diabetic subjects. The development of long-term complications in diabetes is influenced by hyperglycemia. Poor control of hyperglycemia accelerates progression of diabetic complication.

Hyperglycemia, the major defining feature of all forms of diabetes, arises due to absolute or relative insulin deficiency, increased hepatic glucose production (HGP), due to increased hepatic lactate uptake produced by glycolytic cells. It has been reported that the plasma glucose level is maintained by regulation of HGP and glucose uptake in the peripheral tissues (DeFronzo and Ferrannini, 1987; Cherrington *et al*., 1987). Moreover, fasting plasma glucose level has been shown to be directly correlated with the rate of fasting hepatic glucose production (Bogardus *et al*., 1984; Firth *et al*., 1987). Thus, the increase in hepatic glucose production in type 2 diabetes may be due to increased supply of gluconeogenic precursor (lactate) for increase gluconeogenesis. Previous studies have found that increased rates of glucose lactate interconversion termed cori cycle have been observed in diabetic dogs (Stevenson *et al*., 1983). Zawadzki *et al.* (1988) concluded that the rates of endogenous glucose production and that of Cori cycle are increased in subjects with type 2 diabetes. These showed that, increased provision of lactate is of considerable importance for increased hepatic glucose production in type 2 diabetes.

Diabetes is being combated through aggressive treatment directed at lowering circulating blood glucose and inhibiting postprandial hyperglycemic rise mainly by stimulating pancreatic β-cells to secret more insulin, increasing insulin receptor

sensitivity, inhibiting hepatic glucose production and also enhancing glucoseuptake by glucose utilizing tissues. Current strategies to treat diabetes include reducing insulin resistance using glitazones (or thiozolidinediones) which are insulin sensitizers, supplementing insulin supplies with exogenous insulin, increasing endogenous insulin production with oral hypoglycemic such as sulfonylureas and meglitinides, reducing hepatic glucose production through biguanides, and limiting postprandial glucose absorption with alpha-glucosidase inhibitors. Promising therapeutic targets are also emerging such as (1) insulin sensitizers including protein tyrosine phosphatase-1B (PTP-1B) and glycogen synthase kinase 3 (GSK3), (2) inhibitors of gluconeogenesis like pyruvate dehydrogenase kinase (PDH) inhibitors, (3) lipolysis inhibitors, (4) fat oxidation including carnitine palmitoyltransferase (CPT) I and II inhibitors, and (5) energy expenditure by means of beta 3-adrenoceptor agonists.

Current treatments are often inefficient at sustaining glycemic control and may cause undesirable side effects. Therefore, a new and more effective therapeutic target needs to be discovered. Emerging targets for therapy especially for type 2 diabetes presently include inhibition of gluconeogenesis, lipolysis, and fatty acid oxidation, as well as stimulation of β3-adrenergic receptors (Moneva and Dagogo-Jack, 2002).

The mammalian liver is the primary site of gluconeogenesis, where lactate (a gluconeogenic substrate) taken up by the liver is oxidized to pyruvate which is then converted to glucose by pyruvate carboxylase. Bottger and Wieland (1969) reported that the carboxylation of pyruvate by pyruvate carboxylase (PC) occur exclusively in the mitochondria.Previous study by Herbert et al (1983) supported the role of CA in supplying HCO_3^- forpyruvate carboxylation in chameleons and alligators. Garg (1974) and King *et al.* (1974) previously reported the presence of endogenous

carbonic anhydrase (CA) in rat liver.previous studies have shown that, CA provides the HCO_3^- required for the initial steps in glucose synthesis,fatty acid synthesis, general amino acid synthesis, and urea synthesis (Coulson and Herbert, 1984; Dodgson and Contino, 1988; Dodgson and Forster, 1986; Haussinger and Gerok, 1985; Herbert *et al*., 1975).

Carbonic anhydrase is found in the blood of all vertebrates. Some early evidence suggests that the changes in carbonic anhydrase activities in erythrocytes may be an initial step of altered metabolism in diabetes mellitus (Gambhir *et al*., 1997). However, the precise role of carbonic anhydrase activity, in the development of diabetes complication, is currently unknown. It has been reported that inhibition of carbonic anhydrase was found to impair proton secretion into the proximal tubule lumen thereby decreasing bicarbonate reabsorbtion and rate of acidification of urine, producing alkaline urine and eventually metabolic acidosis (Hannedoeche *et al.*, 1991). Carbonic anhydrase inhibitors were previously widely used in clinical practice as diuretics and as antihypertensive drugs (Pastorekova *et al*., 2004). Recent studies showed carbonic anhydrase inhibitors may provide a novel therapy for obesity, cancer and Alzheimer's disease (Supuran, 2008). However, as carbonic anhydrase are ubiquitous enzymes in vertebrates, carbonic anhydrase inhibition in organs other than the target may result in undesired side effects. The most frequent ones are: numbness and tingling of extremities; metallic taste; depression; fatigue; malaise; weight loss; decreased libido; gastrointestinal irritation; metabolic acidosis; renal calculi and transient myopia (Bartlett and Jaanus, 1989; Maren, 1992; Maren, 1995; Barboiu *et al*., 1999), which are all common among diabetic patients.

Previous studies have found out that carbonic anhydrase inhibitors inhibit gluconeogenesis *in vitro*or after acute administration in rats (Cao and Rous, 1978;

Dodgson and Forster, 1986). It has also been reported that changes in CA activity is associated with metabolic diseases such as diabetes mellitus and hypertension (Parui *et al.*, 1991).Carbonic anhydraseis one of the fastest enzymes known and performs well established, major roles in respiration and acid-base balance.The enzyme has been found to play a significant role in metabolic processes by providing bicarbonate as a substrate for gluconeogenesis, ureagenesis, pyrimidine synthesis and fatty acid synthesis (Chegwidden *et al.*, 2000).

Dodgson and Watford (1990)reported that; diabetes results in a twofold increase in the activity of CA. They suggested that the increase in CA during diabetes is due to its importance in provision of HCO_3^- as substrate for hepatic gluconeogenesis and ureagenesis.Dodgson and Forster (1986)concluded that CA is functionally important for gluconeogenesis in the male guinea pig liver.A recent study by Becker and Deitmer, (2004); Becker and Deitmer, (2008) has significantly enhanced our understanding of the role of CA in lactate transport. It was demonstrated that lactate movement in/out of the cells is facilitated by carbonic anhydrase and bicarbonate transporters in various cells and tissue through mono-carboxylate transporters (MCT). We hypothesize the possible linkage between lactate accumulation and reduction in blood glucose through inhibition of carbonic anhydrase;considering the role played by carbonic anhydrase in hepatic glucose production and transport of lactate in/and out of cells via the MCTs.

Cadaba farinosa Forssk belongs to the family capparideae and is a slender shrub with a strongly furrowed stem that grows in the African Sahel and Arabia (Hendrick 1919; Bartha, 1970). It has a variety of common names: in Hausa as *Bagayii*, Fulfulde as*balamji* and in Kanuri asgursimé. Ground dried leaves of *Cadaba farinosa* are also added as a sweetener to a cereal-based porridges called “kunun

zaki" in Hausa (Gaffa *et al*. 2002; Terna *et al*., 2002). The plant is used by locals in some part of Jigawa state for the treatment of cancer and diabetes. According to ethnomedical information the plant *Cadaba farinosa* has been used for treating various ailments, and used as anti- inflammatory, anti-diabetic and in female fertility (Tenpe *et al*., 2006; Nadkarni, 2002).

1.1 Statement of Research Problem

Persistent or uncontrolled hyperglycemia is associated with increased and premature morbidity and mortality. The development of long-term complications in diabetes is influenced by hyperglycemia. Poor control of hyperglycemia accelerates its progression.

Despite the availability of successful prevention strategies and treatments, the number of individuals with diabetes and its associated complications continue to increase. The currently available antidiabetic agents are widely used to control hyperglycemia and hyperlipidemia, but these drugs fail to significantly alter the course of diabetic complications and have limited use because of undesirable side effects and high rates of secondary failure.

The manifestation of diabetes causes considerable human suffering and enormous economic costs. Despite public health efforts, diabetes remains a serious cause of morbidity and mortality. Therefore patients with diabetes mellitus are at especially increased risk of developing macrovascular and microvascular complications, including coronary heart disease, stroke, peripheral vascular disease, hypertension, nephropathy, neuropathy, and retinopathy.

1.2 Justification of the study

Increased hepatic production of glucose is one of the primary causes of persistent hyperglycemia in diabetes. Carbonic anhydrase plays a significant role in hepatic glucose production by providing HCO_3^- as a substrate for the pyruvate carboxylase reaction and also in facilitating hepatic lactate (gluconeogenic substrate) uptake for gluconeogenesis(Chegwidden *et al.,* , 2000). Thus, efforts to better understand the role of carbonic anhydrasein hepatic glucose productionis paramount for development of treatment strategies in diabetes mellitus.In particular, numerous studies have established that elevated levels of blood lactateis associated with diabetes.

Carbonic anhydrase (CA) has been associated with diabetes mellitus and hypertension (Parui *et al*., 1992; Tashian *et al*., 1991; Wistrand and lindquist, 1991; Parui *et al*., 1991). Therefore new biochemical targets are urgently required to reduce circulating blood glucose level. Compounds that can inhibit carbonic anhydrase may be useful in the treatment of diabetes.

Contemporary drug discovery is under increased pressure to identify more suitable small molecules as chemical starting points for drug development and finding novel compounds as starting points for drug discovery research. Natural products already provide a significant portion of approved drugs and have emerged as an effective way to sample chemical diversity.

Thus, it is essential to look for a more effective and safe therapeutic compound with promising antidiabetic activity. The identification of unique CA inhibiting compound might offer possibilities for future rational drug discovery design and development.

1.2 Aim of the study

The studywas aimed at investigatingthe role of carbonic anhydrase in diabetes mellitus, and to determine the beneficial effect of *C. farinosa* in the management of diabetes.

1.3.1 Specific Objectives

The specific objectives of the study were to:

i. Investigate the changes of erythrocyte carbonic anhydrase activity on blood glucose, lactate, cholesterol and triglyceride level in STZ induced diabetic rats.
ii. Determine the i*n vitro* inhibitory activity of Metformin, crude leaf extract and column fraction of *C. farinosa* on carbonic anhydrase activity.
iii. Investigate *in vivo* inhibitory activity of Acetazolamide, Metformin and leaf extract of *C. farinosa* on carbonic anhydrase, HbA1c and other metabolic parameters.
iv. Study the antioxidant potential of leaf extract of *Cadaba farinosa* on STZ induced diabetic rats.
v. Identify the potential component(s) responsible for anti-diabetic activity of leaf extract of *Cadaba farinosa* using FTIR and GC-MS analysis.
vi. Isolate and characterize the active compound(s) responsible for antidiabetic potential of *C. farinosa.*

1.3 Research hypotheses

Inhibition of carbonic anhydrase does not reduce circulating blood glucose.

Methanolic leaf extract of *C. farinosa* does not have anti diabetic activity.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Carbonic Anhydrase

In the beginning of the 20th century, it was not clear how bicarbonate was transported in the blood and released in the lung capillaries; however, it was known that the transit time of blood through the capillaries was only about 1 second. In the late 1920s, it was realized that blood probably contains a catalyst for this reaction (Henriques 1928; Van Slyke and Hawkins 1930). Some years later, it became evident that the catalyst in blood is, indeed, an enzyme (Meldrum and Roughton 1932; Meldrum and Roughton 1933). The name of this enzyme was reportedly suggested by Philip Eggleton to be "carbonic anhydrase" (Davenport 1984). Nevertheless, it was not until 1960 that the carbonic anhydrase CA enzyme was purified for the first time from bovine erythrocytes (Lindskog 1960).

Carbonic anhydrases (CAs) (EC 4.2.1.1) are zinc-containing metalloenzymes that catalyze the reversible hydration of carbon dioxide to bicarbonate and a proton.

$$CO_2 + H_2O \longleftrightarrow H_2CO_3 \longleftrightarrow HCO_3^- + H^+$$

Carbonic anhydrase is one of the fastest enzymes known and performs well established functions. The CAs are produced in a variety of tissues where they participate in several physiological processes, such as pH regulation, CO_2 and HCO_3^- transport, the production of body fluids, bone resorption, and metabolic processes.

In mammals, each characterized CA enzyme is a member of the α - CA family. This family consists of 13 active isozymes, 12 of which are expressed and functional in humans (Hilvo *et al*. 2005). Each isozyme has a characteristic subcellular

localization, distribution within the body, enzymatic activity, and affinity for inhibitors: CA I, II, III, VII, and XIII are cytoplasmic; CA IV, IX, XII, XIV, and XV are anchored to plasma membranes; CA VA and VB are mitochondrial; and CA VI is the only secreted protein.

2.1.1 Catalyticmechanism of carbonic anhydrase

The α-CAs are globular proteins that exhibit a considerable degree of three dimensional similarity and a typical folding structure characterized by a central anti-parallel β-sheet (e.g Cu-Zn Superoxide dismutase) (Di Fiore *et al*., 2009). The active site is located in a large, cone shaped cavity that reaches the center of the protein molecule. The metal ion, which is Zn^{2+} in all α-CAs examined to date, is essential for its catalytic mechanism (Lindskog and Silverman, 2000; Supuran, 2004). The Zn^{2+} ion is coordinated by three histidine residues (His94, His96 and His119 in CA II) and a water molecule or hydroxide ion (Stams and Christianson 2000). The active form of the enzyme is basic with a hydroxide ion bound to Zn^{2+} (Lindskog and Silverman, 2000). This strong nucleophile attacks the CO_2 molecule that binds in a nearby hydrophobic pocket, leading to the formation of bicarbonate coordinated to Zn^{2+}. The bicarbonate ion is subsequently displaced by a water molecule and liberated into solution. This results in the enzyme being in the acidic, catalytically inactive form with a water molecule coordinated to Zn^{2+}. This reaction is shown in Equation (1) (Lindskog and Silverman 2000, Supuran 2004). To regenerate the basic form, a proton transfer reaction from the active site to the environment takes place, which may be assisted either by active-site residues or by buffers present in the medium. This reaction is represented in Equation 2 (Lindskog and Silverman 2000; Supuran, 2004).

Equation (1) $EZn^{2+} - OH^{-} + CO_2 \longleftrightarrow EZn^{2+} - HCO_3^{-} \xleftrightarrow{H_2O} EZn^{2+} - OH_2 + HCO_3^{-}$

Equation (2) $EZn^{2+} - OH_2 \longleftrightarrow EZn^{2+} - HO^{-} + H^{+}$

The rate-limiting step in the catalysis is the proton transfer reaction Equation (2). In isozymes with high catalytic activity, this step is assisted by a histidine residue (His64), which is termed a proton-shuttle residue and is located at the opening of the active site (Tu *et al*., 1989). In addition to His64, a unique cluster of histidine is thought to be important for the high catalytic activity of the CA II isozyme, ensuring an efficient proton transfer process (Di Fiore *et al*., 2009). This property also explains the fact that CA II is one of the most active enzymes known, approaching the limit of diffusion control. Interestingly, in addition to its catalytic function, the intramolecular proton shuttle supports a non-catalytic function of CA II by enhancing the activity of acid- or base-transporting proteins in a direct, non-catalytic manner (Becker *et al*., 2011).

In addition to the reversible hydration of carbon dioxide, α- CAs can catalyze a variety of other reactions, such as the hydration of cyanate to carbamic acid, the hydration of cyanamide to urea, and other less-investigated processes. It is still unclear whether CA-catalyzed reactions other than CO_2 hydration have physiological significance (Supuran, 2004).

2.2 Carbonic anhydrase and lactate uptake

Wetzel *et al.*(2001) presented the first evidence for a facilitated transport of lactate by carbonic anhydrase. Hallerdei *et al*. (2010) shows that gene knock-out of the extracellular CA isoforms IV, IX and XIV in mouse muscle all lead to a reduction in lactate influx and efflux.A recent study by Becker and Deitmer, (2004; 2008) has

significantly enhanced our understanding of the role of CA in lactate transport. It was demonstrated that lactate movement in/out of the cells is facilitated by carbonic anhydrase and bicarbonate transporters in various cells and tissue through mono-carboxylate transporters (MCT). It has also been reported by Becker and Deitmer, 2004; Becker and Deitmer, 2008; Becker et al., 2010, that carbonic anhydrase II (CAII) interaction with MCT1 and MCT4 increased its transport activity.They suggested that CA isoforms can cooperate to modulate rapid shuttling of metabolites through MCTs across the cell membrane. Indeed they were able to show that the presence of both intracellular CAII and extracellular CAIV enhance transport activity of MCT1 and MCT4 in both directions, *i.e.* influx and efflux of H^+/lactate to a higher extant than did either isoform alone.

However, Nguyen and Bonano (2011), found evidence supporting the notion that lactate-H^+ cotransport via monocarboxylate transpoters (MCT) 1,2 and 4 is facilitated by HCO_3^-, Carbonic anhydrase activity, Na^+ /H^+ exchange, and $1Na^+$: 2 HCO_3^- - co transport. It has been clearly demonstrated that lactate is capable of entering cells *via* the monocarboxylate transporter (MCT) protein shuttle system (Andrew *et al*., 2005). Liver is the major organ that is responsible for lactate clearance. Luft (2001) reported that approximately 1400 mmol of lactic acid are produced daily, which are buffered by 1400 mmol of HCO_3^- to form sodium lactate. It has been demonstrated that the enhancing effect of CAII on H^+/lactate influx via MCT1 and MCT4 increased with increasing extracellular proton concentration, but decreased with extracellular lactate concentration (Becker and Deitmer, 2008; Becker *et al*., 2010; Almquist *et al.*, 2010).

2.3 Carbonic Anhydrase in Hepatic Gluconeogenesis

Earlier studies have demonstrated the activity of carbonic anhydrase in the liver. Carbonic anhydrase play significant role in the provision of bicarbonate as substrate for carboxylation in various metabolic pathways which include gluconeogenesis, amino acid synthesis, lipogenesis, ureagenesis and pyrimidine synthesis (Chegwidden*et al.,* 2000). The first mitochondrial CA was isolated from guinea pig liver and called CA V (Dodgson, 1987). It was subsequently identified in mouse, rat, and human through molecularcloning (Nagao *et al*., 1993; Nagao *et al*., 1994).

Garcia-Marín *et al.* (1991) previouslydemonstrated the activity of plasma membrane-associated CA in hepatocytes. However, the study of Seppo*et al.* (2002) demonstrated the expression of CA XIV at the hepatocyte plasma membrane, where they suggest the role of this isozyme in liver pH homeostasis.

An earlier study that came out from Carter *et al*., (1987) laboratory demonstrated localization of both CAII and CAIII isozyme in the perivenous area of the liver. Dodgson and Forster (1986) showed the effect of carbonic anhydrase inhibitor acetazolamide on mitochondrial carbonic anhydrase isozyme five (V) (CA V) and of ethoxzolamide on total hepatocyte carbonic anhydrase inhibition. In a recent study, Carmen *et al*. (2016) showed that carbonic anhydrase isozyme Vforms (CAVA) and (CAVB) provide the bicarbonate required by four mitochondrial metabolic liver enzymes. They reported that defective provision of bicarbonate, which may result from inhibition of hepatic carbonic anhydrase, leads to hyperammonemia, elevated lactate and ketone bodies, metabolic acidosis, hypoglycemia, and excretion of carboxylase substrates.

In 1983 it was reported from a different laboratory that the CA inhibitor acetazolamide decreased rat tubular glucose synthesis; the authors were unaware of the existence of CA V and concluded that this finding was a nonspecific effect of acetazolamide on the proximal tubule (Tannen and Ross, 1983). Recent studies have been aimed at examining the hypothesis that renal CA V is involved in glucose synthesis in proximal convoluted tubules when pyruvate is added as the sole substrate.In order to test their hypothesis that CA V is needed to provide the substrate HCO_3^- for pyruvate carboxylase in the liver,Dodgson and Cherian (1989) starved rats for 48 hrs so they may remove all endogenous glucose from the proximal convoluted tubules and also to maximize the activity of gluconeogenic enzymes. They showed that CA inhibitor ethoxzolamide inhibit CA V activity of intact mitochondria. They determined that glucose synthesis was a linear function of time in both the presence and absence of the inhibitor. They showed that when proximal convoluted tubules were incubated in Krebs-Henseleit buffer (which contains 25 mM $NaHCO_3$) and 10 mM pyruvate with 5% CO_2 and 95% O_2, glucose synthesis was decreased increasingly by increasing the concentrations of ethoxzolamide; 50% was decreased by 0.6 µM concentration of the inhibitor.

In a subsequent study, acetazolamide (100 µM) and benzolamide (600 µM) mediated decrease of glucose synthesis could not be overridden by doubled pCO_2, leading to the conclusion that a non-mitochondrial CA participates in glucose synthesis (Dodgson and Cherian, 1990). Their conclusion of non-mitochondrial participation of CA cannot hold because no CA catalyses the direct synthesis of glucose, increase provision of $NaHCO_3$ and pCO_2 cannot be responsible for glucose synthesis but rather the facilitated lactate uptake by CA is what brings about glucose synthesis through gluconeogenesis.

Dodgson and Watford (1990) reported in one of their diabetic studies that; diabetes results in a twofold increase in the activity of CA V. They suggested that the increase in CA V isozyme during diabetes is due to its importance in provision of substrate for hepatic gluconeogenesis and ureagenesis. In a different study Dodgson and Watford (1990) examined the changes in the activity of CA-III in hepatocytes of acute diabetic rats. They found out that diabetes resulted in 50% reduction in CA-III isozyme activity. Whereas in the liver of chronic diabetic rats an approximately 98% reduction in CA-III content in the liver was observed. This reduction in hepatic carbonic anhydrase activity in chronic diabetic state may probably be an adaptive response by the liver to reduce hepatic lactate uptake for gluconeogenesis. Coulson and Herbert (1984)concluded that mitochondrial CA inhibition has been responsible for decreased urea and glucose synthesis by alligators and chameleons *in vivo,* and also Haussinger and Gerok (1985) and Marsolais *et al.* (1987) concluded that it isresponsible for decreased urea synthesis in isolated perfused rat livers. Decreased urea and glucose synthesis has been reported in isolated rat hepatocytes by (Boon and Meijer, 1988; Rognstad, 1983) and in isolated guinea pig hepatocytes by (Dodgson and Forster, 1986; Dodgson, 1987) after CA inhibition.

2.4 Carbonic Anhydrase Inhibitors

Enzyme inhibitors are of immense importance. Every living organism relies on inhibitors as feedback signals that coordinate metabolic pathway activity: by reducing the catalytic activity of their respective enzyme targets, inhibitors throttle the flux of intermediates through a pathway. Over the past century, biochemists have documented countless naturally occurring and synthetic compounds as well as simple inorganic ions that inhibit enzyme catalysis. The identification and characterization of new enzyme inhibitors is a major intellectual enterprise for

Biochemists. The overarching goal is to develop systematic ways for improving the effectiveness (i.e., potency and specificity) of enzyme inhibitors.Carbonic anhydrase inhibitors were shown to be useful as diuretics, or in the treatment and prevention of a variety of diseases such as glaucoma, epilepsy, congestive heart failure, mountain sickness, gastric and duodenal ulcers, neurological disorders and osteoporosis, among others (Maren, 1991; Supuran, 1994; Puscas and Supuran, 1996; Wistrand and Lindqvist, 1991).Even so, carbonic anhydrase inhibitors (CAI)s have a firm place in medicine, mainly as anti-glaucoma or anti-secretory drugs, diuretics, as well as agents for the treatment/prevention of several neurological disorders. Several such derivatives – acetazolamide, methazolamide, ethoxzolamide and dichlorophenamide - were successfully used in clinical medicine for the last 45 years (Claudiu and Andrea, 2000).

2.4.1 Carbonic anhydrase inhibition

Carbonic anhydrase (CA) plays a fundamental role in regulation of systemic acid-base homeostasis by facilitating urinary acidification in kidney. More than 95% of renal CA activity is cytosolic and corresponds to CA II; only 5% is membrane-associated (McKinley and Whitney, 1976; Wistrand and Kinne, 1977; Alpern and Rector, 1996). Cytosolic CA II facilitates H^+ secretion by catalyzing the formation of HCO_3^- from cellular OH^- generated from the secretion of protons in the presence of CO_2 (Maren, 1967; Pitts and Alexander, 1945;Rector *et al*., 1960). CA II also facilitates the diffusion of CO_2 through aqueous solutions at physiological pH (Enns, 1967; Gros *et al*., 1976), and this function may help mediate renal H^+ secretion. Nonetheless, inhibition studies showed that a non absorbable, large molecular weight CA inhibitor blocked up to 90% of bicarbonate absorption in isolated,

perfused kidney and in vivo micropuncture (Lucci *et al*., 1980; Lucci *et al*., 1983; DuBose and Lucci, 1983).

The fundamental importance of CA II in renal acidification was demonstrated in humans with CA II gene mutations who exhibited renal tubular acidosis (Sly *et al*., 1983). CA II deficient mice produced by chemical mutagenesis also had impaired renal acidification (Lewis *et al*., 1988). However, administration of carbonic anhydrase inhibitors to CA II deficient patients and mice led to bicarbonaturia, supporting a key role for the membrane-associated CA activity in renal bicarbonate reabsorption (lewis *et al*., 1988; Brechue *et al*., 1991).

During metabolic acidosis the kidney responds by increasing H^+ secretion in the proximal tubule and collecting duct (Alpern *et al*., 1991). The renal response to acidosis is complex, involving increases in protein and DNA and RNA synthesis (Lotspeich, 1965). Despite its high turnover rate, CA II activity, as well as mRNA, has also been found to be increased during chronic metabolic acidosis (Brion *et al*., 1991; Schwartz *et al*., 1993). Brion *et al*., (1994) showed that chronic metabolic acidosis (CMA) accomplished by NH_4Cl loading with food restriction induces soluble CA activity in rabbit renal cortical homogenates. They conclude that CMA induces CA activity in the proximal convoluted tubule PCT (CA II and CA IV) and the outer medullary collecting ducts OMCD (most likely CA II) of adult rabbit kidneys. The induction of CA activity accompanies the increase in urinary acidification observed in CMA. Previous studies have established that the erythrocyte CA concentration in chronic kidney disease patients is increased, but the mechanism of this has not been clarified (Anker *et al*., 1977; Goriki *et al*., 1982; Yamakido *et al*., 1982). The acid–base equilibrium is maintained by kidneys through the action of CA, and thus variation in the level of this enzyme can be

expected in kidney disease patients, where the acid might lead to an adaptive increase of the CA.Inhibition of luminal CA activity blocks the majority of bicarbonate absorption of the proximal tubule ((Lucci *et al*., 1980; Lucci *et al*., 1983).

Inhibition of CA causes alkalinization of proximal tubular cells (Kleinman *et al*., 1980), which would in turn inhibit luminal H^+ secretion. Indeed, it has been observed, in decreased HCO_3^- absorption (H^+ secretion) in the presence of basolateral CA inhibition. The mechanism for this decrease in proximal tubular HCO_3^- absorption in the setting of basolateral CA inhibition has been reviewed by Seki *et al*. (1996) and may result from development of alkaline pH disequilibrium in the basal labyrinth due to the accumulation of large amounts of HCO_3^- via the basolateral Na^+- HCO_3^- cotransporter (Muller-Berger *et al*., 1997). Alkalinization of the cell by basolateral CA inhibition also may have a direct effect on electroneutral NaCl and fluid absorption, mediated via the basolateral Na^+ - dependent Cl_2/HCO_3^- exchanger (Alpern and Chambers, 1987), which is known to be functional in the proximal straight tubule (Sasaki and Yoshiyama, 1988). As cell pH is increased, the driving force for chloride exit is decreased, so that overall electroneutral NaCl transport is inhibited. This could further decrease fluid and HCO_3^- absorption.

2.4.2Carbonic anhydrase inhibition in the liver

Carbonic anhydrases are known for their efficient reversible hydration of carbon dioxide to generate bicarbonate (HCO_3^-). In the liver HCO_3^- is required as a substrate for at least three (3) important metabolic pathways namely; urea synthesis, gluconeogenesis and fatty acid synthesis.Carbamoyl phosphate synthetase I utilizes

HCO_3^-rather than CO_2 for thesynthesis of carbamoyl phosphate (Dodgson, 1987). This is the committed step in ureagenesis.Ornithine transcarbamylase utilizes carbamoyl phosphate as a co-substrate in thesynthesis of citrulline (Dodgson and Forster, 1984; Dodgson and Watford, 1990), which is the first intermediate of the urea cycle.Mitochondrial carbonic anhydrase supplies the HCO_3^- required in the carbamoyl-phosphate synthetase I reaction as reported by (Dodgson, 1987; Nguyen and Bonanno, 2011) for the production of urea from ammonia. This indicates that inhibition of mitochondrial carbonic anhydrase will drastically affect urea synthesis from ammonia due to low supply of HCO_3^-, therefore the amount of NH_3 to be disposed will exceeds that of HCO_3^- and this will result in NH_3 accumulation leading to a condition known as hyperammonemia as reported by Carmen *et al.,* (2016).Earlier studies have reported that incubation of mice mitochondria with acetazolamide results in pyruvate carboxylase inhibition (Carmen *et al.,* 2016). They concluded that acetazolamide inhibits pyruvate carboxylase directly. Their data showed that the activity of pyruvate carboxylase is not pH dependent, as the mitochondrial carbonic anhydrase.Acetazolamide inhibits pyruvate carboxylase to around 80 to 85% at the same concentration mitochondrial carbonic anhydrase was fully inhibited (Cao and Rous, 1978). Barrittg *et al*., (1976)found out that injection of carbonic anhydrase inhibitors into alligators and chameleons resulted in inhibition of work-induced hyperglycemia. In a similar study acetazolamide and ethoxyzolamide were found to inhibit gluconeogenesis in vitro or after acute Invivo administration in rats (Dodgson and Forster, 1986; Bode et al., 1994). Dodgson and Forster (1986)concluded that carbonic anhydrase is functionally important for gluconeogenesis in the male guinea pig liver.Previously, Kowalchuk *et al.,* (1992) have observed that the post-exercise increase in lactate concentration in the blood

was significantly lower after treatment with acetazolamide. This indicates that acetazolamide inhibited the carbonic anhydrase facilitated lactate out flux across the MCTs in to the blood.Inhibition of extracellular muscle CA by Geers *et al*., 1997 causes a decrease of lactate release from moderately exercising isolated blood-perfused muscle. DeFronzo *et al.,* (1985)proposed that increased muscle release of lactate and alanine could be responsible for sustaining increased gluconeogenesis in type 2 diabetes. Since lactate is required as a substrate for gluconeogenesis.

Some studies have shown that suppression of hepatic gluconeogenesis is accompanied by increase in blood lactate level like the case with metformin therapy on type II diabetes. Radziuk *et al.,* (1997) reported that high therapeutic metformin levels provoked a reduction in lactate uptake by the liver. This reduction in hepatic uptake may be the reason metformin concentration increases in the blood.It has also been reported byLutz *et al.,* (2001) that the uptake of gluconeogenic substrates, such as alanine and lactate,is reduced in the presence of metformin. Cusi *et al.,* (1996) suggested from clinical studies and animal models that the primary function of metformin is to decrease hepatic glucose production, mainly by inhibiting gluconeogenesis (Hundal, et al., 2000; Natali and Ferrannini, 2006). It has been reported that the overall effect of metformin is that, it inhibits gluconeogenic enzymes and stimulates glycolysis by altering the activity of multiple enzymes in gluconeogenesis, glycogenolysis, fatty acid synthesis(Fulgencio *et al.,* 2001).

2.4.3 First Generation inhibitors

Discovered in the 1950s by Roblin's group (Miller*et al.,* 1950), the heterocyclic sulfonamides such as acetazolamide, methazolamideand ethoxzolamide (Supuran*et al.,* 2004) as well asthe aromatic compound dichlorophenamide, represent the first

generation ofclinically used carbonic anhydrase inhibitors (Maren, 1967). They are very strong inhibitorsof most CA isoforms of the 15 ones presently known in humans.

2.4.4 Second Generation inhibitors

In 1983, in a seminal paper Maren's group (Maren*et al.,* 1983) postulated that a water-solublesulfonamide, possessing a relatively balanced lipid solubility as well as strong enough CA inhibitory properties,would be an effective drug via the topical route, but at that momentno inhibitors possessing such properties existed, as the bio-organic chemistry ofthis class of compounds was rather unexplored at that time (Supuran*et al.,* 2004). Water-solublesulfonamide CAIs started to be developed in several laboratories soon thereafter,and by 1995 the first such pharmacological agents, dorzolamidehas been launchedfor clinical use by Merck, as 2 % eye drops (Sugrue, 2000; Maus*et al.,* 1997).

2.4.5 Third Generation inhibitors

The third generation inhibitors consist in attaching water-solubilizing functionalities tothe molecules of aromatic/heterocyclic sulfonamides incorporating derivatizablemoieties of the amino, imino or hydroxyl type. Such moieties included, amongothers, pyridine-carboximido; carboxypyridine-carboxamido, quinolinesulfonamido;picolinoyl, isonicotinoyl, perfluoroalkyl/arylsulfonyl-, as well as aminoacyl groups, whereas ring systems which have been derivatized by using theabove mentioned moieties included: 2-; 3- or 4-aminobenzenesulfonamides;4-(¨-aminoalkyl)-benzenesulfonamides; 3-halogeno-substituted-sulfanilamides;1,3-benzene-disulfonamides; 1,3,4-thiadiazole-2-sulfonamides; benzothiazole-2-sulfonamides as well as thienothiopyran-2-sulfonamides, and were chosen in sucha

way as to demonstrate that the proposed approach was a general one. Compounds prepared by the tail approach showed 2–3 times more effective topicalintraocular pressure (IOP) lowering effects in rabbits as compared to dorzolamide **(**Scozzafava*et al*., 1996a; Scozzafava*et al*., 1996b; Scozzafava*et al*., 2000; Scozzafava*et al*., 2002a**).**The best studied drug is acetazolamide, which has been frequently administered for years due to its efficient reduction of Intra ocular pressure, greatly reduced toxicity and ideal pharmacokinetic properties (Wistrand and Lindqvist, 1991).

2.4.6 Acetazolamide

In 1956 acetazolamide was the first non-mercurial diuretic to be used clinically, (Barboiu *et al*., 1999). It represents the prototype of a class of pharmacological agents with relatively limited therapeutic usefulness in the present day, but which played a major role in the development of fundamental renal physiology and pharmacology, as well as the design of many of the presently widely used diuretic agents, such as the thiazide and the high ceiling diuretics (Barboiu *et al*., 1999; Jackson, 1996; Supuran *et al*., 1996). CA is highly abundant in the kidney (a total concentration of about 8 - 10 mM has been estimated) (Barboiu *et al*., 1999). It is present in the proximal and distal tubules as well as in the loop of Henle; two isozymes were evidenced, the cytosolic CA II and the membrane bound CA IV (Barboiu *et al*., 1999; Lonnerholm *et al*., 1986), both of which possess very high catalytic activity. CAs present in the kidneys play a crucial function in at least three physiological processes: (i) the acid-base balance homeostasis (by secreting and excreting protons, due to the CO_2 hydration reaction catalysed by these enzymes); (ii) the bicarbonate reabsorption process and (iii) and the $NH4^+$ output (Lassen *et al*., 1987; Lonnerholm *et al*., 1986; Supuran and Manole,1999). These important

physiological functions are well localised in the different segments of the nephron: bicarbonate reabsorption occurs in the proximal tubule, whereas urinary acidification and NH_4^+ output in the distal tubule and collecting duct (Barboiu *et al*., 1999; Jackson, 1996; Supuran and Manole,1999). Following the administration of a CAI, such as acetazolamide, the urine volume promptly increases (the urine secretion rate is generally tripled (Barboiu *et al*., 1999; Jackson, 1996) and it's normally acidic pH (pH 6.0) becomes alkaline (around 8.2) (Barboiu *et al*., 1999; Jackson, 1996). An increased amount of bicarbonate is eliminated into the urine (120 times higher than the amount eliminated normally), together with Na^+ and K^+ as accompanying cations, whereas the amount of chloride excreted is diminished (Barboiu *et al*., 1999; Jackson, 1996). The increased alkalinity of the urine is accompanied by a decrease in the excretion of titratable acid and ammonia as a consequence of metabolic acidosis. This sequence of events is due to the inhibition of CA in the proximal tubule, which leads to inhibition of H^+ secretion by this segment of the nephron. Inhibition of both cytosolic (CA II) as well as membrane-bound (CA IV) enzymes seems to be involved in the diuretic effects of the sulphonamides (Barboiu *et al*., 1999; Jackson, 1996; Lonnerholm *et al*., 1986). This inhibition decreases the availability of protons for the Na^+ - H^+ antiporter, which maintains a low proton concentration in the cell (Jackson, 1996). The net effect of these processes is the transport of sodium bicarbonate from the tubular lumen to the interstitial space, followed by movement of the isotonically obligated water and hence augmented diuresis (Barboiu *et al*., 1999; Jackson, 1996).

However, as CAs are ubiquitous enzymes in vertebrates, administration of systemic sulphonamides leads to CA inhibition in organs other than the target (such as the eye). The most frequent ones are: numbness and tingling of extremities; metallic

taste; depression; fatigue; malaise; weight loss; decreased libido; gastrointestinal irritation; metabolic acidosis; renal calculi and transient myopia (Bartlett and Jaanus, 1989; Maren ,1992; Maren , 1995; Barboiu *et al*., 1999). This and other studies involving the clinically used compounds **(**acetazolamide, methazolamide, ethoxzolamide, and dichlorophenamide**)** only gave negative results.

2.5 Diabetes mellitus

Diabetes mellitus is a global epidemic with an estimated worldwide prevalence of 415 million people in 2015, which is projected to rise to 642 million people by 2040 (IDF, 2015). The very considerable health, social and economic burdens caused by diabetes mellituspresent a major challenge to health-care systems around the world. Diabetes mellitus is a complex endocrine and metabolic disorder in which the interaction between genetic and environmental factors generates a heterogeneous and progressive pathology of defective insulin secretion and / or with varying degrees of insulin resistance and dysfunction of pancreatic β cells and α cells, as well as other endocrine disturbances (Stumvoll *et al*., 2005; Kahn *et al*., 2006; Mulder *et al*., 2009). Insulin resistance results from deficits in signalling pathways at the level of the insulin receptor and downstream, and type 2 diabetes emerges when β cells can no longer secrete sufficient insulin to overcome insulin resistance (Defronzo, 2009; Bailey, 2007). Overweight and obesity are major risk factors for the development of insulin resistance (Altaf *et al.*, 2015; Samuel and Shulman, 2012; Meier and Wagner,2014).

Hyperglycaemia is the fundamental biochemical feature of all forms of diabeets, causing oxidative and nitrosative stress and activation of inflammatory pathways and endothelial dysfunction, as well as precipitating microvascular complications and contributing to macrovascular disease, which are major causes of morbidity and

mortality (Brownlee, 2005). The results of several randomized controlled trials (RCTs) have demonstrated the short-term and long-term benefits of improving glycaemic control in delaying the onset and reducing the severity of diabetes-related outcomes, particularly retinopathy, nephropathy, neuropathy and cardiovascular disease, and also mortality (UKPDS, 1998; Holman *et al.*, 2008; DCCT, 1993). Attaining normal (or nearly normal) levels of blood glucose (where practical) is a major aim of all diabetes treatment. Several strategies are available for this purpose: lifestyle changes, including dietary prudence, weight loss and physical activity, remain the cornerstones of management.

2.6 Drugs used in the treatment of Diabetes

Today's clinicians are presented with an extensive range of oral antidiabetic drugs for type 2 diabetes. The main classes are heterogeneous in their modes of action, safety profiles and tolerability. These main classes include agents that stimulate insulin secretion (sulphonylureas and rapid-acting secretagogues), reduce hepatic glucose production (biguanides), delay digestion and absorption of intestinal carbohydrate (α-glucosidase inhibitors) or improve insulin action (thiazolidinediones). However, most of them exert undesirable side effects, drug interactions and the treatment is expensive since anti diabetic therapy comprise poly pharmacy regimen (Baynes and Thorpe,1996; Dandona *et al*., 2004).

The first attempts to treat human diabetes by orally active drugs were made between 1925 and 1930 with synthalines and their derivatives. However, because of their toxicity, these compounds were never used in clinical practice. The hypoglycemic activity of some of the antibacterial sulfonamides was discovered in the 1940's, with carbutamide and tolbutamide the first sulfonamide derivative antidiabetic drugs in

clinical use. The second-generation sulfonylurea, glibenclamide, has been in clinical use since 1969 and is per milligram 500 times as active as tolbutamide (Loubatières, 1969). The oral antidiabetic drugs form the basis of the modern pharmacological treatment of type II diabetes.

On the basis of their primary mechanism of action, the oral antidiabetic drugs can be divided into those that act by enhancing insulin secretion from the pancreas and those that act through extrapancreatic effects. The former include the sulfonylureas; the meglitinide analogues repaglinide and nateglinide are new short-acting insulin secretagogues (Landgraf, 2000). Nateglinide differs from repaglinide in that it is even shorter-acting (Kalbag *et al*., 2001). Biguanides are an effective treatment in hyperglycemia, acting mainly by promoting glucose utilization and reducing hepatic glucose production (Dunn and Peters 1995). It is not metabolized to a significant extent and is excreted primarily unaltered in the urine (Dunn and Peters 1995).

2.6.1 Metformin

Metformin, a biguanide derivative (dimethylbiguanide), is the most common drug used for type II diabetes treatment (Hundal and Inzucchi, 2003). Several studies have shown that metformin has insulin-sensitizing effects in the major tissues responsible for regulating the blood glucose concentration, including skeletal muscle, adipose tissue and liver (Wiernsperger and Bailey, 1999). Metformin inhibits gluconeogenesis and stimulates glucose uptake by peripheral tissues, processes requiring the activation of AMPK (Musi *et al*., 2002). In addition, it has been shown that metformin has hypoglycemic effects on streptozotocin (STZ) - induced diabetic rats, suggesting that these effects are not dependent on insulin action (Cheng *et al*. 2006). These findings support the increasing use of metformin

for type 1 diabetic patients, providing an additional effect to insulin treatment (Abdelghaffar and Attia, 2009).

2.6.2 Metformin and Carbonic anhydrase inhibition

Increased hepatic glucose production has been reported in several studies to be responsible for hyperglycemia in type 2 diabetes. Clinical studies and studies in animal models have suggested that the primary function of metformin is to decrease hepatic glucose production, mainly by inhibiting gluconeogenesis (Bailey and Turner, 1996; Hundal *et al.*, 2000). The glucose-lowering effect of metformin can be attributed to its ability to suppress hepatic glucose production. Several mechanism have been discussed earlier in this review that tries to explain the exact mechanism of metformin action, but the exact pathway involved in the metformin inhibition of hepatic gluconeogenesis is not well explained. Muller*et al*. (1997) reported that mitochondria seem to be the primary targets of metformin action, still metformin can influence erythrocytes, which lack mitochondria, possibly by affecting membrane fluidity, but he suggested further exploration of this mechanism is needed.Metformin has been reported to inhibit complex I of the respiratory chain in intact cells (El-Mir et al., 2000; Owen et al., 2000) but does not affect the oxidative phosphorylation machinery downstream of complex I (El-Mir et al., 2000).

In 1978, Dr. Peter Mitchell was awarded the Nobel Prize for Physiology and Medicine for his chemiosmotic theory, but Dr. Robert E. Forster believed that something was wrong with Dr. Mitchell's chemiosmotic theory and that a mitochondrial CA is responsible for dissipation of the proton gradient required for the synthesis of ATP. The two experts collaborated for a study in mitochondrial bioenergetics. Dodgson (1991a and 1991b) detected mitochondrial CA V in rat

liver and kidney and in the guinea pig liver and skeletal muscle. Parkkila *et al.*,(1998) suggested that CA V may play a role in the secretion of insulin from the pancreatic B-cells, possibly by providing the energy required for the processes involved in insulin secretion such as the movement of calcium ion through the gated channels. The adverse effect of metformin is lactic acidosis. In fact severe lactic acidosis was the main reason another antidiabetic drug phenformin was withdrawn from the market. The inhibition of respiratory chain by metformin causes a shift from aerobic to anaerobic metabolism which produces lactic acid as the end product.

The implication of CA inhibition will be impairment of the aerobic oxidation of glucose which the body responds by shifting to anaerobic oxidation, which produces lactic acid as the end product. Continued anaerobic oxidation further produces more lactic acid that overwhelms the body's capacity to clear it, therefore lactate accumulate in the blood leading to lactic acidosis. The decrease in hepatic glucose output by metformin may be explained interms of reduced delivery of gluconeogenic substrate (lactate) brought about by CA inhibition which can no longer maintain continues supply of the substrate or otherwise cannot produce more HCO_3^- needed by pyruvate carboxylase for continued hepatic glucose production.

Insulin, sulfonylureas, and thiazolidinediones all have various dangerous side effects, and all three lead to additional weight gain in generally already obese patients. The biguanides have advantage because they do not engender further weight gain. However, many well documented cases of lactic acidosis in diabetic patients using the biguanide phenformin were reported(Dembo *et al.*, 1975). The association caused phenformin to be removed from the market in the United States in 1978. Metformin is a far less dangerous biguanide and has been widely used in

Europe. Putative risk factors for lactic acidosis with biguanide treatment are as follows: age of 60 yrs decreased cardiac, hepatic, or renal function; diabetic ketoacidosis; surgery; respiratory failure; ethanol intoxication; and fasting. Biguanides may inhibit oxidative metabolism and thus increase the concentration of NADH, reduce gluconeogenesis, and suppress the gastrointestinal absorption of glucose. Patients with type 2 diabetes mellitus who develop this complication have numerous concomitant problems, and the literature is difficult to interpret.

2.7 Natural Products as Medicine

In developing countries, due to economic factors, nearly80% of the population still depends on the use of plant extracts as a source of medicine. Natural products also play an important role in the health care system in developed countries. The isolation of the analgesic morphine from the opium poppy, *Papaver somniferum*, in 1816 led to the development of many highly effective pain relievers (Benyhe, 1994). The discovery of penicillin from the filamentous fungus *Penicillium notatum* by Fleming in 1929 had a great impact on the investigation of nature as a source of new bioactive agents (Bennett and Chung, 2001). Natural products can also be used as starting materials for semisynthetic drugs. The main examples are plant steroids, which led to the manufacture of oral contraceptives and other steroidal hormones.

Natural products are the major mine for discovering promising lead candidates, which play an important role in future drug development programs. Ease of availability, least side effects and low cost make the herbal preparations the main key player of all available therapies, especially in rural areas (Arya *et al*., 2011). Since centuries, many plants are considered a fundamental source of potent anti-diabetic drugs. Nowadays synthetic oral hypoglycemic drugs and exogenous insulin

are the main agents for controlling diabetes. However, they exhibited prominent side effects and failed to reverse the course of its complications. This constitutes the major force for finding alternatives, mainly from plant kingdom that are of less severe or even no side effects (Upendra *et al*., 2010).

2.7.1 Natural Products that inhibit Carbonic anhydrase

Almost all reported carbonic anhydrase inhibitors comprise a zinc binding group in their structure of which the primary sulfonamide moiety ($-SO_2NH_2$) is the foremost example and to a lesser extent the primary sulfamate ($-O-SO_2NH_2$) and sulfamide ($-NH-SO_2NH_2$) groups. Natural products (NPs) that comprise these zinc binding groups in their structure are however rare and relatively few natural products have been explored as a source for novel carbonic anhydrase inhibitors.Several classes of novel CA inhibitors have been identified from screening collections of NPs; The most notable ones are coumarin and phenol containing NPs (Supuran, 2011).

a) Coumarins

Coumarin compounds are abundant secondary metabolites in plants and are foundto a lesser extent in microorganisms and animal sources. Plant coumarins arephytoalexins, defense compounds produced when the plant is under threat fromother organisms, and have attracted interest owing to a range of biological activitiesincluding antimicrobial, molluscicidal, acaricidal, antiviral, anticancer, antioxidantand anti-inflammatory properties (Borges et al., 2005). In recent years it was discovered thatNP coumarins inhibit CA enzymes via an alternate and unprecedented mechanismto classical sulfonamides (Vu*et al*., 2008).

b) Phenols

The first single crystal X-ray structure of phenoland a CA protein (hCA II)was reported in 1994 and identified that phenolbinds in an unprecedented way withinthe enzyme active site (Nair*et al.*, 1994). It was shown that the phenolic OH interacts withthe zinc-bound water molecule/hydroxide ion through a hydrogen bond while asecond hydrogen bond formed between the phenolic OH and the NH amide ofThr199, an amino acid critical for inhibition of various CAs.

The phenols have been evaluated for their inhibition of human cytosolicisoforms CA I and II and mitochondrial isozymes CA VA and CA VB. The latter have been recognized as potential targets for designing antiobesityagents that act with a novel mechanism of action (De Simone*et al.*, 2008; Supuran, 2003). The simplephenolic secondary metabolites have also been tested against hCA III, IV,VI, VII, IX, XII, XIII and XIV (Innocenti*et al.*, 2008; Innocenti*et al.*, 2009). The chemical diversityof phenolic NPs is vast; so far investigation of this chemotype for its interaction withCAs is in its infancy.

c) Polyamines

Polyamines belong to an alkaloid structure class and have been reported fromvarious natural sources including terrestrial and marine animals, plants, fungi and bacteria (Dictionary of Natural Products, 2012). Two of the simplest polyamines isolated to date include spermine and spermidine. The polyamine chemotype has been shown to modulate multiplebiological processes including gene expression, cell proliferation, translation, cellsignaling, membrane stabilization and ion channel inhibition as well as antibacterialactivity (Casero and Marton, 2007; Fleidervish *et al*., 2008; Wallace and Niiranen, 2007; Soda *et al*., 2009; Xu *et al*., 2012; Yin *et al*., 2011; Buchanan *et*

al., 2007).Stachydrine and 3-hydroxy-stachydrine are the two spermidine alkaloids isolated from the leaves (Al-Musayeib *et al*., 2013) and stem bark (Ahmad *et al*.,, 1985), of *Cadaba farinosa.*Despite the myriad bioactivities reported for polyamine NPsuntil recently no CA inhibition had been reported. Carta et al. (2010) showed thatspermine,spermidine andseveral semi-synthetic polyamine analogues inhibited hCA I-XIV with *K*i valuesranging from low nanomolar to millimolar.

2.8 *CadabafarinosaForssk*

Cadaba farinosa Forrsk(Capparaceae) also known as Indian Cadaba is widely distributed in arid area worldwide. It is full of minerals and amino acids which is beneficial for cattle's as well as human being. It is extensively used as food by animal. It is ample of pharmacological actions as mentioned in 'Ayurveda and Siddha' – an ancient Indian system of medicine but its medicinal potentials are not satisfactorily studied yet.

2.8.1 Taxonomicalclassification

Kingdom: Plantae

Subkingdom: Tracheobionta

Super division: Spermatophyta

Division: Magnoliophyta

Class: Magnoliopsida

Subclass: Dilleniidae

Order: Capparales

Family: Capparaceae

Genus: Cadaba Forsk.

Species: farinosa (*Garden Guide's plant* 1997-2008)

2.8.2 Vernacularnames/Local Names

English: Indian cadaba

Hindi: Kodhab, katagum, Dabi

Fulani: baggahi

Hausa: bagayi

Niger: Balamji

2.8.3 BotanicalDescription

Cadaba farinosa Forsk belong to the family Capparidaceae (Capparaceae). It is a middle sized family with 45 genera and nearly 600 species which are distributed throughout the world, mostly in tropical and subtropical region. The capparidaceous plants are usually herbs, erect or scandent, shrubs and rarely trees. The genera cadaba is represented by 30 species. *Cadaba farinosa Forrskis* a smooth, purplish, younger, pubescent, yellowish brown Stems. The leaves are entire, simple, silvery gray and with simple scales. Seeds of the plant are covered in a bright orange membrane strait, surrounded by orange red-aril. Cadaba species belongs to hermaphroditic category whose flowers and fruit appear at the end of the rainy season and the beginning of the dry season (Baumer, 1993).

2.8.4 PhytochemicalConstituents

Cadaba farinosa contains large number of active constituent like alkaloids, sugar, carbohydrates, glycoside, protein, Amino acid, flavonoid, saponins, tannins, phenolic compounds, gums, mucilage and Steroids. *Cadaba farinosa* has many active phytoconstituents such as non-tannin, phenolics, and kaempferol (Mangan, 1998), new spermidine alkaloid cadabicine, L-Stachydrine and 3-hydroxystachydrine, 3- hydroxystachydrine (Ahmad *etal*., 1985), three novel spermidine alkaloids one Capparisine and an aromatic acid, ά,β-Dihydroferulic acid, novel sesquiterpenoid Cadabicine methyl ether and Cadabicine diacetate, besides a

sisquiterpene, cadibicilone (Yousif *et al*., 1984). It also contains 3(4-formylphenoxy) -4- methoxybenzaldehyde, Methyl cinnamate, methyl ferulate ether, ether of p-cinnamic acid-m- ferulic acid, Thiazolidine compound (Amber, 1990; Ahmad, 1992). It also shows the presence of quercetin, isoorientin, hydroxybenzoic acid, syringic acid, vanillic acid and 2-hydroxy- 4-methoxy benzoic acid (Khare, 2006).

2.8.5 Mineraland Protein Content

The leaves of *Cadaba farinosa* contain the highest amount of Calcium (12.2 mg/g dry weight) and stems and leaves also contain a reasonable amount of bone building minerals. It also contained nutritionally significant amount of copper, iron, magnesium, manganese, phosphorous, zinc, chromium, and molybdenum. The stems and leaves of *Cadaba farinosa* contain 8.32 and 14.8 % of protein w/w (Glew *et al*., 2010).

2.8.6 Ethno-MedicinalUses of*Cadaba farinosa*

The whole plant is purgative, anthelmintic, anti-syphilitic, emmenagogue, aperients, and stimulant, anti-scorbutic and anti-phlogistic. It is also used in treatment of cough, fever, dysentery and as antidote against poisoning. The boiled leaves are eaten as an anthelmintic; decoction with other ingredients is employed in the treatment of amenorrhea, dysmenorrheal and uterine obstruction, decoction of leaves with myrobalans and ginger or with a senna and Epsom salt given as purgative and anti-phlogistic in syphilis, scrofula and rheumatism. The plant is used by locals in the treatment of cancer and diabetes. According to ethnomedical information, the plant *Cadaba farinosa* has been used for treating various ailments such as anti-inflammatory, anti-diabetic and in female fertility (Tenpe *et al*., 2006; Nadkarni,

2002). The root of plant posses' similar medicinal properties like leaves, the root preparation is used in anthrax. The flower buds are stimulant, antiscorbutic, purgative, emmogogue, anti-phlogistic and anthelmintic especially for round worm (Nadkarni, 2002). The ash of plants is rubbed into skin to relieve general body pains (Amber, 1990).The young leaves and twigs of *Cadaba farinosa* are edible and also used in spicing and flavouring food. Leaves are pounded with cereals and eaten as cake in Nigeria. They are also boiled and made into gruel. The whole plant is used as fodder by manylivestockparticularly during the dry season. Camels are the main consumers, since other species find it difficult to reach the foliage. Buffalo, black rhino and hartebeests also seek the foliage (Hong *et al*., 1996). The fodder has 30% protein content with digestibility *in-vitro* value of 78%. *Cadabafarinosa* also possess high ash content. The plant also provides fuel wood. Crushed leaves mixed with millet flour are used as a medicine against coughs. The fruit are edible (Von Maydell, 1986).

2.8.7 PharmacologicalActivities

At a dose of 250 and 500 mg/kg body weight, ethyl acetate and aqueous fractions of *Cadaba farinosa*, Forsk wereshown to have a significant hepatoprotective effect against CCl_4 induced hepatotoxicity in rats. The activity was evaluated by the estimation of SGOT, SGPT, ALP, Gamma GT, total bilirubin and direct bilirubin in treated rats. The hepatoprotective activity was due to flavonoids and phenols present in the extract (Umesh *et al*., 2010a). The leaf extract (30 mg/kg) of *Cadaba farinosa* exhibits the significant hepatoprotective effect. Simultaneously ethanolic extract of leaf confirmed decreased levels of SGPT, SGOT, ALP, Total bilirubin & reduction in improved liver weight (Sanghi *et al*., 2007). The ethyl acetate extract of *Cadaba farinosa* exerts protective action by decreasing CCl_4-derived free radicals and

significantly decrease the elevated level of serum enzyme activities (Kohli and Jain, 2006). Ethyl acetate and aqueous fraction of *Cadaba farinosa*, Forsk shows significant antioxidant activity (DPPH scavenging method, Nitric oxide scavenging method, Super oxide anion radical scavenging method, Hydroxyl scavenging activity) and suggests that possible mechanism for hepatoprotective activity may be due to free radical scavenging potential caused by the presence of flavonoids in the extracts (Umesh *et al*., 2010a). Decoction of *Cadaba farinosa* is commonly used as antiprotozoal, schistosomicidal and antifungal (Harouna *et al*., 1993). The i*n vitro* study using water, ethanol and acetone as a solvent were effective in reducing the mycelial growth of *Fusariumoxysporum* f. species (Siva *et al*., 2008). Graham *et al*explained potential of *Cadaba farinosa* against cancer through their survey (Graham *et al*. (2000). Currently, all the medicinal plants species are exposed to anthropogenic impact (Habib *et al*., 2004). *Cadaba farinosa* shows potent *in-vitro* cytotoxicity in methanol extracts tested against FL-cells (Al-Fatimi, *et al.,* 2007). The dichloromethane and ethanol extracts of *Cadaba farinosa* Forsk shows potent antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*and *Micrococcus flavus* (Al-Fatimi *et al*., 2007). Alcohol and aqueous extract of *Cadaba farinosa* (leaves) were subjected for hypoglycemic activity in wistar rats (160- 200 g). Phytochemical analysis of ethanol extract revealed the presence of terpenoids, flavonoids, steroids, proteins, alkaloids, gums, sugars and saponins (Arokiyaraj *et al*., 2008).

2.8.8 ToxicologicalandSafetyAssessment

In 1943, Vander and Walt Steyn reported that the genus Cadaba has toxicity (Vander Walt and Steyn, 1943), but Telrandhe *et al.*(2010) reported that the different solvent extracts were administered orally with increasing dose and LD_{50} was found to be

2000 mg/kg for all extract in experimental animals. No clinical signs and mortality of animals was observed (Umesh *et al*., 2010b).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Chemicals/Reagents

Streptozotocin,Bovine erythrocyte carbonic anhydrase enzyme, Thiobarbituric acid (TBA), (−)-epinephrine, P-Nitrophenyl acetate were purchased from Sigma-Aldrich Chemical Company (St. Louis U.S.A.). HbA1c was purchased from spectrum diagnostics (Egypt). Accutrend GCT Meter, with Cobas® test strips was purchased from Roche (Germany). All other chemicals and reagents used in this study were of analytical grade.

3.1.2 Equipment

UV-Vis spectrophotometer (Shimadzu UV-2600 Spectrophotometer, Japan), Centriguge (Beckman, USA), Thermo Scientific Nicolet iS10(England) FTIR Spectrophotometer, assisted by a computer, range 4000–400cm-^{1}. GCMS-QP2010Plus Shimadzu (Japan), Hewlett Packard gaschromatograph (model 6890) equipped with aflame ionization detector and injector MS transferline temperature of 230°C. Incubator (Agilent Technologies USA). Agilent NMR Spectroscopy (400 MHZ).

3.1.3 Plant Sample Collection and Identification

Leafs and stemswere collected from Daushe area of Ringim, Jigawa State, during winter season. The plant was identified and authenticated by malam magaji at the Herbarium Unit, Biological Sciences Department, Ahmadu Bello University Zaria, Nigeria, where a voucher specimen number (2744) was deposited for further reference.

3.1.4Animals

Male Wister albino rats180–220 grams weight from the Department of Veterinary Physiology, Ahmadu Bello University Zaria were used for this study. The rats were allowed to acclimatize for 7 days in laboratory condition. Five rats each were housed in zinc cages beddedwith husk. Standard animal diet (Vitafeeds, Hadejia road Kano, Nigeria) with water in glass bottles *ad libitum* were fed to the animals. The principles of laboratory animal care were followed according to the instructions by National Institute of Health (NRC,1996).

3.2 METHODS

3.2.1 Extraction and Fractionation

Leaves of *C. farinosa*were dried in shade at room temperature and grounded to powder using pestle and mortar. The powder (800g) was defatted with five (5) liters of n-hexane, The residue was then extracted with 10 litres of methanol. The methanolconcentrate was filtered through a Buchner funnel with Whatman filter paper No.1. The filtrate was evaporated to dryness under reduced pressure.The methanol extract was subjected to solvent-solvent partition to obtain fractions.

3.2.2 Column Chromatography

A column of 600 mm height and 15 mm inner diameter packed with neutral silica gel of mesh size 60-120 was used for the separation. 10 g of crude methanol extract of *C. farinosa* leaves was loaded on the column already packed with silica gel. The components of the crude extract was separated by elution on the column using an eluting mixture of appropriate solvent system (Ethyl acetate and methanol in the ratio of 6:4) as eluents, in mixtures of increasing polarity as described by Ejele, *et al*., (2012). Fractions of aliquots (30ml) were collected into different beakers.Each collected fraction was analyzed by TLC method to ascertain the purity.The solvent

was allowed to evaporate.Column fractions with similar TLC profileswere pooled together; after which carbonic anhydrase inhibitory assay was carried out with the various pooled fractions at different concentrations.

3.2.3 Thin Layer Chromatography (TLC)

Thin layer chromatography was carried out on various fractions of column chromatography to determine the profile and best solvent system for column chromatography. A chromatographic plate pre-coated with silica gel was used. The methanol extract (0.5 g) was dissolved in 5ml distilled water and then spotted on the TLC plate using capillary tube. The plates were placed in separate saturated chromatographic tanks with a mixture of different solvents system (Ethyl acetate and methanol in the ratio of 1, 6:4, 3:2 and chloroform and methanol in a ratio of 3:1 and 1:1). Thereafter, the plates were removed, air dried, sprayed with P-anisaldehyde and the color change was observed.

3.2.4 Preparative TLC (Thin Layer Chromatography)

Preparative thin layer chromatography was carried out on the most potent fraction.The Methanol fraction A was loaded on to a preparative TLC (20 x20 cm) plate by streaking a solution of the fraction (0.5g/3ml of distilled water) on a TLC plate. The plate was developed in a chromatographic tank using solvent system (Ethyl acetate and methanol in a ratio of 6:4) and air-dried. A small portion (1cm) of the developed plate was cut off, sprayed with P-anisaldehyde and the color change was observed. The cut off portion was then matched with the developed TLC plate to give an idea of the bands on the TLC plate. The developed plate was carefully scraped into different beakers and transferred into centrifuge tubes;

methanol (5ml) was then added to each tube and centrifuged at 1,500 xg for 30 minutes. The supernatant layer was decanted into beakers and then air-dried.

3.2.5 Gas chromatography- Mass spectroscopic Analysis

Gas Chromatography Mass Spectroscopy (GC-MS) analysis was carried out on the most potent column fraction using helium as the carrier gas, at a flow rate of 1.2ml/min. The injection volume was 1µL. The inlet temperature was maintained at 230°C. The oven temperature was programmed initially at 50°C for 5 minutes, then programmed to increase to 300°C at a rate of 10°C/minute ending with 25 minutes. Total run time was 45 minutes. The MS transfer line was maintained at a temperature of 300°C. The source temperature was maintained at 230°C and the MS Quad at 150°C. The ionization mode used was electron ionization mode at 70eV. Total Ion Count (TIC) was used to evaluate for compound identification and quantitation. The spectrum of the separated compound was compared with the database of the spectrum of known compound saved in the NIST02 Reference Spectra Library. Data analysis and peak area measurement was carried out using Agilent Chemstation Software.

3.2.6 FTIR (Fourier Transform Infrared Spectrophotometer) Analysis

FTIR analysis was also conducted to identify the functional groups present in the fractions.The infrared spectra of the purified fractions for the most potent fraction, was recorded on a Thermo Scientific Nicolet iS10 (England) FTIR Spectrophotometer, assisted by a computer, in the solid state in the 4000–1400 cm^{1}. The reported wave-numbers were estimated to be accurate to within ± 3 cm^{-1}.

3.2.7 Nuclear Magnetic Resonance Analysis

Structural determination of the active compound was madeaccording to spectroscopic analysis. The ^{1}H and ^{13}C NMR spectra were recorded on an agilent 400MHz NMR spectrometer (Agilent, Palo Alto, USA); chemical shifts were reported and expressed as parts per million(d), using the residual dimethyl sulfoxide as an internalstandard, and coupling constant (J) in Hz.

3.2.8 AnimalStudy design

The animals were grouped into 6 groups of 5 rats each. Group I rats (Normal control); Group II (Diabetic control); Group III (Diabetic rats treated orally with Acetazolamide, 3.6mg/kg/day for 28 days); Group IV (Diabetic rats treated with Metformin, 14.3mg/kg/day for 28 days); Group V (Diabetic rats treated with crude methanol leaf extract of *Cadaba farinosa* leaves 1000mg/kg/day for 28 days)and Group VI (Diabetic rats treated with crude methanol extract of *Cadaba farinosa* leaves 500mg/kg/day for 28 days).. Group I (Normal control) and Group II (Diabetic control) rats received only water at the time of treatment of other groups.

3.2.9 Induction of diabetes

Diabetes was induced in all the rats (fasted overnight) except in the normal control (Group I) by Streptozotocin(STZ) at single dose of 60 mg / kg body weight, dissolved in ice cold citrate buffer (0.1 M, pH 4.5), through interperitoneal route. Hyperglycemia was confirmed by the elevated fasting glucose level > 200 mg/dl in blood, determined at 72 h after STZ injection.

3.2.10Collection of blood samples and Hemolysate preparation

The blood samples were collected by cardiac puncture in vials with EDTA and centrifuged at 200 xg for 10 minutes. The plasma was separated from the cells and buffy coat removed. The packed red cells were washed three times with normal saline (0.9% NaCl) and were lysed with isotonic ice cold water, yielding hemolysate which was used for the assay of carbonic anhydrase.

3.2.11 Biochemical Analysis

a) Blood Glucose Analysis

The fasting blood glucose level was determined as described by Raba and Mottola (1995) based on glucose oxidase/ mediator reaction. Cobias® Glucose test strips react specifically with glucose and therefore are not affected by maltose. Each test strip has a test area containing detection reagent: Glucose Oxidase 12.5U/cm; bis-(2-hydroxy-ethyl)-(4-hydroximinocyclo-hexa-2,5-dienylidene)-ammonium chloride 35.0 µg/cm; 2,18-phosphomolybdic acid 191.4 µg/cm; non-reactive substances 8.1 mg/cm. When whole blood is applied, a chemical reaction takes place and causes the test area to change its colour. The meter measures changes in colour and converts it into concentration, which is displayed on the screen (Accutrend, 2009).

b) Blood Lactate Analysis

Blood lactate was determined by lactate oxidase reaction (Accutrend 2009) protocol, each test strip has a test area containing detection reagents: Lactate oxidase (Aerococcus viridians) 1.9U; N,N-Bis-(2-hydroxy-ethyl)-(4-hydroximino-cyclo-hexa-2,5-dienylidene)-ammonium chloride 7.2 µg; 2,18-phosphomolybdate 11.4 µg. In this method the applied blood seeps through the yellow protective mesh into the glass fibre; the erythrocyte are retained and only blood plasma reaches the detection film. Lactate is determined by reflectance photometry at a wavelength of 657nm in a colometric lactate-oxidase mediator reaction(Accutrend, 2009).

L-lactate + $mediator_{form1}$ ⟶ $mediator_{reduced}$

$mediator_{reduced}$ + 2,18-phosphomolybdatemolybdenumblue+ $mediator_{form2}$

c) *Blood cholesterol Analysis*

Blood cholesterol was determined based on the reference method (CHOD-PAP, Roche Diagnostics) on enzymatic cleavage of the cholesterol ester into fatty acids and cholesterol; oxidation of cholesterol into cholestenone with the simultaneous formation of hydrogen peroxide which oxidizes an indicator to its blue radical cation. Strip test components: CHE (microorganisms) 1.44U; CHOD (Nocardia erythroporis) 0.12U; POD (horseradish) 0.20U; 3, 3'5, 5'-tetramethylbenzidine 8.5 µg; non-reactive components 4 mg (Accutrend, 2009).

d) *Blood Triglycerides Analysis*

Blood triglyceride was determined based on triglyceride cleavage by an esterase into glycerol and fatty acids. Two additional enzymatic steps convert glycerol into hydroacetone phosphate and hydrogen peroxide. In the presence of peroxidase, the hydrogen peroxide oxidizes an indicator to a dye and its concentration is then measured by means of reflectance photometry. Strip test components: CHE (Candida cylindracea) 0.15U; GK (*Bacillus stearothermolphilus*) 0.36 U; GPO (E. coli rec.) 0.08U; POD (horsedish) 0.63 U; ATP 12 µg; 4-(4-dimethylaminophenyl)-5-methyl-2-(4-hydroxy-3,5-dimethoxyphenyl)-imidazol-dihydr-chloride 27 µg; non-reactive components 0.43mg(Accutrend, 2009).

3.2.12 Assay of carbonic anhydrase activity

Carbonic anhydrase activity was determined as described by Verpoorte *et al*.(1967), with the modification described by Parui *et al.*,(1991) usingspectrophotometer. In this assay, the esterase activity of carbonic anhydrase was determined from the hydrolysis of 1.5ml of 3mM p-nitrophenyl acetate to p-nitrophenol. The assay system contained 100 µL hemolysate placed in 1 cm spectrometric cuvette containing 1.4 ml 0.05 M Tris- HCl buffer, pH: 7.4 and 1.5ml of 3mM p-nitrophenyl acetate. The change in absorbance at 348 nm was measured over the period of 3 min before and after adding the sample. The absorbance was measured by a UV-Vis spectrophotometer (Shimadzu UV-2600 Spectrophotometer). One unit of enzyme activity was expressed as µmol of p-nitrophenol relased/min/µL from hemolysate at room temperature (25^{o}C) (Parui *et al*., 1991; Gambhir *et al*., 1997).

In Vitro Inhibition Studies

For the inhibition studies of Acetazolamide, Metformin and Crude methanol leaf extract of *Cadaba farinosa*, different concentrations of these compounds were added tothe enzyme, and the above procedure was followed after which absorbance was measured at 348nm. Activity percentage values of CA for differentconcentrations of each were determinedusing the formula. Results areexpressed as percent inhibition calculated from:

CA enzyme % inhibition without inhibitors wasaccepted as 100% activity.

3.2.13 Assay of Glycated Hemoglobin (HBA1C)

Glycosylated haemoglobin determination: HbA1C was measured using Spectrum-diagnostics Egypt, according to the manufacturer's instruction. The assay principle

is based on the principle developed by (Bates, 1978; Gonen, B., and Rubenstein, 1978; Trivelli *et al.,* 1971) wherea hemolysed preparation of whole blood was mixed continuously for 5 minutes with a weakly binding cation-exchange resin. The labile fraction is eliminated during the hemolysate preparation and during the binding. During this mixing, HbA binds to the ion exchange resin leaving glycated hemoglobin free in the supernatant. After the mixing period, a filter separator is used to remove the resin from the supernatant. The percent glycosylated hemoglobin was determined by measuring the ratio of the absorbances at 405nm of the Glycosylated hemoglobin (GHb) and the Total hemoglobin fraction (THb) was determined by addition of 0.02 ml of Hemolysate into 5.0 ml of distilled water into tubes labeled as Control and Test and was mixed well. The absorbance was read against distilled water. The ratio of the absorbances of GHb and THb of the control and test is used to calculate the percent GHb of the sample thus:

Calculations

$$\text{Ratio of Control } (R_C) = \frac{\text{Abs. Control GHb}}{\text{Abs. Control THb}}$$

$$\text{Ratio of Test } (R_T) = \frac{\text{Abs. Test GHb}}{\text{Abs. Test THb}}$$

$$\text{GHb in \%} = \frac{\text{Ratio of Test } (R_T)}{\text{Ratio of Control } (R_C)} \times 10 \text{ (Value of Control)}$$

Rc = Ratio of control sample

R_T= Ratio of test sample

Concentration of standard =10

Principle

This method utilizes the interaction of antigen and antibody todetermine the HbA1c in whole EDTA blood. HbA1c in test samplesis absorbed onto the surface of latex

particles, which react withAnti-HbA1c (antigen-antibody reaction)and gives agglutination. Theamount of agglutination is measured as absorbance. The HbA1cvalue is obtained from a calibration curve.

3.2.14 Assay for Superoxide dismutase (SOD)

Superoxide Dismutase (SOD) activity was measured as described by Martin *et al*. (2000).The reaction mixture contained 0.8 ml of50 mmol/l glycine buffer (pH 10.4), and 0.2 ml serum or washed hemolysate. Thereaction was initiated by the addition of 0.02 ml of a 20 mg/mlsolution of (−)-epinephrine. Absorbance was recorded at 480nmin a spectrophotometer (Shimadzu-1601, Japan). SOD activity wasexpressed as μMol epinephrine/ml packed cell/min protected from oxidation bythe sample compared with the corresponding readings in the blankcuvette. The molar extinction coefficient of $4.02\times10x3M^{-1}$ $cm^{-1}$was used for calculations.

Principle

The O_2^- substrate for SOD is generated indirectly in the oxidation of epinephrine at alkaline pH by theaction of oxygen on epinephrine. As O_2^- builds in the solution, the formation of adrenochromeaccelerates because O_2^- also reacts with epinephrine to form adrenochrome. Toward the end of thereaction, when the epinephrine is consumed, the adrenochrome formation slows down. SOD reacts with the O_2^- formed during the epinephrine oxidation and therefore slows down the rate offormation of the adrenochrome as well as the amount that is formed. Because of this slowing process,SOD is said to inhibit the oxidation of epinephrine.

3.2.15 Assay for Catalase (CAT)

Catalase (CAT) activity was assayed by the method of Claiborne (1985). Briefly, the assay mixture consisted of 0.05Mphosphate-buffer (pH7.0), 0.019Mhydrogen peroxide (H_2O_2), and 0.05 ml serum or washed hemolysatein a total volume of 3.0 ml. Changes in absorbance were recorded at 240 nm. CAT activity was expressed as µM H_2O_2 consumedmg^{-1}protein.

Principle

Catalase catalyses the decomposition of hydrogenperoxide (H_2O_2) to water and oxygen. Hydrogenperoxide is formed in the eukaryotic cell as a by-productof various oxidase and superoxide dismutase reactions.

The disappearance of hydrogen peroxide (H2O2) is measured spectrophotometrically at 240 nm.One unit (U) is defined as the amount of enzyme which decomposes 1 µmol of H2O2 per min at 25°C and pH 7.0

3.2.16 Determination of the degree of lipid peroxidation

The method of Utley *et al*., (1967),was used to estimate the degree of lipid peroxidation. Serum (0.25 ml)was pipetted into 15mm×100mm test tubes and incubated at 37 ◦C in a metabolic shaker for 1 h. After 1 h of incubation, 0.5 ml of 5% (w/v) chilled trichloroacetic acid (TCA), followed by 0.5 ml of 0.67% TBA (w/v) was added to each test tube and centrifuge tube, and centrifuged at 1000×g for 15 min. Thereafter, the supernatant was transferred to other test tubes and was placed in a boiling water bath for 10 min. The absorbance of pink color produced was measured at 535nmin a spectrophotometer (Shimadzu-1601, Japan). The TBARS content was calculated by using a molar extinction coefficient of $1.56\times105M^{-1}$ cm^{-1} and expressed as µMol TBARS/ml packed red cell/min.

3.2.17 Protein assay

Protein content was determined by the method of Lowry *et al*. (1951), using bovine serum albumin (BSA) as a standard.The proteinsolution was mixed with a copper sulphate solution and the Folin reagent (a mixture ofsodium tungstate, molybdate and phosphate) to produces blue purple color. The colorsolution is quantified by measuring the absorbance at 660 nm.

Principle

Under alkaline conditions the divalent copper ion forms a complex with peptide bonds in which it is reduced to a monovalent ion. Monovalent copper ion and the radical groups of tyrosine, tryptophan, and cysteine react with Folin reagent to produce an unstable product that becomes reduced to molybdenum/tungsten blue.

3.3 Statistical Analysis

Results were presented as mean ± standard Deviation (SD). Within and between groups, comparisons were performed by the analysis of variance (ANOVA) (using SPSS 20.0 for windows Computer Software Package). Significant differences were compared by Duncan‟ s new Multiple Range test; a probability level of less than 5% ($P < 0.05$) was considered significant (Duncan, 1955).

CHAPTER FOUR

4.0 RESULTS

4.5 Role of Carbonic anhydrase on blood glucose, lactate, cholesterol and triglycerides levels

The effect of 14 day STZ-induced diabeteson carbonic anhydrase, glucose, lactate, cholesterol and triglycerides is presented in Table 4.1. From the data it can be shown that STZproduced a significant ($p<0.05$) increase in carbonic anhydrase activity, glucose, lactate, triglycerides and cholesterol levels when compared to non-diabetic control (Table 4.1).

In Table 4.2, the effect of 28 day STZ-Induced diabetes on the activity of carbonic anhydrase and the level of glucose, lactate, cholesterol and triglycerides is presented.Carbonic anhydrase activity significantly ($p<0.05$) decreasedin STZ induced diabetic rats when compared to non-diabetic control over the course of 28 days. Furthermore about2.2 fold increase in blood lactate level was seen in untreated STZ induced diabetic rats when compared to non-diabetic control (Table 4.2). Treatment of STZ-induced diabetic rats with Acetazolamide resulted in a significant ($p<0.05$)decrease in blood glucose level and carbonic anhydrase activity. However, inhibition of carbonic anhydrasewith Acetazolamide in STZ induced diabetic rats resulted in 6.9-fold increase in blood lactate level. A significant ($p<0.05$) reduction ofblood cholesterol and increase in triglycerides levelswere observed in STZ-induced diabetic rats, when treated with Acetazolamide (Table 4.2).

Table 4.1**: Effect of (14 day) Streptozotocin (STZ)-Induced diabetes on erythrocyte carbonic anhydrase activity, glucose, lactate, cholesterol and triglyceride**

Parameters/Groups	**Non Diabetic**	**Diabetic (STZ)**
Carbonic anhydrase (µmol/min/µL)	6.3 ± 0.2	**9.1 ± 0.7[a]**
Glucose(mmol/L)	3.5 ± 0.2	**12.7 ± 3.9[a]**
Lactate(mmol/L)	4.2 ± 0.9	**6.2 ± 1.2[a]**
Cholesterol (mmol/L)	3.9 ± 0.07	**4.3 ± 0.02[a]**
Triglyceride (mmol/L)	1.5 ± 0.5	**2.5 ± 0.6[a]**

Metabolic parameters in the early diabetic phase during 14 day diabetic study[a]$P < 0.05$ vs Non Diabetic group; (n=5).Values are expressed as Mean ± SD

Table 4.2:**Effect of (28 days) STZ-Induced diabetes and its treatment with Acetazolamide on erythrocyte carbonic anhydrase activity**

Parameters/Groups	Non-Diabetic	Diabetic (STZ)	Diabetic (STZ) + Acetazolamide
Carbonic Anhydrase (μmol/min/μL)	4.9 ± 0.2	2.8 ± 0.3[a]	1.4 ± 0.4[b]
Glucose (mmol/L)	5.5 ± 0.8	17.1 ± 4.1[a]	5.1 ± 1.9[b]
Lactate (mmol/L)	2.1 ± 0.6	4.8 ± 0.6[a]	14.6 ± 3.8[b]
Cholesterol (mmol/L)	4.0 ± 0.0	4.5 ± 0.0	1.0 ± 0.1[b]
Triglyceride (mmol/L)	1.1 ± 0.2	1.16 ± 0.2	4.1 ± 0.1[b]

Metabolic parameters in the early diabetic phase during 14 day diabetic [b]P < 0.05 vs Diabetic (STZ) group; [a]P < 0.05 vs Non Diabetic group (n=6). Values are expressed as Mean ± SD

4.6 Effect of Metformin, Acetazolamide and methanol Leaf Extract of *C.farinosa* on Erythrocyte Carbonic Anhydrase activity in STZ induced Diabetes

Treatment of STZ induced diabetic rats with Metformin at 14.3mg/kg/day for 28 days, significantly($p<0.05$) reduced erythrocyte carbonic anhydrase activity when compared to normal control (Figure 4.1). As expected Metformin also significantly ($p<0.05$) reducedfasting blood glucose levelwhen compared with normal control (Figure 4.3).

4.6.1 Acetazolamide reduces blood glucose; increases blood lactate and HbA1c level in STZ induced diabetic rats.

Data presented in Figure 4.2showed that treatment of STZ induced diabetic rats with Acetazolamide at 3.6mg/kg/day for 28 days resulted in 3 fold significant ($p<0.05$) increase in HbA1c when compared with normal control rats. However, treatment of STZ induced diabetic rats with the same inhibitor at the same concentration for 28 daysas shown in (Figure 4.3)resulted in a significant reduction in blood glucose level with a 4 fold increase in blood lactate level.

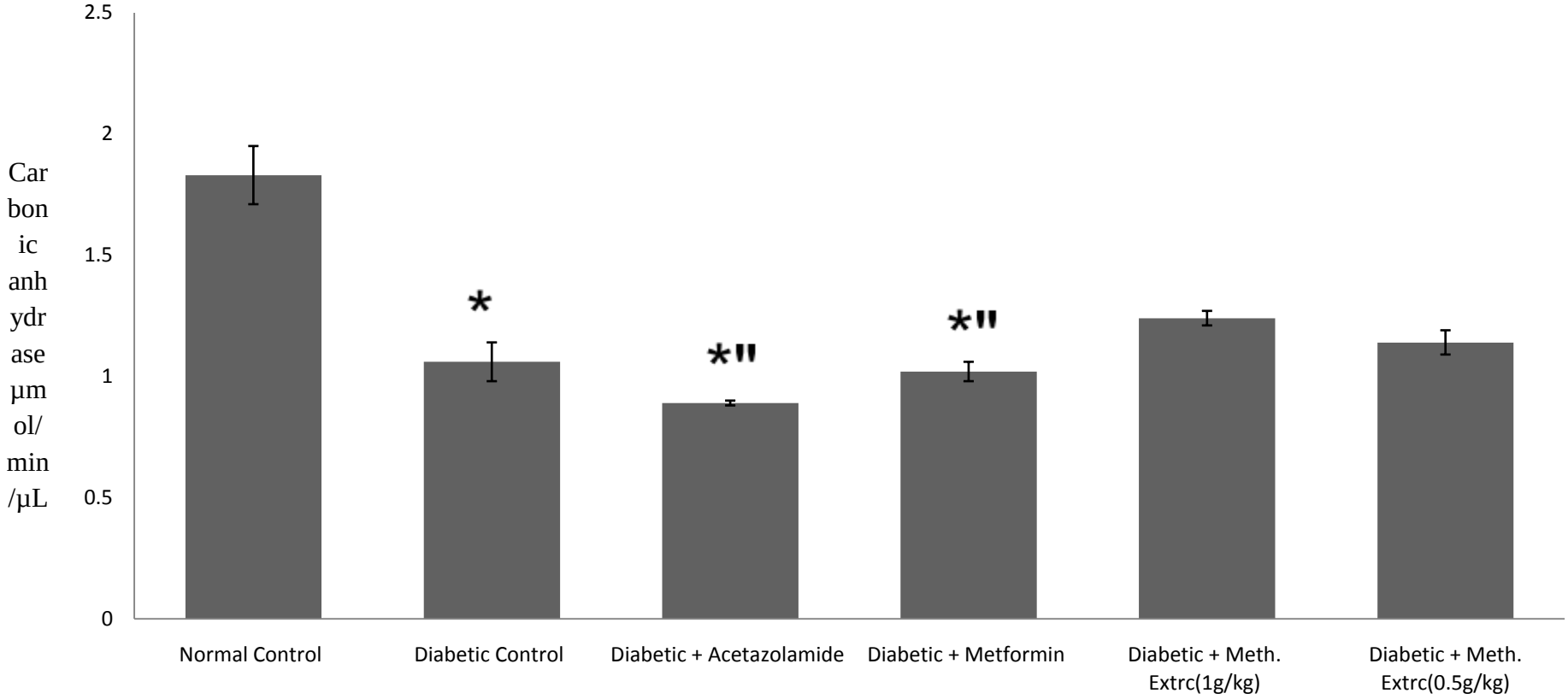


Figure 4.1: The effect of Acetazolamide (at 3.6mg/kg/day), Metformin (14.3mg/kg/day) and Methanol extracts of *Cadaba farinosa* (1000mg /kg/day and 500mg /kg/day) doses for 28 days on Carbonic anhydrase activity in STZ induced diabetic rats treated. *P < 0.05 vs Normal control; **"**P < 0.05 vs Diabetic control (n=5).

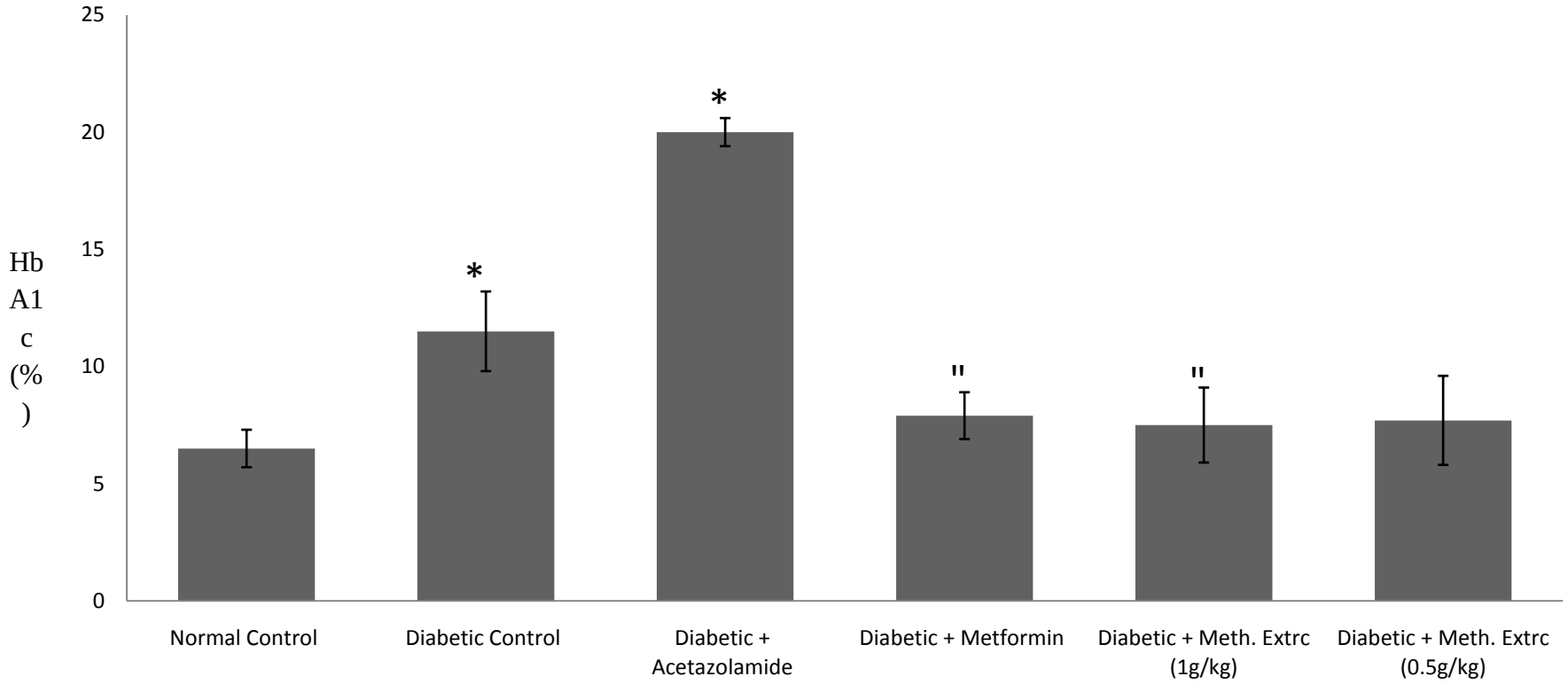


Figure 4.2**:**The effect of Acetazolamide (at 3.6mg/kg/day), Metformin (14.3mg/kg/day) and Methanol extracts of *Cadaba farinosa* (1000mg /kg/day and 500mg /kg/day)doses for 28 days on HBA_{1C}levels in STZ induced diabetic rats treated.**"**P < 0.05 vs Diabetic control, *****P < 0.05 vs Normal control (n=5).

4.2.1 *Cadaba farinosa* extract lowers blood glucose; lactate and glycated hemoglobin (HbA1c) level in STZ induced diabetic rats.

The effect of crude methanol extract of *C. farinosa* on STZ induced diabetic rats, lactate and HbA1c were measured. The extract at 1000mg/kg/day for 28 days significantly ($p<0.05$) reduced the level of blood glucose, lactate and HbA1c level when compared with diabetic control (Figure 4.3 and 4.4).

4.2.2 *Cadaba farinosa* extract affects carbonic anhydrase activity and blood lactate level in STZ induced diabetic rats.

Crude methanol leaf extract of *cadaba farinosa*significantly ($p<0.05$) reducederythrocyte carbonic anhydrase activity in STZ induced diabetic rat when compared with normal control but a significant ($p<0.05$) increase in carbonic anhydrase activity was observed when compared with diabetic controlrats.

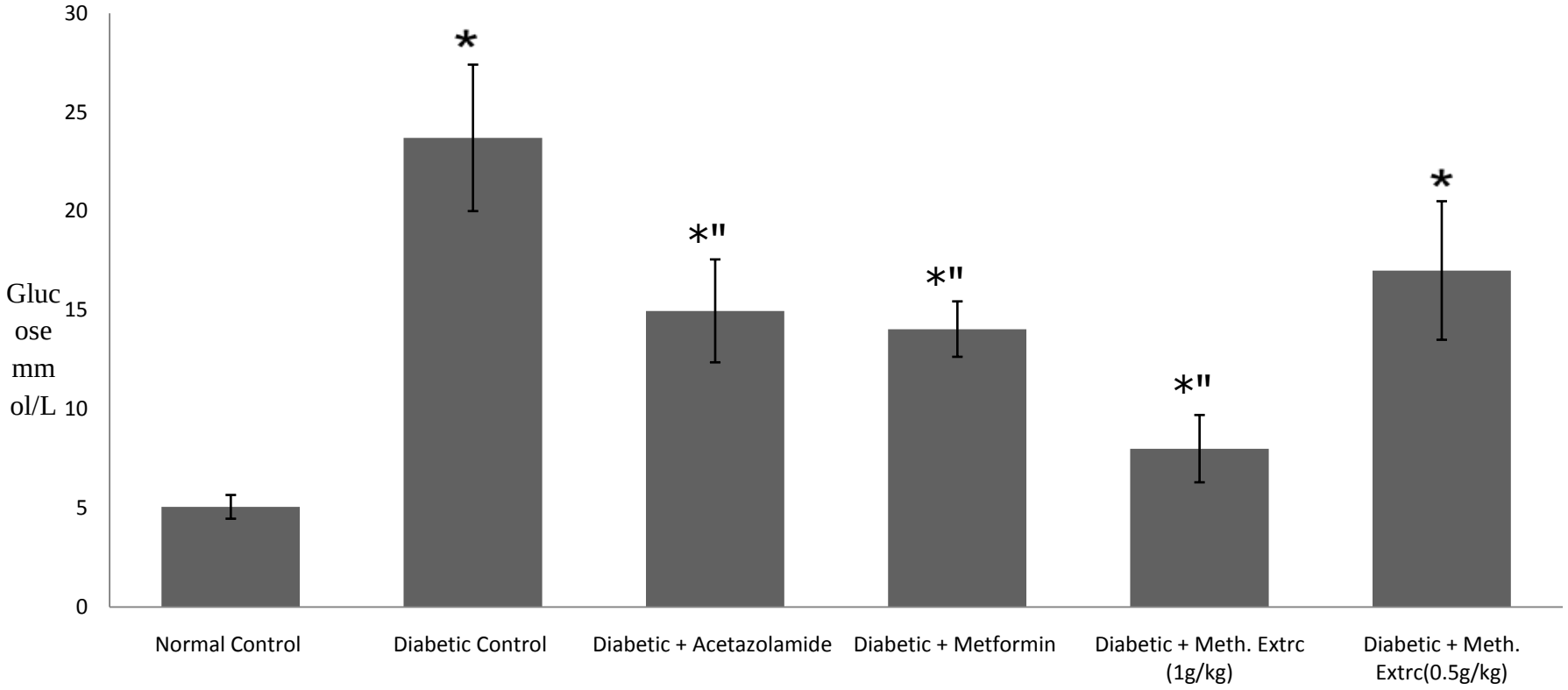


Figure 4.3 The effect of Acetazolamide, Metformin and Methanol extract of *Cadaba farinosa* at 3.6mg/kg/day, 14.7mg/kg/day, 1000mg /kg/day and 500mg /kg/dayThe effect of Acetazolamide (at 3.6mg/kg/day), Metformin (14.3mg/kg/day) and Methanol extracts of *Cadaba farinosa* (1000mg /kg/day and 500mg /kg/day) doses for 28 days on Glucoselevels in STZ induced diabetic rats treated. *P < 0.05 vs Normal control; “P < 0.05 vs Diabetic control (n=5).

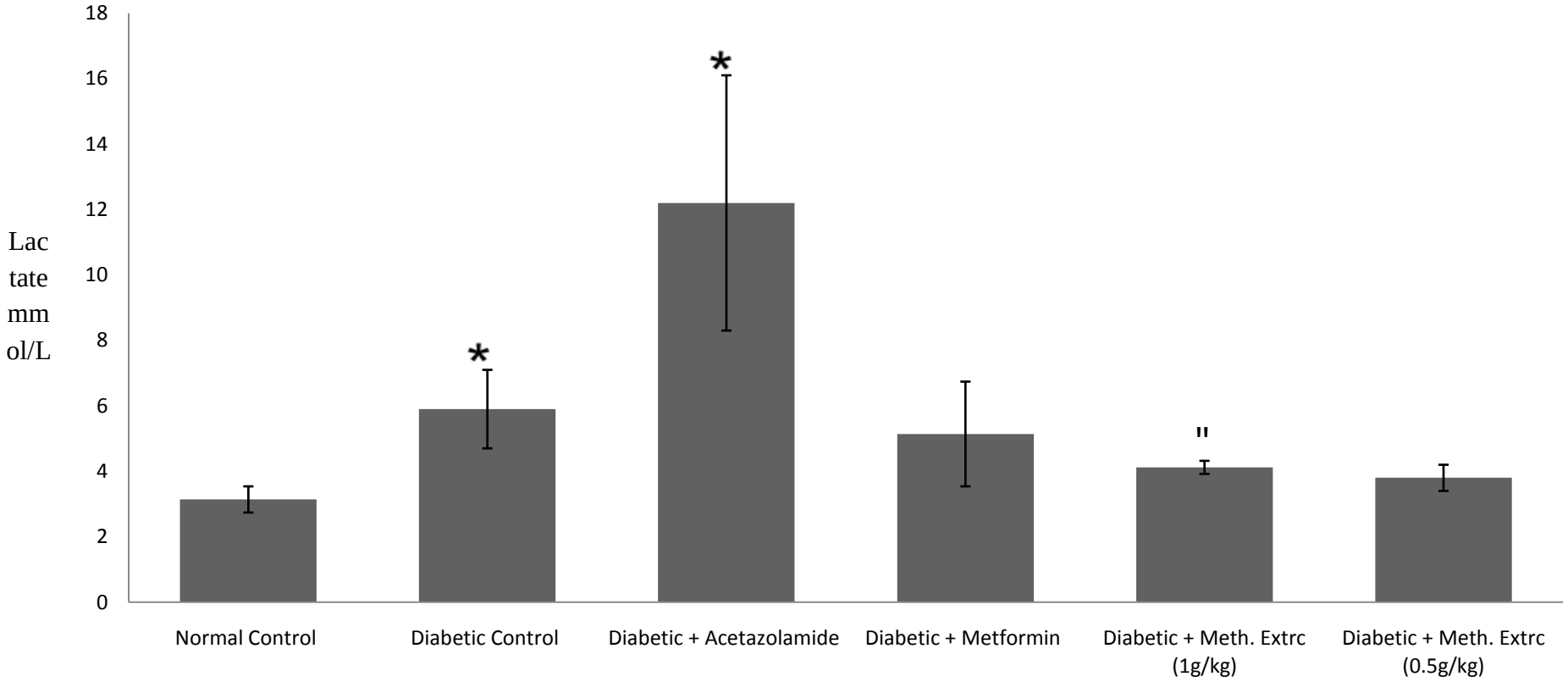


Figure 4.4**:**The effect of Acetazolamide (at 3.6mg/kg/day), Metformin (14.3mg/kg/day) and Methanol extracts of *Cadaba farinosa* (1000mg /kg/day and 500mg /kg/day)doses for 28 days on Lactatelevels in STZ induced diabetic rats treated. *P < 0.05 vs Normal control, (n=5),"P < 0.05 vs Normal control, (n=5).

4.2.3 Acetazolamide, Metformin and *Cadaba farinosa* extract affect blood cholesterol and Triglycerides levels in STZ induced diabetic rats.

Figure 4.5demonstratesthat Acetazolamideand Metformin significantly ($p<0.05$) reducedblood cholesterol level, while blood triglycerides level increased significantly ($p<0.05$)in diabetic rats treated with Acetazolamide.

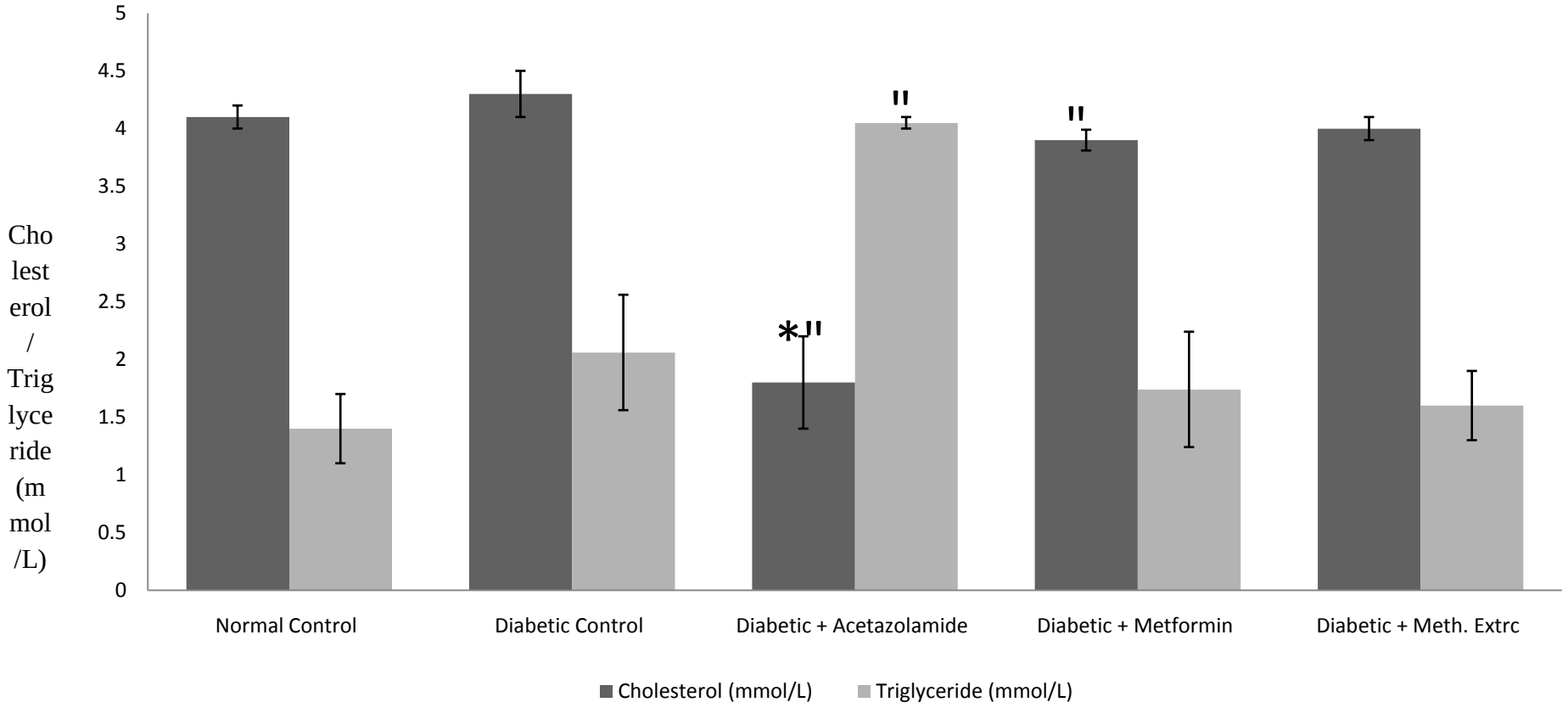


Figure 4.5**:**The effect of Acetazolamide (at 3.6mg/kg/day), Metformin (14.3mg/kg/day) and Methanol extracts of *Cadaba farinosa* (1000mg /kg/day and 500mg /kg/day) doses for 28 days on Cholesterol and Triglyceridelevels in STZ induced diabetic rats treated. *P < 0.05 vs normal control,"P < 0.05 vs Diabetic control, (n=5).

4.3 Changes in antioxidants level in STZ induced Diabetic ratsafter treatment with Acetazolamide, Metformin and Crude leaf extract of *Cadaba farinosa*

Figure 4.7,illustratesthat Acetazolamide significantly ($P < 0.05$) reducesplasma erythrocyte catalase activity in STZ induced diabetic rats when compared with diabetic control. On the other hand Methanol leaf extract of *Cadaba farinosa*was found to significantly ($P < 0.05$) increase the activity of erythrocyte catalase in STZ induced diabetic ratswhen compared with diabetic control.

Figure 4.8 Showed a significant reduction of plasma catalase activity in diabetic groups treated with Acetazolamide when compared with both normal and diabetic control group. Methanol leaf extract of *Cadaba farinosa*was found to significantly ($P < 0.05$) decrease the activity of plasma catalase when compared with diabetic control group.

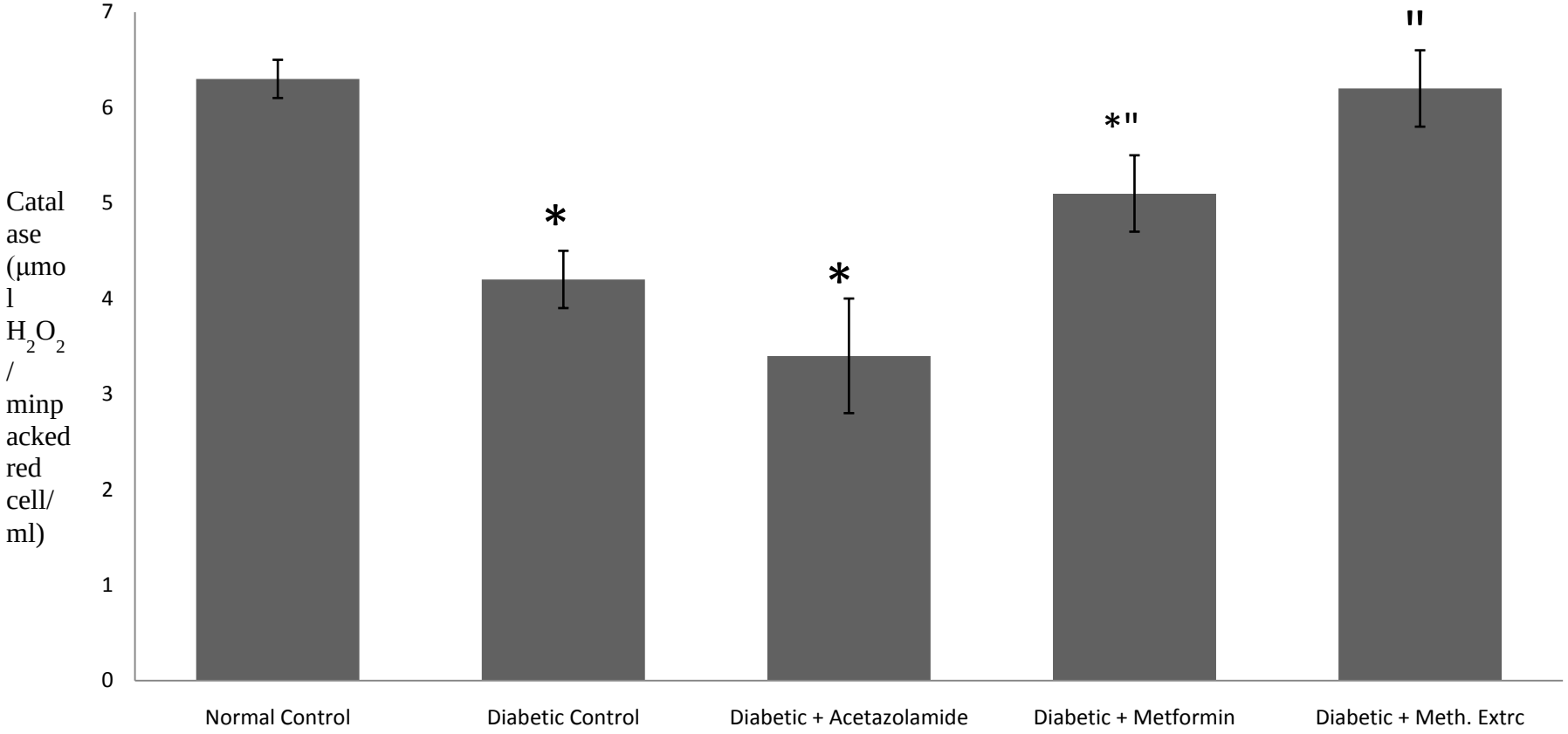


Figure 4.6: Effect of Acetazolamide, Metformin and Methanol extract of *Cadaba farinosa*at 3.6mg/kg/day, 14.7mg/kg/day and 1000mg /kg/day doses for 28 days on Red Blood cell catalase activity levels in STZ induced diabetic rats. *P < 0.05 vs Normal control,"P < 0.05 vs Diabetic control (n=5).

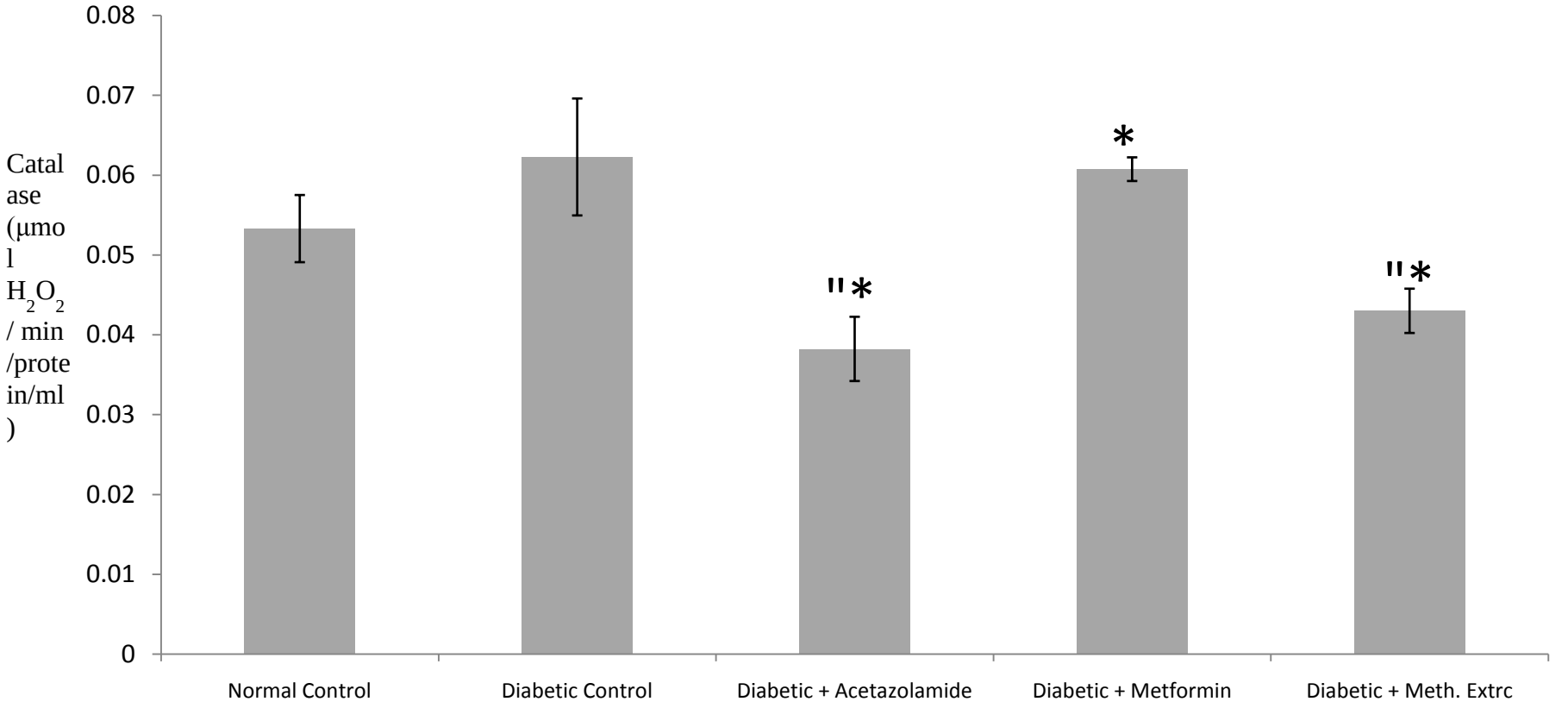


Figure 4.7: Effect of Acetazolamide, Metformin and Methanol extract of *Cadaba farinosa* at 3.6mg, 14.7mg 1000mg /kg/day doses for 28 days on SerumCATALASE activity levels in STZ induced diabetic rats treated. *P < 0.05 vs Normal control. ”P < 0.05 vs

4.3.1 Changes in Erythrocyte and serumSOD Activity after treatment with Acetazolamide, Metformin and Crude leaf extract of *Cadaba farinosa* in STZ-Induced Diabetic rats

Metformin and Crude leaf extract of *Cadaba farinosa* treatment showed a significant decrease ($P < 0.05$) in erythrocyte SOD activity in STZ induced diabetic rats when compared with normal control (figure 4.9). Whereas STZ induced diabetic rats treated with Acetazolamide showed a significant ($P < 0.05$) increase in serum SOD when compared with both normal and diabetic control (figure 4.10).

4.3.2 Carbonic anhydrase inhibition Decreased TBARS Level in STZ-induced diabetic rats

Figure 4.11:STZ induced diabetic rats treated with Acetazolamide, Metformin and Crude leaf extract of *Cadaba farinosa* showed a significant ($P < 0.05$) decreased TBARS level when compared with diabetic control group.

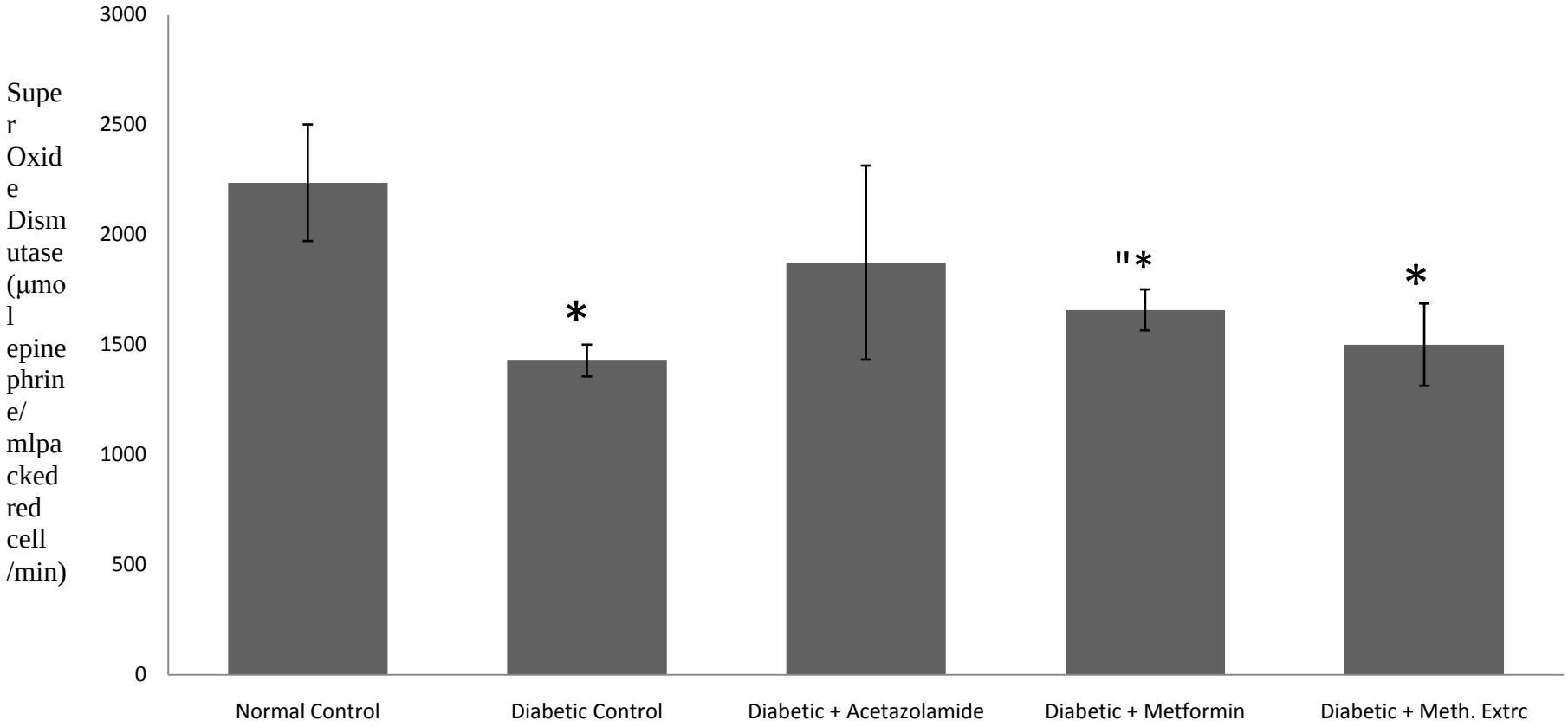


Figure 4.8: Effect of Acetazolamide, Metformin and Methanol extract of *Cadaba farinosa* at 3.6mg, 14.7mg and 1000mg /kg/day doses for 28 days on Red Blood cell Superoxide dismutase activity levels in STZ induced diabetic rats. *P < 0.05 vs Normal control, ”P < 0.05 vs Normal control (n=5).

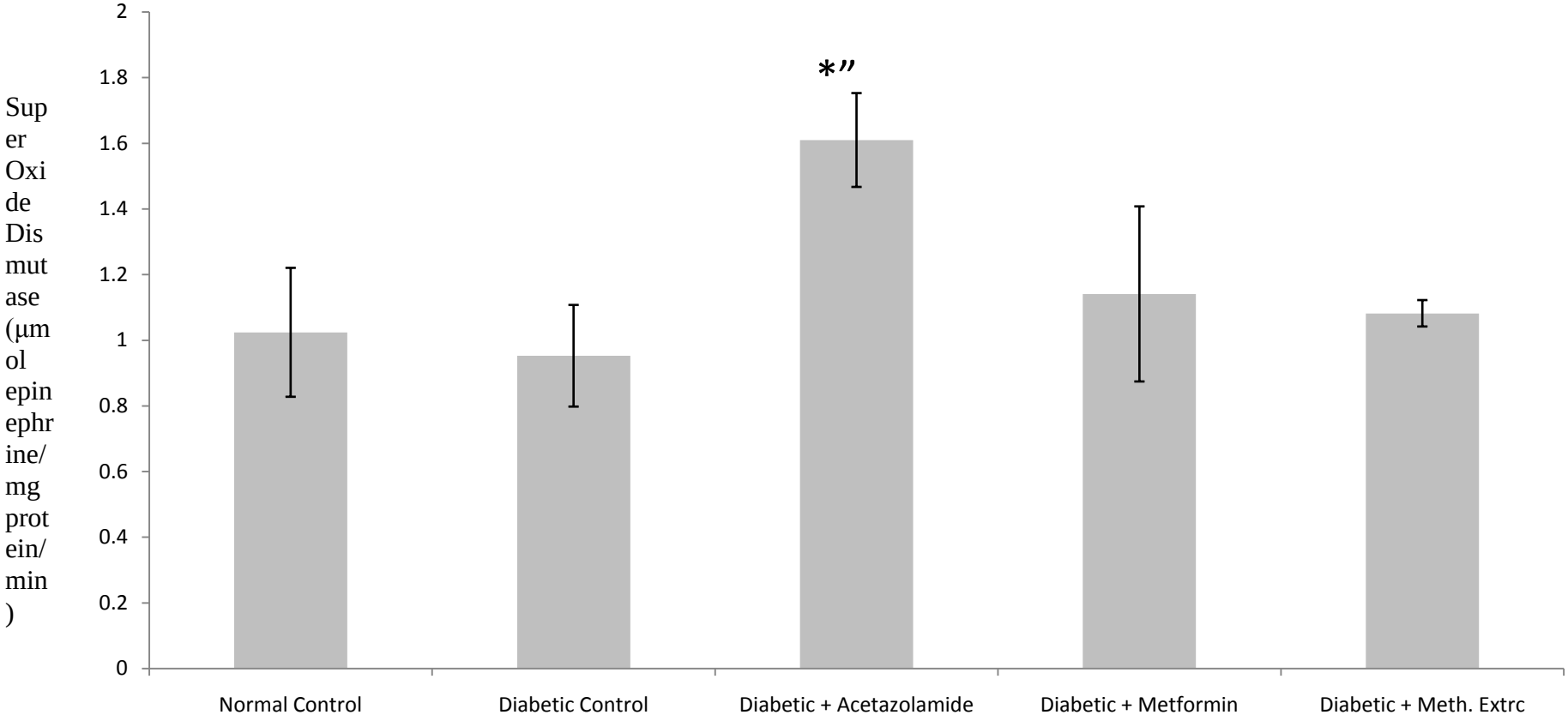


Figure 4.9: Effect of Acetazolamide, Metformin and Methanol extract of *Cadaba farinosa* at 3.6mg/kg/day,14.7mg/kg/day and 1000mg /kg/day doses for 28 days on Plasma Superoxide dismutase activity levels in STZ induced diabetic rats. *P < 0.05 vs Normal control, "P < 0.05 vs Diabetic control (n=5).

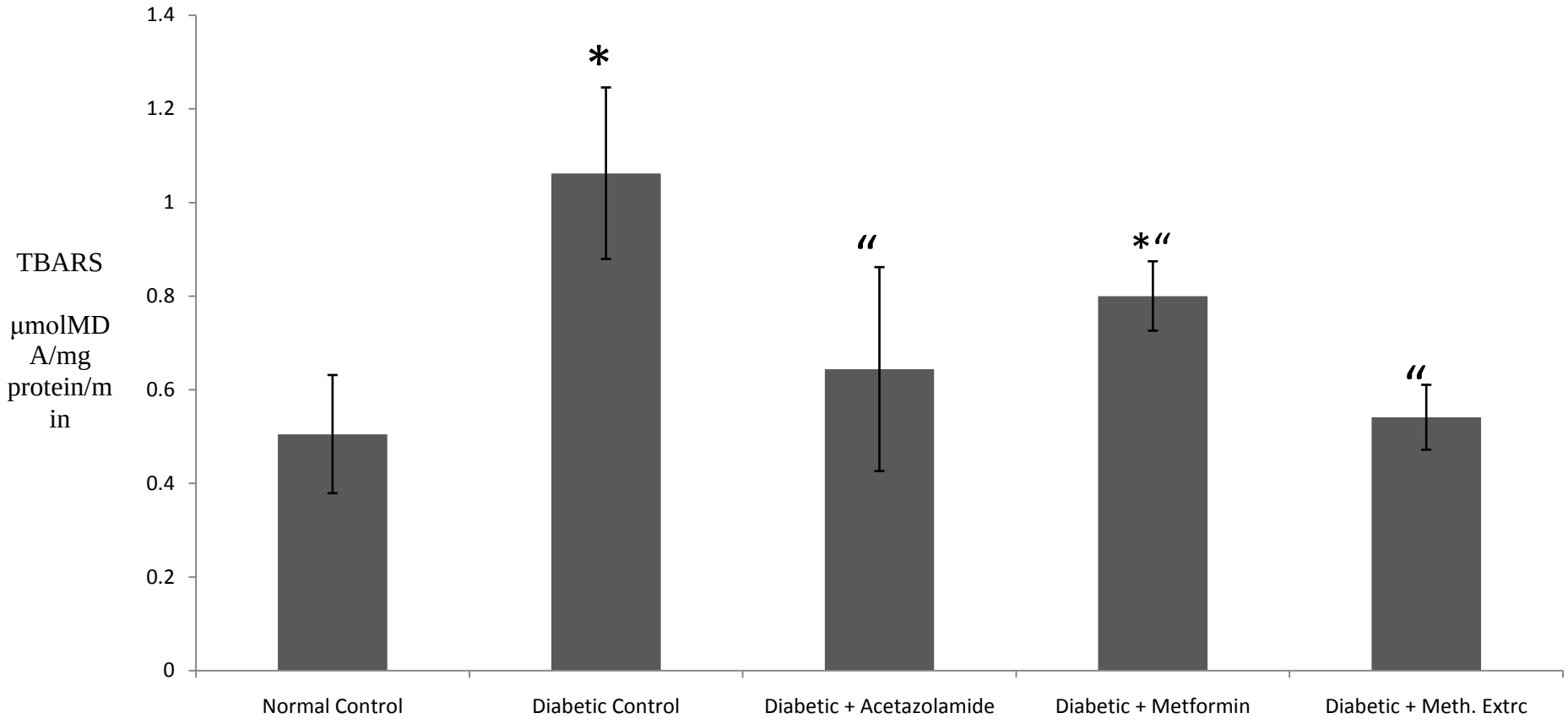


Figure 4.10: Effect of Acetazolamide, Metformin and Methanol extract of *Cadaba farinosa* at 3.6mg, 14.7mg and 1000mg /kg/day doses for 28 days on Red Blood Cell TBARS in STZ induced diabetic rats. *P < 0.05 vs Normal control; “P < 0.05 vs Diabetic control (n=5).

4.4**Analysis of extracted fractions**

The air-dried powdered leaves (800g) were defatted and extracted with methanol (10 L). The extract was concentrated under reduced pressure to obtain a dark green residue weighing 100.1 g. The crude methanol extract (5.0 g) was subjected to column chromatography over a silica gel column (100 g × 50 × 3 cm) using a methanol: ethyl acetate (6:4) gradient producing five fractions A to E, which gives Fraction A (25 mg, brown powder), fraction B (29 mg, light green powder), fraction C (32 mg, black green powder), fraction D (23 mg, green powder) and fraction E (19 mg, yellowish green powder). The five fractions, (A to E) were tested *invitro* against carbonic anhydrase activity.

4.41*In-Vitro* Inhibitory effect of Methanol Leaf Extract of *Cadaba farinosa*, Acetazolamide and Metformin on carbonic anhydrase activity

Figure 4.12 showed the effect of crude methanol leaf extract of *C. farinosa*, Acetazolamide and Metformin on carbonic anhydrase activity. Among the three groups acetazolamide showed the highest inhibition being the standard inhibitor of carbonic anhydrase24.3 µg/100 µL followed by Metformin 18.5 µg/100 µL and lastly crude methanol leaf extract of *C. farinosa*6.3 µg/100 µL.

Among the five fractions obtained from the methanol extract, fraction A showed the lowest activity of 21.8when compared with fraction B, C and D which showed 33, 36 and 35 µmol/min/µL inhibition respectively.However, only fraction E showed increased carbonic anhydrase activity of 41.2 µmol/min/µL (Figure 4.13)**.**

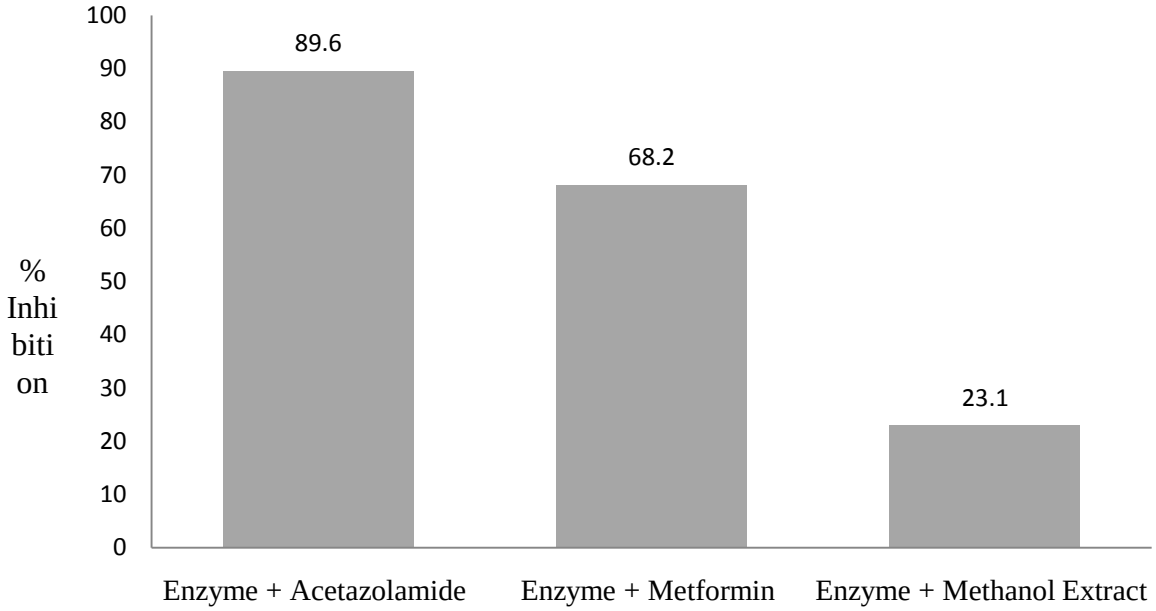


Figure 4.11**:** In vitro inhibitory effect of Acetazolamide, Metformin and crude methanol leaf extract of *Cadaba farinosa* at 15µg/100µl on Bovine erythrocyte carbonic anhydrase activity.

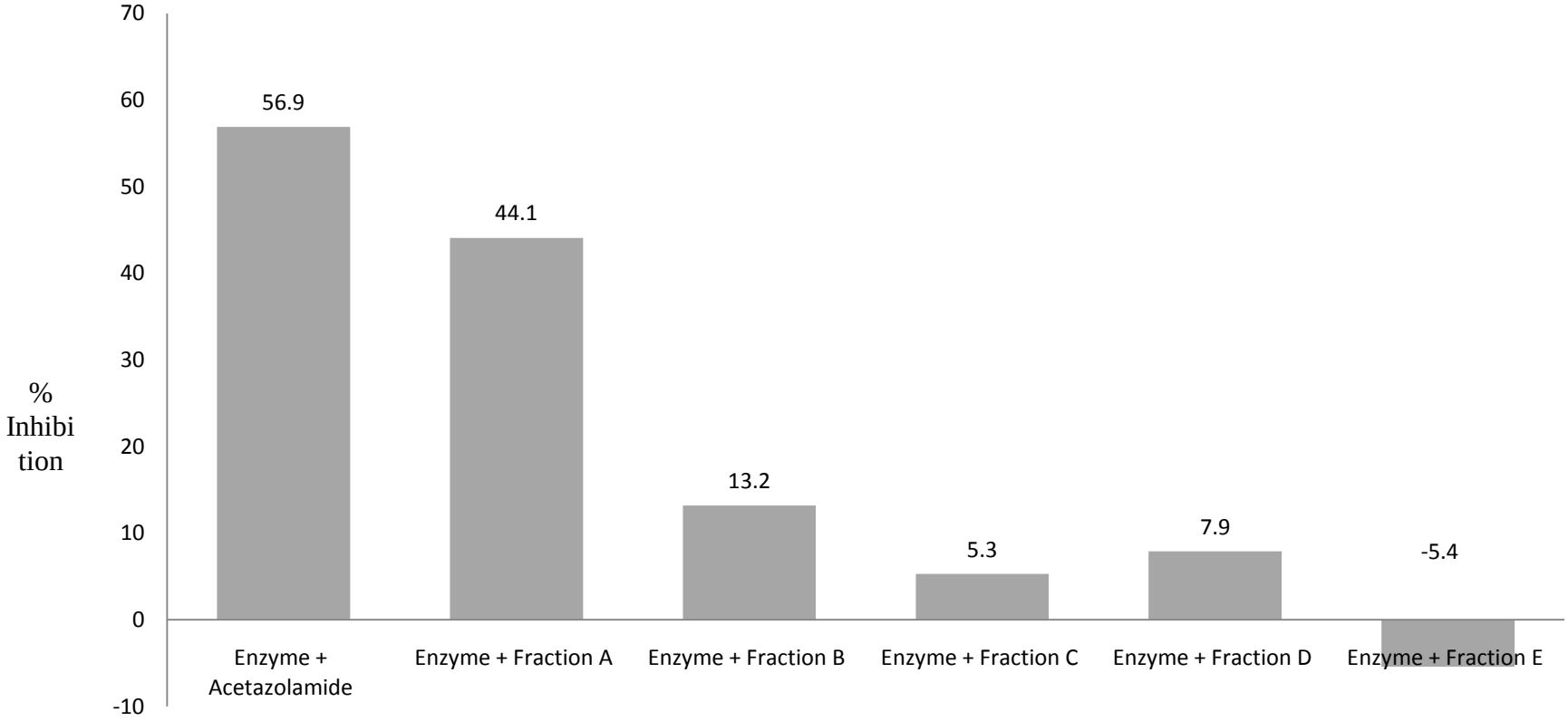
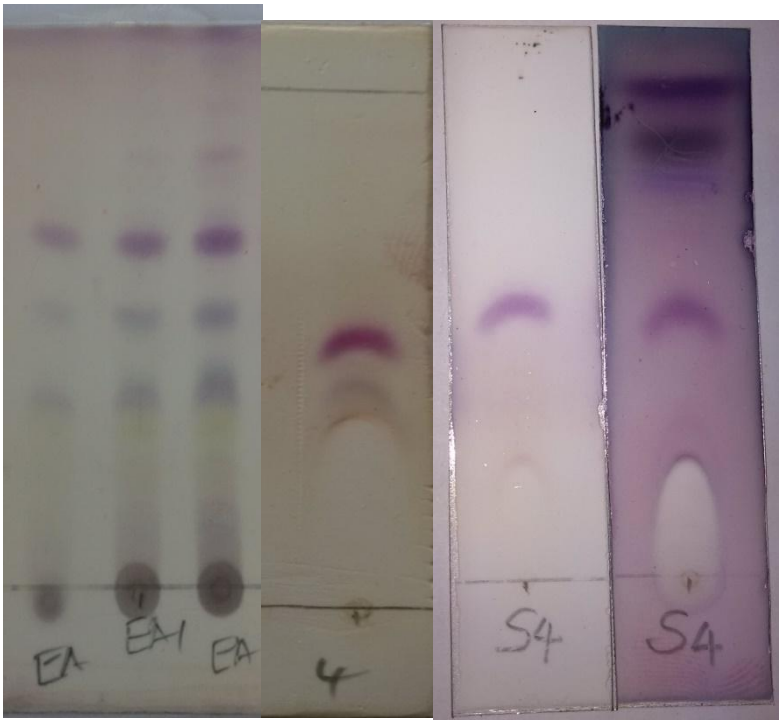


Figure 4.12**:** In vitro inhibitory effect of Acetazolamide, Metformin and purified fractions of methanol leaf extract of *Cadaba farinosa* at 15µg/100µl on Bovine erythrocyte carbonic anhydrase activity.

4.4.2 Thin-Layer Chromatography of the highest inhibitory fraction

Plate I (a) revealed five spots on the TLC chromatogram of the crude methanol leaf extract. Individual spot was tested against CA activity; the one with the highest inhibition was run as Plate 1(b). Plate I (b) revealed the TLC chromatogram of the spot with the highest inhibition against carbonic anhydrase activity *invitro.*



Crude Methanol extract (a) 4 Fraction A (b)

Plate I: The TLC chromatogram of crude methanol extract leaf extract of *Cadaba farinosa* (a) and Fraction A (b)

EA,EA1 = Crude methanol chromatogram(6:4): R_f value = 0.61

4= Column chromatogram of the active fraction (6:4): R_f value = 0.53

S4= Preparative TLC chromatogram of the active fraction (6:4): R_f value = 0.52

4.4.3 Infrared spectra analysisof compound 1

The infrared spectra of purified fraction A of *C. farinosa* leaf extract showed a single band at 3486.91 cm^{-1} which was assigned to symmetric vibration of NH_2 group. The corresponding NH_2 appeared in the 3400-3500 cm^{-1} region characteristic band of Amine group present in the spectrum. A single band at 2226.31 cm^{-1} region is tentatively assigned to Nitrile group (C≡N) which appeared in the 2220-2240 cm^{-1} region. The infrared spectral data of fraction A in **(**Table 4.3**)** showed some characteristic bands related to α- aminonitriles; which could be attributed to C≡N group.

Table 4.3:Functional Groups identified using FTIR spectra analysis for the inhibitor (fraction A) of methanol leaf extract of *cadaba farinosa.* Wavenumbers (cm^{-1})

S/N	Absorbance	Absorption ranges	Functionalgroupnames
1	3486.91	3400-3500	N-H, Amine
2	2932.73	2850-3000	C-H, Alkane (strech)
3	2226.31	2220-2240	CN, Nitrile
4	1719.05	1710-1720	>C=O, Ketone
5	1653.86	1640-1670	C=C, Alkene

4.4.4 Gas chromatography-mass spectra analysis

The GC-MS result of the purified fraction A shown in (Table 4.4**)**revealed the presence of D-ribonitrile, 2,3,4,5-tetraacetate ($C_{13}H_{17}NO_{8)}$, phenol, 4-methyl-3-(2-methylhydrazino)- ($C_8H_{12}N_2O$), 5-ethyl-2-methylpyridine 1-oxide ($C_8H_{11}NO$), 1,2-Dimethyl-3-nitro-4-nitroso-benzene ($C_8H_8N_2O_{3)}$and Octanamide ($C_{18}H_{37}NO$).

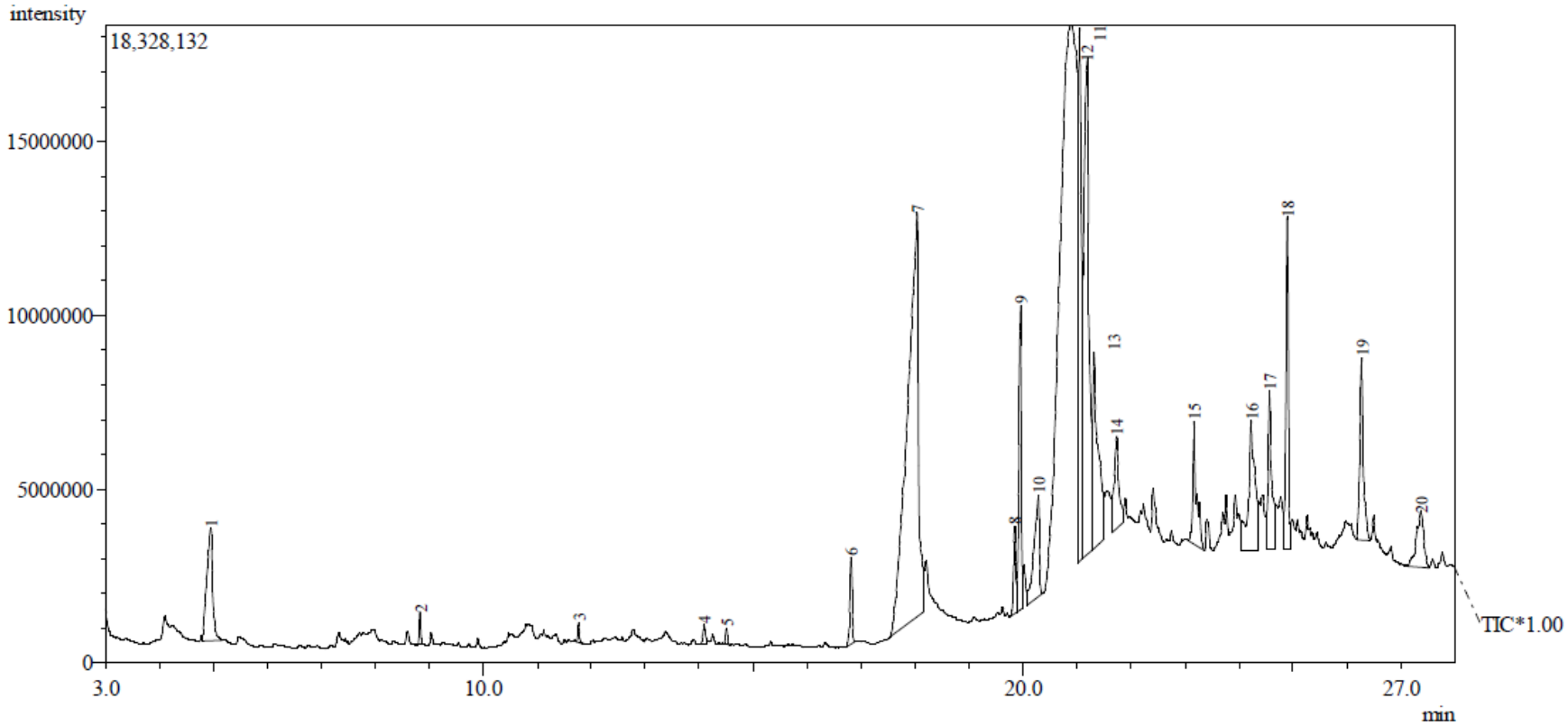


Plate 2:GC-MS chromatogram of the fraction, Column Oven Temp. :80.0 °CInjection Temp. :250.00 °C

Table 4.4**:** Compounds identified using GC-MS analysis from the inhibitor (fraction A) of methanol leaf extract fraction of *cadaba farinosa.* Wavenumbers (cm^{-1})

S/N	Compound Name	Peak	Retention Time	Formula	Molecular Weight.
1	N-Ethoxyisobuten-3-imine	1	4.950	$C_6H_{11}NO$	113
2	D-Ribonitrile, 2,3,4,5-tetraacetate	2	8.833	$C_{13}H_{17}NO_8$	315
3	Phenol, 4-methyl-3-(2-methylhydrazino)-	79	11.767	$C_8H_{12}N_2O$	152
4	5-Ethyl-2-methylpyridine 1-oxide	79	11.767	$C_8H_{11}NO$	137
5	1,2-Dimethyl-3-nitro-4-nitroso-benzene	3	11.767	$C_8H_8N_2O_3$	180
6	9-Octadecenamide	15	23.167	$C_{18}H_{35}NO$	281

4.4.5 Structural elucidation of compound (1)

Compound 1, the most potent fraction against carbonic anhydrase in the methanol extract, was obtained as a colorless crystalline solid. The compound showed a positive purple spot on a TLC plate sprayed with p-anisaldehyde. The ^{1}H- and ^{13}C-NMR (in DMSO) spectrum is shown in (Table 4.). The planar structure was deduced to be 6-acetyloxy-3-hydoxy-5-hexenoic acid (figure 4.15).

Chemical formula = $C_8H_{12}O_5$

Molecular weight = 188

Melting point = 149.94^{o}C

Boiling point = 409.12 ^{o}C

% Yield = 12.5

Table 4.5. ^{1}H and ^{13}C NMR data of compounds 1 isolated from *C. farinosa*

Pos.	**1 (^{13}C)**	**^{+}H**
1	172.21	8.49
2	29.64	8.44
3	71.91	4.77
4	27.98	3.26
5	129.48	3.02
6	132.37	2.95
7	167.73	1.98
8	19.64	1.68

6-acetyloxy-3-hydroxy-5-hexenoic acid

Figure 4.13 Structure of compound 1

CHAPTER FIVE

5.0 DISCUSSION

The effect of short term STZ induced diabetes results presented in this study indicated that diabetes provides a stable pattern of increased carbonic anhydrase activity with glucose, lactate, cholesterol and triglycerides. Analysis of these metabolic parameters (Table 4.1) indicated that the STZ-induced diabetes exhibit all the hallmark indicators of a mild to severe diabetes (Alberti and Zimmet 1998), such as attenuated weight gain, hyperglycemia and hyperlipidemia. One interesting finding is the rise in blood lactate level in the early phase of diabetes.

Previous studies showed that lactate is not only increased in the early stages of diabetes but has also been shown to predict its occurrence in the future (Crawford *et al*. 2010;and Nicky *et al*. 2012). Forbath *et al*. (1967) and DeMeutter and Shreeve (1963); have also reported that there is an increase in lactate production, in diabetic dogs and humans. Becker and Deitmer, (2008); Wetzel *et al*. (2001); Svichar and Chesler, (2003); Becker *et al*. (2004); Becker *et al*. (2005); Becker *et al*. (2010); Becker and Deitmer, (2004) have reported that monocarboxylate transporters (MCT) dependent lactate-H^+ flux is facilitated by bicarbonate transporters and carbonic anhydrase activity in various cells and tissues.

Red blood cells produce lactic acid as a byproduct of the regeneration of ATP during anaerobic glycolysis but cannot use lactic acid. The result of the present study suggests that blood lactate might have increased due to increased anaerobic oxidation of glucose in STZ induced diabetic rats which produced lactate as the end product. Nguyen and Bonanno, (2011) found evidence supporting the notion that lactate-H^+ cotransport via monocarboxylate transpoters (MCT) 1,2 and 4 is facilitated by HCO_3^-, Carbonic anhydrase (CA) activity, Na^+ /H^+ exchange, and $1Na^+ : 2HCO_3^-$ cotransport. Thus the

increase in carbonic anhydrase activity seen in STZ induced diabetic rats (Table 4.1) may have come as an adaptive response to increase the facilitated efflux of lactate from the glycolytic cells leading to increased blood lactate concentration.

It was reported in early 1983 that CA inhibitor acetazolamide decreased rat tubular glucose synthesis; the authors were unaware of the existence of carbonic anhydrase isozyme V (CA V) and concluded that this finding was a nonspecific effect of acetazolamide on the proximal tubule (Tannen and Ross, 1983). Other studies were aimed at examining the hypothesis that renal CA V is involved in glucose synthesis in proximal convoluted tubules when pyruvate is added as the sole substrate. Among such studies is that of Dodgson and Cherian (1989) who starved rats for 48 hours so they may remove all endogenous glucose from the proximal convoluted tubules and also to maximize the activity of gluconeogenic enzymes. They showed that CA inhibitor ethoxzolamide inhibit CA V activity of intact mitochondria. They determined that glucose synthesis was a linear function of time in both the presence and absence of the inhibitor. They showed that when proximal convoluted tubules were incubated in Krebs-Henseleit buffer (which contains 25 mM $NaHCO_3$) and 10 mM pyruvate with 5% CO_2 and 95% O_2, glucose synthesis was decreased increasingly by increasing the concentrations of ethoxzolamide; 50% was decreased by 0.6 µM concentration of the inhibitor. Acetazolamide inhibits pyruvate carboxylase around 80 to 85% inhibition at the same concentration mitochondrial carbonic anhydrase was fully inhibited Barrittg *et al* (1976).

A novel finding of the current study was the reduction in blood glucose concentration by Acetazolamide in STZ induced diabetic rats (Table 4.2).

The result showed an increase in blood lactate and triglyceride level, a decrease in blood glucoseand cholesterol level in the diabetic group treated with acetazolamide compared with the the diabetic control group. This indicates a consistency between results obtained in this study and that of others, such as that of Carmen *et al* (2016) who showed that carbonic anhydrase isoforms (CAVA) and (CAVB) provide the bicarbonate required by four mitochondrial metabolic liver enzymes. They reported that defective provision of bicarbonate, which may result from inhibition of carbonic anhydrase, leads to hyperammonemia, elevated lactate and ketone bodies, metabolic acidosis, hypoglycemia, and excretion of carboxylase substrates. Previously Acetazolamide and ethoxyzolamide were found to inhibit gluconeogenesis in vitro or after in vivo administration in rats (Dodgson and Forster, 1986; Bode *et al*., 1994; Cao and Rous, 1978). Dodgson and Forster (1986) concluded that carbonic anhydrase is functionally important for gluconeogenesis in the male guinea pig liver when there is a requirement for bicarbonate as substrate.

It may therefore be suggested that carbonic anhydrase plays a crucial role in gluconeogenesis, due to the fact that carbonic anhydrase inhibition does not only lower blood glucose level but increases blood lactate level as well. Many studies have demonstrated that suppression of hepatic gluconeogenesis is accompanied by increased blood lactate level as seen in metformin treatment of type II diabetes. A potentially life-threatening complication of Metformin is the occurrence of lactic acidosis, metformin-associated lactic acidosis (MALA). Radziuk *et al*., (1997) reported that high therapeutic metformin levels provoked a reduction in lactate uptake by the liver. This reduction in hepatic uptake may be the reason metformin concentration increases in the blood. It has also been reported by Lutz *et al*., (2001) that the uptake of gluconeogenic substrates, such as alanine and lactate,is reduced in the presence of metformin.

To reproduce the result, STZ induced diabetic rats were treated with Metformin 14.7mg/kg/day for 28 days in order to investigate the effect of Metformin on carbonic anhydrase activity and blood lactate level. For the first time the present study not only demonstrates that Metformin significantly ($p<0.05$) reduced carbonic anhydrase activity but also significantly increased blood lactate level.

However, the magnitude of the efflux of lactate in the bloodstream, which is the anionic form of lactic acid, may override the body's ability to utilize protons, hence converting hyperlactatemia into lactic acidosis. Indeed, it has been proposed that increased muscle release of lactate and alanine could be responsible for sustaining increased gluconeogenesis in NIDDM (DeFronzo *et al*., 1985). Increased provision of lactate is of considerable importance for the increased gluconeogenesis in diabetes. In earlier studies, carbonic anhydrase has been found to facilitate lactic acid transport in rat skeletal muscle fibers (Wetzel *et al*., 2001). It was also reported that monocarboxylate transporters MCT1 and MCT4 transport activity is increased by interaction with carbonic anhydrase II (CAII) (Becker and Deitmer, 2008; Becker *et al*., 2005; Becker *et al*., 2010).

The result obtained in the present study that untreated diabetic rats for 28 days showed a decrease in CA activity is consistent with previous findings. Gambhir *et al*. (1997), observed decrease in carbonic anhydrase activity in type II diabetic patients. They therefore speculated that the change in the CA activity may be of fundamental importance in the regulation of intracellular; pH_i for the basic control of metabolism in diabetes mellitus. Some early evidence suggest that the change in carbonic anhydrases activities in erythrocytes may be an initial step of altered metabolism in diabetes mellitus (Gambhir *et al*., 1997),. Dodgson and Watford, (1990), examined previously the changes in the activity of CA-III in hepatocytes of acute diabetic rats. Diabetes

resulted in 50% reduction in the activity of CA-III. They observed an approximate 98% reduction in CA-III content in the liver of chronic diabetes mellitus rats relative to controls. A 75% reduction in serum CA-III content relative to control values after the administration of streptozotocin was observed.

It may therefore be suggested that, the low blood glucose concentration and increased blood lactate level may not come as a coincidence given lactate's association with glucose in diabetes and the accumulating evidence linking lactate flux with carbonic anhydrase as described previously.

Possible explanation in terms of potential mechanism for the increased blood lactate concentration may be due to reduction in carbonic anhydrase activity, which has been reported to facilitate lactate influx and efflux across the MCTs. Klier *et al*. (2014) showed that transport activities of MCT1 and MCT4 are enhanced by cytosolic carbonic anhydrase. They suggested that MCT4 is the main pathway to export lactate out of glycolytic cells, which may produce larger amount of lactate during metabolic demand, while MCT1 can both serve as a lactate importer and exporter. The question remains whether inhibition of carbonic anhydrase is responsible for blood lactate accumulation and decreased hepatic gluconeogenesis? Could it be the same reason why metformin reduces blood glucose by inhibiting hepatic gluconeogenesis while leading to blood lactate accumulation?

Hems *et al*. (1966), showed that gluconeogenesis from lactate in the isolated perfused rat liver was markedly inhibited when the pH of the perfusate was less than 7.1. Subsequently Lloyd *et al*. (1973), showed that, under similar conditions of simulated metabolic acidosis in the perfused rat liver, lactate uptake was also inhibited and that this occurred when hepatic mean intracellular pH had fallen below 7.05. It may therefore be suggested that in diabetic condition the increase in blood lactic acid

concentration and decreased blood glucose level was probably caused by inhibition of carbonic anhydrase. Already, carbonic anhydrase inhibition has been found to be associated with metabolic acidosis. It may therefore be suggested that carbonic anhydrase plays an important role in gluconeogenesis, due to the fact that carbonic anhydrase inhibition does not only lowers blood glucose level but increases blood lactate level as well. It can be reported that inhibition of carbonic anhydrase may impair lactate flux across the MCT's in various cells and tissues. It may therefore be suggested that the reported incidence of lactic acidosis that occur with Metformin therapy in type II diabetes might be the result of decreased carbonic anhydrase activity. It has been stated previously that the activity of carbonic anhydrase was reduced significantly in the liver of both acute and chronic streptozotocin induced diabetic rats (Dodgson and Watford, 1990). The ability of Metformin to inhibit carbonic anhydrase in diabetic rats points towards a negative change that indicates a powerful effect on aspects of the complex metabolic disturbance in diabetes.

The ability of metformin to lower circulating blood glucose levels in type II diabetic patients has been explained by various mechanisms, such as increased glucose uptake in liver and muscle, reduced gluconeogenesis, decreased lactate and alanine uptake, improved GLP-1 and reduced glucagon functions. The overall effect of the metformin action is that, metformin inhibits gluconeogenic enzymes and stimulates glycolysis by altering the activity of multiple enzymes in gluconeogenesis, glycogenolysis, ketogenesis and β-Oxidation pathways (Fulgencio *et al*., 2001). Metformin was reported to reduce hepatic glucose output by up to 75% (Stumvoll *et al*., 1995). The uptake of gluconeogenic substrates, such as alanine and lactate, is reduced in the presence of metformin (Lutz *et al*., 2001). The effects of metformin on hepatic glycogen metabolism are not well-established; however, *in vitro* treatment of

hepatocytes decreased glycogen synthesis (Otto *et al*., 2003). With these various proposed mechanisms of metformin action, it may therefore be proposed that metformin may reduce circulating blood glucose level by inhibition of carbonic anhydrase, which facilitate lactate (a gluconeogenic substrate) uptake by the hepatocytes for the synthesis of glucose.

The result of the present study showed a 3 fold significant ($p<0.05$) increase in HbA1c level in STZ induced diabetic rats treated with carbonic anhydrase inhibitor (Acetazolamide). This increase may be as a result of inhibition of carbonic anhydrase *in-vivo*. Several factors have been reported that can influence the rate of glycation of hemoglobin: which are pH (Higgins and Bunn, 1981; Lowrey *et al*., 1985; Gil *et al*., 1991), glucose concentration (Higgins and Bunn, 1981), carbonate (Gil *et al*., 2004) and catalysis by 2,3-diphosphoglycerate (Lowrey *et al*., 1985; Gil and Uzcategui, 1993; Baynes *et al*., 1984). Low intracellular pH (pHi) has been reported to increase glycation of hemoglobin as low erythrocyte pHincreases HbA1c formation (Speeckaert *et al*., 2014). It may therefore be suggested that, the increased HbA1c level seen in diabetes may be attributed to the increased anaerobic glycolysis in erythrocyte which produces lactate as the end product of glycolysis.

Red blood cells produce lactic acid as a byproduct of the regeneration of ATP during anaerobic glycolysis but cannot use lactic acid (Luft, 2001). The rate of production can increase 50-fold if either glucose or glycogen is required to generate ATP in the absence of oxygen (Luft, 2001). Lactate is transported out of the cell via monocarboxylate transporters (MCT) in an electroneutral transport mode of 1 H^+: 1 Lactate (Klier *et al*., 2014). H^+ transport system under conditions of exclusively aerobic metabolism is used by the cell to maintain a facilitation of CO_2 diffusion, whereas under conditions of dominating anaerobic glycolysis and low intracellular pH,

it is mainly used to transport H^+ along with the lactate anion through the monocarboxylate transporters (MCT), a prerequisite for the elimination of lactic acid from the cell. Inhibition of carbonic anhydrase may result in increased intracellular lactate level as has been previously shown, which will lead to lactic acidosis and consequently decreased intracellular pH*i*. The presence of carbonic anhydrase significantly buffers pH*i*. On the other hand, disruption of pH*i* via inhibition of Carbonic anhydrase may decrease lactate efflux and result in intracellular lactate accumulation which decreased pH*i,* and increased HbA1c formation.

Based on the study reported by Speeckaert *et al*., (2014) that, low intracellular pH (pHi) increases HbA1c formation. It may therefore be concluded that inhibition of intracellular erythrocyte carbonic anhydrase will lead to accumulation of intracellular lactate and result in lactic acidosis, hence the decreased pH (pH*i*). At a low pH even glucose cannot be utilized anaerobically due to the fact that cellular energy production can be compromised in metabolic acidosis (Halperin *et al*., 1996) as the activity of 6-phosphofructokinase, a critical enzyme in glycolysis, is pH dependent. The net effect is increased formation of HbA1c favored by low pH.

Moreover, the present study also demonstrates relationship of carbonic anhydrase inhibition with dyslipidemia. The results of the present study show a significant decrease in total cholesterol ($p < 0.05$), and significant increase in triglyceride ($p < 0.05$) in STZ induced diabetic rats treated with Acetazolamide. Dyslipidemia is a common finding in diabetes mellitus. Diabetic dyslipidemia is associated with high plasma triglycerides, low HDL-Cholesterol and increased LDL-Cholesterol particles (Mooradian, 2009).Hypertriglyceridemia is more common in diabetics as compared to non-diabetics, and it has been found to increase VLDL triglyceride up to four (4) fold

(Arbeeny *et al*., 1989). The result of the present study is consistent with the findings above. It was reported that carbonic anhydrase plays a role in hepatic lipogenesis at the level of pyruvate carboxylation (Biswas and Kumar, 2010) and it also plays a role in providing bicarbonate as substrate for carboxylation in lipogenesis (pyruvate carboxylase and acetyl coA carboxylase), (Chegwidden *et al*., 2000).

The finding from this study may probably suggest that inhibition of carbonic anhydrase may be responsible for the altered level of catalase and superoxide dismutase activity in both the erythrocyte and plasma of STZ induced diabetic rats. Inhibition of carbonic anhydrase has been reported to cause metabolic acidosis, which is a condition of low pH. Catalase and superoxide dismutase activities may be affected by low pH as most enzymes have optimum pH at which they catalyze reactions. To our knowledge, this is the first study demonstrating an association between decreased catalase activity; elevated SOD activity and carbonic anhydrase inhibition in both the erythrocyte and plasma of diabetic rats. Red blood cell SOD activity is frequently measured in humans as an index of defense against superoxide radical in the blood.

Hyperglycemia enhances intracellular acidosis and accumulation of intracellular calcium (Levine *et al*., 1988; OuYang *et al*., 1994). In addition, decrease in blood pH is often observed in metabolic acidosis. A pH-dependent reduction in the affinity of hemoglobin for O_2 (Bohr effect) has been reported in metabolic acidosis (Bellingham *et al*., 1971). The final effect on the affinity of hemoglobin for O_2 will depend on the duration of the acidosis. Complications associated with diabetes are augmented by increased O_2 consumption, ROS production, lipid peroxidation and decreased mitochondrial antioxidant substrates (GSH, NADH, CoQ, *etc*) (Duchen, 2004; Green *et al*., 2004). It has been reported that diabetic rats display higher O_2 consumption and reduced mitochondrial antioxidant GSH and coenzyme Q pools than non-diabetic rats

(Duchen, 2004). It may therefore be suggested that, the enhanced SOD activity and decreased TBARS level in diabetic rats treated with Acetazolamide (carbonic anhydrase inhibitor) might be due to decreased O_2 delivery and consumption brought about by inhibition of carbonic anhydrase.

Any increase in SOD catalytic activity produces an excess of H_2O_2 that must be efficiently neutralized by catalase or glutathione peroxidase (Casado *et al*., 2007). In tissues lacking significant catalase activity, detoxification of hydrogen peroxidase becomes critically dependent on Glutathione peroxidase (Ishibashia *et al*., 1997). In the present study catalase activity was found to have significantly ($p<0.05$) decrease when carbonic anhydrase was inhibited, suggesting that CA and CAT might influence each other. Ra¨isa¨nen *et al*. (1999) demonstrated that CA III overexpressing cells grew faster and were more resistant to cytotoxic concentrations of H_2O_2 than control cells. Their results suggested that CA III functions as an oxyradical scavenger and thus protects cells from oxidative damage.

It may be suggest that catalase and carbonic anhydrase together can be more effective in mediating defence against free radical damage. Kim *et al*. (2004), suggest that CA would mediate detoxification of peroxide.

$$H_2O_2 + CO_2 \longleftrightarrow H_2CO_4$$

Recently glutaraldehyde-crosslinked Poly-SFHb-SOD-CAT-CA was prepared by Bian *et al*. (2011. They suggested that the incorporation of CA resulted in a soluble nanodimension complex of PolySFHb- SOD-CAT-CA that has enhanced antioxidant properties in addition to acting as carrier for both O_2 and CO_2 (Bian *et al*., 2011).

Lack of convincing evidence on the decreased catalase activity due to inhibition of carbonic anhydrase does not mean that there is no link or interaction. However,

assessing oxidative stress induced by inhibition of carbonic anhydrase is not easy. Carbonic anhydrase (CA) is the enzyme in RBC responsible for the transport of CO_2. CA inhibitor can block RBC–CA activity, resulting in marked decrease in CO_2 transport (Geers and Gros, 2000). The result is a decrease in blood pH due to increased CO_2 concentration. Decreased levels of CA in STZ induced diabetic rats may provide a convenient media for reduced catalase activity by creating an acidic media.

Methanol leaf extract of *Cadaba farinosa*, lowers blood glucose level significantly ($p<0.05$) in STZ induced diabetic rats when compared with diabetic control. This similar trend has been observed with metformin treatment, an established hypoglycemic agent. Leaf extract of *Cadaba farinosa* is being used for the treatment of cancer and diabetes in some parts of northern Nigeria as folk medicine. FTIR spectra analysis, revealed characteristic absorption bands related to α- aminonitrile at 2226.31 cm^{-1} region which can be assigned to Nitrile group (C≡N). Several amino nitriles have been developed as reversible inhibitors of dipeptidyl peptidase (DPP IV) for treating diabetes (Khun *et al*., 2007). Vildagliptin is a recently released aminonitrile-containing antidiabetic drug that inhibits dipeptidyl peptidase IV (DPP-IV). It is therefore suggested that, the reduction in blood glucose level may be due to the presence of aminonitrile containing compounds in the leaves, but further research is required on the plant.

*In vitro*study of the Crude Methanol extract of *Cadaba farinosa* against carbonic anhydrase shows that the extract inhibits the activity of carbonic anhydrase. Five distinct fractions were obtained after fractionation. Both fractions were tested against carbonic anhydrase activity *in vitro.* Among the five fractions only fraction A showed 44.1% inhibition while fractions C-E showed mild inhibition as well. Fraction A was subjected to further analysis to see the compounds responsible for activation and

inhibition.The FTIR spectra data showed the presence of aminonitrile functional groups in the fraction A, suggesting the presence of the aminonitrile functional component which apparently may be responsible for the inhibitory activity of fraction A. The methanol extract was however found to inhibit carbonic anhydrase activity *in vivo,* and also reduce blood glucose concentration in diabetic rats. This may not be un expected considering the functional group of the recently released aminonitrile-containing antidiabetic drug called Vildagliptin (He *et al*., 2009).Several nitrile-containing drugs are in use for a variety of medical treatments. The nitrile containing drug such as the block-buster drug Anastrazole, marketed by Astra-Zeneca under the trade name Arimidex, is considered the drug of choice for treating oestrogen-dependent breast cancer, etravirine is a nitrile containing anti HIV agent, while (rilpivirine) are among the many etravirine analogs under development, which is beingtouted as among "the most potent anti-HIV agent(s) ever discovered." (Khun *et al*., 2007) and Entacapone used for treating Parkinson's disease is a potent inhibitor of catecholamine-*O*-methyltransferase. And numerous candidates are being pursued in clinical trials (De Clercq, 2005). Diamines and polyamines occur in the plants and animal kingdom as free bases (biogenic amines) as well as their derivatives. Polyamines are a class of alkaloid compounds which have been shown to modulate multiple biological processes including gene expression, cell proliferation, translation, cell signaling, membrane stabilization and ion channel inhibition as well as antibacterial activity (Casero and Marton, 2007; Fleidervish *et al*., 2008; Wallace and Niiranen, 2007; Soda *et al*., 2009; Xu *et al*., 2012; Yin *et al*., 2011; Buchanan *et al*., 2007). Polyamines have been reported in plants (Dictionary of Natural Products, 2012). Two of the simplest polyamines isolated to date include spermine and spermidine**.** Stachydrine and 3-hydroxy-stachydrine are the two spermidine alkaloids isolated from the leaves (Al-

Musayeib *et al*., 2013) and stem bark (Ahmad *et al*., 1985), of *Cadaba farinosa.* Recently Carta *et al*. (2010) showed that both spermine and spermidine and several of their semi-synthetic analogues inhibited hCA I-XIV isozymes with *K*i values ranging from nanomolar to millimolar. These studies confirm the inhibitory activity of the leaf extract of *cadaba farinosa* against carbonic anhydrase both *in vitro* and *in vivo.*

GC-MS analysis of fraction A revealed the presence of compounds (ribonitrile, methyl hydrazine, methyl pyridine, dodecanamide and octanamide) which are suggestive for the discovery of novel antidiabetic compounds and for selectively targeting either human or bacterial CAs. A compound with chemical similarity to dodecanamide ($C_{12}H_{25}NO$); 12 amino-dodecanoic acid ($C_{12}H_{26}NO_2$) was isolated from the leaves of *cadaba farinosa* by Al-Musayeib *et al*., (2013) the first compound reported to be isolated from plant source.

Hydrazine derivatives are reported to be environmental and food pollutants but are also important because of their use in medicine for the treatment of tuberculosis and cancer, and are also reported to pose significant health risks to humans as they are mutagenic and carcinogenic (Sinha and Mason, 2014). Hydrazine and its derivatives, which are used as high energy rocket fuel, induce a variety of toxic insults, including hypoglycemia, disorders of the CNS, induction of systemic lupus erythematosus, and cancer (Trohalaki *et al*., 2002; Fortney, 1966; Toth, 1978). Hydrazines are also found in tobacco and in edible mushrooms, and in Isoniazid and iproniazid, monoamine oxidase inhibitors, are in use for the treatment of tuberculosis and, until recently, as an antidepressant, respectively (Mitchell *et al*., 1976; Nelson *et al*., 1976). The derivative of hydrazine identified in the present study may also bring about reduction in blood glucose concentration, as it has been reported earlier to cause hypoglycemia.

The ^{13}C NMR spectra revealed the presence of carboxylic acid moiety at dC 172.21 (C-1), OH-bonded methyl at dC 71.91 (C-3), and acetyloxy bonded to methylene adjacent to carbonyl dC 132.37 (C-6). On the basis of these findings, compound 1 was assigned as 6-acetyloxy-3-hydroxy-5-hexenoic acid. Hexenoic acid derivatives have been reported to be used as antidiabetic agents. A novel compound of 6-(1,3-dihydro-4-hydroxy-6-methoxy-7-methyl-3-oxo-5-isobenzofuranyl)-4-methyl-4-hexenoic acid and N, N-dimethyl- imidodicarbonimidic diamide, has been reported to be used as an immunosuppressant and anti-hyperglycemic agent for the treatment of diabetic nephropathy (http://www.google.ga/patents/WO2005033089A1?cl=en). It has also been reported that Hexanoic acid derivatives can act as dipeptidyl peptidase inhibitors ("DPP-IV inhibitors") which are useful in the treatment or prevention of diseases in which the dipeptidyl peptidase-IV enzyme is involved, particularly in the treatment of type 2 diabetes (https://www.google.tl/patents/US20080051452). These data obtained in this study were very similar to that of 5-hydroxyl-5-methyl-2-hexenoic acid described by Zhang et al. (2010). The difference was that the 6-acetyloxy was replaced by 5- hydroxyl methyl in 5-hydroxyl- 5-methyl-2-hexenoic acid. This is the first information ever reported on the isolation of 6-acetyloxy-3-hydroxy-5-hexenoic acid by *Cadaba farinosa.* The carbonic anhydrase inhibitory activity results showed that compound 1 possessed inhibitory activity against carbonic anhydrase (IC_{50} = 13.57 µg/100 µL), The inhibitory effect was reduced with increased concentrations, indicating that the inhibitory activity of compound 1 was not concentration- dependent. The findings were in agreement with the results obtained from the *in vitro* inhibitory assay of crude methanol extract, and it further may further confirm that compound 1 may be the main carbonic anhydrase inhibitory compound of *Cadaba farinosa.*

Thus the claims made on the antidiabetic activity of *Cadaba farinosa* leaves may be justified to have the potential for lowering of blood glucose concentration mainly by inhibition of carbonic anhydrase. This plant warrants further investigation that will provide future opportunities to study promising compounds for CA inhibition and antidiabetic.

CHAPTER SIX

6.0 SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary

i. In the early phase of STZ induced diabetic rats, erythrocyte carbonic anhydrase activity increased.

ii. However, prolonged untreated STZ induced diabetes resulted in decreased erythrocyte carbonic anhydrase activity.

iii. Inhibition of erythrocyte carbonic anhydrase activity with Acetazolamide resulted in reduced circulating blood glucose level, whereas blood lactate level increased significantly ($P<0.05$).

iv. Metformin was found to inhibit carbonic anhydrase activity both *in-vitro* and in vivo.

v. Acetazolamide was found to increase the activity of super oxide dismutase and bring about decrease in TBARS level.

vi. *Cadaba farinosa* methanol leaf extract was found to significantly reduce circulating blood glucose level and increased the activity of catalase in STZ induced diabetic rats.

vii. Partially purified Fraction A of *Cadaba farinosa* was found to inhibit carbonic anhydrase activity *in-vitro.*

viii. FTIR spectra of the purified fractions of methanol leaf extract of *Cadaba farinosa* revealed the presence of characteristic band absorption of alpha aminonitrile.

ix. GC-MS analysis revealed the presence of derivatives of hydrazine, pyridine and ribonitrile.

6.2 Conclusion

- The results of the present study have suggested that inhibition of carbonic anhydrase may be responsible for the reduction of blood glucose in the extract treated diabetic rats, because carbonic anhydrase provide bicarbonate required by pyruvate carboxylase for hepatic synthesis of glucose at the same time facilitates hepatic lactate uptake for glucose production.
- Inhibition of carbonic anhydrase may be the causal mediators of increase blood lactate level in diabetes because carbonic anhydrase has been found to facilitate hepatic uptake for gluconeogenesis.
- Based on the results obtained, it can be proposed that increased lactate may be responsible for prompting the increased carbonic anhydrase activity in the early phase of diabetes.
- Furthermore, inhibition of carbonic anhydrase might be responsible for the reduction of TBARS level and increased SOD activity in diabetes.
- Based on the present findings it can be proposed that Metformin may probably reduce hyperglycemia by inhibiting carbonic anhydrase activity.
- The anti-diabetic effect of methanol leaf extract of *Cadaba farinosa* might be due to the presence of aminonitrile functional component and also its ability to inhibit carbonic anhydrase.

6.3 Recommendations

i. Hepatic carbonic anhydrase activity should be studied to understand the linkage between hepatic lactate uptake and glucose production.

ii. Future study is required to further evaluate the n-Hexane and ethyl acetate extract fractions of *cadaba farinosa* both *in vitro* and *in vivo* in models of type 1 and type 2 diabetes.

iii. The results of the present study suggest that carbonic anhydrase inhibition is a causal event in the reduction of circulating blood glucose and increased blood lactate level; however, it is unknown whether carbonic anhydrase directly impairs hepatic glucose production or is indirectly involved by reducing hepatic lactate uptake. Further studies are required in this line.

iv. Further studies should explore the link between inhibition of carbonic anhydraseand hepatic gluconeogenesis. As that will particularly be helpful when researchers want to answer the question, of what is the exact mechanism of Metformin action?

v. In this regard, future work should examine whether inhibition of hepatic carbonic anhydrase will prevent hepatic gluconeogenesis in type 2 diabetic model.

REFERENCES

Abdelghaffar, S. and Attia, A.M. (2009). Metformin added to insulin therapy for type 1 diabetes mellitus in adosescents. *CochraneDatabase. Systematic Review*, (1): CD006691.

Accutrend plus Glucose performance evaluation results, (2009), Roche Diagnostics.

Ahmad (1992). Alkaloids of plants. *Pakistan Journal of Science and Research*, 35:475.

Ahmad Viqar, U.A. Rahman, A. Chen, M. Clardy, H.M (1985). Cadabicine, an alkaloid from *Cadaba farinosa. Phytochemistry*, 24(11): 2709-2710.

Alberti, K. G. and P. Z. Zimmet (1998). Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. *Diabetic Medicine*15(7): 539-53

Al-Fatimi, M. Wurster, M. Schroder, G. and Lindequist, U. (2007). Antioxidant, antimicrobial and cytotoxic activities of selected medicinal plants from Yemen. *Journal of Ethnopharmacology*, 111:657-666.

Almquist, J. Lang, P. Pr¨atzel-Wolters, D. Deitmer, J.W. Jirstrand, M. and Becker, H.M (2010). A Kinetic Model of the Monocarboxylate Transporter MCT1 and its Interaction with Carbonic Anhydrase II. *Journal of Computational and Scientific System Biol*ogy. 3:107–116

Al-Musayeib, N.M. Mohamed G.A. Ibrahim, S.R.M, and Ross, S.A. (2013). Lupeol-3-O-decanoate, a new triterpene ester from Cadaba farinosa Forssk., growing in Saudi Arabia. *Medicinal Chemistry Research,* DOI 10.1007/s00044-013-0536-1 1-6.

Alpern, R.J. and Chambers, M. (1987). Basolateral membrane Cl/HCO3 exchange in the rat proximal convoluted tubule Na-dependent and -independent modes. *Journal of General Physiology,* (89): 581–598.

Alpern, R.J. and Rector, F.C. (1996). Renal acidification mechanisms, in *Brenner and Rector's The Kidney* (5th ed), edited by Brenner BM, Rector FC, Philadelphia, Saunders, pp 408–471

Altaf, Q. A., Barnett, A. H. and Tahrani, A. A (2015). Novel therapeutics for type 2 diabetes: insulin resistance. *Diabetes Obes. Metab.* 17, 319–334

Amber, R. A. (1990). Studies on the Chemical Constituents of *Cadaba Farinosa,* PhD thesis (University of Karachi, Pakisthan):1-147.

Andrew, P. Adam, L.M. and Peter, W.W (2005). Lactate – a signal coordinating cell and systemic function. *The Journal of Experimental Biology.* 208: 4561-4575

Arbeeny, C.M. Nordin, C. Edelstein, D. Stran, N. Gibbons, N. and Eder, H.A (1989). Hyperlipoproteinimia, in spontaneously diabetic, guinea pigs. *Metabolism,*38:895–900.

Arokiyaraj, S. Radha, R. Martin, S. and Perinbam, K. (2008). Phytochemical analysis and anti-diabetic activity of Cadaba fruticosa R.Br. *Ind J Sci & Tech*; 1(6): 1-4.

Arya, V. Gupta, V.K. and Ranjeet, K. (2011). A review on fruits having anti-diabetic potential. *Journal of Chemical and Pharmaceutical Research,* (3): 204-212.

Bailey, C. J (2007). Treating insulin resistance: future prospects. *Diab. Vasc. Dis. Res.* 4, 20–31

Bailey, C.J. Turner, R.C (1996). Metformin. *New England Journal of Medicine* .334(9), 574–579.

Barboiu, M. Supuran, C.T. Menabuoni, L. Scozzafava, A. Mincione, F. Briganti, F. and Mincione, G. (1999). Carbonic anhydrase inhibitors. Synthesis of topically effective intraocular pressure lowering agents derived from 5-(w-aminoalkylcarboxamido)-1,3,4-thiadiazole-2- sulfonamide. *Journal of Enzyme Inhibition*, (15): 23-46.

Barrittg, J. Zander, G.L. and Utter, M.F. (1976). Gluconeogenesis: Its Regulation in Mammalian Species. R. W. Hanson and M. K. Mehlman, Eds. John Wiley and Sons, Inc. New York.

Bartha, R. (1970). Fodder plants in the sahel zone of Africa. Munchen, Weltforum Verlag, FAO. Available online: http:// www.fao.org/ag/agr/agap/frg/AFRIS/refs/45.HTM 3

Bartlett, J.D. and Jaanus, S.D. (Boston (1989). *Clinical ocular pharmacology*. Butterworth, 254-263.

Bates, H.M (1978). Lab. Mang., 16

Baumer, M. (1993). Notes on trees and shrubs in arid and semi-arid regions. Rome.

Baynes, J. Thorpe, S. and Murtiashaw, M. (1984). Nonenzymatic Glucosylation of Lysine Residues in Albumin. *Methods in Enzymology.106*:88-98.

Baynes, J.W and Thorpe, S.R. (1996). Role of Maillard reaction in diabetes mellitus and diseases of aging. *Drugs Aging* 9(2): 69-77.

Becker, H.M. and Deitmer, J. W. (2004). Voltage dependence of H+ buffering mediated by sodium bicarbonate cotransport expressed in Xenopus oocytes. *Journal of Biological Chemistry*, 279:28057–28062.

Becker, H.M. and Deitmer, J.W. (2008). Nonenzymatic proton handling by carbonic anhydrase II during H+-lactate cotransport via monocarboxylate transporter 1. *Journal of Biological Chemistry*, 283: 21655–21667.

Becker, H.M. Broer, S. and Deitmer, J. W. (2004). Facilitated lactate transport by MCT1 when coexpressed with the sodium bicarbonate cotransporter (NBC) in Xenopus oocytes. *Biophysical Journal,* 86:235–247.

Becker, H.M. Hirnet, D. Fecher-Trost, C. Sultemeyer, D. and Deitmer, J. W. (2005). Transport activity of MCT1 expressed in Xenopus oocytes is increased by interaction with carbonic anhydrase. *Journal of Biological Chemistry,*280:39882–39889.

Becker, H.M. Klier, M. and Deitmer, J. W. (2010). Nonenzymatic augmentation of lactate transport via monocarboxylate transporter isoform 4 by carbonic anhydrase II. *Journal of membrane Biology,*234:125–135.

Becker, H.M. Klier, M., Schuler, C. McKenna, R. and Deitmer, J.W. (2011): Intramolecular proton shuttle supports not only catalytic but also noncatalytic function of carbonic anhydrase II. *Proceedings of the Natonal Academy of Science,* USA (108):3071-3076.

Bellingham, A. J. Detter, J. C. and Lenfant, C. (1971). Regulatory mechanisms of hemoglobin oxygen affinity in acidosis and alkalosis. *Journal of Clinical Investigation,*50: 700-706.

Bennett, J. W. and Chung, K. T (2001). Alexander Fleming and the discovery of penicillin. *Advance in Applied Microbiology*. (49): 163-184

Benyhe, S. (1994). New aspects in the study of an ancient compound. *Life Sciences***,** (*55*):969-979.

Bian, Y. Rong, Z. and Chang, T.M.S. (2011). A novel biotechnology-based blood substitute that transports both oxygen and carbon dioxide and also acts as an antioxidant. *Artificial Cells Blood Substitute and immobilisation Biotechnology,* 39:127–136.

Biswas, U.K. and Kumar, A. (2010). Study on Lipid Profile, Oxidative Stress and Carbonic Anhydrase Activity in Patients With Essential Hypertension. *Journal of Clinical and Diagnostic Research.* 4(6): 3414-3420.

Bode, A.M. Foster, J.D. and Nordlie, R.C. (1994). Glycogenesis from glucose and ureagenesis in isolated perfused rat livers. Influence of ammonium ion, norvaline, and ethoxyzolamide. *Journal of Biological Chemistry,* 269:7879–7886

Bogardus, C. Lillioja, S. Howard, B. Reaven, G. and Mott, D (1984). Relationships between insulin secretion, insulin action, and fasting plasma glucose concentration in nondiabetic and non-insulin-dependent diabetic subjects. *Journal of Clinical Investigation*. 74: 1238–1246.

Boon, L. and Meijer, A.J (1988). Control by pH of urea synthesis in isolated rat hepatocytes.*European Journal of Biochernistry.* 171:465-469.

Borges, F. Roleira, F. Milhazes, N. Santana, L. and Uriarte, E (2005). Simple Coumarins and Analogues in medicinal chemistry: occurrence, synthesis and biological activity. *Current Medicinal Chemistry.* 12:887–916

Bottger, I. and Wieland, O (1969). Intracellular localization of pyruvate carboxylase and phosphoenolpyruvate carboxykinase in rat liver. *European Journal of Biochemistry*. 8**,**113-19.

Brechue, W.F. Kinne-Saffran, E. Kinne, R.K. and Maren, T.H. (1991). Localiza- tion and activity of renal carbonic anhydrase (CA) in CA-II deficient mice. *Biochimica et Biophysica Acta* (1066):201–207.

Briganti, F. Mangani, S. Orioli, P. Scozzafava, A. Vernaglione, G. and Supuran, C.T. (1997). Carbonic anhydrase activators: X-ray crystallographic and spectroscopic

investigations for the interaction of isozymes I and II with histamine. *Biochemistry,* (36):10384-10392.

Briganti, F. Scozzafava, A. and Supuran, C.T. (1999). Novel carbonic anhydrase isozymes I, II and IV activators incorporating sulfonyl-histamino moieties. *Bioorganic and Medicinal Chemistry Lett*ers, (9):2043-2048.

Brion, L.P. Zavilowitz, B.J. Rosen, O. and Schwartz, G. J. (1991). Changes in soluble carbonic anhydrase activity in response to maturation and NH4Cl loading in the rabbit. *American Journal of Physiology,*. 261 (*Regulatory Integrative Comparative Physiology*, (30): R1204–R1213.

Brion, L.P. Zavilowitz, B.J. Suarez, C. and Schwartz, G.J. (1994). Metabolic acidosis stimulates carbonic anhydrase activity in rabbit proximal tubule and medullary collecting duct. American Journal of Physiology - *Renal Physiology,*266(2): F185-F195D

Brownlee, M (2005). The pathobiology of diabetic complications: a unifying mechanism. *Diabetes* 54, 1615–1625.

Buchanan, M.S. Carroll, A.R. Fechner, G.A. Boyle, A. Simpson, M.M., Addepalli, R., Avery, V.M., Hooper, J.N.A., Su, N., Chen, H.W., and Quinn, R.J (2007). Spermatinamine, the first natural product inhibitor of isoprenylcysteine carboxyl methyltransferase, a new cancer target. *Bioorganic and Medicinal Chemistry Letters*, 17:6860–6863

Cao, T.P. and Rous, S. (1978). Action of acetazolamide on liver pyruvate carboxylase activity, glycogenolysis and gluconeogenesis of mice. *International Journal of Biochemistry*, 9:603–605

Carmen, D.Véronique, R.Saikat, S.Allan, M.L.René, S.and Martin, L. (2016). Defective hepatic bicarbonate production due to carbonic anhydrase VA deficiency leads to

early-onset life-threatening metabolic crisis, Genetics in Medicine.doi:10.1038/gim.2015.201

Carta, F. Temperini, C. Innocenti, A. Scozzafava, A. Kaila, K. and Supuran, C.T. (2010). Polyamines inhibit carbonic anhydrases by anchoring to the zinc-coordinated water Molecule. *Journal of Medicinal Chemistry,* 53:5511–5522

Carter, N. Jeffery, S. Legg, R. Wistrand, P. and Lonnerholm, G (1987). Expression of Hepatocyte Carbonic anhydrase in vitro and Invivo. *Biochemical Soiety Trans.* 15:667-668.

Casado, A. de la Torre, R. and López-Fernández, M.E. (2007). Copper/zinc superoxide dismutase activity in newborns and young people in Spain. *Indian Journal of Medical Research,*125**:** 655 – 660.

Casero, R.A. and Jr, Marton, L.J. (2007). Targeting polyamine metabolism and function in cancer and other hyperproliferative diseases. *Nature Review Drug Discovery.* 6:373–390.

Chegwidden, W.R. and Carter, N.D. (2000) *Introduction to the carbonic anhydrases*. In Chegwidden, W.R., Carter, N.D. and Edwards, Y.H. (eds), The Carbonic Anhydrases: New Horizons. Birkhauser Verlag, Basel, vol. 90, pp. 13–28.

Chegwidden, W.R. Dodgson, S.J. and Spencer, I.M. (2000). The roles of carbonic anhydrase in metabolism, cell growth and cancer in animals. In: Chegwidden, W.R. *et al*. (Eds.). The Carbonic Anhydrases, *Birkhäuser Basel*. (90): 343-363.

Cheng, J.T. Huang, C.C. Liu, I.M. Tzeng, T.F. and Chang, C.J (2006). Novel Mechanism for plasma glucose-lowering action of Metformin in Streptozotocin induced diabetic rats. *Diabetes,* 55(3): 819– 825.

Cherrington, A.D. Stevenson, R.W. Steiner, K.E. Davis, M.A. Myers, S.R. Adkins, B.A. Abumrad, N.N. and Williams, P (1987). Insulin, glucagon and glucose as

regulators of hepatic glucose uptake and production in vivo. *Diabetes and Metabolism Review*.3:307–332.

Claiborne, A. Catalase activity, in: R.A. Green Wald (Ed.), Handbook of Methods for Oxygen Radical Research, CRC Press, Boca Raton, FL, 1985, pp. 283–284.

Clare, B.W. and Supuran, C.T. (1994). Carbonic anhydrase activators. 3. Structure-activity correlations for a series of isozyme II activators. *Journal of Pharmaceutical Science,*83:768-773.

Claudiu, T.S. and Andrea, S. (2000). Carbonic anhydrase inhibitors and their therapeutic potential. *Expert Opinion on Therapeutic Patents, 10(5):575-600*

Consoli, A. and Nurjhan, N. (1990). Contribution of gluconeogenesis to overall glucose output in diabetic and nondiabetic men. *Annals of Medicine,* 22, 191–195.

Coulson, R.A., and Herbert, J.D (1984). A role for carbonic anhydrase in intermediary metabolism. *Annalls of New York Academy of Science.* 429**,** 505-15

Crawford, S.O. Hoogeveen, R.C. and Brancati, F. L (2010). Association of blood lactate with type 2 diabetes: the atherosclerosis risk in communities' carotid MRI study. *International Journal of Epidemiology* 39(1): pp. 1647–1655.

Cusi, K. Consoli, A. and DeFronzo, R.A (1996). Metabolic effects of metformin on glucose and lactate metabolism in noninsulin-dependent diabetes mellitus. *Journal of Clinical Endocrinology and Metabolism.* 81 : 4059 - 4067

Dandona, P. Aljada, A. Chaudhuri, A. Mohanty, P. and Rajesh, G (2004). *Metabolic. Syndrome and Related Disorders,* (2): 2-8.

Davenport, H.W. (1984). The early days of research on carbonic anhydrase. *Annals of New York Academy of Science,* 429:4-9.

Davies, A.O. (1984). Rapid desensitization and uncoupling of human beta adrenergic receptors in an *in vitro* model of lactic acidosis. *Journal of Clinical Endocrinology and Metabolism,* 59:398-404.

De Clercq, E. (2005). Emerging anti-HIV drugs. *Expert Opinion on Emerging Drugs*, 10:241–274.

De Simone, G. Di Fiore, A. and Supuran, C.T (2008). Are carbonic anhydrase inhibitors suitable for obtaining antiobesity drugs? *Current Pharmaceutical Des* 14:655–660

Defronzo, R. A (2009). Banting Lecture. From the triumvirate to the ominous octet: a new paradigm for the treatment of type 2 diabetes mellitus. *Diabetes* 58, 773–795.

DeFronzo, R. Golay, A. and Felber, J. (1985). Glucose and lipid metabolism in obesity and diabetes mellitus. In: Substrate and Energy Metabolism. editors J. Garrow, and D. Hallidaed, London. 70-81.

DeFronzo, R.A. and Ferrannini, E (1987). Regulation of hepatic glucose metabolism in humans, *Diabetes and Metabolism Review*. 3(2):415-459.

Dembo, A.J. Marliss, E.B. and Halperin, M.L. (1975). Insulin therapy in phenformin-associated lactic acidosis: A case report, biochemical considerations and review of the literature. *Diabetes* 24: 28–35.

DeMeutter, R.C. and Shreeve, W.W. (1963). Conversion of DL-lactate-2-C ^{14} or -3-C^{14} or pyruvate-2-C ^{14} to blood glucose in humans: effects of diabetes, insulin, tolbutamide, and glucose load. *Journal of Clinical Investigation*, 42:525-33.

Di Fiore, A. Monti, S.M. Hilvo, M. Parkkila, S. Romano, V. Scaloni, A. Pedone, C. Scozzafava, A. Supuran, C.T. and De Simone, G. (2009): Crystal structure of

human carbonic anhydrase XIII and its complex with the inhibitor acetazolamide. Proteins 74:164-175.

Dictionary of Natural Products (DVD) (2012), Vol version 21.1. Taylor & Francis Group/CRC Press, London

Dodgson S.J, Forster R.E (1986). Inhibition of CA V decreases glucose synthesis from pyruvate. *Archive of Biochemistry and Biophysics,* 251:198–204.

Dodgson, S.J (1987). Inhibition of mitochondrial carbonic anhydrase and ureagenesis: a discrepancy examined. *Journal of Applied Physiology.*63:2134–2141

Dodgson, S.J (1991a).. Carbonic anhydrases in the kidney. In: SJ Dodgson, RE Tashian, G Gros, ND Carter (eds): The Carbonic Anhydrases. Cellular Physiology and Molecular Genetics, First Edition, Plenum Press, New York, 345-350.

Dodgson, S.J (1991b). Liver mitochondrial carbonic anhydrase (CA V), gluconeogenesis, and ureagenesis in the hepatocyte. In: SJ Dodgson, RE Tashian, G Gros, ND Carter (eds): The Carbonic Anhydrases. Cellular Physiology and Molecular Genetics, First Edition, Plenum Press, New York, 297-306.

Dodgson, S.J. and Cherian, K (1990). Rat renal proximal tubular gluconeogenesis: Possible involvement of non-mitochondrial carbonic anhydrase isozymes *Archives of Biochemistry and Biophysics.* 282:1-7

Dodgson, S.J. and Cherian, K. (1989). Mitochondrial carbonic anhydrase is involved in rat renal glucose synthesis. *American Journal of Physiol*ogy, 257:E791-E796

Dodgson, S.J. and Contino, L.C (1988). Rat kidney mitochondrial carbonic anhydrase. *Arch Biochemica et Biophysica.* 260, 334-41.

Dodgson, S.J. and Watford, M. (1990). Differential regulation of hepatic carbonic anhydrase isozymes in the streptozotocin-diabetic rat. *Archive of Biochemistry and Biophysics*, 277, 410-414.

DuBose, T.D. and Lucci, M.S. (1983). Effect of carbonic anhydrase inhibition on superficial and deep nephron bicarbonate reabsorption in the rat. *Journal of Clinical Investigation,* 71:55–65.

Duchen, M.R (2004). Mitochondria in health and disease: Perspectives on a new mitochondrial biology. *Molecular Aspect of Medicine,25:* 365–451.

Duncan, D.B. (1955). Multiple range and multiple F-test. *Biometrics,* 11, 1–42.

Dunn, C.J. and Peters, D.H. (1995). Metformin. A review of its pharmacological properties and therapeutic use in non-insulin-dependent diabetes mellitus. *Drugs*, 49:721-49.

Ejele, A.E. Enenebaku, C.K. Akujobi, C.O. and Ngwu, S.U. (2012). Effect of Microbial storage on Phytochemistry, anti-sickling and anti-microbial potential of *New Bouldia laevis* leaf extract. *International Research Journal of Microbiology,* 3(4), 113-116.

El-Mir, M.Y. Nogueira, V., Fontaine, E., Averet, N., Rigoulet, M., and Leverve, X (2000). Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I. *Journal of Biological Chemistry*. 275(1), 223–228.

Enns, T. (1967). Facilitation by carbonic anhydrase of carbon dioxide transport. *Science* 155: 44–47.

Firth, R. Bell, P. and Rizza, R (1987). Insulin action in non-insulin-dependent diabetes mellitus; the relationship between hepatic and extrahepatic insulin resistance and obesity. *Metabolism*.36: 1091–1095.

Fleidervish, I.A. Libman, L. Katz, E. and Gutnick, M.J. (2008). Endogenous polyamines regulate cortical neuronal excitability by blocking voltage-gated NaCl channels. *Proceedings of the National Academy of Science, U S A.* 105:18994–18999

Forbath, N. Kenshole, A.B. and Hetenyi, G. (1967). Turnover of lactic acid in normal and diabetic dogs calculated by two tracer methods. *American Journal of Physiol*ogy, (212):1179-84.

Fortney, S.R. (1966). Effect of hydrazine on liver glycogen, arterial glucose, lactate, pyruvate and acid-base balance in the anesthetized dog. *Journal of Pharmacology and Experimental Therapy,* 153: 562-568.

Fulgencio, J.P. Kohl, C. Girard, J. and Pegorier, J.P. (2001). Effect of metformin on fatty acid and glucose metabolism in freshly isolated hepatocytes and on specific gene expression in cultured hepatocytes. *Biochemical Pharmacology,* 62, 439–446.

Gaffa, T. Jideani, I.A. and Nkama, I. (2002) Traditional production, consumption and storage of kunu – a nonalcoholic cereal beverage. *Plant foods for human Nutrition,* 57:73-81.

Gambhir, K.K. Oates, P. Verma, M. Temam, S. and Cheatham, W. (1997). High fructose feeding enhances erythrocyte carbonic anhydrase 1mRNAlevels in rat. *Annals of New York Acad*emy of *Science,* (827): 163–169.

García Marín, J.J. Urbieta, A.T. Barriocanal, F.P. Barbero, E.R. and Eleno, N (1991). Plasma membrane-bound carbonic anhydrase activity in the regenerating rat liver. *Biochimica Biophysica Acta*. 1061: 9-14.

Garden Guide's plant, Hill climbs Media 1997-2008.

Garg, L.C (1974).. Induction of hepatic carbonic anhydrase by estrogen.*Pharmacology and Experimental Therapeutics.* 189, 557-562.

Geers, C. Bertram, O. and Gros, G (1997). Extracellular carbonic anhydrase and blood pH equilibrium in exercising skeletal muscle (Abstract). *Pflugers Archives.* 433: P449.

Geers, C. and Gros, G. (2000). Carbon dioxide transport and carbonic anhydrase in blood and muscle. *Physiology Reviews*,80(2): 681–715.

Gil, H. Mata-Sagreda, J. and Schowen, R. (1991). Isotope Effects in the Nonenzymatic Glucation of Hemoglobin Catalyzed by Phosphate. *Actual. Fisico-quím. Org.*286-306.

Gil, H. and Uzcategui, J. (1993). Isotope Effects in the Nonenzymatic Glycation of Hemoglobin Catalyzed by DPG. *Actual. Físico-quím. Org.*109-121.

Gil, H. Vásquez, B. Peña, M. and Uzcategui, J. (2004). Effect of Carbonate and Arsenate on the Kinetics of Glycation of Human Hemoglobin. *Journal of Physiology and Organic Chemistry,17*:537-540

Glew, R.H. John, K.G. Hernandez, M. Pastuszyn, A. Ernst, J. and Djomdi, N. (2010). The amino acid, Mineral and Fatty acid content of three species of human plant foods in Cameroun. *Food,* 4(1):1-6.

Gonen, B., and Rubenstein, A.H(1978). *Diabetologia* 15, 1

Goriki, K. Wada, K. and Hata, J. (1982). The relationship between carbonic anhydrases and zinc concentration of erythrocytes in patients under chronic hemodialysis. *Hiroshima Journal of Medical Science,* 31:123–7.

Graham, J.G. Quinn, M.L. Fabricant, D.S. and Farnsworth, N.R. (2000). Plants used against cancer – an extension of the work of Jonathan Hartwell. *J of Ethno pharmacol.* 73**:**347-377.

Green, K. Brand, M.D. and Murphy, M.P. (2004). Prevention of mitochondrial oxidative damage as a therapeutic strategy in diabetes. *Diabetes,* 53: 110-118

Gros, G.W. Moll, H. Hoppe, and Gros, H. (1976). Proton transport by phosphate diffusion: a mechanism of facilitated CO2 transfer. *Journal of General Physiology*, 67: 773–790.

Habib, A. Ghulam, R.B. and Abdul, L. (2004). Medicinal Flora of the Thar Desert: An overview of Problems and their Feasible Solutions. *Zones Aridas Pakistan,*73-83.

Hallerdei, J. Scheibe, R.J. Parkkila, S. Waheed, A. Sly, W.S. Gros, G. Wetzel, P. and Endeward, V (2010). Tubules and surface membranes provide equally effective pathways of carbonic anhydrase facilitated lactic acid transport in skeletal muscle. *PLoS One.* 5:e15137

Halperin, F.A. Cheema-Dhadli, S. Chen, C.B. and Halperin, M.I. (1996). Alkali therapy extends the period of survival during hypoxia: studies in rats. *American Journal of Physiology,* 271:R381-R387.

Hannedoeche, T. Lazaro, M. Delgado, A.G. Boitard, C. Lacour, B. and Gr¨unfeld, J.P. (1991). Feedback- mediated reduction in glomerular filtration during acetazolamide infusion in insulin-dependent diabetic patients. *Clinical Sciences* (81**):** 457–64.

Harouna, H. Favel, A. Delmas, F. Elias, R. Saadou, M. and Balansard, G. (1993). Screening of some plants used in traditional medicine in Niger for anti-parasitic and antifungal activities. Medicament *et al*iments: *Lapproche ethnopharmacogique*; 285-287.

Haussinger, D. and Gerok, W (1985).Hepatic urea synthesis and pH regulation: role of CO_2, HCO_3^-, and the activity of carbonic anhydrase. *European journal of Biochemistry.* 152**,** 381-386.

He, H. Tran, P. Yin, H. Smith, H. Batard, Y. Wang, L. Einolf, H. Gu, H. Mangold, J.B., Fischer, V. and Howard, D. (2009). Absorption, metabolism, and excretion of [14C] vildagliptin, a novel dipeptidyl peptidase 4 inhibitor, in humans. *Drug Metabolism and Disposition*. 37:536–544.

Helms, R. Ross, B. D. Berry, M. N. and Krebs, H. A. (1966). Gluconeogenesis in the perfused rat liver. *Biochemical Journal*, 101; 284-292

Hendrick, U.P. (1919) sturtevant's notes on edible plants of the world. J.B. Lyon Co., Albany NY. Available at: http:// www.sws bm.com

Henriques O (1928): Die Bindungsweise des Kohlendioxyds im Blute. *Biochem Ztschrft*, 200:1-24.

Herbert, J.D. Coulson, R.A. and Hernandez, T (1983). Inhibition of pyruvate carboxylation in alligators and chameleons by carbonic anhydrase inhibitors. *Comparative Biochemical Physiology* 75A**,** 185-92

Herbert, J.D., Coulson, R.A., Hernandez, T., and Ehrensvard, G. A (1975). Carbonic anhydrase requirement for the synthesis of glutamine from pyruvate in the chameleon. *Biochemical and Biophysical Research Communication* 65**,** 1054-60.

Higgins, P. and Bunn, H. (1981). Kinetics Analysis of Nonenzymatic Glycosylation of Hemoglobin. *Journal of Biological Chemistry,256,* 5204-5208.

Hilvo, M. Tolvanen, M. Clark, A. Shen, B. Shah, G.N. Waheed, A. Halmi, P. Hanninen, M. Hamalainen, J.M. Vihinen, M. Sly, W.S. and Parkkila, S. (2005):

Characterization of CA XV, a new GPI-anchored form of carbonic anhydrase. *Biochemical J*ournal, 392:83-92.

Holman, R. R., Paul, S. K., Bethel, M. A., Matthews, D. R. and Neil, H. A (2008). 10-year follow-up of intensive glucose control in type 2 diabetes. *N. Engl. J. Med.* 359, 1577–1589.

Hong, T.D. Linington, S. and Ellis, R.H. (1996). Seed storage behavior: a compendium. *Handbooks for Genebanks*: No.4. IPGRI.

http://www.google.ga/patents/WO2005033089A1?cl=en

https://www.google.tl/patents/US20080051452

Hundal, R.S. and Inzucchi, S.E (2003). Metformin: new understandins, new uses. *Drugs* 63(18): 1879–1894.

Hundal, R.S. Krssak, M. Dufour, S. Laurent, D. Lebon, V. Chandramouli, V. Inzucchi, S.E. Schumann, W.C. Petersen, K.F. Landau, B.R. and Shulman, G.I (2000). Mechanism by which metformin reduces glucose production in type 2 diabetes. *Diabetes*. 49 : 2063 – 2069

Ilies, M.A. Banciu, M.D. and Ilies, M. (1997). Carbonic anhydrase activators. Part 17. Synthesis and activation study of a series of 1-(1,2,4-triazole-(1H)-3-yl)- 2,4,6-trisubstituted-pyridinium salts against isozymes I, II and IV. *European Journal of Medicinal Chemistry*,32:911-918.

Innocenti, A. Sarikaya, S.B.O. Gulcin, I. and Supuran, C.T (2009). Carbonic anhydrase inhibitors. Inhibition of mammalian isoforms I-XIV with a series of natural product polyphenols and phenolic acids. Bioorg Med Chem 18:2159–2164

Innocenti, A. Vullo, D. Scozzafava, A. and Supuran, C.T (2008). Carbonic anhydrase inhibitors: inhibition of mammalian isoforms I-XIV with a series of substituted

phenols including paracetamol and salicylic acid. Bioorg Med Chem 16:7424–7428

Internation Diabetic Federation (2014) Diabetes Atlas Sixth Edition Poster Update

Internation Diabetic Federation (2015) Diabetes Atlas Seventh Edition Poster Update

Ishibashia, M. Akazawaa, S. Sakamakia, H. Matsumotoa, K, Yamasakia, H. Yamaguchia, Y. Gotob, S. Uratab, Y. Kondob, T. and Nagatakia, S. (1997). Oxygen-Induced embryopathy and the significance of glutathione-dependent antioxidant system in the rat embryo during early organogenesis. *Free Radical Biology and Medicine,* 22:447-454.

Jackson, E.K. (1996). Diuretics In: *Goodman and Gilman's The Pharmacological Basis of Therapeutics, 9th Ed.* Hardman JG, Limbird LE, Molinoff PB, Ruddon RW, Gilman AG (Eds.), McGraw-Hill, New York. 685-713.

Kahn, S. E., Hull, R. L. and Utzschneider, K. M (2006). Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature* 444, 840–846.

Kalbag, J.B. Walter, Y.H. Nedelman, J.R. and McLeod, J.F. (2001). Mealtime glucose regulation with nateglinide: comparison with repaglinide and placebo. *Diabetes Care,* 24:73-7.

Khare, C.P. (2006). Indian Medicinal Plants: An Illustrate Dictionary. (Springer-VerlagBerlin/Heidelberg publication. New York- USA):107.

Khun, B. Hennig, M. and Mattei, P. (2007). Molecular recognition of ligands in dipeptidyl peptidase IV. *Current Topic in Medicinal Chemistry,* 7:609–619.

Kim, G. Lee, T.H. and Wetzel, P. (2004). Carbonic anhydrase III is not required in the mouse for normal growth, development, and life span. *Molecular Cell Biology,* 24: 9942–9947.

King, R. W. Garg, L.C, Huckson, J. and Maren, T.H (1974). The isolation and partial chracterisation of sulfonamide-resistant carbonic anhydrases from the liver of the male rat. *Molecular Pharmacology* 10, 335-343.

Kleinman, J.G. Brown, W.W. Ware, R.A. and Schwartz, J.H. (1980). Cell pH and acid transport in renal cortical tissue. *American Journal of Physiology and Renal Fluid Electrolyte Physiology,* 239: F440–F444.

Klier, M. Fabian, T. Andes, J. Deitmer, W. and Holger, M. (2014).Intracellular and Extracellular Carbonic Anhydrases Cooperate Non-enzymatically to Enhance Activity of Monocarboxylate Transporters. *The Journal of Biological Chemistry*, 289;5. pp. 2765–2775.

Kohli K. and Jain, G. K. (2006). Herbal Drugs: Regulation across the Globe. Jaypee Brothers Medicinal, New Delhi: 624.

Kowalchuk, J.M. Heigenhauser, G.J.F. Sutton, J.R. and Jones, N.L (1992). Effect of acetazolamide on gas exchange and acid base control after maximal exercise. *Journal of Applied Physiology.* 72: 278–287.

Landgraf, R. (2000). Meglitinide analogues in the treatment of type 2 diabetes mellitus. *Drugs and Aging*, (17): 411-25.

Lassen, N.A. Friberg, L. Kastrup, J. Rizzi, D. and Jensen, J.J. (1987). Effects of acetazolamide on cerebral blood flow and brain tissue oxygenation. *Postgraduate Medical Journal,*63:185-187.

Levine, S.R. Welch, K.M. Helpern, J.A. Chopp, M. Bruce, R. Selwa, J. and Smith, M.B. (1988). Prolonged deterioration of ischemic brain energy metabolism and acidosis associated with hyperglycemia: human cerebral infarction studied by serial 31P NMR spectroscopy. *Annals of Neurology,* 23; 416–418.

Lewis, S.E. Erickson, R.P. and Barnett, L.B. (1988). N-ethyl-N-nitrosourea induced null mutation at the mouse Car-2 locus: An animal model for human carbonic anhydrase II deficiency syndrome. *Proceedings of NationalAcademy of Science, USA* 85:1962–1966.

Lindskog, S. (1960): Purification and properties of bovine erythrocyte carbonic anhydrase. *Biochimica Biophysica Acta* 39:218-226.

Lindskog, S. and Silverman, D.N. (2000): The catalytic mechanism of mammalian carbonic anhydrases. In: The Carbonic Anhydrases: New Horizons, pp. 175-195. Eds. Chegwidden WR, Carter ND and Edwards YH, Birkhäuser Verlag, Basel.

Lloyd, M.H. Iles, R.A. Simpson, B. R. Strunin, J.M., Layton, J.M. and Cohen, R.D (1973). The effect of simulated Metabolic acidosis on intracellular pH and Lactate Metabolism in the isolated perfused rat liver. *Clinical Science and Molecular Medicine*. 45: 543-549.

Lonnerholm, G. Wistrand, P.J. and Barany, E. (1986). Carbonic anhydrases isoenzymes in the rat kidney. Effects of chronic acetazolamide treatment. *Acta Physiology and Scandinivia,*126:51-60.

Lotspeich, W.D. (1965). Renal hypertrophy in metabolic acidosis and its relation to ammonia excretion. *American Journal of Physiology*, 208: 1135– 1142.

Loubatières, A. (1969). The discovery of hypoglycemic sulfonamides and particularly of their action mechanism. *Acta Diabetica lat*, (6): (suppl 1):20-56.

Lowrey, C. Lyness, S. and Soeldener, J. (1985). The Effect of Hemoglobin, Ligands on the Kinetics of Human Hemoglobin Formation. *Journal Biological Chemistry,260*:11611-11618.

Lowry, O.H. Rosebrough, N.J. Farr, A.L. and Randall, R.J (1951). Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 ;265–275.

Lucci, M.S. Pucacco, L.R. and DuBose, T.D. (1980). Direct evaluation of acidification by rat proximal tubule: Role of carbonic anhydrase. *American Journal Physiology*, 238:F372–F379.

Lucci, M.S. Tinker, J.P. Weiner, I.M. and DuBose, T.D. (1983). Function of - proximal tubule carbonic anhydrase defined by selective inhibition. *American Journal Physiology*, 245:F443–F449.

Luft, F.C. (2001). Lactic Acidosis Update for Critical Care Clinicians. *Journal of American Society of Nephrol*ogy, (12): S15–S19.

Lutz, T.A. Estermann, A. Haag, S. and Scharrer, E. (2001). Depolarization of the liver cell membrane by metformin. *Biochimica Biophysica Acta.* 1513, 176–184.

Mak, R.H. (2008). Insulin and its role in chronic kidney disease. *Pediatric Nephrology,* 23,355-362.

Mangan, J.L. (1998). Nutritional Effects of Tannins in Animal Feeds. *Nutrition Research Reviews* 1: 209-231.

Maren, T.H. (1967). Carbonic anhydrase: chemistry, physiology and inhibition. *Physiology Review,* 47: 595–781.

Maren, T.H. (1991). The links among biochemistry, physiology and pharmacology in carbonic anhydrase mediated systems. In: *Carbonic Anhydrase - From Biochemistry and Genetics to Physiology and Clinical Medicine*. Botré F, Gros G, Storey BT (Eds.), VCH, Weinheim :186-207.

Maren, T.H. (1992). Role of carbonic anhydrase in aqueous humor and cerebrospinal fluid formation. In: *Barriers and Fluids of the Eye and Brain*. Segal, MB (Ed.), MacMillan Press, London. 37-48.

Maren, T.H. (1995). The development of topical carbonic anhydrase inhibitors. *Journal of Glaucoma,* 4:49-62.

Maren, T.H. Jankowska, L. Sanyal, G. and Edelhauser, H.F (1983). The transcorneal permeability of sulfonamide carbonic anhydrase innhibitors and their effect on aqueous humor secretion. *Experimental Eye Research*. 36:457–480

Marsolais, C. Huot, S. David, F. Garneau, M. and Brunengraber, H (1987). Compartmentation of $14CO_2$ in the perfused rat liver. *Journal of Biological. Chemistry*. 25:262(6):2604-2607

Martin, J. Stevens, I. Obrosova, X. Cao, C. and Van Huysen, D.A. (2000). Greene, Effects of DLa- lipoic acid on peripheral nerve conduction, blood flow, energy metabolism, and oxidative stress in experimental diabetic neuropathy. *Diabetes,* 49: 1006–1115.

Maus, T.L. Larsson, L.I. McLaren, J.W. and Brubaker, R.F (1997). Comparison of dorzolamide and acetazolamide as suppresors of aqueous humor flow in humans. *Archives of Ophthalmology* 115:45–49

McKinley, D.N. and Whitney, P.L. (1976). Particulate carbonic anhydrase in homogenates of human kidney. *Biochimica Biophysica Acta* 445:780–790.

Meier, B. C. and Wagner, B. K(2014). Inhibition of $HDAC_3$ as a strategy for developing novel diabetes therapeutics. *Epigenomics* **6**, 209–214

Meldrum, N. and Roughton, F. (1932): Some properties of carbonic anhydrase, the carbon dioxide enzyme present in blood. *Journal of Physiology,* 75:15-16.

Meldrum, N.U. and Roughton, F.J. (1933): Carbonic anhydrase. Its preparation and properties. *Journal of Physiology,* 80:113-142.

Miller, W.H. Dessert, A.M. and Roblin, R.O Jr (1950). Heterocyclic sulfonamides as carbonic anhydrase inhibitors. *Journal of American Chemical Society*. 72:4893–4896

Mitchell, J.R. Zimmerman, H.J. Ishak, K.G. Thorgeirsson, U.P. and Timbrell, J.A. (1976). Isoniazid liver injury: clinical spectrum, pathology, and probable pathogenesis. *Annals of Internal Medicine,* 84: 181-192.

Moneva, M.H. and Dagogo-Jack, S (2002). Multiple Drug Targets in the Management of Type 2 Diabetes. *Current Drug Targets*. 3; (3) 203-221(19)

Mooradian, A.D. (2009). Dyslipidemia in type 2 Diabetes Mellitus. *Nature Clinical Practice and Endocrinology Metabolism,* 5:150–159.

Mulder, H., Nagorny, C. L., Lyssenko, V. and Groop, L (2009). Melatonin receptors in pancreatic islets: good morning to a novel type 2 diabetes gene. *Diabetologia* **52**, 1240–1249.

Muller-Berger, S. Nesterov, V.V. and Fromter, E. (1997). Partial recovery of in vivo function by improved incubation conditions of isolated renal proximal tubule. II Change of Na-HCO3 cotransport stoichiometry and of response to acetazolamide. *Pflugers Archives*, 434: 383–391.

Musi, N. Hirshman, M.F. Nygren, J. Svanfeldt, M. Bavenholm, P. Rooyackers, O. Zhou, G. Williamson, J.M. Ljunqvist, O. Efendic, S. Moller, D.E. Thorell, A. and Goodyear, L.J (2002). Metformin increases AMP-Activated protein kinase activity in skeletal muscle of subjects with type 2 diabetes. *Diabetes,* 51: 2074–2081.

Nadkarni, A.K. (2002). Indian Materia Medica. 3 rd ed. Bombay. *PopularPrakashan* (I): 225-226.

Nagao, Y. Platero, J.S. Waheed, A. and Sly, W.S (1993). Human mitochondrial carbonic anhydrase: cDNA cloning, expression, subcellular localization, and mapping to chromosome 16. *Proceedings of National Academy of Science* U S A; 90:7623–7627

Nagao, Y. Srinivasan, M. Platero, J.S. Svendrowski, M. Waheed, A. and Sly, W.S (1994). Mitochondrial carbonic anhydrase (isozyme V) in mouse and rat: cDNA cloning, expression, subcellular localization, processing, and tissue distribution. *Proceedings of National Academy of Science* USA. 91:10330–10334

Nair, S.K. Ludwig, P.A. and Christianson, D.W (1994). Two-site binding of phenol in the active site of human carbonic anhydrase II: structural implications for substrate association. *Journal of American Chemical Society*. 116:3659–3660

Natali, A. and Ferrannini, E (2006). Effects of metformin and thiazolidinediones on suppression of hepatic glucose production and stimulation of glucose uptake in type 2 diabetes: a systematic review. *Diabetologia*. 49 : 434 – 441

Nelson, S.D. Mitchell, J.R. Timbrell, J.A. Snodgrass, W.R. and Corcoran, G.B. (1976). 3rd Isoniazid and iproniazid: activation of metabolites to toxic intermediates in man and rat. *Science*, 193: 901-903.

Nguyen, T.T. and Bonanno J.A. (2011). Bicarbonate, NBCe1, NHE, and Carbonic Anhydrase Activity Enhance Lactate-H^+Transport in Bovine Corneal Endothelium. *Investigative Ophthalmology and Visual Science,* 52;11; p 8086-8093.

Nicky, K. Juan, C.M. Sean L.M. Briana S. Tim, C. Melissa, V. Stephen, W. Richard, F. Shona, M. Courtney, S. Sharon, J. Adrian, C. Lucia, G. Victoria, C.F. Guy, K. Sofianos, A. and Ken, R.W. (2012). Methazolamide Is a New Hepatic Insulin Sensitizer That Lowers Blood Glucose In Vivo. *Diabetes,* (61): 2146–2154.

NRC [National Research Council]. 1996. Laboratory Animal Management: Rodents: National Academy Press.

Otto, M. Breinholt, J. and Westergaard, N. (2003). Metformin inhibits glycogen synthesis and gluconeogenesis in cultured rat hepatocytes. *Diabetes Obesity and Metabolism*, 5, 189–194.

OuYang, Y.B. Mellergård, P. Kristián, T. Kristiánova, V. and Siesjö, B.K. (1994). Influence of acid–base changes on the intracellular calcium concentration of neurons in primary culture. *Experimental Brain Research*, 101; 265–271.

Owen, M. R., Doran, E., and Halestrap, A.P (2000). Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. *Biochemical Journal*. 348**,** 607–614.

Parkkila, A.K. Scarim, A.L. Parkkila, S. Waheed, A. Corbett, J.A. and Sly, W.S (1998). Expression of carbonic anhydrase V in pancreatic ,a-cells suggests role for mitochondrial carbonic anhydrase in insulin secretion. *Journal of Biological Chemistry*.273, 24620-24623

Parui, R. Gambir, K.K. Cruz, I. and Hosten, A.O. (1992). Erythrocyte carbonic anhydrase: A major intracelluler enzyme to regulate cellular sodium metabolism in chronic renal failure patients with diabetes and hypertension. *Biochemistry International*, (26): 809-20.

Parui, R. Gambir, K.K. and Mehrotra, P.P. (1991). Changes in carbonic anhydrase may be the initial step of altered metabolism in hypertension. *Biochemistry International*, (23): 779–89.

Pastorekova, S. Parkkila, J. Pastorek, S. and Supuran, C.T. (2004). Carbonic anhydrases: current state of the art, therapeutic applications and future

prospects, *Journal of Enzyme Inhibition and Medicinal Chem*istry, (19) 199–229.

Pitts, R.F. and Alexander, R.S. (1945). The nature of the renal tubular mechanism for acidifying the urine. *American Journal of Physiology*, 144: 239–254.

Puscas, I. and Supuran, C.T (1996). Farmacologia clinica da ulcera peptic (1996). In: *Aparelho Digestivo*. Coelho J (Ed.), MEDSI, Rio de Janeiro.1704-1734.

Ra¨isa¨nen, S.R. Lehenkari, P. Tasanen, M. Rahkila, Pha¨rko¨nen P.L. and V¨Aa¨na¨nen H.K. (1999). Carbonic anhydrase III protects cells from hydrogen peroxide-induced apoptosis. *Faseb*, 13: P 513-522

Raba, J. and Mottola, H. (1995). *Crititcal Reviews in Analytical Chemistry*, 25(1): 1-42.

Radziuk, J.D. Zhang, Z. and Wiernsperger, N. (1997). Effects of metformin on lactate uptake and gluconeogenesis in the perfused rat liver. *Diabetes*, 46: 1406–1413

Ramesh, B.K., Maddirala D.R. Vinay K.K. Shaik S.F., Tiruvenkata K. E.G., Swapna, S. Ramesh, B., and Rao, C.A. (2010). Antihyperglycemic and antihyperlipidemic activities of methanol:water (4:1) fraction isolated from aqueous extract of Syzygium alternifolium seeds in streptozotocin induced diabetic rats. *Food and Chemical Toxicology,* (48) 1078–1084.

Rector, F.C. Seldin, A.D. Roberts, J. and Smith, J.S. (1960). The role of plasma CO_2 tension and carbonic anhydrase activity in the renal reabsorption of bicarbonate. *Journal of Clinical Investigation*, 39: 1706–1721.

Reichard, G.A. Moury, N.F. Hochella, N.J. Patterson, A.L. and Weinhouse, S (1963). Quantitative Estimation of the Cori Cycle in the Human. *The Journal of Biological Chemistry.*238:495-501.

Rognstad, R (1983). Carbondioxide metabolism in the liver. *Archives of Biochemistry and Biophysics*. 442-448.

Samuel, V. T. and Shulman, G. I (2012). Mechanisms for insulin resistance: common threads and missing links. *Cell* 148, 852–871

Sanghi, D.K. Jaiswal, S.B. Shamsudin, J.S.B. and Saini, V. (2007). Hepatoprotective activity of *Cadaba farinose. International Journal of Green Pharmacy,* 1; (2):41-42.

Sasaki, S. and Yoshiyama, N. (1988). Interaction of chloride and bicarbonate transport across the basolateral membrane of rabbit proximal straight tubule Evidence for sodium chloride/bicarbonate exchange. *Journal of Clinical Investigation,* 81: 1004–1011.

Schwartz, G.J. Winkler, C.A. Zavilowitz, B.J. and Bargiello, T. (1993). Carbonic anhydrase II mRNAis induced in the rabbit kidney cortex during chronic metabolic acidosis. *American Journal of Physiol*ogy, 265 (*Renal Fluid Electrolyte Physiology*, 34): F764–F772.

Scozzafava, A. and Supuran, C. T. (2002a). Carbonic anhydrase activators: high affinity isozymes I, II, and IV activators, incorporating a beta-alanyl-histidine scaffold. *Journal of Medicinal Chemistry*, 45: 284–291.

Scozzafava, A. and Supuran, C.T. (2002b). Carbonic anhydrase activators: human isozyme II is strongly activated by oligopeptides incorporating the carboxyterminal sequence of the bicarbonate anion exchanger AE1. *Bioorganic and Medicinal Chemistry Letters,* 12: 1177–1180.

Scozzafava, A. Briganti, F. Mincione, G. Menabuoni, L. Mincione, F. and Supuran, C.T (1999b). Carbonic anhydrase inhibitors. Synthesis of water-soluble, amino acyl/dipeptidyl sulfonamides possessing long lasting-intraocular pressure lowering properties via the topical route. *? Journal Medicinal Chemistry.* 42:3690–3700

Scozzafava, A. Menabuoni L. Mincione, F. Briganti, F. Mincione, G. and Supuran, C.T (1999a) Carbonic anhydrase inhibitors: synthesis of water-soluble, topically effective intraocular pressure lowering aromatic/heterocyclic sulfonamides containing cationic or anionic moieties: is the tail more important than the ring*? Journal Medicinal Chemistry*. 42:2641–2650

Scozzafava, A. Menabuoni, L. Mincione, F. Briganti, F. Mincione, G. and Supuran, C.T (2000). Carbonic anhydrase inhibitors: perfluoroalkyl/aryl-substituted derivatives of aromatic/heterocyclic sulfonamides as topical intraocular pressure lowering agents with prolonged duration of action. *? Journal Medicinal Chemistry*. 43:4542–4551

Scozzafava, A. and Supuran, C.T. (2000c). Carbonic anhydrase activators. Part 21. Novel activators of isozymes I, II and IV incorporating carboxamido- and ureido histamine moieties. *European Journal of Medicine Chemistry*,35:31-39.

Scozzafava, A. and Supuran, C.T. (2000d). Carbonic anhydrase activators. Part 24. High affinity isozymes I, II and IV activators, derivatives of 4-(4-chlorophenylsulfonylureido- amino acyl)ethyl-1*H*-imidazole. *European Journal of Pharmaceutical Science*, 10: 29-41.

Seppo, P. Antti, J.K. Kari, K. Anna-Kaisa, P. Jukka, H. Hannu, R. Abdul ,W. and William, S.S (2002). The plasma membrane carbonic anhydrase in murine hepatocytes identified as isozyme XIV. *BMC Gastroenterology*. 2:3

Seki, G. Coppola, S. Yoshitomi, K. Burckhardt, B.C. Samarzija, I. Muller-Berger, S. and Fromter, E. (1996). On the mechanism of bicarbonate exit from renal proximal tubular cells. *Kidney International*, 49: 1671–1677.

Sinha, B.K. and Mason, R.P. (2014). Biotransformation of Hydrazine Derivatives in the Mechanism of Toxicity. *Journal of Drug Metabolism and Toxicol*ogy, 5: 3, p 2-6.

Siva, N. G. and Banumathy N. M. (2008). Antifungal effect of leaf extract of some Medicinal Plants against *Fusariumoxysporum* causing wilt disease of *Solanummelogena*L.(Tamil Nadu, India) 1-8.

Sly, W.S. Hewett-Emmett, D. and Whyte, M.P. (1983). Carbonic anhydrase II deficiency identified as the primary defect in the autosomal recessive syndrome of osteopetrosis with renal tubular acidosis and cerebral calcification. *Proceedings of Natural Academy of Science, USA* 80: 2752–2756.

Soda, K. Dobashi, Y. Kano, Y. Tsujinaka, S. and Konishi, F. (2009), Polyamine-rich food decreases age-associated pathology and mortality in aged mice. *Experimental Gerontology*, 44:727–732.

Speeckaert, M. Van Biesen, W. Delanghe, J. Slingerland, R. Wiecek A. Heaf, J. Drechsler, C. Lacatus, R. Vanholder R. and Nistor, I. European Renal Best Practice Guideline Development Group on Diabetes in Advanced CKD (2014). Are there better alternatives than haemoglobin A1c to estimate glycaemic control in the chronic kidney disease population? *Nephrology and Dialysis Transplant,*10: 1–11

Stams, T. and Christianson, D.W. (2000): X-ray crystallographic studies of mammalian carbonic anhydrase isozymes. In: The Carbonic Anhydrases: New Horizons, pp. 159-174. Eds.

Stevenson, R.W. Parsons, J.A. George, K. and Alberti, K.G.M (1983). Effect of Intraportal and Peripheral Insulin in Glucose Turnover and Recycling in Diabetic Dogs. *American Journal of Physiology.*244: E190-E195.

Stumvoll, M. Nurjhan, N. Perriello, G. Dailey, G. and Gerich, J.E. (1995). Metabolic effects of metformin in non-insulin-dependent diabetes mellitus. *New England Journal of Medicine,* 333, 550–554.

Stumvoll, M., Goldstein, B. J. and van Haeften, T. W (2005). Type 2 diabetes: principles of pathogenesis and therapy. *Lancet* 365, 1333–1346.

Sugrue, M.F (2000) Pharmacological and ocular hypotensive properties of topical carbonic anhydrase inhibitors. *Progressive Retinal Eye Research*. 19:87–112

Sun, M.K. and Alkon, D.L. (2001). Pharmacological enhancement of synaptic efficacy, spatial learning, and memory through carbonic anhydrase activation in rats. *Journal of Pharmacology and Experimental Therapy,* 297**:** 961–967.

Sun, M.K. and Alkon, D.L. (2002). Carbonic anhydrase gating of attention: memory therapy and enhancement. *Trends in Pharmacological Science,*23:83–89.

Supuran, C.T (2003) Carbonic anhydrase inhibitors in the treatment and prophylaxis of obesity. *Expert Opinion in Therapeutics Patents.* 13:1545–1550

Supuran, C.T (2011). Carbonic anhydrase inhibition with natural products: novel chemotypes and inhibition mechanisms. *Molecular Diversity* 15:305–316

Supuran, C.T. (1994). Carbonic anhydrase inhibitors. In: *Carbonic Anhydrase and Modulation of Physiologic and Pathologic Processes in the Organism*. Puscas I (Ed.) Helicon, Timisoara, Romania :29-111.

Supuran, C.T. (2004): Carbonic anhydrases: catalytic and inhibition mechanisms, distribution and physiological roles. In: Carbonic Anhydrase: Its Inhibitors and Activators, pp. 1-23. Eds.

Supuran, C.T. (2008). Carbonic anhydrases: novel therapeutic applications for inhibitors and activators, *Nature Review Drug Discovery*, (7): 168–181.

Supuran, C.T. and Clare, B.W. (1994). Carbonic anhydrase activators. Part 3. Structure–activity correlations for a series of isozyme II activators. *Journal of Pharmaceutical Sciences*, 83: 768–773.

Supuran, C.T. and Scozzafava, A. (2000). In *The Carbonic Anhydrases — New Horizons* (eds Chegwidden, W. R., Carter, N. D. and Edwards, Y. H.) 197–219 (Birkhauser, Basel.

Supuran, C.T. Balaban, A.T. Cabildo, P. Claramunt, R.M. Lavandera, J.L.and Elguero, J. (1993). Carbonic anhydrase activators. Part 7. Isozyme II activation with bis-azolylmethanes, -ethanes and related azoles. *Biological and Pharmaceutical Bulletin,*16:1236-1239.

Supuran, C.T. Barboiu, M. Luca, C. Pop, E. Brewster, M.E. and Dinculescu, A. (1996). Carbonic anhydrase activators. Part 14. Synthesis of mono- and bis- pyridinium salt derivatives of 2-amino-5-(2-aminoethyl)- and 2-amino-5-(3-aminopropyl)-1,3,4-thiadiazole and their interaction with isozyme II. *European Journal of Medicinal Chemistry,*31:597-606.

Supuran, C.T. Claramunt, R.M. Lavandera, J.L. and Elguero, J. (1996a). Carbonic anhydrase activators. Part 15. A kinetic study of the interaction of bovine isozyme II with pyrazoles, bis- and tris-azolylmethanes. *Biological and Pharmaceutical Bulletin,*19:1417-1422.

Supuran, C.T. Conroy, C.W. and Maren, T.H. (1996b). Carbonic anhydrase inhibitors: synthesis and inhibitory properties of 1, 3,4-thiadiazole-2,5-bissulfonamide. *European Journal of Medicinal Chemistry,*. 31:843-846.

Supuran, C.T. and Manole, G. (1999). *Carbonic Anhydrase Inhibitors: Synthesis, reactions and therapeutic applications.* Roumanian Academy Publishing House, *Bucharest* :11-178.

Supuran, C.T. and Puscas, I. (1994): Carbonic anhydrase activators. In: *Carbonic anhydrase and modulation of physiologic and pathologic processes in the organism.* Puscas I (Ed.), Helicon, *Timisoara*, 113-145.

Supuran, C.T. and Scozzafava, A. (1999). Carbonic anhydrase activators. Amino acyl/dipeptidyl histamine derivatives bind with high affinity to isozymes I, II and IV and act as efficient activators. *Bioorganic and Medicinal Chemistry*, 7: 2915-2924.

Supuran, C.T. Scozzafava, A. and Conway, J. (2004). *Carbonic Anhydrase — Its inhibitors and Activators* 1–363 (CRC, Boca Raton,)

Svichar, N. and Chesler, M. (2003). Surface carbonic anhydrase activity on astrocytes and neurons facilitates lactate transport. *Glia*, 41: 415–419.

Tambutte, S. Tambutte, E. Zoccola, D. Caminiti, N. Lotto, S. Moya, A. Allemand, D. and Adkins, J (2006). Characterization and role of carbonic anhydrase in the calcification process of the azooxanthellate coral Tubastrea aurea. *Marine Biology*. DOI 10.1007/s00227-006-0452-8

Tannen, R.L. and Ross, B.L. (1983). Effect of pH on ammonia production by renal mitochondria. *Journal of Laboratory and ClinicalMedicine*, 102:536-542

Tashian, R.E. Hewett-Emmet, D. and Venta, P.J. (1991). Diversity and evolution in the carbonic anhydrase gene family. In: *Carbonic anhydrase: from biochemistry and genetics to physiology and clinical medicine*. Botr`e F, Gros G, Storey BT, eds. Weinheim: VCH Verlagsgesellschaft, 151–61.

Telrandhe, U. Umesh, B. Hemalatha, S. and Modi, A. (2010). Pharmacognostic and phytochemical investigation on root of cadaba farinosa forsk. *International Journal of Pharma and Bio Sciences*, 1: 2, 1-13

Temperini, C. Scozzafava, A. Vullo, D. and Supuran, C.T. (2006) Carbonic anhydrase activators. Activation of isozymes I, II, IV, VA, VII and XIV with L- and D-histidine and crystallographic analysis of their adducts with isoform II: engineering proton transfer processes within the active site of an enzyme. *Chemistry*, 12:7057–7066

Tenpe, C.R. Upaganlawar, A.B. and Yeole, P.G. (2006). Antidiabetic activity of *Cadaba indica* in Alloxan induced diabetic rats. *Indian Journal of Natural Product*, 22 (2): 14-17.

Terna, G. Jideani, I.A. and Nkama, I. (2002). Nutrient and sensory quality of kunun zaki from different saccharification agents. *International journal of food science and nutrition*, (53): 109-115.

The Diabetes Control and Complications Trial Research Group (1993). The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N. Engl. J. Med.* 329, 977–986.

Toth, B. (1978). Tumorigenic effect of 1-hydrazinophthalazine hydrochloride in mice. *J Natural Cancer Inst,* 61: 1363-1365.

Trivelli, L.A., Ranney, H.M., and Lai, H.T (1971). *New England Journal of Medicine* 284, 353

Trohalaki, S. Zellmer, R.J. Pachter, R. Hussain, S.M. and Frazier, J.M. (2002). Risk assessment of high-energy chemicals by in vitro toxicity screening and quantitative structure-activity relationships. *Toxicology Science,* 68: 498-507.

Tu, C.K. Silverman, D.N. Forsman, C. Jonsson, B.H. and Lindskog, S. (1989): Role of histidine 64 in the catalytic mechanism of human carbonic anhydrase II studied with a site-specific mutant. *Biochemistry,* 28:7913-7918.

UK Prospective Diabetes Study (UKPDS) Group (1998). Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). *Lancet* 352, 837–853

Umesh, B.T. Anuj, Vaibhav,M. Avinash, U. Hemalatha, G. and Goswami D. V (2010a). Hepatoprotective and Antioxidant activity of Root of *Cadaba farinosa*, Forsk against CCl4 induced hepatotoxicity in rats. *Journal of Pharmaceutical Research,* 3; (6):1-5

Umesh, B.T. Hemlatha, S. and Modi, A. (2010b). Pharmacognostic and phytochemical investigation of root of *Cadaba fariosa*forsk. *International Journal of Pharma and Bioscience,* 1(2): 1-13.

Upendra, R.M. Sreenivasulu, M. Chengaiah, B. Jaganmohan, R.K. and Madhusudhana, C.C. (2010). Herbal medicines for diabetes mellitus: a review. *International Journal of Pharmaceutical Technology Research,* (2): 1883-1892.

Utley, H.C. Bernheim, F. and Hochslein, P. (1967). Effect of sulfhydryl reagent on peroxidation in micrisome, *Archive of Biochemistry and Biophysics*, 260: 521–531.

Van Slyke, D. and Hawkins, J. (1930): Studies of gas and electrolyte equilibrium in blood. XVI. The evolution of carbon dioxide from blood and buffer solutions. *Journal of Biological Chemistry,* 87:265-279.

Vander, Walt. S.J. and Steyn, D.G. (1943). Onderstepoort, *Journal of veterinary Science,* 18: 207.

Verpoorte, J.A. Mehta, S. and Edsall, J.T. (1967). Esterase activities of human carbonic anhydrase. *Journal of Biological Chemistry*, 242: 18: 4221-4229.

Von Maydell, H. J. (1986). Trees and shrubs of the Sahel - their characteristics and uses. GTZ 6MBH,Eschborn.

Vu, H. Pham, N.B. and Quinn, R.J (2008). Direct screening of natural product extracts using mass spectrometry. *Journal of Biomolecular Screening.* 13:265–275

Wallace, H.M. and Niiranen, K. (2007). Polyamine analogues – an update. *Amino Acids*, 33:261–265.

Wetzel, P. Hasse, A. Papadopoulos, S. Voipio, J. Kaila, K. and Gros, G. (2001). Extracellular carbonic anhydrase activity facilitates lactic acid transport in rat skeletal muscle fibres. *Journal of Physiology*, 531:743– 756.

Wiernsperger, N.F. and Bailey, C.J. (1999). The anti-hyperglycemic effect of Metformin: Therapeutic and cellular mechanism. *Drugs*, (58): (Suppl. 1) 31–39.

Wistrand, P.J. and Kinne, R. (1977). Carbonic anhydrase activity of isolated brush border and basal-lateral membranes of renal tubular cells. *Pflugers Archives*, 370:121–126.

Wistrand, P.J. and Lindqvist, A. (1991). Design of carbonic anhydrase inhibitors and the relationship between the pharmacodynamics and pharmacokinetics of acetazolamide. In: *Carbonic Anhydrase - From Biochemistry and Genetics to Physiology and Clinical Medicine.* Botrè F, Gros G, Storey BT (Eds.), VCH, Weinheim :352-378.

Xu, M. Davis, R.A. Feng, Y. Sykes, M.L. Shelper, T. Avery, V.M. Camp, D. and Quinn, R.J. (2012). Ianthelliformisamines A-C, antibacterial bromotyrosine-

derived metabolites from the marine sponge *Suberea ianthelliformis*. *Journal of Natural Product,* 75:1001–1005.

Yamakido, M. Yorioka, N. Goriki, K. Wada, K. Hata, J. and Nishimoto, Y. (1982). Carbonic anhydrase I and II levels in erythrocytes of chronic kidney disease patients. *Hiroshima Journal of Medical Sciences,* 31: 1– 6.

Yin, S. Davis, R.A. Shelper, T. Sykes, M.L. Avery, V.M. Elofsson, M. Sundin, C. and Quinn, R.J. (2011). Pseudoceramines A-D, new antibacterial bromotyrosine alkaloids from the marine sponge *Pseudoceratina* sp. *Organic and Biomolecular Chemistry,* 9:6755–6760.

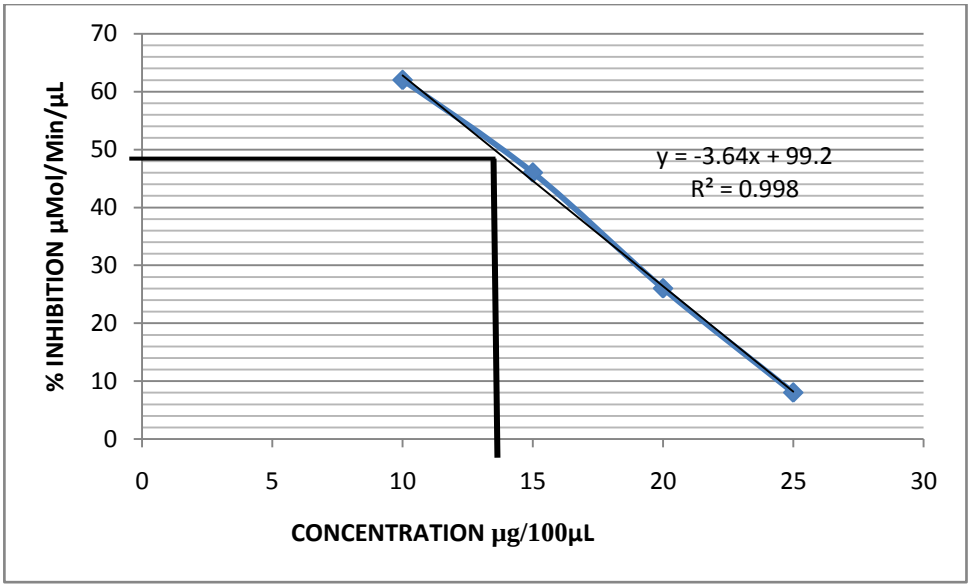
Yousif, G. Iskander, G.M. and Eisa, E. B. (1984). Alkaloid components of the Sudan Flora. II. Alkaloid *Cadaba farinosa* and *C. rotundifolia. Fitoterapia,* 55 (2):117-118.

Zawadski. J. Wolfe, R. Mott, D. Lillioja, S. Howard, B. and Bogardus, C (1988). Increased Rate of Cori Cycle in Obese Subjects with NIDDM and Effects of Weight Reduction. *Diabetes*. 37:154-159.

Zhang J, Wang X, Yan Y, Jiang L, Wang J, Li B, and Xiang W(2010). Isolation and identification of 5-hydroxyl-5-methyl-2-hexenoic acid from Actinoplanes sp. HBDN08 with antifungal activity. Bioresource Technology 101 :8383–8388

APPENDIX

50% INHIBITORY CONCENTRATION (IC_{50}) OF PURIFIED FRACTION OF *CADABA FARINOSA* METHANOL LEAF EXTRACT ON CARBONIC ANHYDRASE ACTIVITY.



IC_{50}= 13.57 µg/100 µL

Cadaba farinosa Forssk