EFECTS OF STEVIOSIDE-SUPPLEMENTED BISCUITS ON THE KIDNEY FUNCTION OF RABBITS

A THESIS
BY

MD. MASUD RANA
Student ID: 1805349
Session: 2018-2019
Semester: July- December, 2019

MASTER OF SCIENCE

IN

BIOCHEMISTRY AND MOLECULAR BIOLOGY

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY
HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY, DINAJPUR-5200

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December, 2019

Dedicated
To my
Beloved Parents

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The Authors

ABSTRACT

Diabetes is one of the four major type of non-communicable diseases that make the largest contribution to morbidity and mortality worldwide. According to WHO, about 422 million people globally had diabetes, with most living in the developing countries. Diabetes are closely associated with chronic kidney disease (CKD). Worldwide, the prevalence of kidney disease and the metabolic syndrome is becoming a significant medical concern and public health burden. The prevalence of CKD has reached epidemic status in 10–12% of the populations, and more than 50% of elderly worldwide. The prevalence of diabetes is increasing in Bangladesh in both urban and rural areas. Stevia (*Stevia rebaudiana*) plant is an exotic plant in our country. Stevia leaf is sweet in taste. Its leaves contains different types of steviol glycosides such as 'stevioside' which is 250 to 300 times sweeter than sucrose. It is used as a natural sweetener and has no adverse effects on health. Stevioside provides zero calories in a wide range of beverages and foods. Some diabetic foods (*e. g*., cake, candy, and bread) prepared with artificial sweetener such as saccharin, aspartame etc. are available in Bangladesh, but these sweeteners has serious adverse effects on health. Therefore, we aimed to formulate a stevioside-supplemented biscuit using stevioside as a natural sweetener and to evaluate the effects of this biscuit on kidney function of the rabbit. We have prepared a stevioside-supplemented biscuit using stevioside as a sugar substitute and also prepared other two types of biscuit using sugar and saccharin to compare the results. Then we fed these three types of biscuits to three separate groups of rabbits and observed their effects on the kidney function of rabbits. We measured levels of creatinine, albumin, uric acid, urea and serum electrolytes (sodium, potassium, and chloride). Biochemical tests were performed by spectrophotometer and automatic electrolyte analyzer. The results revealed that the creatinine level was in normal range (0.97–1.13 mg/dl) in stevioside-supplemented biscuit. Stevioside-supplemented biscuit showed the high albumin content (3.50- 3.24 g/dl) than that of sugar (3.34-3.17 g/d) and saccharin (3.33- 3.02 g/dl). Stevioside-supplemented biscuit also contained less urea level (36.36 mg/dl) and uric acid (0.61 mg/dl) than that of sugar (urea= 47.2; uric acid =1.03 mg/l) and saccharin (urea=50; uric acid =1.4). Our electrolytes analysis showed that stevioside fed rabbit's had good level of sodium (143-139 mmol/l), potassium (3.6-3.7 mmo/l), and chloride (103.6-102.5 m moil/l) than that of sugar and saccharin fed rabbits. Altogether, stevioside-supplanted biscuit showed the best results than sugar and saccharin-supplemented biscuits. Therefore, stevioside biscuit can be considered as the best sugar alternative biscuit for the diabetic patients.

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ABBREVIATION

CKD	Chronic kidney disease
DNA	Deoxyribonucleic acid
BCG	Bromocresol Green
FEP	Flame Emission Photometry
WHO	World Health Organization
FDA	Food And Drug Administration
PKU	Phenylketonuria
ALT	Alkaline phosphatase
AST	Aspartate aminotransferase
ALP	Alanine aminotransferase
ATP	Adenosine triphosphate
TEAC	Trolox equivalent antioxidant capacity
TRAP	Total radical absorption potentials
ORAC	Oxygen radical absorption capacity
GTP	Glutamyl transpeptidase
NAG	n-acetyl-d-glucuronidase
MDA	Malondialdehyde
HSD	Honest significant differences

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CHAPTER I

INTRODUCTION

Stevia (*Stevia rebaudiana*) plant is an exotic plant in our country. It is also known as “honeyleaf. Stevioside is a glycoside isolated from the plant Stevia, which has been widely used as a sweetening agent in Japan for 20 years (Ms melis *et al.,* 1997, 1985). It is a natural sweetener plant having medicinal and commercial importance is being used all over the world (B Ahmed *et al.,* 2011). The main glycosidic compound accumulated in this plant is stevioside which is a secondary metabolite and 250-300 times sweeter than sucrose (Brandle *et al.,* 2004). The stevioside and rebaudioside A are the most abundant in the stevia leaf (JECFA *et al.,* 2005). Both compounds are diterpenoid glycosides that differ only by the presence of a single glucose moiety. In developed countries, raw, leaf-dried and powdered pure natural product is preferred for stevioside diabetics as they prove to be safer than widely used artificial sweeteners such as saccharin, aspartame, and cyclamate etc. Nowadays serious health care and social concerns related to overweight have increased the importance of sugar free products (Cross *et al.,* 2007). Stevia is a sweet herb indigenous to South America, confirmed promising results as the remedy of diabetes, hypertension, sexual dysfunction and other metabolic disorders (P.B. Jeppesen *et al.,* 2003, M. Ghaheri *et al.,* 2010). Potentiating insulin secretion, lowering blood pressure, treating obesity and decreasing blood sugar are exclusive therapeutic characteristics of stevioside (Wangh *et al.,* 2012). Antioxidant and anti-inflammatory are the common effects of stevia, which help to lessen the cardiovascular and metabolic disorders (C. Boonkaewwan *et al.,* 2006). In pure form, stevioside is a white crystalline material, an optical rotation of -39.3^0 in water, and elemental composition of $C_{38}H_{60}O_{18}$ (Boeckh *et al.,* 1998). Nowadays serious health care and social concerns regarding excess weight has raised the importance of sugar-free products (Cross *et al.,* 2007). The stevioside molecule is stable under dry conditions and in aqueous food systems. Its stability is significantly better than other artificial sweeteners like aspartame (Hamzah *et al.,* 2013). Several studies performed on substituting stevia in sugar containing bakery products such as cake (Schirmer *et al.,* 2012) , yoghurt (Abdel-Salam *et al.,* 2009) and bread (Parimalavalli *et al.,* 2007) but no research have been done on stevioside-supplimented biscuits. Therefore in this

study, we addressed the effect of stevioside on the kidney function of rabbit‘s. On the other hands, there is a few processed food for diabetics in the markets.

Diabetes are closely associated with chronic kidney disease (CKD), it has up raised the interest to investigate the renoprotective effect of stevia in CKD. Worldwide, the prevalence of kidney disease and the metabolic syndrome is becoming a significant medical concern and public health burden (Farhana Rizwan *et al.,* 2019). The prevalence of CKD has reached epidemic status in 10–12% of the populations, and more than 50% of elderly worldwide. Increasing body weight, hypertension and insulin resistance all contribute to the chance of the increasing the prevalence of CKD with high morbidity and mortality rate (Pestana *et al.,* .,2015). Approximately, more than 450,000 patients in the United States and more than 175,000 patients in Europe suffer from end-stage renal disease and more than one million globally. However, the overall prevalence of (CKD) is expected to be 30 to 50 time’s greater worldwide (Satko S et at., 2015).

Formulation of stevioside-supplemented biscuits using stevioside as an alternative to sugar is not available in our country and the number of diabetes patients is increasing day by day. They are always concerned about their food choices. We conducted our research considering diabetes patients and make biscuits with stevioside. Several studies have reported hypoglycemic and hypotensive effects of stevioside and Stevia extracts, particularly among individuals with type 2 diabetes and hypertension.

The kidney function test is an indicator of the kidney's condition, which is a suitable modality to estimate kidney dysfunction. Since the kidney performs a variety of activities, no single test is sufficient for a complete estimate of kidney function. They are helpful to recognize the pattern of kidney disease. It helps to distinguish between acute kidney and (CKD). Creatinine is a chemical waste product in the blood that passes through the kidneys to be filtered and eliminated in urine. The chemical waste is a by-product of normal muscle function. The more muscle a person has, the more creatinine they produce. Levels of creatinine in the blood reflect both the amount of muscle a person has and their amount of kidney function. When there is kidney damage or kidney disease, and the kidneys are not able to filter waste efficiently, there will likely be a rise in creatinine levels in the blood. Albumin is a protein made by the liver. Albumin helps keep fluid in the bloodstream so it doesn't leak into other tissues. It also carries various substances throughout the body,

including hormones, vitamins, and enzymes. Low albumin levels can indicate a problem with the kidney. Uric acid is a normal body waste product. It forms when chemicals called purines break down. Purines are a natural substance found in the body. They are also found in many foods such as liver, shellfish, and alcohol. They can also be formed in the body when DNA is broken down. Serum uric acid concentrations increase in (CKD) and may lead to tubular injury, endothelial dysfunction, oxidative stress, and intra renal inflammation. Urea is a nitrogen-containing compound formed in the liver as the end product or protein metabolism and urea cycle. About 85% of urea is eliminated via kidneys; the rest is excreted via the gastrointestinal tract. Serum urea is increased in conditions where renal clearance decreases in acute and chronic renal failure. Sodium is an essential electrolyte that helps maintain the balance of water in and around your cells. It's important for proper muscle and nerve function. It also helps maintain stable blood pressure levels. Sodium is essential for the body functions listed above; too much sodium can be harmful for people with kidney disease because kidneys cannot eliminate excess sodium and fluid from the body. As sodium and fluid buildup in tissues and bloodstream, increases blood pressure. Potassium is a mineral that is vital to cell metabolism. It helps transport nutrients into cells and removes waste products out of cells. It is also important in muscle function, helping to transmit messages between nerves and muscles. Potassium is also necessary for maintaining fluid and electrolyte balance and pH level. When kidneys fail they can no longer remove excess potassium, so the level builds up in the body. Chloride is an electrolyte. It is a negatively charged ion that works with other electrolytes, such as potassium, sodium, and bicarbonate, to help regulate the amount of fluid in the body and maintain the acid-base balance. An increased level of blood chloride (called hyperchloremia) usually indicates dehydration, but can also occur with other problems that cause high blood sodium, such as Cushing syndrome or kidney disease.

This study discusses the current knowledge of existing alternatives to sugar replacement and the studies done to identify enhanced featured substances. Here, we also describe the benefits and potential applications of sweet taste enhancers in the food industry.

This study was aimed to evaluate the safe use of stevioside as a low-calorie sugar substitute, as well as its protective role on kidney function of rabbits. Therefore,

regarding the utilization of stevioside and its numerous health benefits against different degenerative chronic diseases, the present study was conducted with the following objectives:

Objectives

- To measure creatinine level to know kidney condition in rabbits.
- To analyze the effects of stevioside supplemented -biscuit on serum albumin level in the blood in rabbits.
- To analyze the effects of stevioside- supplemented biscuit on uric acid in the blood in rabbits.
- To measure the level of urea in the blood in rabbits.
- To analyze the effects of stevioside- supplemented biscuit on electrolytes in rabbits**.**

CHAPTER II

REVIEW OF LITERATURE

2.1 Introduction

Stevia rebaudiana is a perennial plant of tribe Eupatorieae and family Asteraceae. Stevia genus comprises of about 150-200 species of herbs and shrubs (Gentry, 1996) and native to Brazil and Paraguay regions of South America (Soejarto *et al.,* 2002; Ramesh et al.,2006). Stevia is also known as sweet leaf, sweet herb, sweet weed and honey leaf (Carakostas *et al.,* 2008; Inamake *et al.,* 2010). In the native state it occurs on the edges of marshes or in grassland communities on soils with shallow water table (Shock *et al.,* 1982) with semi-humid subtropical climate, temperatures ranging from -6 to 43.8°C, with an average of 23.8°C and rainfall ranging from 1500 to 1800 mm per annum (Yadav *et al.,* 2011). Stevia is a short day plant that grows up to height of 1m (Mishra et al.,2010). It has 2 to 3cm long and elliptical leaves having alternate arrangement and bears a brittle stem and an extensive root system. Flowers are white in color with a pale purple throat. They are small in size and arranged in the form of small corymbs (Madan *et al.,* 2010; Yadav and Guleria *et al.,* 2012). The fruit is a five-ribbed spindle shaped achene (Katayama et al.,1976; Blumenthal et al,, 1996). The plant has gained commercial importance as a natural low calorie sweetener, due to the presence of high concentration of stevioside and rebaudioside-A in leaves (Kinghorn *et al.,* 2002; Ramesh *et al.,* 2006). Natural low caloric sweeteners not only create a calorie deficit but are also an appropriate tool against the health problems (Surana *et al.,* 2006). Stevioside passes through the digestive processes without chemical break down, thus making stevia safe for the diabetic peoples (Yadav *et al.,* 2011). Historically, plant has been used for various purposes throughout the world (Goyal *et al.,* 2010). Leaves of stevia has therapeutic properties like anticariogenic (Yabu *et al.,* 1977; Gardana *et al.,* 2010), antimicrobial (Satishkumar *et al.,* 2008), antiviral (Kedik *et al.,* 2009), antifungal (Silva *et al.,* 2008), anti-hypertensive (Chan *et al.,* 1998; Lee *et al.,* 2001;Hsieh *et al.,* 2003), anti-hyperglycaemic (Jeppesen *et al.,* 2002; Benford *et al.,* 2006), anti-tumour (Satishkumar *et al.,* 2008; Kaushik *et al.,* 2010), anti-inflammatory (Ghanta *et al.,* 2007; Arya *et al.,* 2012), hepatoprotective (Mohan and Robert *et al*,. 2009), diuretic, anti-diarrhoeal, anti-human rotavirus activities (Das *et al.,* 1992; Takahashi *et al.,*

2001), anti-HIV (Takahashi et *al*.,1998) and immunomodulatory (Chatsudthipong and Muanprasat *et al.,* 2009). In recent times, plant has gained significance in the pharmaceutical, food and cosmetic industries (Kienle *et al.,* 2007; Hansen *et al.,* 2010; Kienle *et al.,* 2010; Kroyer *et al.,* 2010; Herranz *et al*.,2010). Several studies have shown steviol glycosides as a substitute for sugar (Crammer and Ikan *et al.,* 1986; Anton et al.,2010; Gasmalla et al.,2014).There are numerous extraction, purification and estimation methods of steviol glycosides (Vanek *et al*.,2001; Choi *et al.,* 2002; Yoda *et al*.,2003; Erkucuk *et al.,* 2009) developed in the world. So in this regard, this paper is an attempt to summarize the scattered literature and reports on a single podium.

Figure 2.1 Stevia Plant

2.2 Review on selected stevia

2.2.1 History of stevia

Stevia leaves were used by indigenous peoples in Paraguay and Brazil since before recorded history (Lee *et al.,* 1979; Soejarto *et al.,* 2002). In the 1887, M. S. Bertoni, a Botanist was the first European to document stevia and later on in 1931, French chemists extracted stevioside, the main sweet component in the form of an extremely sweet, white crystalline compound. Afterwards stevia was considered to utilize as a sweetener for food shortages experienced by Britain during World War II, conversely, interest faded when sugar again became available. Japan used stevia in replace of saccharin after it was banned in the 1970 s and stevia sweeteners have been consumed in Japan in large amount than in any other country. In North America and Europe, stevia began to use as herbal product and started available in the market In the 1970s and 1980s and its extracts have been allowed for using as a dietary enhancement in the US in 1994 (FDA *et al*, 1995). In Europe it was not permitted in market or for other use due to the lack of proper reports or documentation. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) reviewed steviol glycosides in the 58th, 63rd, and 68th meetings. JECFA established both temporary specifications and a temporary ADI for steviol glycosides of 0-2 mg/kg bw/day. Along with this JECFA requested human studies conducted in normotensive and hypotensive subjects to answer questions about potential blood pressure lowering effects and also in relation to insulin-dependent and insulin-independent diabetes to know the effects on glucose homeostasis. After getting sufficient information, the European Commission's Scientific Committee on Food (SCF) reviewed and suggested that it would reduce the safety factor to 100 and make the ADI permanent (SCF *et al.,* 1985, 1999).

2.2.2 Stevia rebaudiana– origin and distribution

Stevia is native to the Amambay region of Northeastern Paraguay and has been reported to occur in neighboring parts of Brazil and Argentina as well (Soejarto, 2002). Although Stevia continues to be a rare plant in its native habitat, agricultural production in South America and Asia, and ornamental use in Europe and North America have made its occurrence in the world perhaps more common than it ever was in the past. *Stevia rebaudiana* belongs to the Asteraceae family and it and

Stevia phlebophylla are the only members of the 230 species in this genus to produce steviol glycosides (Kinghorn and Soejarto, 1985). The only other non-Stevia species found to have SGs is Rubus chingii, a member of the Rosaceae native to China , which contains rubusoside, a SG that is not found in *S.rebaudiana* (Tanaka *et al.,* 1981). There has been very little investigation into the adaptive role of SGs, but there is some evidence that stevioside and its derivatives have a deterrent effect on aphid feeding suggestive of a classical role in chemical defense against pests (Nanayakkara *et al.,* 1987; Wink., *et al.,* 2003). Other work has shown that the sweetness of SGs attracts aquatic animals, which when extrapolated to herbivorous land animals, could be associated with negative effects on survival and fitness of Stevia (Harada *et al.,* 1993). That being said it simply may be that SGs have no real adaptive role and instead they are part of some unselected chemical diversity whose contribution to fitness and therefore the propagation of the species is as a sweetening agent. The attraction to humans has allowed Stevia, an otherwise obscure species, to spread throughout the world (Firn and Jones *et al*, 2003)

2.2.3 Chemical constituents of stevia

The complete chemical composition of Stevia species is not yet available. However, a variety of Stevia species has been tested for their chemical compositions. The useful part of this shrub is the leaves. Out of 110 species tested for sweetness, only 18 we refound to possess this characteristic (Soejarto *et al.,* 1982). Eight ent-kaurene glycosides namely dulcoside A, rebaudiosides AE, steviolbioside, and stevioside produce the sweet taste sensation (Kinghorn *et al.,* 1984). These glycosides are mainly compounds of the diterpene derivative steviol (Shibata *et al.,* 1995). *S. rebaudiana* Bertoni, the sweetest species, contains in its leaves all of the eight ent-kaurene glycosides (Kinghorn *et al.,* 1984), with stevioside being the major constituent (38% by weight of the dried leaves) (Melis *et al.,* 1992). In addition, S. rebaudiana Bertoni contains stigma sterol, b-sitosterol, and campesterol (D'Agostino *et al.,* 1984). The same species also contains steviol, a product formed by enzymatic hydroxylation within the plant (Kim *et al.,* 1996). Other chemicals with no sweet taste are also found in Stevia species and some may even be bitter in taste. Stevis alioside A (from the roots of Stevia salicifolia) (Mata *et al.,* 1992), longipinane derivatives in the roots of Stevia connate (Sanchez-Arreola *et al.,* 2000), epoxylabdane diterpenes and a clerodane derivative in the leaves of Stevia

subpubescens (Roman *et al.,* 2000), flavonoids from the leaves of *S.rebaudiana* (Soejarto *et al.,* 1982), Stevia nepetifolia (Rajbhandari and Roberts,1983), stevia microchaeta, Stevia monardifolia, *Stevia.origanoides* (Rajbhandari and Roberts *et al.,* 1985) and Stevia procumbens (aerial parts) (Sosa *et al.,* 1985), and sesquiterpene lactones from the aerial parts of S. Procumbens and the leaves of *S. origanoides* (Calderon *et al.,* 1987) are in this group.

2.2.4 Metabolism of stevia

S. rebaudiana leaves contain a zero-calorie ent-kaurene diterpene glycosides (stevioside and the rebaudiosides) 250-300 times sweeter than sucrose with superior solubility in water and a positive taste profile that are safely metabolized by the body without any effect (Soejarto *et al.,* 1982; Megeji *et al.,* 2005; Geuns *et al.,* 2007). The major compounds of Stevia as steviol glycosides are metabolized and eliminated through similar pathways in both humans and animals, has been studied by (Genus *et al.,* 2003, 2007). Rebaudioside A in the digestive tract is first metabolized by microbes in the colon to stevioside which is further converted into glucose molecule and steviol. The released glucose molecule is used by the bacteria in the colon and is not absorbed into the blood stream. The metabolized components essentially leave the body and there is no accumulation. The metabolism of steviol glycosides to steviol means that the metabolic equivalency of the different steviol glycosides permits to apply the findings from studies with stevioside to the safety evaluation of rebaudioside A, and thus to the safety of Stevia (Koyama *et al.,* 2003). There Stevia species possess some differences between, it has been demonstrated that the conversion rate of rebaudiside A and stevioside are similar between rats and humans, with the conversion from stevioside to steviol more rapid than that of rebaudioside A to stevioside in both species. Moreover, quantitative and qualitative similarities have been found between the organisms in the gut (microflora) of rat and the human body (Wingard *et al.,* 1980). A study on the human digestive tract demonstrates that steviol is not altered or changed at either high or low concentrations as observed through human faeces, indicating that steviol is in fact the final product of Stevia metabolism (Koyama *et al.,* 2003). The study also showed that the majority of steviol glycosides are absorbed and glucuronidated (a bond intended to help them clear out of the blood) in the liver. The newly bonded glucuronide is released in the blood and filtered by the kidneys into the urine. Small

amounts of glucuronidate that remain in the colon are excreted through fecal matter. Tests with stevioside compounds and the effect of gastric juices and digestive enzymes on them show their failure to degrade or rearrange the compounds (Wingard *et al.,* 1980). In vitro digestibility of steviosides by various digestive enzymes was examined by (Hutapea *et al*.,1997), it was found that none of the enzymes digested the stevioside and intestinal microflora hydrolyzed it to both steviol and steviol-16, 17 alphaepoxide. Later, steviol 16, 17 alphaepoxide was then completely converted back into steviol, which further excreted from the body in urine as steviol glucuronide (Chatsudthipong and Muanprasat *et al*, 2009).

2.2.5 Steviol glycosides components and their features

Stevioside obtained from stevia leaves is sweeter than sucrose and includes rebaudiosid A, B, C, D, E and dulcosid-A glycosides (Carneiro *et al.,* 1997). The dried leaves and dust extract of this plant are sweeter than sugar 15-20 times and 300 times respectively and they are zero-calorie (Singh and Rao *et al*, 2005).

2.3. Diabetes consideration

According to WHO (OMS) Diabetes has been contextualized as pandemic and it is often considered a "disease of civilization‖. Indeed, it has been set the "World Diabetes Day" because it is very serious and increasingly brings to the state, the family and the patient high costs of purchasing power in treatment. WHO in 2013 WHO (OMS) have reports that 347 million people worldwide suffer from diabetes Type 2. In an interview with the Bulletin, it has been reported that Type 2 diabetes, which accounts for over 90% of cases of diabetes, is a product of modern technology, and it will continue increasing, and it shows a wide range of variation in prevalence around the world, and it is expected to affect around 600 millions of people by the year 2035. It is why, the IDF in its introduction reports several keys messages as follows:

- 382 million people have diabetes; by 2035 this will rise to 592 million
- The number of people with type 2 diabetes is increasing in every country
- 80% of people with diabetes live in low- and middle-income countries
- The greatest number of people with diabetes are between 40 and 59 years of age

- 175 million people with diabetes are undiagnosed
- Diabetes caused 5.1 million deaths in 2013; Every six seconds a person dies from diabetes
- Diabetes caused at least USD 548 billion dollars in health expenditure in 2013 – 11% of total spending on adults
- More than 79,000 children developed type 1 diabetes in 2013
- More than 21 million live births were affected by diabetes during pregnancy in 2013.

This federation also report, the diabetes prevalence in the regions:

- In Africa, 76% of deaths due to diabetes are in people under the age of 60
- Europe has the highest prevalence of type 1 diabetes in children
- In the Middle East and North Africa, 1 in 10 adults has diabetes
- More was spent on healthcare for diabetes in North America and the Caribbean than in any other region
- In South and Central America, the number of people with diabetes will increase by 60% by 2035
- In South-East Asia, almost half of people with diabetes are undiagnosed
- In the Western Pacific, 138 million adults have diabetes–the largest number of any region.

While, the type 1 diabetes (T1DM) is associated with the complex insulin regimens (Kourtoglou, 2011) in the type 2 diabetes (T2DM), the improvement in blood glucose control is based primarily on behavioralchanges (reduced calorie and carbohydrate intakes, increased physical activity)(Sievenpiper and Dworatzek, 2013).

Consumption of sugar-sweetened food may be one of the dietary causes of metabolic disorders, such as obesity. Since the World Health Organization (WHO) in 1997 WHO World Health Report have declared obesity as a major public health problem, diabetic prevalence has been documented during the last two decades (Santonja and Shaikhet, 2014). Therefore, substituting sugar with low calorie sweeteners may be an efficacious weight management strategy. In view of these postulates previously discussed, strategies involving the use of steviol glycosides in the development of food for the consumption of diabetics must be applied, and these foods should be validated through large-scale population trials, considering validated

surrogate end points to evaluate its effect in prevention of chronic diseases such as type 2 diabetes mellitus.

2.3.1 Stevia and their potential uses diabetic-directed processed food

There exist in the market only a limited diabetic-directed processed foods, those available generally are non-autochthonous, versatile, and expensive. Several researching have been done for to use the stevia refined products, Narayanan*et al.,*2014 in sensorial studies of the appropriated concentration of stevia used for yogurt, underline the importance of careful selection of stevia type and concentration as well as optimizing yogurt cultures and fermentation conditions before product launch. Other authors (Tejo *et al.,* 2013, Jones *et al.,* 2014) have shown that stevioside and rebaudioside A isolated from stevia leaves are sweetening compounds of interest for promoting healthy foods. On the other hand, stevia with 97% rebaudioside A did not show off-flavor and presented similar acceptance and sensory profile in relation to control of mango nectar (Silva Cadena *et al.,* 2013) formulated chocolates containing stevia leaves and peppermint exhibited the best sensory properties (especially with regard to mouth feel, sweetness and herbal aroma), as well as the highest polyphenolic content and antioxidant capacity (Belščak-Cvitanović*et al.,* 2015). (Melo*et al.,* 2009) studies have shown the crucial attributes which determine consumer acceptability of chocolate using stevia. They have found the sweet aroma, melting rate, and sweetness as attributes of acceptance; whereas the bitterness, bitter aftertaste, adherence, and sandiness were drivers of disliking. In order to analyze the ideal and relative sweetness of a prebiotic chocolate milk dessert, Morais*et al.,* 2014 have studied the of sweetness level in this product with different types of artificial and natural sweeteners. They have concluded that stevia was the lower with sweetening power as compared to neotame, sacarose, sucralose and aspartame. (Lothrop *et al.,* 2012) had conducted a research to determine the physicochemical and sensory effects of replacing a mixture of rebaudioside-A and erythritol for sucrose at varying levels (0, 25, 50, 75 and 100%) in chiffon cake. The author has concluded that functional properties specific gravity, cake volume) some chemical (moisture content) and physical properties (crumb and crust color) were not affected by the sucrose substitution. However, there are impacts on the water activity of the chiffon cake, and nutrition analysis showed a decrease in both calories and sugars as the sucrose replacement

level increased. All other nutrient levels remained constant among treatments. These findings suggest a chiffon cake formulated with 50% sucrose and 50% rebaudioside-A and erythritol results in a product with high overall consumer acceptability and 20% fewer calories than one formulated with 100% sucrose. Kienle in 2014, of UniversitätHohenheim have recommend stevia natural sweetener for use in different food stuff, providing different recipes and concentrations of the sweetener. Prakash, *et al.,* 2012 have studied the degaradation of rebaudioside A under acidic conditions, they author have concluded that rebaudioside A yielded six minor degradation compounds. At industrial level there several products strictly directed to diabetic consumers, such as: dessert, cookies, jam, and the sweeteners, but the most common use of the non-caloric sweeteners in the carbonated beverages Mattes and Popkin (Mattes and Popkin *et al.,* 2009). (ChromaDex *et al.,* 2014) is a company that offers a large number presentation of stevia and its derivatives for use in different food products. (Trini *et al.,* 2014) in Argentina commercialize a group of product for diabetic with stevia as sweetener, among they there are a powder for preparing diet ice with stevia, in three flavors: Chocolate, Strawberry and Vanilla, which provide in average 31.7 Kcal /133 KJ (unprepared product), and Papaphilippou& Patisserie Panayiotis Ice Cream Ltd (2014) is sold the fat free ice cream with Paraguayan stevia. Truvia Company offer Truvía® sweetener and baking blends products and also provide its product to their partner to produce a gamma of products such beverages (sodas, vitaminwater, juices, tea), yogurt, bars and iSKream. The Truvía® natural sweetener commercialized in USA (2014) is safe and recommended for use by people with diabetes, because in the label it is declared stevia leaf extract and erythritol. Truvía® stevia leaf extract and erythritol have both been studied in short and long term clinical studies to evaluate safety for use by people with diabetesTruvía® stevia leaf extract and erythritol have both been studied in short and long term clinical studies to evaluate safety for use by people with diabetes. This Truvía® natural sweetener has little or no effect on blood glucose or insulin. However, the Truvía® sweetener commercialized in Venezuela contains sucrose (azúcarmorena) and then it cannot be recommended for diabetic uses.

2.3.2. Studies on safe consumption of the stevia and its use by consumers with regimen especial feeding

At the first time there was some discrepancy for consumption safety of the stevia,

(Geuns *et al.,* 2007) have pointed out that stevia and stevioside have no effect on mammalian reproduction or fertility, and they are safe for use as sweeteners and acceptable for both diabetic and phenylketonuria patients. Posterior research performed by (Geuns, *et al.,* 2007) have shown that steviol or related metabolites do not accumulate in the human body, and that at least in healthy human subjects pure stevioside taken at a dose of 750 mg/day had no effect on either blood pressure of insulin levels. Following those reviews, clinical evidence emerged that suggested stevioside can be used by diabetics and hypertensive patients and they may also offer therapeutic benefits, as they have anti-hyperglycemic, anti-hypertensive, anti-inflammatory, anti-tumor, anti-diarrheal, diuretic, and immunomodulatory actions (S. K. Yadav and P. Guleria, 2012). Stevia extracts, besides having therapeutic properties, contain a high level of sweetening compounds, known as steviol glycosides, which are thought to possess antioxidant, antimicrobial and antifungal activity. Stevioside and rebaudioside A are the main sweetening compounds of interest. Moreover the glycoside from estevia y are thermostable even at temperatures of up to 200 °C, changes of pH, and fluorescence, even during long storage periods, making them suitable for use in cooked foods (Lemus-Mondaca *et al.,* 2012). As (González *et al.,* 2014) have reported stevioside and rebaudioside. A have multiple advantages as dietary supplements. They are non-metabolizable (non-caloric) (Atteh *et al.,* 2008) non acidogenic, and do not cause dental caries (Brambilla *et al.,* 2014). Another notable benefit is that the consumption of both steviol glycosides is safe for human health and there are no restrictions on their use by people suffering from diabetes (Gregersen *et al.,* 2004). In fact, high doses of stevioside (750–1500 mg per day) have been used with favorable results for the treatment of hypertension and type 2 diabetes (Jeppesen *et al.,*2003). The therapeutic value of stevioside consists of the fact that it substitutes ssugar whilst at the same time stimulating the secretion of insulin in the pancreas during the treatment of diabetes and other carbohydrate metabolism disorders (Erkucuk *et al.,* 2009, Geuns *et al.,* 2007).

2.4 Stevioside-supplemented biscuits

Biscuits are flour-based bakery product which attracts consumers owing to their various tastes, long shelf life and relatively low cost (Manley *et al.,* 2000). Due to competition in the market and increased demand for health promoted natural

products, attempts are being made to improve biscuits' nutritional value as well as functionality by modifying their nutritive composition (Rahaie *et al.,* 2012). Since a very important aspect of biscuits is its sugar content, the granulated sugar can have desirable effects on flavor, dimensions, color, hardness and surface finish of biscuits. However, today a high level of sugar consumption is not desirable (Vitali *et al.,* 2009). Consumption of excessive quantities of granulated sugar increases the energy intake which can lead to harmful effects on the body, including obesity and chronic diseases. Nowadays serious health care and social concerns regarding excess weight, has raised the importance of sugar-free products (Cross *et al.,* 2007). Replacement of natural materials and artificial components has been employed to provide sweetness for the diet. Sorbitol, mannitol, xylitol, erythritol, lactitol, maltitol, stevioside, thaumatins, etc. are natural sweeteners which exhibit an appropriate potential for food applications. Also, artificial sweeteners including sucralose, aspartame and acesulfame-K are frequently used as sugar substitutes (Manisha *et al.,* 2012). Stevioside, the major component of *Stevia rebaudiana* Bertoni, is extensively used as a non-caloric natural sweetener in food industry which is approximately 300 times sweeter than sucrose (Manisha *et al.,* 2012; Wang *et al.,* 2012). Lowering blood pressure, treating obesity, decreasing blood sugar and potentiating insulin secretion are exclusive therapeutic characteristics of stevioside (Wang *et al.,* 2012). Stevioside provides zero calories in a wide range of beverages and foods and well suited for blending with other non-calorie or carbohydrate sweeteners. The stevioside molecule is stable under dry conditions and in aqueous food systems. Its stability is significantly better than other artificial sweeteners like aspartame (Hamzah *et al.,* 2013). Several studies performed on substituting stevia in sugar containing bakery products such as pound cake (Schirmer *et al.,* 2012), muffins (Zhan *et al.,* 2012), yoghurt cake (Abdel-Salam *et al.,* 2009) and bread (Parimalavalli, 2007) but no extensive research have been done on high caloric sugar-contain biscuits. Therefore in this study we sought to address the effect of stevioside substituting on some physical, chemical and sensory properties of biscuit.

2.5. Effects of steviosides on biochemical parameters

2.5.1. Effects of stevia or aspartame administration on food consumption, body weight, and blood glucose levels of control and diabetic rats

Stevia and Aspartame treated rats showed a significant reduction in food consumption by 23% and 9 %, respectively in comparison to control rats ($P<0.05$). Stevia and Aspartame treated rats showed a significant reduction in body weight by 27 % and 18 % respectively, compared to control rats ($P<0.05$). However, serum blood glucose didn't changed ($P>0.05$) when compared control rats with both treatments. Alloxan administration to control rats resulted in significant increase in food consumption by 20% and increase in serum blood glucose levels by 333%. These changes in diabetic group were associated with a slight decrease in body weight compared to control.

Treatment of diabetic group with stevia or Aspartame significantly decreased food consumption by 24% and 13% respectively. Serum blood glucose was significantly decreased by 38% with Stevia treatment and 50% with Aspartame treatment when compared to diabetic group.

However, this improvement did not reach basal levels of their corresponding groups ($P<0.05$). Notably, food consumption is significantly higher in diabetic rats treated with Aspartame than in diabetic rats treated with stevia. Although, serum blood glucose in diabetic rats treated with aspartame is lower than diabetic rats treated with stevia.

2.5.2. Effects of stevia or aspartame administration on liver functions (ALT, AST, ALP) of control and diabetic rats

To investigate the effect of Stevia or Aspartame administration on liver function, the serum levels of ALT, AST and ALP were measured. Stevia-treated rats showed no change in ALT, AST and ALP levels when compared to control rats, while Aspartame-treated rats showed significant increase in ALT, AST and ALP levels by 3.3, 4.2 and 1.8 folds respectively. Liver enzymes were highly elevated in diabetic rats in comparison to control rats. Treatment of diabetic rats with Stevia significantly lowered ALT, AST and ALP levels. However, Aspartame had no effect on the levels of these enzymes. Stevia treatment of diabetic rats lowered ALT by 62%, AST by

57% and ALP by 41%. Furthermore, by comparing the liver functions in both Stevia-treated diabetic and Aspartame-treated diabetic groups, we found that Stevia had significantly prominent reduction in serum ALT, AST, ALP levels ($P<0.05$).

2.5.3. Effects of stevioside or aspartame administration on kidney functions (Urea and Creatinine) of control and diabetic rats

Both serum urea and creatinine levels were estimated to investigate the effect of Stevia or Aspartame administration on kidney function. Aspartame administration, unlike Stevia, showed a significant elevation ($P<0.05$) in urea and creatinine levels in comparison to control. Alloxan induced diabetic group showed a significant deterioration in kidney function, as demonstrated by the elevation of urea levels 2 folds and creatinine levels 10 folds, compared to control rats.

Treatment of diabetic rats with Stevia significantly ameliorated renal function. The serum urea levels decreased by 16% and creatinine decreased by 65%. Aspartame treatment of diabetic rats did not improve kidney function.

2.5.4 Stevioside effects on renal function

Abundance of information was offered by traditional herbalism regarding the treatment of the kidney disease. The presence of diterpene glycosides in the herb *S. rebaudiana* exhibits a high degree of natural antioxidant activity and is used as high potency sweeteners.(Toskulkao *et al*.,1994) studied the interaction between urinary enzyme levels and changes in plasma creatinine, and blood urea nitrogen levels in rats treated with stevioside by means of concurrent changes of the kidney. There is an increase in blood urea nitrogen after subcutaneous injection with stevioside (1.5 g/kg BW) at 3 h onward. The blood urea nitrogen and creatinine level increases to a maximum after stevioside injection with approximately 180 and 132% at 9 hours. At this point in time stevioside causes a significant increase in alkaline phosphatase (AP), glucosuria and glutamyl transpeptidase (GTP), however, no significant changes in proteinuria, n-acetyl-d-glucuronidase (NAG), or glutathione-S-transferase (GSH-S-TF). Degeneration of the proximal convoluted tubule cells was detected after histopathological examination of the kidney induced by stevioside but no lipid peroxidation was found. Results revealed that stevioside causes nephrotoxicity at the proximal convoluted tubules rather than at the other tubules or glomeruli. It was most probably by a defect of the cell volume regulation due to depletion of

intracellular ATP and disruption of microvilli, and nuclear dysfunction. In another study by (Melis *et al.,*1992) the effect of stevioside from *S. rebaudiana* leaves on the renal function of normal and hypertensive rats was studied. The examined stevioside functions as a systemic vasodilator which aggravates hypotension, diuresis, and natriuresis in both the normal and hypertensive rats. The administration of stevioside continuously to both normal and hypertensive rats increased the renal plasma flow and glomerular filtration rate, which was due to the vasodilation of both the afferent and efferent arterioles. A similar study shows that long-term oral intake or acute intravenous administration of stevioside lead to a decreased plasma volume producing diuresis and natriuresis. On the other hand, the infusion of stevioside directly into rats' artery induces diuresis. This reaction was due to decreased proximal tubular reabsorption as indicated by lithium clearance (Chatsudthipong & Thongouppakarn *et al.,* ., 1995), signifying that stevioside targets at the proximal tubule of the kidneys. This research was designed to investigate the effect of stevioside on the transepithelial transport of p-aminohippurate in isolated S_2 segments of rabbit proximal renal tubules using in vitro micro-perfusion. The result shows that stevioside, at a concentration of 0.70 mM, inhibits the transepithelial transport of p-aminohippurate by interfering with the basolateral entry step, the rate-limiting step for transepithelial transport. The absence of the effect of stevioside on the transepithelial transport of p-aminohippurate on the luminal side and its reversible inhibitory effect on the basolateral side indicate that stevioside does not permanently change the p-aminohippurate transport and does not harm the renal tubular function at normal human intake levels (Jutabha *et al.,* 2000). (Hashemi *et al.,* 2014) investigated the feasible protective effects of rebaudioside A on acetaminophen (APAP)-induced oxidative stress in the kidney of mice. The oxidative stress was induced in the kidney of BALB/c mice by the intraperitoneal (i.p.) administration of a single dose of 300 mg/kg acetaminophen. Thirty minutes after acetaminophen injection a number of these mice were treated with rebaudioside A (700 mg/kg) (i.p.). Later, after two and six hours of acetaminophen injection all BALB/c mice were sacrificed and glutathione (GSH), malondialdehyde (MDA), free acetaminophen, and glutathione conjugated acetaminophen (APAP-GSH) were determined in the kidney tissues of sacrificed mice. Thus, findings suggest that though rebaudioside A was not successful in preventing the initiation of acetaminophen induced oxidative stress, as indicated by GSH depletion and lipid

peroxidation in kidneys of mice, but after wards it attenuated lipid peroxidation by reducing acetaminophen conversion to its activated metabolite, specifically n-acetyl-p-benzoquinone imine (NAPQI), which produced APAP-GSH conjugate in kidneys of mice. Thus, rebaudioside A acts as a principal compound in the alleviation of acetaminophen induced oxidative stress in kidneys of mice after acetaminophen overdoses.

2.6. Medicinal uses of the stevia plant

Studies on food safety, including an extensive review of the literature, under taken prior to 1982 (Lee 1979; Kinghorn 1982) concluded that Stevia leaves and extracts are safe; studies since then confirm this. Possible medicinal uses have been investigated often by using Stevia extracts as intravenous infusions in rats; possible effects on glucose metabolism, diuresis, organ weights, endocrine function, and so on, have been studied in this way (Kinghorn *et al.,* 1987; Nunes and Pereira *et al.,* 1988; Oliveira Filho *et al.,* 1988; Suanar-unsawat and Chaiyabut *et al.,* 1996, 1997). Stevia extract infusions have also shown some anti-androgenic activity in rats (Sincholle *et al.,* 1989). Likely beneficial effects of Stevia extracts, as antioxidants and to relieve blood pressure and hypertension, have also been shown (Chan *et al.,* 1998; Xi 1998; Xi *et al.,* 1998). Steviol (aprecursor in the biosynthesis of steviosides) can be produced from steviosides experimentally using specific bacteria but not in sit in the human body. Steviol can exhibit some toxic and mutagenic activity (Tateo 1990). Investigations of the effect of aqueous extract of *S. rebaudiana* leaves on glucose tolerance have been carried out by (Curi *et al.,* 1986) on volunteers. Aqueous extract of 5 g leaves were administered to volunteers at regular 6-hourly intervals for 3 days, with glucose tolerance tests performed before and after extract administration. The extract increased glucose tolerance; it significantly decreased plasma glucose evels during the test and after overnight fasting in all volunteers. In Japan, where artificial chemical sweeteners are not approved, many toxicology safety studies have been conducted (Elton Johnson *et al.,* 1990). Among studies carried out are some to investigate carcinogenicity and mutagenicity (if any) in animal testing (Oliveira Filho *et al.,* 1988; Toruan-Mathius *et al.,* 1995; Toyoda 1997), to show dental benefits in the form of plaque inhibition and cavity reduction (Elton-Johnson *et al.,* 1990), to confirm the safety of Stevia for diabetic use (Polyanskii *et al.,* 1997; Thamolwan and Narongsak *et al.,* 1997). The safety of

feeding to animals, chickens and humans has also been confirmed by a wide range of studies (Sincholle and Marcorelles *et al.,* 1989; Smolyar *et al.,* 1993; White *et al.,* 1994; Melis 1995, 1997; Suanarunsawat and Chaiyabut 1996, 1997; Wood 1996; Polyanskii *et al.,* 1997). The traditional method of use by the Paraguayan Guarani Indians was to dry the leaves and to use them to sweeten tea and medicines or to chew the leaves as a'sweet treat'. Stevia was regularly used in drinks many times a day, not just occasionally, with no side effects. The use of dried leaves (pieces or powdered) is unacceptable in domestic cooking and does leave a sediment in clear drinks, and so forth, and can also leave a green color. There may also be an unpleasant aroma associated with the dried leaves. Appropriate processing of the dry herbage may remove this aroma, which is due to specific leaf compounds (not steviosides) (Tsanava *et al.,* 1991). Although stevia has been used without any problems for many years in its native Paraguay and in other countries for lesser periods, health and safety issues have been receiving considerable attention in the past 20 years. There has been considerable media attention in the USA, including claims and counterclaims before the US FDA. Many of these claims relate to its potential competitive position in relation to aspartame. Stevia products have been approved for use in the USA as nutrition supplements although many protagonists claim it should be granted generally regarded as safe status in the same manner as tea, coffee, sugar and fruit and vegetables, and so on. The general safety of steviosides could be largely due to the fact that they are not broken down nor are absorbed in the digestive tract (Hutapea *et al.,* 1997). Bacteriological studies on hot water extract from *S. Rebaudiana* have been carried out by (Tomita *et al.,* 1997). Lactobacilli were not killed on exposure to the fermented extract; however, under acidic conditions, the extract was found to be bactericidal. In Japan, artificial sweeteners were banned some 40 years ago so Stevia has been their chosen alternative to sweeten their food and beverages. The Japanese have performed over 40,000 clinical studies and found Stevia to be safe. Stevia in its raw form, although incredibly sweet, has a very subtle liquor rice essence to it. A sign of an excellent Stevia product is one that is free of this liquor rice essence and still not bitter (Tateo *et al.,* 1998). (Genus Jan *et al.,* 2002) concluded that Stevia and stevioside are safe when used as a sweetener. Stevia is suited for both diabetics and Phenylketonuria(PKU) patients, as well as for base persons intending to lose weight by avoiding sugar supplements in the diet. No allergic reactions to it seem to exist.

(Midmore and Rank *et al.,* 2002) found that the aqueous extracts of the leaves boiled in water, cooled, then strained (filtered) are preferred in many situations and are better suited for controlled levels of sweetening. Crystalline powders and extracts are preferred in commercial situations as they have a fixed known sweetening value. Fixed concentration liquids are also acceptable. (Kumar *et al.,* 2007) reported that the Stevia is sweetest plant in the world because leaves contain diterpene glycoside that has a sweet taste but it is not metabolized and contains no calories. It is native to a relatively small area of eastern Paraguay (on the Brazilian border) where its leaves have been used by the local Guarani Indians as a sweetener for many hundreds of years. They specially used it in the local green tea (Mate tea-Hex sp.), as well as with other unpalatable medicinal and other drinks. The leaves are 30 times sweeter than cane sugar and can be safely used by diabetic patients. (Sharma and Mogre *et al.,* 2007) observed the effect of consumption of Stevia extract on 20 selected hypercholestronic women: 20 ml extract was used to intervene in one subject in a glass of water (200 ml). They found the consumption of Stevia extract reduces the levels of cholesterol, triglyceride and low density lipoprotein cholesterol significantly while an increase in high density lipoprotein cholesterol was noted, which is desirable. They concluded that Stevia extract had a hypolipidaemic effect used to reduce the resistance of cardiovascular disease. The documented properties of Stevia are anti-bacterial, anti-fungal, anti-inflammatory, anti-microbial, anti-viral, anti-yeast, cardiotonic, diuretic, hypoglycaemic, hypotensive and as a vasodilator. Stevia has an advantage over artificial sweeteners because it is stable at high temperatures and has a pH range 39. Stevia extract is used as a sweetener or flavour enhancer in many countries such as China, Japan, Korea, Israel, Brazil and Paraguay. It is also used in soft drinks, ice creams, cookies, pickles, chewing gum, tea and skincare products (Lee *et al.,* ., 1979; Kinghorn 1982, 1987; Elton Johnson et al.,1990; Tateo *et al.,* 1990). Stevia plant and its extract both are used in weight loss programmes because of their ability to reduce the craving for sweet and fatty foods (Jain *et al.,* 2007).

2.7 Therapeutic benefits

2.7.1 Hypoglycemic effect

Stevia rebaudiana Bertoni has been used as a hypoglycemic substance for hundreds of years in Paraguayan and Brazilian medicine. In 1986, observations were conducted on 16 healthy individuals drinking aqueous extracts of 5 g *S. Rebaudiana* leaves or arabinose solution for 3 days at 6-h intervals. Glucose tolerance tests were conducted before and after intervention. An increased glucose tolerance was observed among the individuals administered stevia extracts (Curi *et al*.,1986). The hypoglycemic action of stevia glycosides was confirmed in a study by (Gregersen *et al.,* 2004) conducted with twelve participants (four women and eight men). The analyses were conducted on patients with at least 4-year history of type 2 diabetes, who were supplemented daily with 1 g stevioside or 1 g maize starch added to a meal. A lesser increase in postprandial glycemia and the insulin index was observed in the group of individuals administered steviosides. Similar conclusions were presented by Taiwanese researchers, who investigated the effect of steviol glycosides on the metabolism of glucose and insulin in two groups of diabetic rats (Chen *et al.,* 2005). In the first group, hyperglycemia was induced using Streptozotocin, while in the other group diabetes was induced by administering fructose (insulin-dependent diabetes). Stevioside intake resulted in a reduced insulin resistance in these animals and a simultaneous suppression of glucagon secretion. Stevioside is capable of reducing the amount of glucagon secreted, probably thanks to the increased mRNA expression of carnitine palmitoyl transferase, peroxisome proliferator activated receptor alpha (PPAR-alpha) and stearoyl-CoA desaturase (Chen *et al.,* 2005; Thomas and Glade *et al*.,2002). A study by (Jeppesen *et al.,* 2003) conducted on GK rats (a non-obese Wistar substrain which develops type 2 diabetes mellitus early in life) showed that intake of 0.025 g/kg b.w. stevioside has a hypoglycemic effect. This effect is a scribed to an increased secretion of insulin and induction of genes associated with glycolysis. Stevioside does not only turn out to be a substance exhibiting a hypoglycemic action, but a similar effect may also be ascribed to rebaudioside A, which was confirmed by Danish researchers when investigating the effect of this compound on the release of insulin from pancreatic islets (Abudula *et al*.,2004). It was found that stimulation of insulin secretion is dependent simultaneously on the dose of stevioside and the presence of

extracellular Ca^{2+}ions. (Mohd-Radzman *et al.,* 2013) observed that stevioside has direct effects on 3T3-L1 insulin sensitivity via increase glucose uptake and enhanced expression of proteins involved in insulin-signaling pathway. Increased insulin secretion is connected with the closure of ATP-dependent potassium channels, which results in the depolarisation of pancreatic beta cells and activation of Ca^{2+} channels. (Saravanan *et al*.,2012) evaluated the effect of rebaudioside A on the activity of hepatic enzymes participating in carbohydrate metabolism in rats with induced diabetes. Those authors showed that rebaudioside A considerably reduces blood glucose concentration and exhibits a protective action towards the pancreas. The hypoglycemic action of rebaudioside A and stevioside may be explained by similarities in their structure. (Holvoet *et al.,* (2014) observed that glycosides of stevia improve metabolism of glucose, fat catabolism, bile acids metabolism, storage and transport of lipids in the liver of insulin resistant obese mice.

2.7.2. Antihypertensive effect

Physiological and Pharmacological experiments have suggested that stevioside from the leaves of stevia act as a typical systemic vasodilator. (Melis *et al*.,01996) in their studies have demonstrated that stevioside from *stevia rebaudiana* leaves provoked hypotension, diuresis and natriuresis in both normal and hypertensive rats. An increase in the renal plasma flow and glomerular filtration in rats had been observed in normal rats and the effect was attributed to the vasodilatation of afferent and efferent arterioles (Melis *et al.,* 1996, 1995). Human studies have also suggested its beneficial role in hypertension for its vasodilator property. It was suggested that 750 – 1500 mg/ day of stevioside, reduces systolic blood pressure by 10 – 11 mmHg and diastolic blood pressure by 6 -14 mmHg within one week of starting the treatment (Ferri *et al.,* 2006 and Maki *et al.,* 2008). It is found that stevioside causes vasorelaxation by inhibition of Ca^{++} influx into the blood vessels (Ulbricht *et al.,* 2010). Therefore stevia could prove to be beneficial in hypertensive patients.

2.7.3. Antioxidant activity

Free radicals are considered to be the causative agents in the development of neurological diseases, inflammations, reduced immunity, ageing, ischaemic heart disease, stroke, Alzheimer's and Parkinson's disease as well as cancer (Hou*et al.,* 2003; Parejo*et al.,* 2002). Leaves of *Stevia rebaudiana* were reported to contain

polyphenolic compounds having antioxidant properties (Muanda *et al.,* 2011; Shukla *et al.,* 2009; Tadhani *et al.,* I., 2007). Antioxidants are compounds that have gained importance in recent years dueto their ability to block the action of free radicals (Devasagayam *et al.,* 2004). Varieties of antioxidants were obtained from the extracts of Stevia rebaudiana, they include, opigenin, kaempferol, and quereitrin that inhibited DNA strand damage (Ghanta *et al.,* ., 2004 and Stoyanova *et al.,* 2011). Phenolic compounds have antioxidant activity. Contents of flavonoid and other phenolic substance act against the development of cancer and heart disease (Kahkonen *et al.,* 1999). Antioxidants are compounds that interact with and neutralize free radicals. Therefore, in recent years, considerable attention has been directed towards the identification of plants with antioxidant potential (Shukla *et al.,* 2011). There are many different antioxidants present in plants and it is very difficult to measure each antioxidant component separately. Therefore, several methods have been developed to measure the antioxidant activity. Among them, Trolox equivalent antioxidant capacity (TEAC), total radical absorption potentials (TRAP), oxygen radical absorption capacity (ORAC), as well as the ferric reducing ability of plasma (FRAP) are commonly used and are the representative methods frequently used in scientific investigations (Tadhani *et al.,* 2007; Evelson *et al.,* 2001; Ou *et al.,* 2001; Benzie and Strain *et al*, 1996). The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay is another method that can accommodate a large number of samples in a short period of time and is sensitive enough to detect natural compounds at low concentrations (B Ahmad *et al.,* 2010), where the antioxidant activity is determined as the percentage inhibition of the DPPH free radical (Turkmen *et al.,* 2005). A recent study assessing the in vitro potential of ethanolic leaf extract of *S. rebaudiana* indicates that it has a significant potential for use as a natural antioxidant (Shukla *et al.,* 2009).

2.7.4. Antimicrobial activity

There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action due to an alarming increase in the incidence of new and re-emerging infectious diseases and development of resistance to the antibiotics in current clinical use (Cowan *et al.,* 1999). The screening of plant extracts has been of great interest to scientists in the search for new drugs for effective treatment of several diseases. Therefore, plant

extracts and phytochemicals with known antimicrobial properties can be of great significance in therapeutic treatments (Jayaraman *et al.,* 2008). The results of an investigation performed in the late 19th and 20th century and the advent of streptomycin and other antibiotics provide the ground for experimentation of a vast number of plants for antibiotic or antimicrobial activities that are useful to man (Doss & Dhanabalan *et al*, 2009). Many plant leaves have antimicrobial principles such as tannins, essential oils and other aromatic compounds. In addition, many biological activities and antibacterial effects have been reported for plant tannins and flavonoids. Plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen substituted derivatives. These compounds protect the plant from microbial infection and deterioration. Some of these phytochemicals can significantly reduce the risk of cancer due to polyphenol antioxidant and anti-inflammatory effects. Some preclinical studies suggest that phytochemicals can prevent colorectal cancer and other cancers (Jayaraman *et al.,* 2008).Stevia is thought to inhibit the growth of certain bacteria and other infectious organisms (Patil *et al.,* 1996; Sivaram & Mukun-dam *et al*, 2003). Some people even claim that using Stevia helps to pre-vent the onset of colds and flu. The ability of Stevia to inhibit growth of certain bacteria helps to explain its traditional use intreating wounds, sores and gum disease. It may also explain why the herb is advocated for anyone who is susceptible to yeast infections or reoccurring streptococcal infections, two conditions that seem to be aggravated by white sugar consumption (Debnath *et al.,* 2008). Antimicrobial activities of various herbs and spices in plant leaves, flowers, stems, roots, or fruits have been reported by manyresearchers. In some studies the antimicrobial activity of various extracts of *S. rebaudiana* (with water, acetone, chloroform, methanol, ethyl acetate or hexane as solvents) have been investigated and its effect on some selected microorganisms such as Salmonella typhi, Aeromonas hydrophila, Vibrio cholerae, Bacillus subtilis, Staphylococcus aureusand others have been examined (Debnath *et al.,* 2008; Ghosh, Subudhi, & Nayak *et al.,* 2008; Jayaraman *et al.,* 2008; Seema,2010; Tadhani & Subhash *et al*, 2006b). The biological activity for Stevia compounds has been studied by (Tomita *et al*.,1997); they studied the bactericidal activity of a fermented hot water extract from *S.rebaudiana* Bertoni towards entero haemorrhagic Escherichia coli and other food borne pathogenic bacteria. Other microorganism's

like Salmonella typhimurium, B. subtilis, and S. aureushas also been found to be inhibited by the fermented leaf extract (Debnath *et al.,* 2008; Ghosh *et al.,* 2008).

2.7.5. Anti-inflammatory and immune-modulatory effect

Stevia has been found to attenuate synthesis of the inflammatory mediators in LPS stimulated THP-1 cells by interfering with the I Kappa B kinases (IKKbeta) and Kappa B signaling pathway thus beneficial as anti-inflammatory and immunomodulatory substance (Bookaewan *et al.,* 2006). Stevia is also rich in beta carotene, ascorbic acid, protein, calcium, iron, magnesium, phosphorus and numerous other phytochemicals. Hence the herbal derivative apart from its sweetening property also is beneficial with its nutritive value. Other proposed uses include alcohol abuse, anti-inflammatory, anti-mutagenic, antitumor, diuretic, digestive aid, food additive, immunomodulation and obesity (Chatsudthipong *et al.,* 2009).

2.7.6. Cariogenic and mutagenic effects

Since Stevia products are used as sugar substitutes by many populations, a study was conducted to test whether stevioside and rebaudioside A may have the potential of causing dental caries from prolonged use. Rats were fed a diet containing 0.5%stevioside or 0.5% rebaudioside A for 5 weeks. Neither compound showed a potential of increasing the risk of developing dental caries (Das *et al.,* .,1992). Several researchers investigated the risk of mutagenicity. In two studies (Matsui *et al.,* 1986; Pezzuto *et al.,* 1996), steviol produced a dose related positive mutagenic effect in some tests. In the same studies, stevioside was found to be devoid of this effect. Other reports indicated lack of mutagenicity of both compounds (Suttajit *et al.,* 1993; Klongpanichpak *et al.,* 1997). Because of these contradictory reports, the Food and Drug Administration is still cautious in introducing this herb as a sugar substitute until its safety is completely established (FDA *et al.,* 1999).

2.7.7. Anti-tumor activity

An antitumor study of stevioside was examined and stevioside suppressed 12-O tetradecanoylphorbol-13-acetate (TPA) induced tumor promotion in a skin carcinogenesis in mice (Nakamura *et al.,* 1995). (Jayaraman *et al.,* 2008) used MTT

(3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay to evaluate cytotoxicity based on metabolic reduction of MTT.

2.8. Biochemical and nutritional aspects of stevia

(Savita, and Ramakrishna et al.,2004) analysed Stevia leaves on a dry weight basis and calculated an energy value of 2.7 kcal g1 . This means that Stevia may be granted the status of a low calorie sweetener, since its sweetness is intense and comparable to that of other commercial sweeteners. Intense sweeteners include acesulfame K (calorie-free), aspartame (4 kcal g1), saccharin (calorie-free) and sucralose (calorie-free) (Savita *et al.,* 2004). Calorie contribution to the diet by the commonly used saccharose, which is considered high since it is metabolized completely by the body, has a potential to escalade towards overweight status. In this context, the use of Stevia as a low-calorie sweetener could be of immense help in restricting or controlling calorie intake in the diet.

2.8.1 Functional properties of stevia leaf powder

According to (Mishra *et al.,* 2010) Stevia leaf presents values of bulk density of 0.443 g ml1 , water holding capacity of 4.7 ml g1 , fat absorption capacity of 4.5 ml g1 , emulsification value of 5.0 ml g1 , swelling index of 5.01 g g1 , solubility of 0.365 g g1 and pH of 5.95. Bulk density of Stevia leaf powder appeared to be low in comparison to protein rich pulses. Higher bulk densities are usually desirable for the purpose of reducing paste thickness, an important factor in child feeding where bulk is of concern. However, Stevia leaf powder appears to lack this property. On the other hand, the study of (Mishra *et al.,* 2010) showed an increased water holding capacity of the Stevia leaf powder, which appears to be advantageous and may be due to high protein content. Proteins would increase water holding capacity, thus enhancing the swelling ability, an important function of protein in preparation of viscous foods such as soups, gravies, dough and baked products. The ability of protein to aid the formation and stabilization of emulsion is also critical in many foods applications, such as cake, batters, coffee whiteners, milks, frozen desserts and others. This property depends heavily on composition and stress under which the product is subjected during processing (Savita *et al.,* 2004). Fat absorption capacity has been attributed to the physical entrapment of oil. Stevia leaf powder seems to possess an adequate fat absorption capacity, allowing it to play an important role in

food processing, since fat acts on flavor retainers and increases mouth feel of foods. (Crammer and Ikan .,1986) affirmed that since stevioside is stable at 95 C it is a suitable sweet additive for cooked or baked foods. The leaves, as well as the pure stevioside extracts, can be used in their natural state or cooked, and are thermostable at temperature up to 200 C (Serio *et al.,* 2010). Incubation of the solid sweetener stevioside at elevated temperatures for 1 h showed good stability up to 120 C, whilst at temperatures exceeding 140 C forced decomposition was seen which resulted in total decomposition by heating at 200 C (Abou-Arab, Abou-Arab, & Abu-Salem *et al.,* 2010). (Chang and Cook *et al*.,1983) reported that Stevia sweeteners have high heat stability after 1 h heating at 100 C. Besides, it was also reported that stevioside and rebaudioside A are reasonably thermally stable under the elevated temperatures used in food processing and do not undergo browning or caramelization when heated (Abou-Arab *et al.,* 2010).

2.8.2. Carbohydrates

Carbohydrates perform numerous essential roles in living beings. Thus, monosaccharides are the major source of energy in human metabolism, while polysaccharides serve as the storage of energy and can act as structural components. Other beneficial health effects have also been linked to these compounds. This includes a prebiotic effect as well as other less common antioxidant or anti-inflammatory activities (Bernal, Mendiola, Ibáñez, & Cifuentes *et al.,* 2011). The benefits associated to Stevia leaf are mainly due to their nutritional composition, which is a good source of carbohydrates, protein and crude fibre, that promotes wellness and reduces the risk of certain diseases. In S. rebaudiana roots and leaves, inulin type fructooligosaccharides, a naturally occurring plant polysaccharide with important functional properties related to prebiotics, dietary fibre, role lipid metabolism and diabetes control, have been isolated by (Braz de Oliveira *et al.,* 2011). They obtained from the roots and leaves of the plant a yield of purified fructooligosaccharides of 4.6% and 0.46%, respectively. This indicates a possible application of extracts as a dietary supplement (Braz de Oliveira *et al.,* 2011).

2.8.3. Proteins

Proteins, peptides and or amino acids are found in a great variety of matrices including animals, fungi, vegetables, cereals, etc. (Bernal *et al.,* 2011). Proteins are

molecules composed of amino acids necessary for growth and repair of body tissues. Their importance lies mainly in that they are an essential constituent of cells and need to be replaced over time, which makes protein intake indispensable. To determine the protein quality of a food it is necessary to know the total protein content as well as the kinds of amino acids present, especially the content of the essential amino acids (Latham *et al.,* 2002). (Mohammad, Sher, Habib, and Iqbal et al.,2007) identified nine amino acids in Stevia leaves, namely glutamic acid, aspartic acid, lysine, serine, isoleucine, alanine, proline, tyrosine and methionine. (Abou-Arab *et al.,* 2010) found still more amino acids in the Stevia leaves. Altogether seventeen amino acids were determined and classified as essential and non-essential amino acids, unfortunately including arginine as one of the indispensable amino acids. According to the report of a joint FAO/WHO/UNU Expert Consultation (WHO *et al.,* ., 2007), the indispensable amino acids are leucine, isoleucine, valine, lysine, threonine, tryptophan, methionine, phenylalanine and histidine. The daily requirements of these amino acids in human nutrition are also summarized. This shows that Stevia leaves contained almost all of the indispensable amino acids, including tyrosine and cysteine. Only the amino acid tryptophan is missing. This means that after extraction of stevioside from the leaves, the residue could be a valuable source of indispensable amino acids for health products. Their content can match the protein requirements recommended by the World Health Organization (WHO *et al.,* 2007).

2.8.4. Minerals

Minerals have many important functions in the human body. Some mineral elements are needed only in very small amounts in human diets, but are vital for metabolic purposes, and are thus called essential trace elements (Latham *et al*, 2002). The elements considered essential or required for the normal functioning of the body, are classified according to their relative amounts or requirements. The main elements are sodium, magnesium, phosphorus, sulphur, chlorine, potassium, and calcium which are classified as macronutrients and the minor elements, considered micronutrients, are chromium, manganese, iron, cobalt, copper, zinc, selenium, molybdenum and iodine (Adotey *et al*, 2009; Szefer & Nriagu *et al.,* 2007). The presence of macro and micronutrients in foods is important for the development and maintenance of vital body functions. They are involved in all aspects of growth,

health and reproduction, participating also in the formation of cells, tissues and organs (Szefer & Nriagu *et al.,* 2007). Stevia contains substantial amounts of these important nutrients, which further establishes it as a mineral loaded ingredient needed to protect the body, regulate and maintain the various metabolic processes. Potassium, calcium, magnesium, and sodium which are nutritionally important, were found in reasonable amount in Stevia leaves. The high concentration of these minerals would be very beneficial to health (Choudhary & Bandyopadhyay *et al.,* 1999). As reported by some authors, the mean concentrations of macro and micro elements that have been determined in dried Stevia leaves are show the high content of potassium determined in all studies is remarkable, although the amount of potassium found by (Abou-Arab *et al.*,2010) seems to be very low compared to that of the other studies, which may be explained by different growth conditions, as described by (Rahmesh, Singh, and Megeji *et al.,* 2006). Zinc and iron are found in foods of plant and animal origin and are present in Stevia leaves. According to (Wu et al.,2005), zinc is a mineral that acts as a non-enzymatic antioxidant, so that its consumption would help in preventing oxidative damage of the cell. The main biological function of iron is the transport of oxygen to the body and consequently a lack of this mineral in the diet leads to anaemia. The high amount of iron in Stevia leaves could again be helpful in contributing to the maintenance of a normal haemoglobin level in the body. Furthermore, Stevia leaves could also be used to prepare various sweet preparations to combat iron deficiency in anaemia which is a major nutritional disorder in developing countries (Abou-Arab *et al.,* 2010).

2.8.5. Lipids

Lipids are a large group of natural compounds. Their main biological functions include energy storage, structural components of cell membranes and important signalling molecules. Although humans and other mammals use various biosynthetic pathways to both break down and synthetize lipids, some essential lipids cannot be made in this way and must be obtained from diet. Interestingly, many papers have discussed the health benefits that can be derived from some of these lipids (Bernal *et al.,* 2011). Fatty acids are carboxylic acids with a variable unbranched aliphatic tail (chain), which is either saturated or unsaturated. They are important as nutritional substances in living organisms. Long chain polyunsaturated fatty acids (PUFA), especially those of the n-3 series, such as a linolenic acid (18:3 n-3), are essential

for human metabolism and have many beneficial effects including the prevention of a number of diseases, such as coronary heart diseases, inflammation, autoimmune disorders, hypertension, hypotriglyceridemic effects (Bernal *et al.,* 2011). Linolenic acid, which is as healthy as the linoleic acid, is considered an essential fatty acid (EFA) necessary for good health. EFAs are important in the synthesis of many cellular structures and several biologically important compounds (Latham *et al.,* 2002). Moreover, other polyunsaturated fatty acids are essential for the human body, performing many functions such as maintenance of cell membranes and production of prostaglandins (regulators of many body processes, including inflammation and blood clotting). Fats are also needed in the diet as input for fat soluble vitamins in foods (A, D, E and K) and can be absorbed to regulate cholesterol metabolism (Pinazo *et al*.,2008). In the leaf oil of Stevia, (Tadhani and Subhash *et al.,* 2006) identified six fatty acids using methyl ester standards. Palmitic, palmitoleic, stearic, oleic, linoleic and linolenic acids were identified in the leaf oil. Among the identified fatty acids, palmitic acid content was found to be highest, whereas stearic acid content was least. Stevia leaf oil proves to be a rich source of linolenic acid. This high value of linolenic acid may contribute to maintain an ideal fatty acid ratio in human diet.

2.8.6. Vitamins

Vitamins are organic substances present in very small quantities in food, but necessary for metabolism. They are grouped together not because they are chemically related or have similar physiological functions, but because they are vital factors in the diet and they all were discovered in connection with the diseases that were caused owing to their deficiency (Latham *et al*, 2002). They are classified as either water soluble or fat soluble. There are 13 vitamins: 4 fat soluble (A, D, E and K) and 9 water soluble (8 vitamins of the B group and vitamin C). These compounds have diverse biochemical roles. Some have hormone like functions as regulators of mineral metabolism (e.g., vitamin D), or regulators of cell and tissue growth and differentiation (e.g., some forms of vitamin A). Others work as antioxidants (e.g., vitamin E and sometimes vitamins B and C). The largest numbers of vitamins (e.g. B complex vitamins) work as precursors of enzyme cofactors (Bernal *et al.,* 2011). The protective effects of plant products are due to the presence of several components that have distinct mechanisms of action; some are enzymes and proteins, and others

are low molecular weight compounds like vitamins (Halliwell, Gutteridge, & Arurma *et al.,* 1987). It has been reported that the levels of plasma antioxidant vitamins and minerals such as vitamin C, E, folic acid, and zinc declined as oxidative damage increased in stressed animals (Sahin, Kucuk, Sahin, & Sari, 2002). (Kim, Yang, Lee, and Kang *et al.,* 2011) studied the amounts of water-soluble vitamins in the Stevia leaf and callus extracts, and determined that the contents of folic acid, vitamin C and vitamin B2 in the leaf extracts were significantly higher than those of the callus extracts. In the leaf extract, folic acid was found to be the major compound, followed by vitamin C. In the callus extract, vitamin C was the major compound, followed by vitamin B. vitamins) work as precursors of enzyme cofactors (Bernal *et al.,* 2011). The protective effects of plant products are due to the presence of several components that have distinct mechanisms of action; some are enzymes and proteins, and others are low molecular weight compounds like vitamins (Halliwell, & Arurma *et al.,* 1987). It has been reported that the levels of plasma antioxidant vitamins and minerals such as vitamin C, E, folic acid, and zinc declined as oxidative damage increased in stressed animals (Sahin & Sari *et al*, 2002). (Kim, Yang, Lee, and Kang *et al.,* 2011) studied the amounts of water soluble vitamins in the Stevia leaf and callus extracts , and determined that the contents of folic acid, vitamin C and vitamin B2 in the leaf extracts were significantly higher than those of the callus extracts. In the leaf extract, folic acid was found to be the major compound, followed by vitamin C. In the callus extract, vitamin C was the major compound, followed by vitamin B.

2.9 Conclusion

Stevia rebaudiana is a sweet diterpenoid glycosides containing plant of Asteraceae family. Stevioside and rebaudioside-A are two major sweet diterpene glycosides present mostly in the leaves. Stevioside is 300 times sweeter than sugar but with bitter after taste however, rebaudioside-A is having more sweeteness than stevioside. There are thousands of patents, literature available for extraction, purification and quantitative estimation of steviol glycosides. In this review, we have tried to provide the all available information regarding different extraction, purification and estimation processes of steviol glycosides. Extraction methods are generally categorised into conventional and non-conventional methods. Modern extraction or non-conventional extraction methods are the most popular methods for the same

because of its merits like reduction in extraction time, higher extract yield and less solvent consumption. But, still conventional methods are being used for steviol glycosides extraction in small scale industries due to its lower cost.

CHAPTER III

MATERIALS AND METHODS

The study was conducted in Biochemistry and Molecular Biology Laboratory, Hajee Mohammad Danesh Science and Technology University, Dinajpur, Bangladesh.

3.1 Materials

3.1.1 Experimental materials

Wheat flour, salt, milk powder, vanilla, stevioside powder, baking powder, food-grade oils were used to make biscuits. These ingredients were collected from the local market in Dinajpur.

3.1.2 Stevioside powder

We have collected stevioside powder from Japan. We prepared diabetic biscuit using this powder.

3.1.3 Animals

12 male rabbits weighing between 650 and 700 g were used for this study. The rabbits were housed under normal temperature (22 ± 2°C) and available light facilities. The animals were allowed to acclimatize for three weeks before commencement of the experiment.

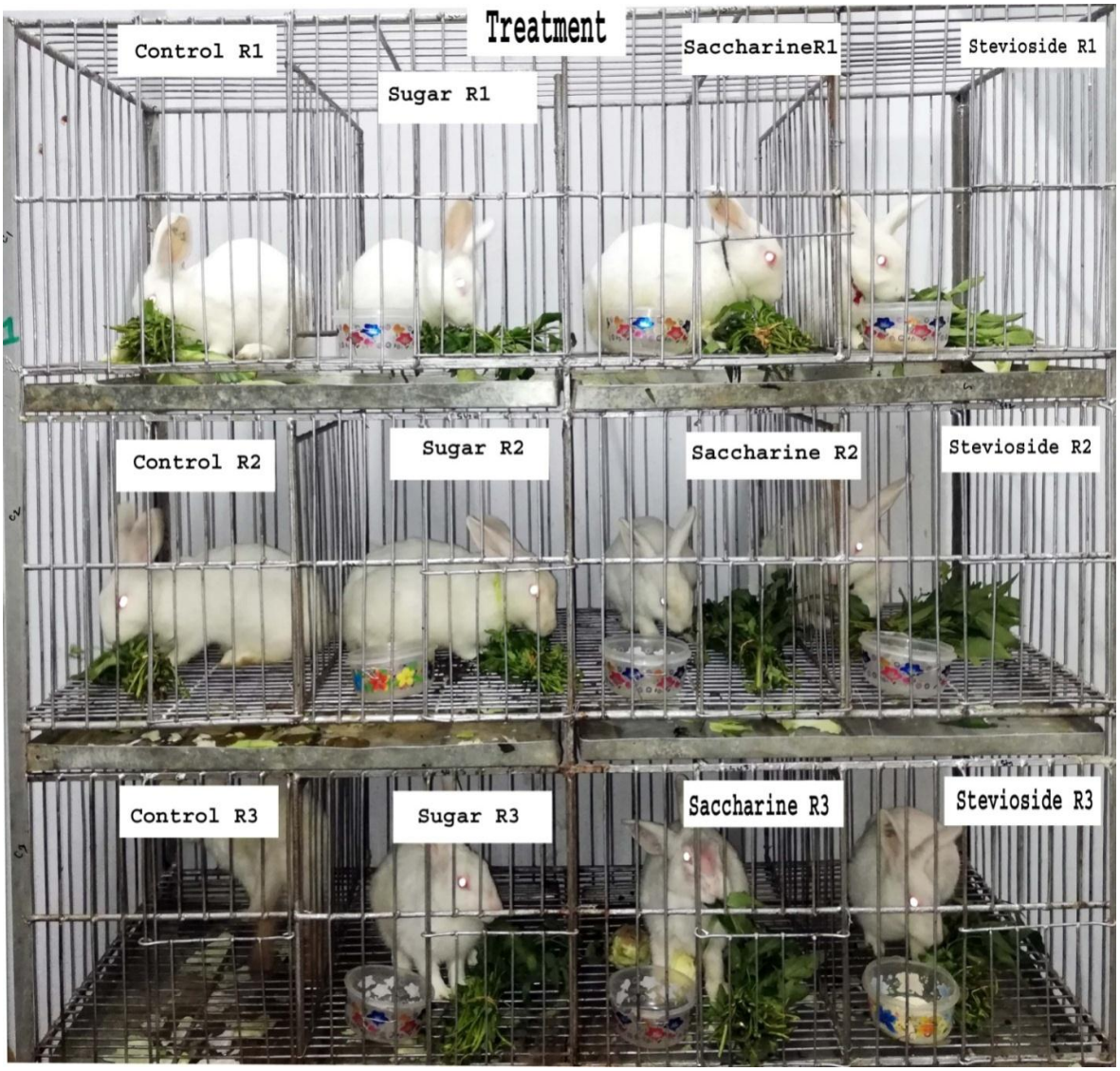


Figure 3.1: Experimental animals

3.1.4.Statistical analysis

Data were analyzed statistically to examine the treatment effect using the **R** programming language software. The mean differences were adjusted by Tukey test/ Honest Significant Differences (HSD) test and ranking was indicated by letters.

3.2 Methods

3.2.1 Biscuits were prepared according to the following procedure as shown in the flowchart**:**

All the ingredients were thoroughly mixed with a hand mixture for 2 minutes

↓

Stevioside was poured into warm water (50±1 °C)

↓

Added the stevioside solutions to the powdered ingredients

↓

Food-grade oil and shortening were added to the mixture

↓

All of the ingredients were mixed for few minutes until gaining a homogeneous mix

↓

Then the biscuit dough was flattened and moulded uniformly

↓

Biscuits were baked at 170 °C for 20 min using microwave-oven

↓

After baking, the biscuits were cooled for 10 min at room-temperature

↓

Packaged and maintained in a dry place to carry out various experiments.

Stevioside biscuits

Sugar biscuits

Saccharin biscuits

Figure 3.2: Different types of biscuits prepared in Biochemistry and Molecular Biology laboratory.

By following the same procedure, we also made the sugar and saccharine biscuits by using sugar and saccharin.

3.2.2. Treatments

We have collected 12 rabbits and divide them into four groups (control, stevioside, sugar, saccharin,) each group contain 3 rabbits

Control = we feed it usual food like vegetable, grass, rice etc.

Stevioside = we fed the rabbits with stevioside-formulated biscuits (20 ± 2) g / day with usual food.

Sugar = we fed the rabbits with sugar -formulated biscuits (20 ± 2) g / day with usual food.

Saccharin = we fed the rabbits with saccharin formulated biscuit (20±2) grams/day with usual food.

3.2.3. Collection of sample:

We initially collected the blood sample from the twelve rabbits and tested the biochemical parameters before starting the treatment. Seven (7) days after treatment we again collected the blood sample and tested the same biochemical parameters. The similar job was repeated for next two times after 14 days and 21 days.

Figure 3.3: Blood collection from rabbits

3.2.4 Sample preparation

After collection of the whole blood,

↓

Allow the blood to clot by leaving it undisturbed at room temperature.

↓

This usually takes 15–30 minutes. Remove the clot by centrifuging at 1,000–2,000 x g for 10 minutes in a refrigerated centrifuge.

↓

The resulting supernatant is designated serum.

↓

Serum was collected in another test tube

Figure 3.4 Sample preparation

3.3. Biochemical analysis

The following biochemical parameters were measured:

A. Creatinine

B. Albumin

C .Uric acids

D. Blood Urea

E. Electrolytes

I. Seurm sodium

II. Serum potassium

III. Serum chloride

A. Determination of creatinine:

Introduction

Creatinine is the result of the degradation of the creatine, component of muscles; it can be transformed into ATP, that is a source of high energy for the cells. The creatinine production depends on the modification of the muscular mass, and it varies little and the levels usually are very stable. Is excreted by the kidneys. With progressive renal insufficiency there is retention in blood of urea, creatinine and uric acid. Elevate creatinine level may be indicative of renal insufficiency. Clinical diagnosis should not be made on a single test result; it should integrate clinical and other laboratory data.

Principles of the method

The assay is based on the reaction of creatinine with sodium picrate as described by Jaffe. Creatinine reacts with alkaline picrate forming a red complex. The time interval chosen for measurements avoids interferences from other serum constituents. The intensity of the color formed is proportional to the creatinine concentration in the sample.

Reagents:

R1	Picric reagent	Picric acid 17.5 mmol/L
R2	Alkaline Reagent	Sodium hydroxide 0.29 mol/L
	Creatinine Cal	Creatinine aqueous primary standard 2 mg/dL

Precaution

R1 (Picric acid): Corrosive (C),Causes severe burns

R2 (NaOH): Irritant (Xi): R36/38: Irritating to eyes and skin.S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. S37/39: Wear suitable gloves and eye/face protection. S45: In case of accident or if you feel unwell, seek medical advices immediately.

Procedure:

1. Assay conditions
   Wavelength: 492 nm. (490-510)
   Cuvette: (1 cm light path)
   Temperature: 37°C
2. We adjusted the instrument to zero with blank of reagent.
3. Pipetted into a cuvette:

	Blank	Standard	Sample
Working Reagent (mL)	1.0	1.0	1.0
Standard (µL)	--	100	--
Sample (µL)	--	--	100

4. Mixed the working reagent with sample by following kit box instructions and started the stopwatch.

5. The absorbance (A_1) was taken after 30 seconds and after 90 seconds (A_2) of the sample addition.

6. Then we calculated: $\Delta A=A_2-A_1$.

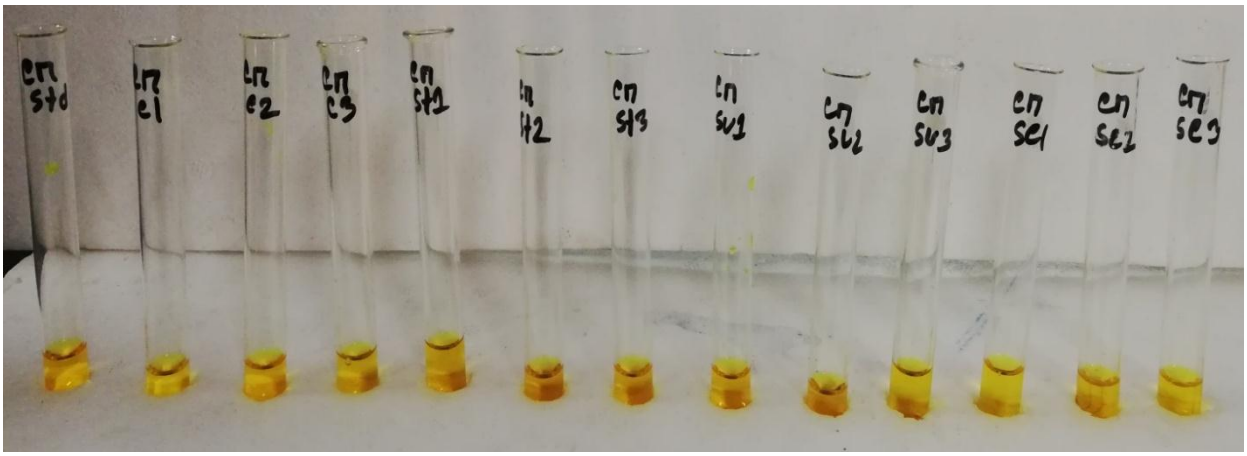


Figure 3.5**:** Test procedure of creatinine

Calculation:

$$\text{Creatinine (mg/dL)} = \frac{(\Delta A)\text{Sample}}{(\Delta A)\text{Calibrator}} \times 2(\text{calibrator conc.})$$

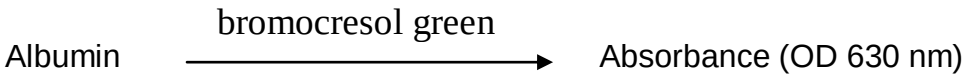
Conversion factor = Mg/dL**×**88.4 = Mmol/L.

B. Determination of albumin

Introduction:

Albumin is the most abundant protein in human blood and is highly conserved among vertebrates. It plays a pivotal physiological role in maintenance of plasma osmotic pressure, vascular permeability, and transport of cholesterol, bile pigments, nitric oxide, metals, and other small molecules in the body. It also functions as a free radical scavenger of reactive oxygen and nitrogen species, triggers cell signaling processes, possesses anti-inflammatory and coagulatory effects. Albumin assay Kit is a simple high through put assay that detects albumin concentration in serum. The assay is based on the selective interaction between Bromocresol Green (BCG) and Albumin forming a chromospheres that can be detected at 630 nm. The signal is directly proportional to the amount of Albumin present in the serum. BCG does not

react with other abundant plasma proteins like IgG. The assay can detect as low as 5 µg (0.01 g/dl) of albumin in serum samples.



Principles of procedure

The method is based on the specific binding of bromocresol green (BCG), an anionic dye, and the protein at acid pH produce a color change of the indicator from yellow – green to green –blue with the resulting shift in the absorption wavelength of the complex. The intensity of the color formed is proportional to the concentration of albumin in the sample.

Reagents

Albumin BCG is supplied as a liquid, ready to use,

Test procedure

1. Assay conditions

- Wavelength (630nm)
- Cuvette (1 cm light path)
- Temperature (15-25^{0}C)

2. We adjusted the instrument to zero with distilled water.

3. Pipette into cuvette.

	Blank	Standard	Sample
Reagent 1 (ml)	1.0	1.0	1.0
Standard (µl)	--	5 µl	--
Sample (µl)	--	--	5 µl

4**.** Mixed the samples with working reagent by following kit box instruction and incubated for 5 or 10 minutes at room temperature.

5. The absorbance (A) of the samples was taken and standard, against the blank. The color was stable 1 hour at room temperature (15-25^0 C)

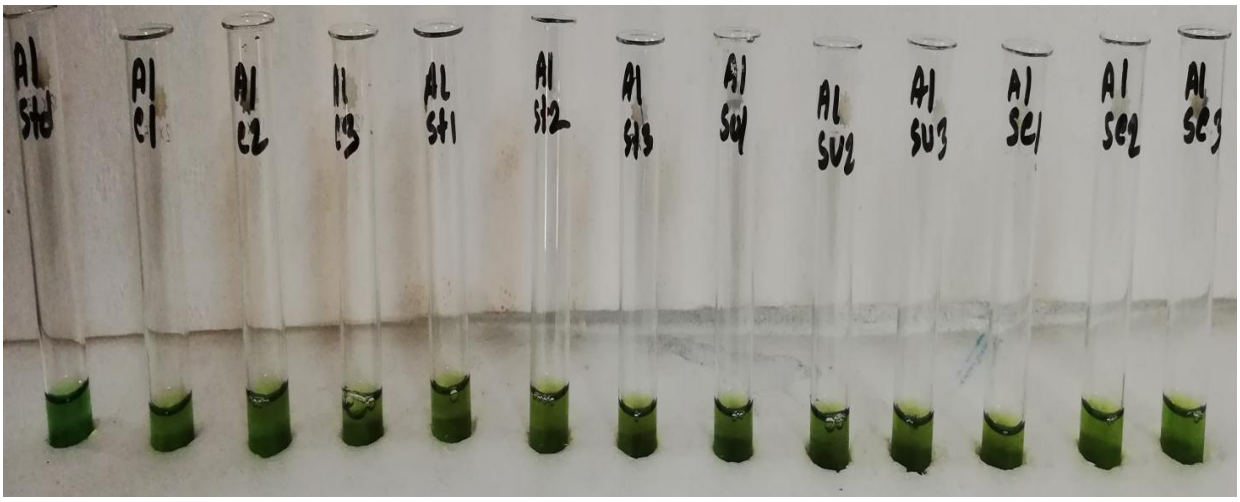


Figure 3.6: Test procedure of albumin

Calculations

$$\text{Albumin (g/dl)} = \frac{(\mathbf{A})\mathbf{samples}}{(\mathbf{A})\mathbf{standard}} \times 5(\text{calibrator conc.})$$

C. **Determination of uric acid**

Introduction

Uric acid and its salts are end products of the purine metabolism. In gout, the most common complication of hyperuricemia, increased serum levels of uric acid lead to formation of monosodium urate crystals around the joints. Further causes of elevated blood concentrations of uric acid are renal diseases with decreased excretion of waste products, starvation, drug abuse and increased alcohol consume as well as use of certain medicaments. High uric acid levels also constitute a indirect risk factor for coronary heart disease. Hypouricemia is seldom observed and associated with rare hereditary metabolic disorders.

Principle

$$\text{Uric Acid} + O_2 + 2H_2O \xrightarrow{\text{Uricase}} \text{Allantoin} + CO_2 + H_2O_2$$

$$2H_2O_2 + \text{4-AAP} + \text{HDCBS} \xrightarrow{\text{peroxidase}} \text{Chromagen} + 4H_2O$$

Uric Acid is oxidized by Uricase to allantoin and hydrogen peroxide. HDCBS + 4-AAP + hydrogen peroxide, in the presence of peroxidase, produces a red chromagen that is measured at 520nm. The absorbance at 520nm is proportional to the concentration of uric Acid in the sample.

Reagent composition

Uric Acid reagent: 4-AAP >0.2mM, HDCBS 2mM, Uricase (Microbial) >150 U/L, Peroxidase (horseradish) >2,500 U/L, Buffer, pH 8.1 ± 0.1, Non-reactive stabilizers.

Reagent preparation

The reagent is ready to use.

Precautions

1. This reagent set is for in vitro diagnostic use only.

2. The reagent should not be used if: The reagent is turbid or contains obvious microbial growth. The reagent blank has an absorbance of 630nm. A pink color is normal for this reagent.

3. All specimens and controls should be handled as potentially infectious, using safe laboratory procedures.

Test procedure

1. Assay conditions

- Wavelength (630nm).

- Cuvette (1 cm light path).

- Temperature (15.25^0 C).

2. We adjusted the instrument to zero with distilled water.

3. Pipette into cuvette.

	Blank	Calibrator	Sample
Reagent (ml)	1.0	1.0	1.0
Calibrator(µl)	--	25	--
Sample(µl)	--	--	25

4. Mixed the sample with reagent by following kit box instruction and incubated for 5 minutes at 37^{0}C or 10 minutes at room temperature (15-25^{0}C)

5. The absorbance (A) of the samples was taken and standard against the blank, the color was stable at least 5 minutes

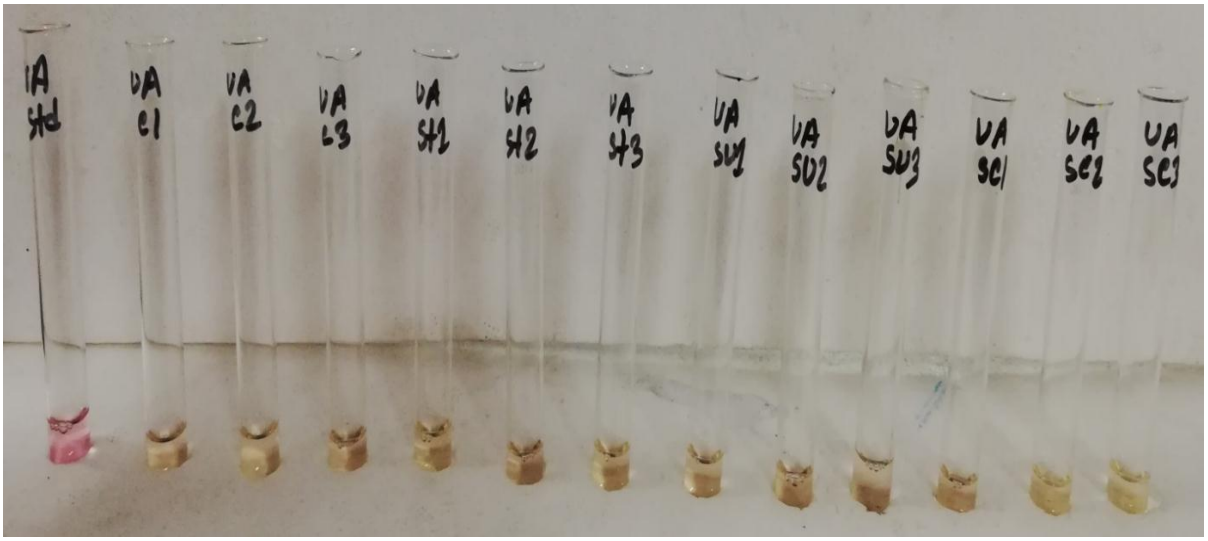


Figure 3.7 : Test procedure of uric acid

Calculations

Uric acid (mg/dl) = $\frac{(A)\text{Sample}}{(A)\text{Sandard}}$ x 6 (calibrator conc.)

Determination of blood urea

Introduction

Urea is the end product of protein nitrogen metabolism and is the primary vehicle for removing toxic ammonia from the body. Urea is synthesized in the liver from the ammonia produced from the catabolism of amino acids via the hepatic urea cycle. The conversion from ammonia to urea is regulated by N-acetylglutamate, which activates carbamoyl phosphate synthetase in the urea cycle. Urea is transported in the blood to the kidneys where it is excreted in the urine. In addition to its role as a carrier of waste nitrogen, urea also has a role in the countercurrent exchange system of the nephrons in which water and ions are re-absorbed from excreted urine. It is freely filtered by the glomeruli and partially passively resorbed as filtrate transverses the renal tubules. Urea reabsorption is inversely proportional to urine flow rate. Consequently, urea concentration depends upon protein intake, protein catabolism, and kidney function.

Urea quantitation is one of the most widely applied tests for kidney function evaluation. The analysis of urea in serum, plasma and urine is an important clinical test for renal disease and dysfunction. The test is frequently tested in conjunction with creatinine determination for diagnosis of pre-renal, renal, and post renal uremia. Toxic urea levels are associated with renal, liver, or other system dysfunction. Prerenal uremia relates to water depletion, increased protein catabolism, infection, hypovolemia, or cardiac decomposition. Glomerulonephritis, tubular necrosis, nephrosclerosis, chronic nephritis, and polycystic kidney are examples of renal uremia, while post renal uremia is predominantly urinary tract obstructions or leakage. Increased urea levels can also be linked to other disease states such as liver disease, diabetes, and congestive heart failure. High plasma urea levels are known as Azotemia. Decreased urea levels are associated with acute hepatic insufficiency or excess parenteral fluid therapy.

Principles

Urease hydrolyzes urea to ammonia and CO_2. The ammonia formed 2 further reacts with a phenolic chromogen and hypochlorite to form a green coloured complex. Intensity of the colour formed is directly proportional to the amount of urea present in the sample.

Ragents :

1. Assay conditions

    - Wavelength (510nm).

    - Cuvette (1 cm light path).

    - Temperature (15.25^{0}C).

2. We adjusted the instrument to zero with distilled water.

3. Pipette into cuvette.

	Blank	Calibrator	Sample
Reagent 1 (ml)	1.0	1.0	1.0
Calibrator (µl)	--	25	--
Sample(µl)	--	--	25

4. Mixed the sample with reagent and waited 1 minutes.

Reagent 2 (ml)	1.0	1.0	1.0

5. Mixed the sample with working reagent 1 by following kit box instruction and incubated for 15 minutes and room temperature 20 minutes (15-20)

6. The absorbance (A) was taken of the samples and standard, against the blank

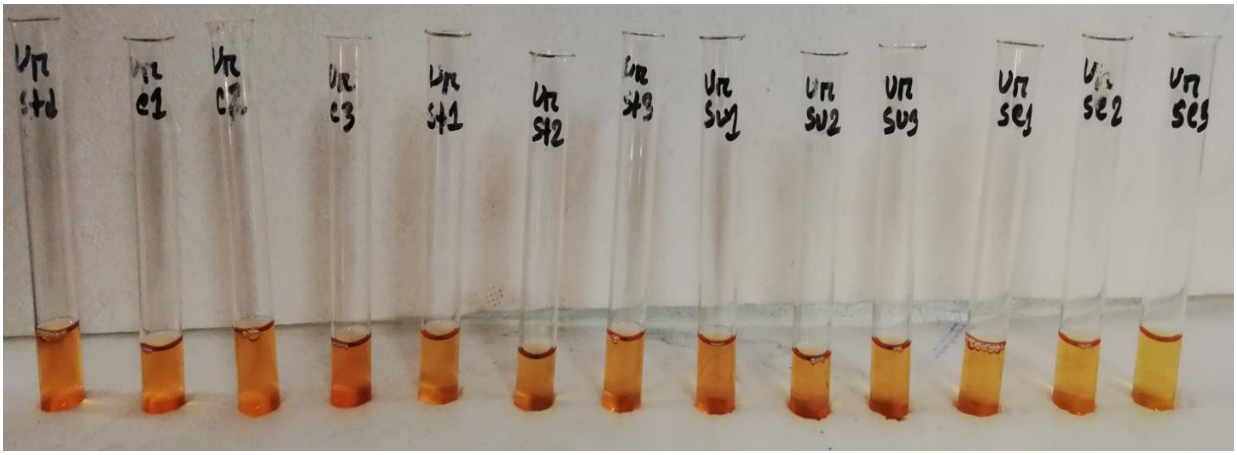


Figure 3.8: Test procedure of urea

7. Calculations: Urea mg/dl = $\frac{(A)\text{Sample}}{(A)\text{Sandard}}$ x 50 (calibrator conc.)

E. Determination of electrolytes.

Introduction

Electrolytes are positively and negatively charged molecules called ions that are found within the body's cells and extracellular fluids, including blood plasma. A test for electrolytes includes the measurement of sodium, potassium, chloride, and bicarbonate. These ions are measured to assess renal (kidney), endocrine (glandular), and acid-base function, and are components of both renal function and comprehensive metabolic biochemistry profiles. Other important electrolytes routinely measured in serum or plasma include calcium and phosphorus. These are measured together because they are both affected by bone and parathyroid diseases, and often move in opposing directions. Magnesium is another electrolyte that is routinely measured. Like calcium, it will cause tetany (uncontrolled muscle contractions) when levels are too low in the extracellular fluids.

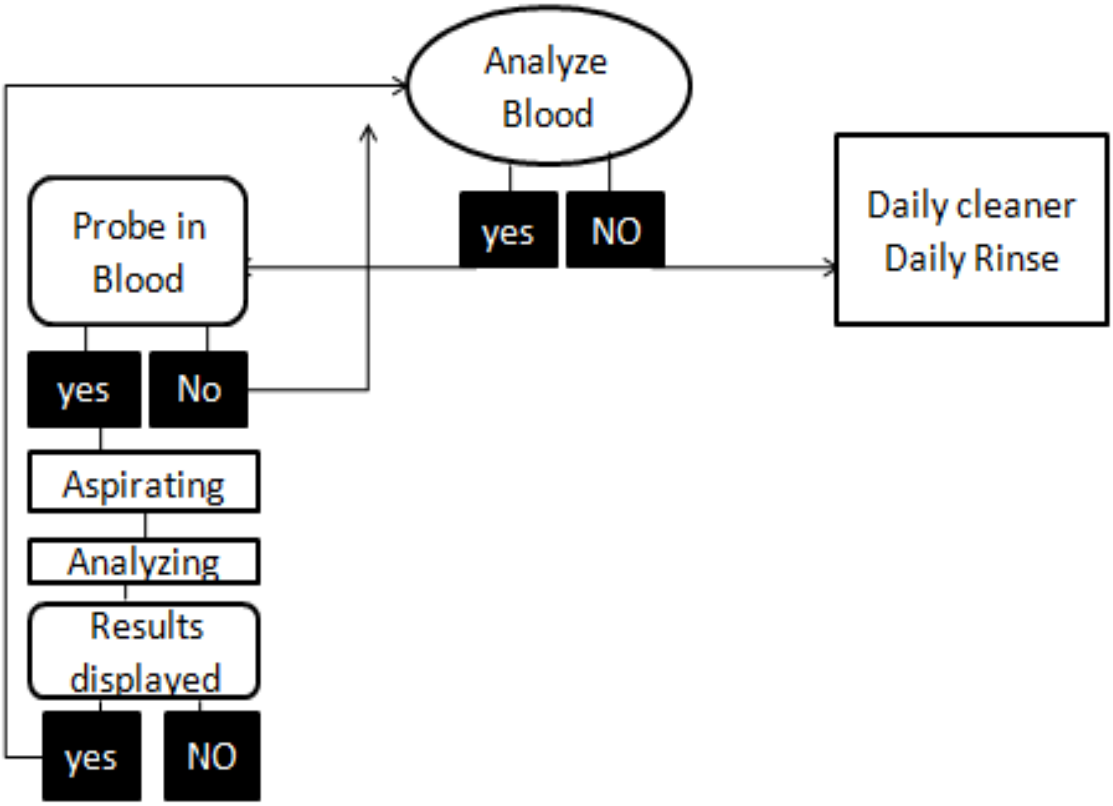
Working principles of electrolytes

Electrolytes play multiple roles in the maintenance of body functions such as sustaining proper body Ph, regulating function of the heart and other muscles, and participating in enzymatic functions. Electrolytic imbalances can result in congestive heart failure, diabetes insipidus, and kidney diseases. For these reasons electrolytic

analysis is a key factor in patient diagnosis and treatment. Electrolyte analyzers measure electrolytes in serum, plasma and urine. Major components of an electrolyte analyzer are – reagents, electrode module, peristaltic pump, and sample probe. Automated systems feature comprehensive test menu, a high throughput as well as STAT testing.

The most common methods of analysis are flame emission photometry and Ion selective electrode. Flame Photometry can be used to measure Na+, K+ and Li+. It provides an indirect measurement, while ISE methods offer direct measurements. Most analyzers use ISE technology to make electrolyte measurements.

Flow chat of electrolytes determination



CHAPTER IV

RESULTS AND DISCUSSION

The current study was conducted to effects of stevioside -supplemented biscuits with sugar and saccharin on the kidney function of rabbits. The results obtained from the study are presented and discussed in this chapter under the following headings.

4.1 Biochemical parameters:

The important bio-chemical parameters of the formulated biscuits were discussed separately in below:

4.1.1 Creatinine:

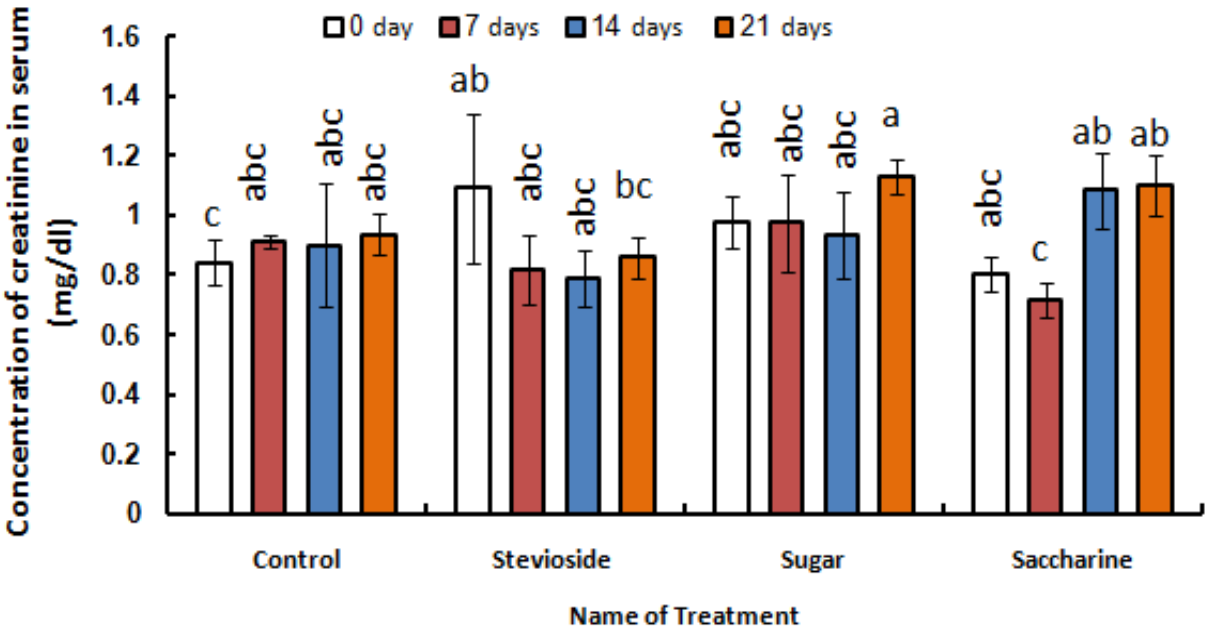


Figure 4.1: Effect of stevioside, sugar and saccharine on creatinine level of rabbits

The above diagram showed that in the control group, there was no significant change in creatinine levels after 21 days of treatment. In the stevioside group, the 7days creatinine level decreased than 0 days, but the creatinine levels at 7, 14 days were found to be approximately same and 21days were found to be a significant change. n sugar-fed rabbits creatinine level, there is no significant change in 0, 7, and 14 days. The lowest creatinine level was found on 14 days, but creatinine levels increased significantly at day 21. The creatinine level in saccharin fed rabbits was

significantly reduced at 7 days compared to 0 days, whereas creatinine levels increased significantly after 14 and 21 days treatment. From the above figure, stevioside fed rabbit showed good creatinine level than sugar and saccharin. High levels of creatinine warn of possible malfunction or failure of the kidneys. In stevioside fed rabbits creatinine level was not so high as sugar and saccharin that indicated that stevioside has no adverse effects on renal function.

4.1.2.Albumin

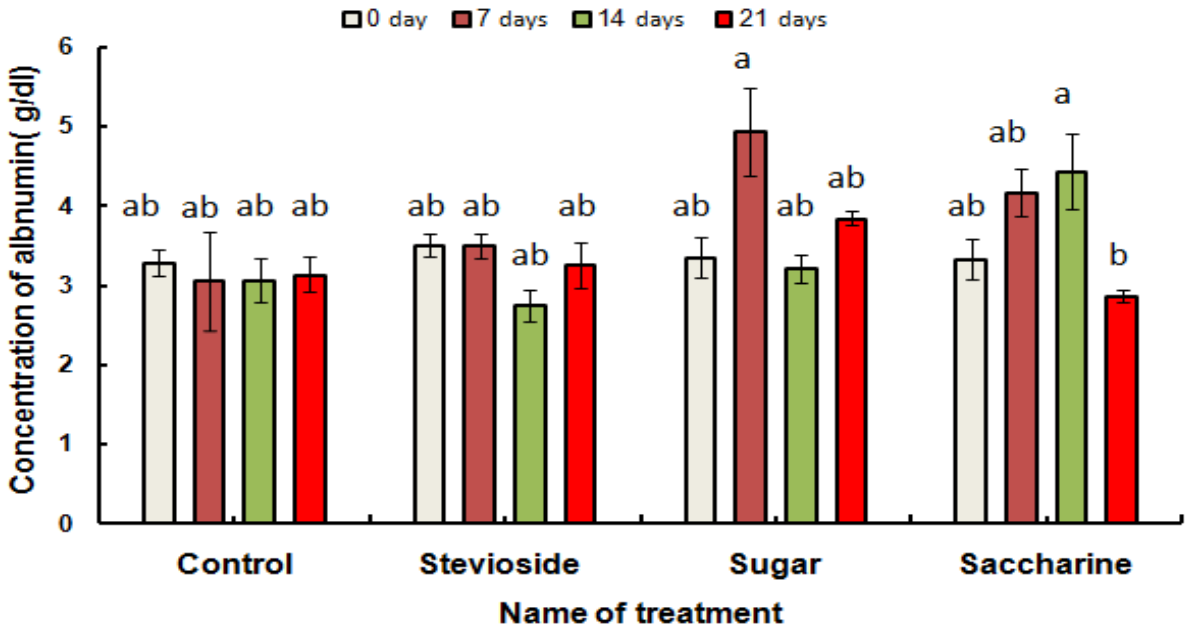


Figure 4.2: Effect of stevioside, sugar and saccharine on albumin level of rabbits

The above diagram showed that in the control group, there was no significant change in albumin level after 21 days of treatment. There are almost similar. In stevioside group at 0 and 7 days albumin concentration similar but significantly decreased in 14 days compared to 0 and 7 days and the albumin concentration increased significantly 21 days. In sugar fed rabbits albumin concentration was significantly higher at 7 days than 0 days and the albumin concentration significantly decreased 14 days and 21 days was found almost same albumin level. The albumin levels of saccharin-fed rabbits were significantly higher at day 7 than at 0 days, and albumin levels were increased significantly at day 14 compared to 7 days. finally, The albumin level was significantly reduced by 21 days.. Albumin is a protein found in the blood. A healthy kidney doesn't let albumin pass from the blood into the urine.

A damaged kidney lets some albumin pass into the urine. Low levels of albumin indicate possible malfunction or failure of the kidneys. In stevioside fed rabbits albumin level was not so low as saccharin that indicated that stevioside has no adverse effects on renal function.

4.1.3 Uric acid

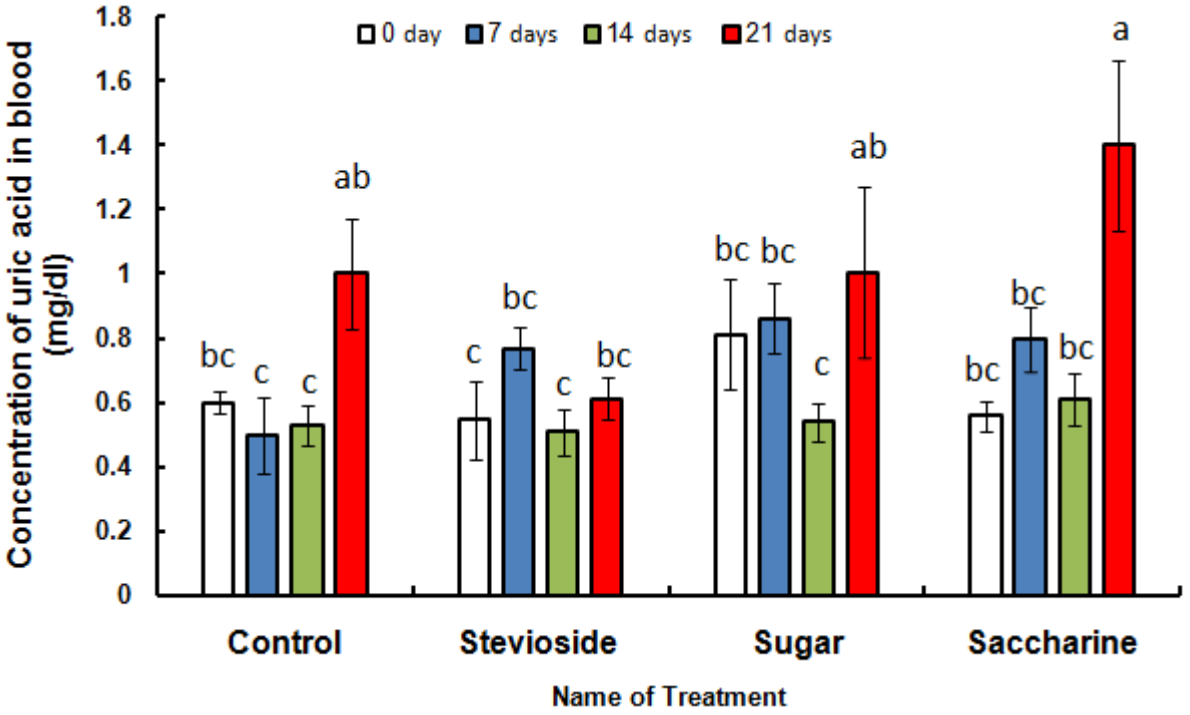


Figure 4.3: Effect of stevioside, sugar and saccharine on uric acid level of rabbits.

The above figure showed that uric acid content was significantly higher in sugar fed rabbits at 0 day. In 7 days, uric acid levels were significantly decreased in control than sugar, stevioside and saccharin group whereas stevioside, and saccharin increased significantly. There was no significant difference among the control, sugar and saccharin group at 14 days but stevioside decreased significantly. In 21 day the level of uric acid concentration significantly increased in saccharin, control, sugar group but stevioside remain almost similar with 14 days. The maximum uric acid (1.4 mg/dl) was found in saccharin group after 21 days treatment and minimum Uric acid (0.53mg/dl) level in control group after 14 days treatment. Uric acid is a waste product that is naturally found in the blood. When we have kidney disease, our kidneys cannot filter out uric acid as well as they should. Too much uric acid building

up in the body may cause gout. Our study indicated that stevioside showed a better result than sugar and saccharin.

4.1.4.Urea

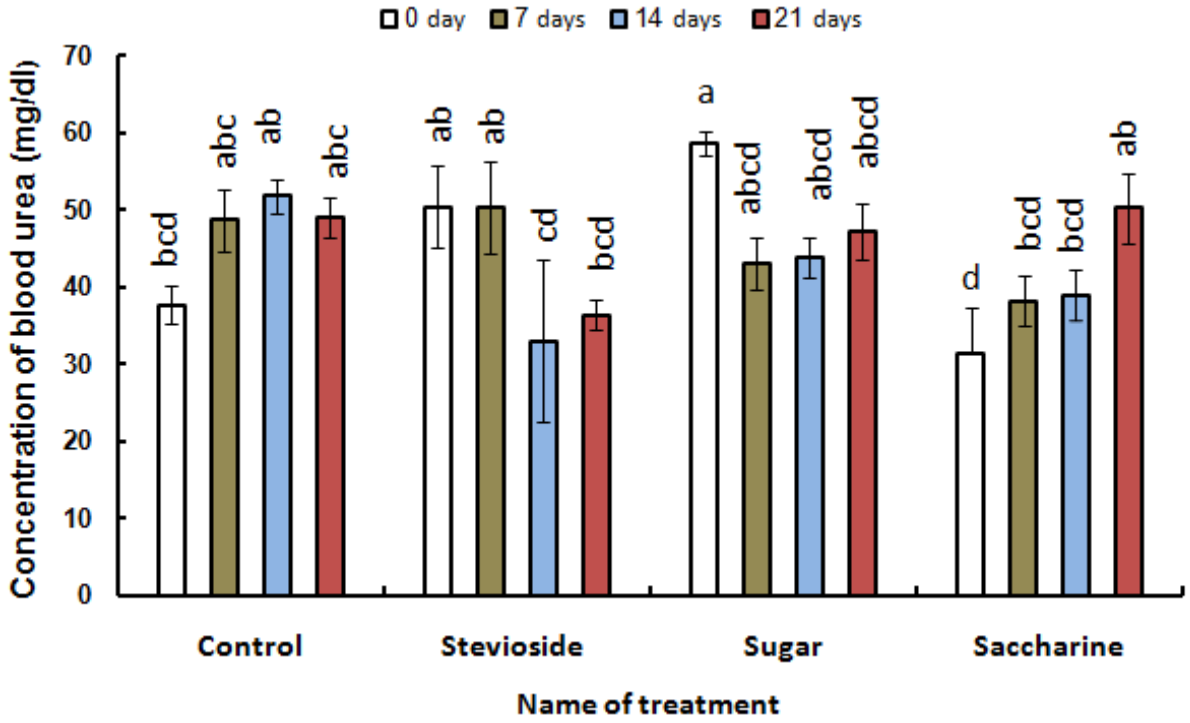


Figure 4.4: Effect of stevioside, sugar and saccharine on urea level of rabbits.

The image proved that in the control group the urea levels were significantly lower at 0 days than 7,14,21 days. There is no significant difference among the 7, 21, day in the control group but the urea levels were significantly in increased at 14 days of treatment. In the stevioside fed rabbits group, the level of urea was significantly lower at 14 days compared to 0,7 days. In 21 days there was a significant change in the urea level. In the sugar group, urea level was significantly reduced after 7 days of treatment and there was no significant difference between days 7 and 14 but urea level increased at 21days compared to 7, 14 days.The urea levels were significantly increased at 7 days in saccharin fed rabbits. There is no significant difference among the 7 and 14 days and after 21 days of treatment, the urea level was increased significantly. High levels of urea warn of possible malfunction or failure of the kidneys. Urea is a nitrogen-containing substance normally cleared from the blood by the kidney into the urine. Diseases that compromise the function of the kidney often

lead to increased blood levels of urea, as measured by the blood urea test. From this study, we can see that stevioside gives better results than sugar and saccharin.

4.1.5 Potassium

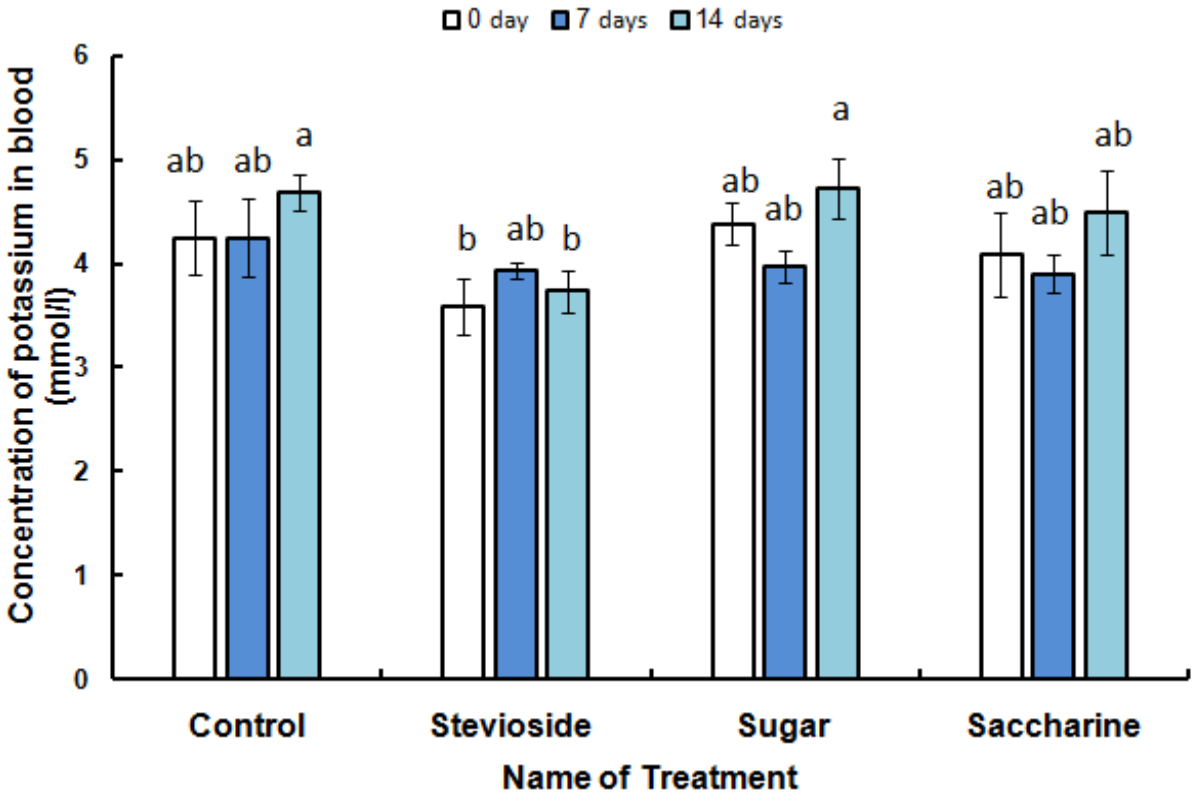


Figure 4.5: Effect of stevioside, sugar and saccharine on potassium level of rabbits.

The above figure showed that potassium content almost similar control, steviosidee, sugar saccharin at 7 days. Potassium levels were significantly increased in sugar after 14 days of treatment but with 0 days the control, and saccharin was equal and stevioside changed significantly. The potassium level significantly decreased was observed in stevioside at 14 days but increased significantly in control, sugar, saccharin group over 14 days. When kidneys fail they can no longer remove excess potassium, so the level builds up in the body. High potassium in the blood is called hyperkalemia, which may occur in people with advanced stages of chronic kidney disease . From this study, we know that Stevioside does not affect potassium levels so we can use stevioside as an alternative to sugar.

4.1.6 .Sodium

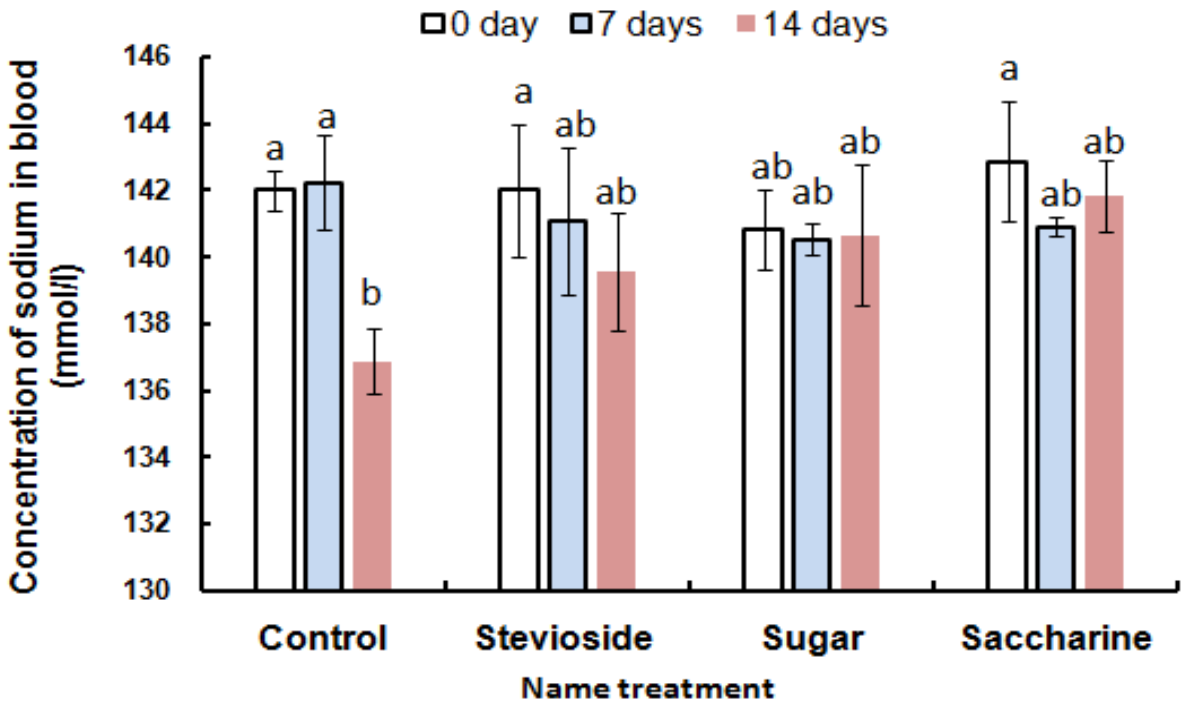


Figure 4.6: Effect of stevioside, sugar and saccharine on sodium level of rabbits.

The above diagram showed that in the control group, there was no significant change in sodium concentration at 0,7 days but significantly decreased at 14 days than 0,7days . In the stevioside group, after treatment of 7 days, the level of sodium concentration was significantly decreased than 0 days. In the 14 days, sodium concentration levels were decreased compared to 0 and 7 days. Sugar group, no significant change was observed after 14 days of treatment. In rabbits fed saccharin, sodium levels were significantly lower at 7 days than 0 days but increased at 14 days. The maximum sodium (142.8mmo/l) was found in saccharin group at 0 days and minimum sodium (136.9mmol/l) level in control group after 14 days treatment. Sodium is important for controlling your blood pressure, but you need the right amount. Too much sodium can increase your blood pressure, and this is bad for your heart and your kidneys. If you have kidney disease, your kidneys can not remove excess salt and fluid so they build up in your body and can cause high blood pressure, Swelling of ankles, feet, hands, and puffiness under your eyes. After 14 days of treatments, we observed stevioside can not affect kidney so we can eat it without any worried.

4.1.7 Chloride

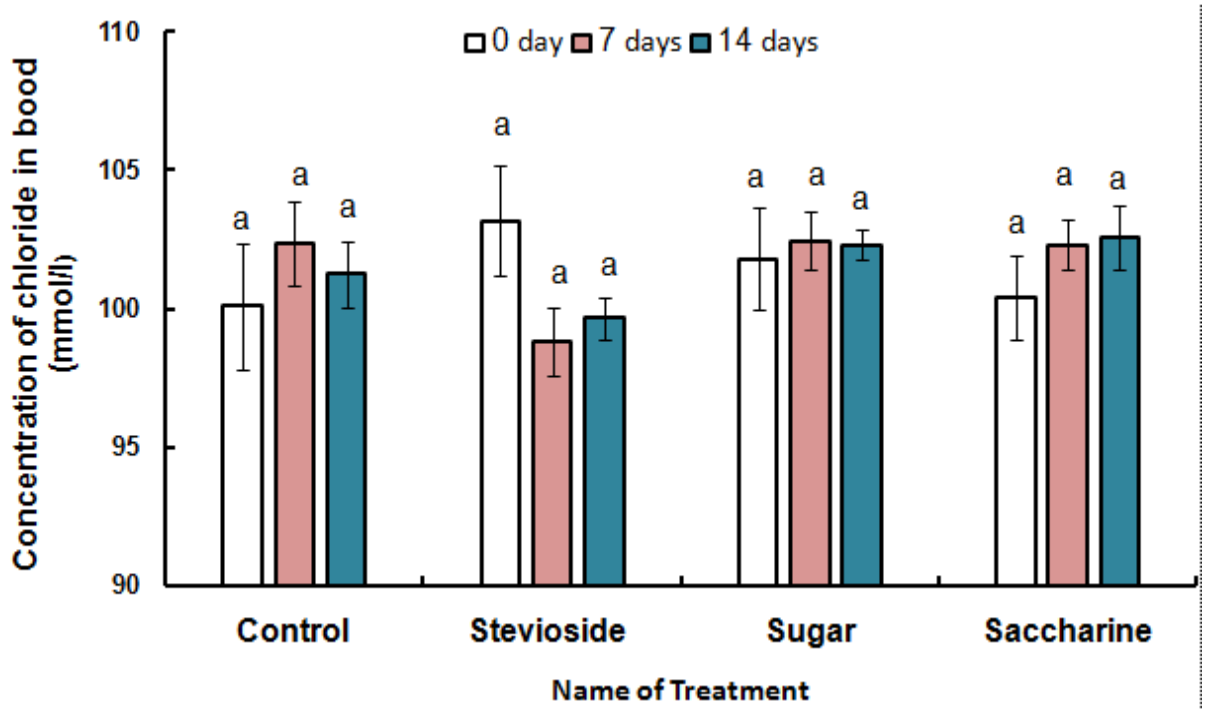


Figure 4.7: Effect of stevioside, sugar and saccharine on chloride level of rabbits.

The above figure showed that there was no significant difference after 21 days of treatment in all groups. In the control rabbits group, the level chloride was increased at 7 days compared to 0 days, whereas the level of chloride was decreased by 14 days than 7 days. In the stevioside group, after 7 days of treatment chloride slightly lower than at 0 days and there was no significant difference among the 7 and 14 days. There was no significant change after 14 days of treatment in the sugar rabbits group. The chloride level was significantly increased in 7 days but there is no significant difference between 7 and 14 days after treatment in the saccharin group.

CHAPTER V

SUMMARY AND CONCLUSION

Stevia (*Stevia rebaudiana*) plant is a foreign plant in our country. Stevia leaf is sweet in taste. Its leaves contains different types of steviol glycosides (stevioside sand rabeuadioside A) such as 'stevioside' which is 300 times sweeter than sucrose. It has both economical and medicinal importance. It is used as a natural sweetener and has no adverse effects on health. Stevioside provides zero calories in a variety of drinks and foods. The number of diabetic patients in our country is increasing day to day. Diabetes are closely associated with chronic kidney disease (CKD), it has up-raised the interest to investigate the renoprotective effect of stevia in CKD. Worldwide, the prevalence of kidney disease and the metabolic syndrome is becoming a significant medical concern and public health burden. Some diabetic foods (e. g., cake, candy, and bread) prepared with artificial sweetener such as saccharin, aspartame etc are available in Bangladesh, but these sweeteners has serious adverse effects on health. Therefore, we aimed to formulate a stevioside-supplemented biscuits using stevioside as a natural sweetener and to evaluate this biscuit based on the analysis of kidney function tests in the rabbits. We prepared a stevioside-supplemented biscuit using stevioside as a sugar substitute and also prepared other two types of biscuit using sugar and saccharin to compare the results. We then fed these three types of biscuits to three different groups of rabbits and observed its effect on the rabbit kidney function tests. We measured levels of serum creatinine, albumin, uric acid ,urea, and serum electrolytes (sodium, potassium, chloride). The kidney functions tests were performed by spectrophotometer and automatic electrolytes analyzer. The results revealed that the serum creatinine level was in normal range (1.09-0.86 mg/dl) in stevioside biscuit than that of sugar (0.97 – 1.13 mg/dl) and saccharin (0.861-1.4 mg/dl). Stevioside biscuit also contain high albumin (3.50 g/dl - 3.24g/dl) than sugar (3.34- 3.17 g/d) and saccharin (3.33- 3.02 g/dl). Stevioside-supplemented biscuit maintain serum potassium level (3.6- 3.7mmol/dl) than sugar (4.3- 4.5 mmol/dl) and saccharin (3.92-4.49 mmol/dl). Stevioside also contain less urea level in blood (52.93- 36.36 mg/dl) than sugar (63.21- 47.2 mg/dl) and saccharin (36.59- 50.2 mg /dl).

Overall, stevioside-supplemented biscuits show the best results from sugar and saccharine-supplemented biscuits.

Therefore, stevioside biscuits may be considered as the best sugar substitute biscuit for patients with diabetes and kidney diseases.

CHAPTER VI

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APPENDICES

Appendix I: Analysis of variance for creatinine

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	3	0.11594	0.038647	2.8742	0.04957 *
Genotype	3	0.10487	0.034958	2.5998	0.06712
REP	2	0.06522	0.032608	2.4251	0.10279
Trt: days	3	0.38336	0.127786	9.5035	9.237e-05 ***
Residuals	35	0.48406	0.013446		

Df= Degrees of freedom, Treatment = Control, Sugar, Steviocide, Saccharine, Genotype= days,

Signif.codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Appendix II: Analysis of variance for albumin

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	3	2.4303	0.81009	3.2538	0.03276 *
Genotype	3	2.0140	0.67134	2.6964	0.06030
REP	2	0.0770	0.03848	0.1545	0.85738
Trt: days	3	0.8372	0.27906	1.1209	0.35346
Residuals	36	8.9630	0.24897		

Df= Degrees of freedom, Treatment = Control, Sugar, Steviocide, Saccharine, Genotype= days

Signif.codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Appendix III: Analysis of variance for uric acid

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	3	0.45331	0.15110	6.6180	0.001123 **
Genotype	3	1.42346	0.47449	20.7812	5.501e-08 ***
rep	2	0.04798	0.02399	1.0507	0.360180
trt:days	3	0.52531	0.17510	7.6691	0.000435 ***
Residuals	36	0.82197	0.02283		

Df= Degrees of freedom, Treatment = Control, Sugar, Steviocide, Saccharine, Genotype= days

Signif.codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Appendix IV: Analysis of variance for blood urea

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	3	551.15	183.72	6.2528	0.001578 **
Genotype	3	95.73	31.91	1.0860	0.367381
rep	2	22.38	11.19	0.3809	0.685949
trt:days	3	1404.11	468.04	15.9298	9.364e-07 ***
Residuals	36	1057.72	29.38		

Df= Degrees of freedom, Treatment = Control, Sugar, Steviocide, Saccharine, Genotype= days

Signif.codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Appendix V: Analysis of variance for potassium

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	3	2.32616	0.77539	8.0643	0.0006321 ***
Genotype	3	1.09347	0.54674	5.6862	0.0092171 **
rep	2	0.24207	0.12104	1.2588	0.3013793
trt:days	3	0.07235	0.02412	0.2508	0.8599916
Residuals	25	2.40378	0.09615		

Df= Degrees of freedom, Treatment = Control, Sugar, Steviocide, Saccharine, Genotype= days

Signif.codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Appendix VI: Analysis of variance for sodium

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	3	3.016	1.0052	0.4112	0.7463443
Genotype	3	45.962	22.9811	9.4017	0.0009024 ***
rep	2	3.937	1.9686	0.8054	0.4581821
trt:days	3	29.218	9.7394	3.9845	0.0189491 *
Residuals	25	61.109	2.4444		

Df= Degrees of freedom, Treatment = Control, Sugar, Steviocide, Saccharine, Genotype= days

Signif.codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Appendix VI: Analysis of variance for Chloride

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	3	13.432	4.4773	1.6803	0.19670
Genotype	3	0.101	0.0503	0.0189	0.98132
rep	2	2.351	1.1753	0.4411	0.64826
trt:days	3	28.405	9.4682	3.5533	0.02866 *
Residuals	25	66.615	2.6646		

Df= Degrees of freedom, Treatment = Control, Sugar, Steviocide, Saccharine, Genotype= days

Signif.codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1