Original Research Article

Bioactivity and Safety of hydro-ethanolic Lyophilised Carrisa edulis

ABSTRACT

Carissa edulis (C. edulis), from the *Apocynaceae* family is a widespread plant species prevalent in tropical and subtropical regions of Africa. The plant is extensively utilized in traditional medicine for various ailments, particularly diabetes mellitus (DM), a chronic, non-communicable disease whose complications lead to over 70% of all amputations globally, and considerable deaths across all adult age groups and resource settings. The endpoint biomarkers of the diseased state include inflammation and oxidative degeneration of cells and tissues. The current therapeutic approaches for management of DM are characterized by several limitations such as adverse side effects and prohibitive costs. This, therefore necessitates the need to explore safer, effective, and affordable alternatives that can inhibit, ameliorate, and/or reverse the biological endpoints of DM. Our current study qualitatively and quantitatively determined the phytoconstituents of *C. edulis*, as well as its activity as an antioxidant and anti-inflammatory agent. The phytoscreening confirmed the presence of numerous biomedically relevant secondary metabolites with high polyphenolic and flavonoid yields The biosafety study using Wistar rat models confirmed that *C. edulis* is nontoxic with an LD50 above 5000mg/kg. The hydroethanolic extract demonstrated high anti-oxidant and high anti-inflammatory activities, which were comparable to the standards, ascorbic acid and diclofenac, respectively. It was therefore concluded that the presence of bioactive secondary metabolites in lyophilized extracts of the aerial parts of *C. edulis* possess satisfactory anti-inflammatory and anti-oxidant profiles. The extracts were also safe, thereby supporting their continued use as an adjunct therapy for known biological end points of DM in traditional medical practices in Southern Africa.

Key words: C*arrisa edulis*, anti-inflammatory, antioxidant, diabetes mellitus, polyphenols, secondary metabolites

Introduction

1.1 Carissa edulis

Carissa edulis (*C. edulis*) is a rapidly propagating, evergreen scrambling shrub, belonging to the *Apocynaceae* family. It blooms with sweet jasmine scented bouquets of fragrant white petals just before the summer rains in subtropical Southern Africa[1]. The red fruits which appear in summer are edible, thus the source of the species name '***edulis'***, a sharp contradiction to the genus name *Carissa*, which is consequent to the presence of Carrisin, a poisonous glucoside that has been isolated from the bark of plants belonging to the genus[2]. The multi stemmed, spiny shrub or occasional dwarf tree is heavily branched with multiple appendages leading to a dense glossy evergreen canopy. The plant oozes a milky, nontoxic latex from all its structures and appendages. The simple long spines are rigid, making the shrub an ideal occasional refuge for hiding or resting small mammals and reptiles. The foliage also provides an appropriate nesting location for birds due to the security offered by the spines as well as the edible fruits[3]. In areas with dense plant population, *C. edulis* plants growing next to each other can form thick effective hedges against intruding game providing excellent self-preservations since the leaves are a delicacy for most herbivores including impala, giraffe, kudu, primates and bush bucks[3]. In traditional medicinal practices in Southern Africa, root and bark decoctions, and infusions based on *C. edulis* plant parts have widely been used as treatments for a broad spectrum of human health conditions including diabetes, sexually transmitted infections, malaria, rheumatoid arthritis and migraines.[4]

Figure 1: Images of C edulis plant, aerial parts, foliage and fruit

1.2 Secondary metabolites and diabetes

Diabetes mellitus (DM) is a non-communicable, chronic, metabolic disorder characterized by erratic and uncontrollable plasma glucose levels[5]. Whilst type 1 DM is an autoimmune disease characterized by the loss of insulin producing pancreatic beta cells, type 2 DM arises from insulin resistance and its decreased secretion[5]. Both hyperglycemia and hypoglycemia are associated with irregularities in the pathways that metabolise starch, carbohydrates, lipids and proteins leading to degenerative microvascular and macrovascular complications. In normal metabolism, triggered by the rise in plasma glucose after food consumption, the pancreas releases insulin. Insulin regulates blood glucose levels by increasing absorption uptake of the glucose into cellular structures[6]. When this process is impaired, the blood plasma fails to offload the glucose leading to hyperglycemia. This is the primary condition that affects diabetics. Lifestyle choices including choice and amount of food intake, physical activity and genetic predisposition, are the leading factors contributing to hyperglycemia in diabetics. In insulin dependent diabetes (type 1 DM), oral therapy using insulin is not possible since insulin is a hormone and will be metabolised before reaching the blood plasma[7]. Therefore, subcutaneous and intravenous insulin injections are the most appropriate alleviation routes during hyperglycemic episodes. However in non-insulin-dependent diabetes (type 2 DM), sulfonylureas and biguanides are the two chemical classes of drugs mostly used[8]. Hypoglycemia, weight gain, increased risk to secondary infections, and gastrointestinal irritation are some of the most common side effects reported with use of anti-diabetic medications[8]. Apart from the well documented side effects, the cost of these conventional treatments, especially insulin, is very high and unaffordable to populations in low economic tier environments like sub Saharan Africa. The development of cheaper and safer alternatives is therefore imperative.

The use of indigenous plants in the treatment of DM is quite prevalent in Southern Africa, and has been for eons. Many local plants including *C. edulis*, *Annona stenophylla, Vernonia amygdalina* and many others are employed as anti-diabetic treatments within the traditional health care system[9]. Although *C.*

edulis is widely used traditionally to cure many ailments, its bioactivity and safety profiles are not well characterised. The activity of anti-hyperglycemic plants is a consequent of their richness in secondary metabolites including polyphenols. Secondary metabolites are organic compounds produced by plants which are not directly involved in growth, development, or reproduction, but are crucial for environmental adaptation[9]. Compounds such as alkaloids, flavonoids, terpenoids, phenolic acids, and saponins have demonstrated significant anti-diabetic effects through a variety of mechanisms. These include antioxidant activity, modulation of insulin signaling pathways, inhibition of carbohydrate digestion and absorption, and anti-inflammatory effects[10]. Terpenoids such as ginsenosides, and flavonoids such as quercetin, have been shown to increase insulin receptor phosphorylation and activate downstream signaling pathways, notably PI3K/Akt, that facilitate glucose absorption in muscle and adipose tissues[11]. This modulation of insulin signaling is crucial for maintaining glucose homeostasis, and preventing hyperglycemia. Alkaloids improve insulin sensitivity, reduce hepatic glucose production, and enhance glucose uptake in peripheral tissues. They exert their effect by activating AMP-activated protein kinase (AMPK), a key regulator of energy metabolism[12]. Saponins have been shown to exert antidiabetic effects by modulating glucose metabolism, enhancing insulin secretion, and improving its sensitivity. For example, the saponin Pseudoginsenoside F11, found in *Panax quinquefolium*, has demonstrated antidiabetic effects in animal models of type 2 DM[13]. Phenolic acids can inhibit enzymes involved in carbohydrate digestion, such as alpha-amylase and alpha-glucosidase. This inhibition slows down the breakdown of complex carbohydrates into simple sugars, leading to reduced glucose absorption and lower postprandial blood glucose levels[13]. By targeting these enzymes, secondary metabolites can help manage blood glucose levels, particularly after meals. By enhancing insulin sensitivity, modulating glucose metabolism, and reducing postprandial glucose levels, these compounds can help achieve better blood glucose control and reduce the need for conventional pharmacological interventions. Several clinical trials have demonstrated the efficacy of secondary metabolites, such as berberine and ginsenosides, in lowering blood glucose levels in diabetic patients[14]. The antioxidant, anti-inflammatory, and insulin-sensitizing properties of secondary metabolites make them promising candidates for the management of DM. The present study seeks to investigate the biosafety and bioactivity of the hydroethanolic extract of the aerial parts of *C. edulis*, in order to validate its use in the management of DM in traditional medicine in Zimbabwe.

1.3 Antioxidants and diabetes

Antioxidants are biomedically significant phytocompounds that are recognized for their numerous activities in health. These novel properties include the ability to neutralize or scavenge free radicals, lower oxidative stress, and protect against oxidative damage[15]. An imbalance between the antioxidant defense system and free radicals, such as the reactive species of nitrogen, sulphur and oxygen, results in oxidative stress[16]. This imbalance is linked to the etiology and pathophysiology of numerous diseases, including cancer, DM, cardiovascular disease, neurodegenerative disorders, and other inflammatory diseases. Oxygen is utilized by cells in regular energy production mechanisms, and this is neutralized by the innate oxidative system. High glucose levels, on the other hand, induce changes in mitochondria, increase oxidative phosphorylation, and result in increased free radical production in conditions such as DM[16]. Furthermore, in DM, reactive oxygen species (ROS) are produced by a variety of mechanisms, including an activated polyol pathway, increased formation of advanced glycated end products (AGEs), stimulation of the AGE receptor, activation of protein kinase C (PKC), and hexosamine pathway overactivity[17]. All of these processes contribute to DNA damage, protein and lipid peroxidation, insulin resistance, decreased antioxidant levels, and impaired insulin secretion and glucose utilization[18]. Several studies have demonstrated evidence of the relationship between oxidative stress, which results in increased production of ROS in DM, and the pathogenesis and progression of diabetic nephropathy, diabetic retinopathy and diabetic neuropathy[19]. Individuals with diabetes have reduced plasma antioxidant levels[20], and thus the failure of the body's innate antioxidant scavenging system to neutralize free radicals causes oxidative stress and, ultimately, cellular damage. Naturally occurring dietary antioxidants, including polyamines, carotenoids, flavonoids, glutathione, alpha-lipoic acid, and vitamins E, A, and C, clearly offer substantial protection against DM. Research indicates that antioxidant therapy prevents oxidative stress-induced beta-cell apoptosis, maintains beta-cell function, and minimizes complications of DM[15].

1.4 Anti-inflammatory role in diabetes treatments

Inflammation is a major factor in the etiology and progression of both type 1 and type 2 DM[21]. An inflammation known as insulitis in the pancreatic Langerhans beta cell islets appears to be a hallmark among all types of diabetes. This results in a decrease in beta cell quantity and function. Insulin-

producing beta cells are destroyed in type 1 DM due to autoimmune inflammation whereas insulin resistance is a result of chronic low-grade inflammation associated with obesity in type 2 DM[22]. Interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ are examples of pro-inflammatory cytokines that have been implicated in diabetes[23]. These cytokines are secreted by immune cells infiltrating the pancreatic islets, leading to beta-cell apoptosis and impaired insulin function[24]. Environmental triggers and viral infections, also play a role in initiating the inflammatory response that leads to the autoimmune destruction of beta cells[25]. Type 2 DM is closely associated with obesity, which is itself a state of chronic low-grade inflammation. Adipose tissue in obese individuals secretes various pro-inflammatory cytokines, including IL-6, TNF-α, and C-reactive protein (CRP), which contribute to insulin resistance[26]. These cytokines interfere with insulin signaling pathways, leading to reduced glucose uptake by cells and elevated blood glucose levels. Inflammation is both a cause and a consequence of insulin resistance. Insulin-resistant adipose tissue releases more free fatty acids, which can activate toll-like receptors (TLRs) on immune cells, further promoting the secretion of pro-inflammatory cytokines[27]. This creates a vicious cycle where inflammation exacerbates insulin resistance, leading to the progression of type 2 DM. Free fatty acids (FFAs) can induce the production and secretion of IL-1β and other IL-1-dependent proinflammatory cytokines in pancreatic islets, contributing to inflammation. IL-1β, once secreted, further stimulates its own production in beta cells, increasing nitric oxide levels and reducing mitochondrial ATP, which exacerbates beta cell dysfunction and decreases insulin secretion[21]. Hyperglycemia, initially triggered by inflammation in beta cells, promotes apoptosis through mechanisms involving IL-1β, as proposed by Shoelson *et al*[28]. Experimental studies have also shown that IL-6 plays a role in inducing apoptosis in pancreatic islets and serves as a marker for the progression of type 2 DM. Additionally, TNF- α is crucial in linking insulin resistance, obesity, and islet inflammation[29]. Its overproduction in adipose tissue perpetuates inflammation, beta cell death, and insulin resistance in peripheral tissues. The identification of potential pathways connecting inflammation to diabetes has produced growing interest in targeting inflammation to help prevent and control diabetes and related conditions, as well as improving risk stratification for diabetes by using inflammatory biomarkers as potential indexes[30].
Aiming to broaden the scope of orally administered hypoglycaemic agents, the present study was carried out to confirm the bioactivity of lyophilised hydroethanolic aerial extracts of *C. edulis* against known biological endpoints associated with hyperglycemic conditions including inflammation and oxidative cell damage. The study also serves to evaluate its safety in animal models, in order to complement earlier studies in different geographical set ups on this cardinal antidiabetic medicinal plant.

2 Materials and methods

2.1 Materials, equipment and facilities

All chemicals, associated reagents, equipment and facilities for the *in-vivo* laboratory animal toxicity investigations and the bioactivity assays were obtained from the University of Zimbabwe, Faculty of Medicine and Health Sciences laboratories, and the Harare Institute of Technology, Pharmaceutical Technology Department.

2.1.1 Animal use approval

Prior to the investigations, animal use and research ethics approvals were obtained from the Joint Parirenyatwa Research Ethics Committee (JREC) which is the local research Institutional Review board for the University of Zimbabwe.

2.1.2 Carisa edulis Plant material collection and preparation

Plant material was collected from the Beatrice area of Zimbabwe, 60 km Southwards from the City of Harare (18.2493° S, 30.8556° E). In observing the rules for Zimbabwe's Sustainable Harvesting of Traditional Medicinal Plants, the material was collected from 5 different plants. The plant material was authenticated as *C. edulis* by the National Herbarium and Botanical Garden in Harare, Zimbabwe. The leaves were thoroughly washed using clean water to remove debris and other contaminants, shade dried at room temperature to constant weight for three weeks, and then pulverized using mortar and pestle. The pulverized material was ground into a fine powder using a coffee grinder (Hamilton Beach Coffee Grinder Model- 80410).
The phyto-extraction was done by adding 500g plant powder into 1200ml of 70% (v/v) hydro-ethanolic mixture in a 2-litre sterile amber bottle and macerated for 5 days, with 3 minutes physical shaking twice a day. A muslin cloth was used to obtain a filtrate from the solution, which was further clarified by filtration using Whatman filter paper number 1. The filtrate was then evaporated under vacuum and low pressure (Rotavapor® R-300, Buchi, Switzerland), followed by lyophilization (Lyovapor l-200, Buchi,

Switzerland) under 140Pa pressure and -50 °C. The lyophilized extract was stored in an airtight sample bottle, at 4 °C in a refrigerator until required.

2.2 Phytochemical Screening of *C. edulis*

In a 200ml round bottomed flask, 10g of the lyophilized hydro-ethanolic extracts of *C. edulis* were dissolved in 100ml of distilled water and subjected to various phyto-screening techniques to confirm the presence or absence of relevant phytoconstituents of pharmacological interest. The following qualitative tests were conducted on the extract liquor.

2.2.1 Detection for alkaloids by the Iodine test

The Iodine test was used to determine the presence of alkaloids. In this assay, to 3ml of the lyophilized extract solution, a few drops of iodine solution were slowly added along the sides of the test tube. The presence of alkaloids was then identified by the appearance of a blue colour, which disappears on boiling and reappears on cooling [31].

2.2.2 Detection of tannins by the Braymer's test

The simplified Braymer's test was used to detect the presence of tannins. To 1ml lyophilised extract solution, 3 drops of a 10% Ferric chloride solution were added. The presence of tannins was confirmed by conversion of the solution to a blue-green colour[32].

2.2.3 Detection of flavonoids by the Ammonia test

Flavonoids were detected by means of the Ammonia test where 5ml dilute ammonia solution was added to 5ml of the lyophilised solution followed by a few drops of conc. H_2SO_4. The emergence of a yellow colour indicates the presence of flavonoids[33].

2.2.4 Detection of Glycosides by the Keller-Killani test

The presence of glycosides was done by the Keller-Killani test[34]. To 1mL of the lyophilised solution, 1.5mL glacial acetic acid was added and a few drops of 5% ferric chloride were added as well as conc. H_2SO_4 (along the side of test tube). The presence of glycosides was confirmed by the emergence of a blue coloured solution in mixture acetic acid layer.

2.2.5 Detection of Phenolic compounds by the Gelatin test

Phenolic compounds were detected using the Gelatin test. In this assay, 2ml the lyophilised extract solution was added to 5ml of a 1% gelatin solution and 5 drops of a 10% NaCl were further added. Phenolics were identified by the appearance of a white precipitate[35].

2.2.6 Detection of saponins by the simplified foam test

The simplified foam test was used to determine the presence of saponins. In this assay 2ml of the extract was added to 20ml distilled water. The mixture was shaken in a graduated cylinder for 15 minutes. The presence of saponins would be confirmed by the formation of form with a head height of at least 1cm[36].

2.2.7 Quantification of Total Phenolic and Tannins content

The total phenolic and tannins content of the lyophilised *C. edulis* were determined according to an amended Folin-Ciocalteu spectrophotometric method[37]. To prepare a calibration curve, phenol (Gallic acid) stock solution (5 mg/mL) was added into 100mL volumetric flasks, and then diluted to volume with distilled water. From each calibration solution, 0.25 mL was mixed with 1.25 mL of 10-fold diluted Folin-Ciocalteu's phenol (1 mL Folin reagent and 9 mL deionized water) reagent and allowed to react for 5 minutes. Then, 2 mL of 7.5% Na_2CO_3 solution was added, and the final volume was made up to 5 mL with deionized water. After 1 hour of reaction at room temperature, the absorbance at 760 nm was determined spectrophotometrically (Lambda 35 UV/Vis-Spectrometer, Perkin Elmer Instruments). The test was done in triplicate. Calibration curves were plotted to determine the level of phenolics and tannins in the samples. The total phenolic contents were calculated on the basis of the calibration curve of gallic acid and expressed as gallic acid equivalents (GAE), in milligrams per gram of the sample (comparison was made between ethanolic and methanolic extracts in this assay).

2.2.8 Quantification of total flavonoids

The total flavonoid content of the lyophilised *C. edulis* was estimated spectrophotometrically at 510 nm. In the test, 1mg of extract was dissolved in 2mL of distilled water. To this solution, 0.5mL of 1M sodium nitrite was added together with 2ml of a 1M, NaOH solution. Distilled water was then added to make up to 10ml volume. The solution was shaken and allowed to stand at room temperature for 15 min and the absorbance was subsequently measured. The total flavonoid content was estimated as mg of quercetin equivalent (mg QE/g extract) on a dry weight basis using the standard curve[38].

2.3 Anti-inflammatory activity of *C. edulis* using the egg albumin denaturation test

The anti-inflammatory activity of the lyophilised leaf extract of *C. edulis* was determined using the egg albumin protein denaturation assay, with slight modifications as described by Chifamba *et a.l* (2024)[39]. The samples and reagents used for this assay include 0.4 mL of egg albumin (fresh) from a free-range domesticated hen (*Gallus domesticus*), 10 mL of phosphate-buffered saline (PBS) at pH 7.2, and 5ml solutions of varying concentrations of the lyophilised leaf extracts in 0.4% DMSO. The concentrations of the lyophilized extracts in the total reaction solution ranged from 50 to 1000 µg/mL. The samples were incubated (Shel lab SRI3 Low Temperature BOD Incubator) for 20 minutes at 37°C; by heating at 65°C in a water bath for an additional 30 minutes to induce denaturation of the egg albumin. After cooling the mixture, the absorbance was measured at 660 nm (UV spectrophotometer, Lambda 35 UV/Vis-Spectrometer, Perkin Elmer Instruments) using the vehicle as blank. Negative controls consisting of 0.4 mL of fresh egg albumin, 0.5 mL of 0.4% DMSO, and 3 mL of PBS were included in the experiment. Diclofenac sodium was used as a positive control for the study at similar concentrations. The percentage of inhibition, which translates to the anti-inflammatory activity of the extracts and standards, was calculated by the following equation

Equation 1

$$Inflamation\ inhibition\ percentage\ effect = \frac{Abs_{sample}}{Abs_{control} - 1}\ x\ 100$$

where,

Abs_{sample} = absorbance of sample, $Abs_{control}$ = absorbance of control.

2.4 Anti oxidancy evaluation of *C. edulis* using the DPPH scavenging assay

The antioxidant activity of the lyophilised *C. edulis* hydroethanolic extracts was determined using the DPPH free radical scavenging assay[40]. Carefully measured 50 µL of the lyophilised plant extract solution in concentrations ranging from 5 to 150 mg/ml was added to identical bottles, and to each 5 ml of a 0.004% (w/v) solution of DPPH was added. The resultant mixture was agitated and incubated at 22°C (Shel lab SRI3 Low Temperature BOD Incubator) in darkness for 30 minutes. Discoloration was measured and readings taken in triplicate at 517 nm using a UV spectrophotometer (Lambda 35 UV/Vis-Spectrometer, Perkin Elmer Instruments). The blank was 70% (v/v) methanol. Ascorbic acid was used as a reference standard. The DPPH scavenging effect was calculated using the following equation,

Equation 2

$$DPPH\ Svavenging\ percentage\ effect = \frac{A^o - A}{A^o}\ x\ 100$$

Where A^o is the absorbance of negative control (0.004% DPPH solution), and A is the absorbance in the presence of extract.

The results were reported as IC_{50} values and ascorbic acid equivalents (AAE, mg/g) of *C.edulis.*

2.5 Acute oral toxicity evaluation of *C. edulis*

The acute oral toxicity evaluation of *C. edulis* lyophilized extract was done using a modified OECD technical guideline 425 (The up and down test)[41]. Female nulliparous Wistar rats (24) were used, which were acclimatized to the test environment for 10days prior to the commencement of the test protocols. The participating animals were fed with a commercial standardized rodent pellet from Agrofeeds® and were given water *ad libitum*. The animal habitat was kept at an average ambient temperature of 25°C throughout the study with a relative humidity level of 40% and an artificially controlled photoperiod of

12-h light and 12-h darkness. The animal welfare, observations and care were supervised by a practicing veterinary officer.
In our test, sequential ordered progressions of doses were orally administered to the animals at 48-hour intervals. The animals were divided into 2 groups of 12 female rats each; the first group (group 1) received distilled water and served as the control group. The second (group 2) received incremental doses of the *C. edulis* solution. The selected animals were marked so as to facilitate individual identification. . The experimental animals were fasted for 18 hours with water prior to dosing. Initial starting doses were chosen based on related toxicological studies. The first animal received a dose of 250mg/kg body weight, which was below a randomly selected estimated LD50. When animals survived the dose, the next dose was doubled, subject to our observations of the test animals over a period of 48 hours. The *C. edulis* was orally gavaged in a water solution in 4 different sets of doses of: 250, 500, 1000, 2500 and 5000 mg/kg body weight. The female rats were observed by a veterinary specialist for morbidity and mortality twice daily. In the absence of mortality the rats were observed for any visible changes and clinical signs and symptoms of toxicity every 1 hour, and up to 12 hours on day 1, and thereafter, once daily up to a maximum of 14 days. The animals were also weighed daily.

3 Results and discussion

3.1 Phytochemical screening

Table 1 : Qualitative screening of C. edulis secondary metabolites

Test	Presence in hydro-ethanolic extract	Presence in distilled water extract
Alkaloids	++	+
Phytosterols	+++	++
Flavonoids	+++	+
Saponins	-	-
Proteins and Amino Acids	++	+
Fixed oils and fats	-	-
Phenolic compounds	+++	+
Tannins	++	+
Carbohydrates	++	+
Glycosides	++	+
Terpenoids	+++	+
Lignans	+++	

(-): Indicates the absence of the phytochemical
(+): Indicates the presence of the phytochemical
(++): Indicates moderate presence of the phytochemical
(+++): Indicates strong presence of the phytochemical

From the phytochemical screening protocols, our investigations confirmed the abundance of primary and secondary metabolites of biomedical relevance with regards to diabetes (Table 1). The compounds with the strongest presence in the plant were phenolics (especially flavonoids), phytosterols, terpenes and lignans. These results correlate with studies by Elfiky *et al*[42], and Nedi *et al*[47] who identified terpenes, tannins, flavonoids, benzenoids, phenylpropanoid, lignans, sesquiterpenes, and coumarins among other compounds in *C. edulis*. The presence of proteins also corelates with observations by Muruthi *et a*l[43] where *C. edulis* exhibited varying protein content and glutathione reductase activity, thereby indicating enzymatic activity potential. This proliferation of medically relevant phytoconstituents validates the numerous uses of the plant in traditional medical practice in general, and the management of diabetes in particular.

3.1.1 Total phenolic content

All the extracts from the 3 different solvents (methanol, ethanol, and water), had considerable total phenolic content (TPC). The results of the TPC of the hydro-ethanolic extract was higher than both the hydromethanolic and aqueous extracts (Figure 2). This result can be explained by the fact that ethanol has a higher London dispersion force than methanol due to its higher molecular weight which leads to stronger intermolecular attraction than methanol.

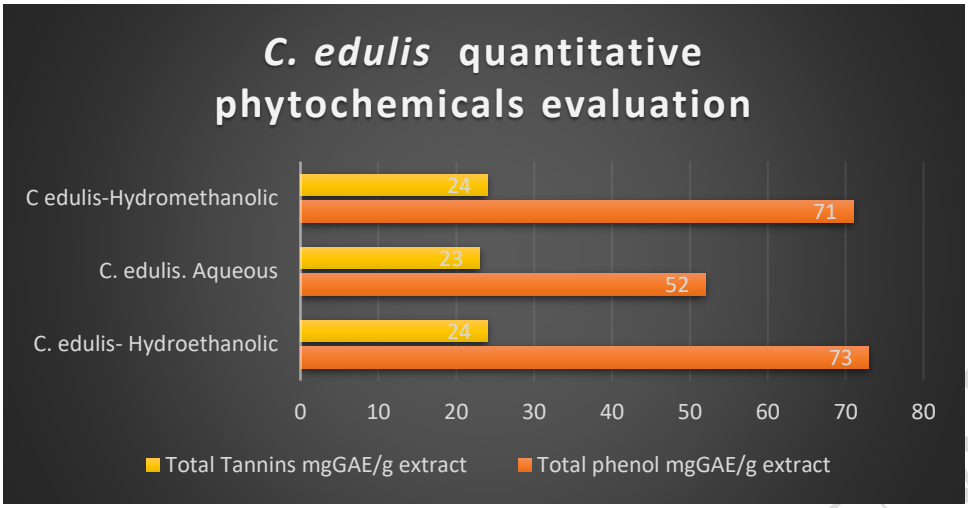

Figure 2: Total phenolic content for carissa edulis extract

The TPC represent the largest, diverse group of related secondary metabolites produced by plants which share a related sub structure composed of an aromatic-OH moiety[44]. Phenolics are produced mainly as a defence mechanism in response to hostile environmental biotic or abiotic stresses. Plant phenolics are mostly hydroxybenzoic and hydroxycinnamic acid derivatives. There has been increased interest towards their medicinal use as antioxidant, photoprotective, anti-inflammatory and wound healing activities[39]. Plant phenolics are the main group of secondary metabolites that function as antioxidants or reactive species scavengers. To this regard, these plant polyphenols mostly operate as singlet oxygen scavengers as well as reducing agents. In our study we preferred the Folin-Ciocalteu method compared to the others. In the present study the results were observed through the formation of a blue-coloured solution after the addition of the lyophilised extracts to the Folin-Ciocalteau reagent in the conducive medium. The colour change was due to the *in-situ* formation of the phospho molybdic-phosphotungstic-phenol complex. The TPC was calculated aided by the use of the regression equation of the calibration curve (R2 =0.989, y = 0.009x + 0.0464), expressed in GAE as milligrams per gram of the extract or fraction (mg GAE/g extract or fraction). As shown in Figure 2 and Figure 3, the 3 extracts yielded phenolic compounds in the following order: hydroethanolic > hydromethanolic> distilled water.

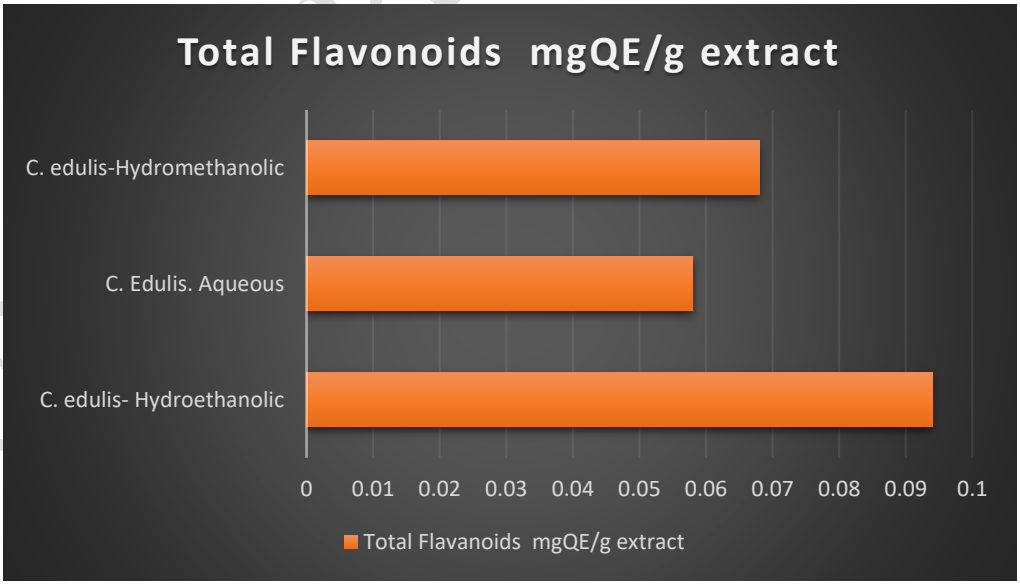

Figure 3: Total flavonoid content for carissa edulis extract

Flavonoids are a class of polyphenolic compounds that are differentiated by the chemical structures yielding subgroups such as flavones, flavonols, flavanones and flavanols[45]. Flavonoids possess potent antioxidant activities. These contribute to the scavenging of 2,2-diphenyl-1- picrylhydrazyl (DPPH) as shown in this study (Figure 4). Other related studies have demonstrated their effectivity against super oxides and ABTS radicals; and strong iron chelating activity. The most abundant flavonoids isolated

from the aerial parts of *C. edulis* in published studies include rutin, kaempferol and quercetin[43]. These flavonoids have displayed anti-inflammatory, arterial blood pressure and anti-diuretic activities in other studies[43].

3.2 Antioxidant Assay

DPPH scavenging activity was 93.4% for ascorbic acid (the standard used) at 150 µm/ml. While *C. edulis* hydro-ethanolic extract was 78.8% and 57.5% for the aqueous extract. Activity was determined from the absorbance obtained at specific concentrations (Figure 4). The IC50 values were 28.5µg/ml, 61.33µg/ml and 116.95µg/ml for ascorbic acid, *C. edulis* hydro-ethanolic extract and the aqueous extract respectively. Antioxidants exert their free radical scavenging activities via diverse mechanisms including reducing power, chelating transition metal, radical scavenging activities, and disintegrating radicals. In our studies we used the DPPH radical. The DPPH is a methanol soluble compound characterized by a deep-violet colour that exhibits maximum UVR absorption at 515 nm. The test principles are based on the fact that reactive species are able to reduce DPPH to 2,2-diphenyl-1-hydrazine (DPPH-H) or a substituted analogous hydrazine (DPPH-R) characterized by colourless or pale-yellow colour[46]. This colour change can easily be tracked spectrophotometrically. Previous scholars established that the participating metabolites in antioxidant activities of *C. edulis* include polyphenols, lignans, phytosterols, as well as enzymes. These metabolites are able to inhibit the oxidation related colour change. In our studies, although both aqueous and hydro-ethanolic extracts showed good inhibitory performance with respect to the DPPH radical, the hydro-ethanolic extract was more superior. The % inhibition was directly proportional to the extract concentration as shown in Figure 5.

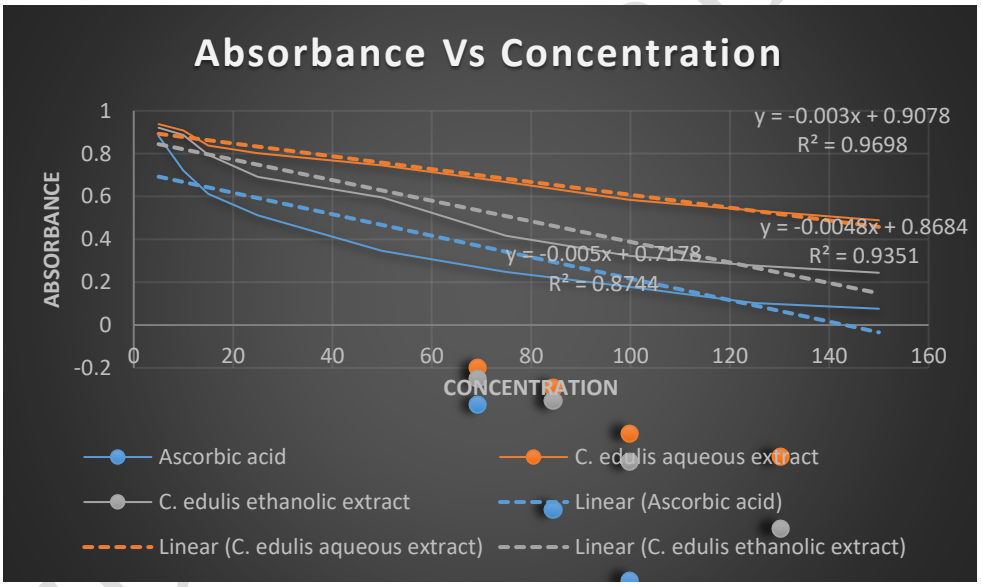

Figure 4: Absorbance vs Concentration of C. edulis extract

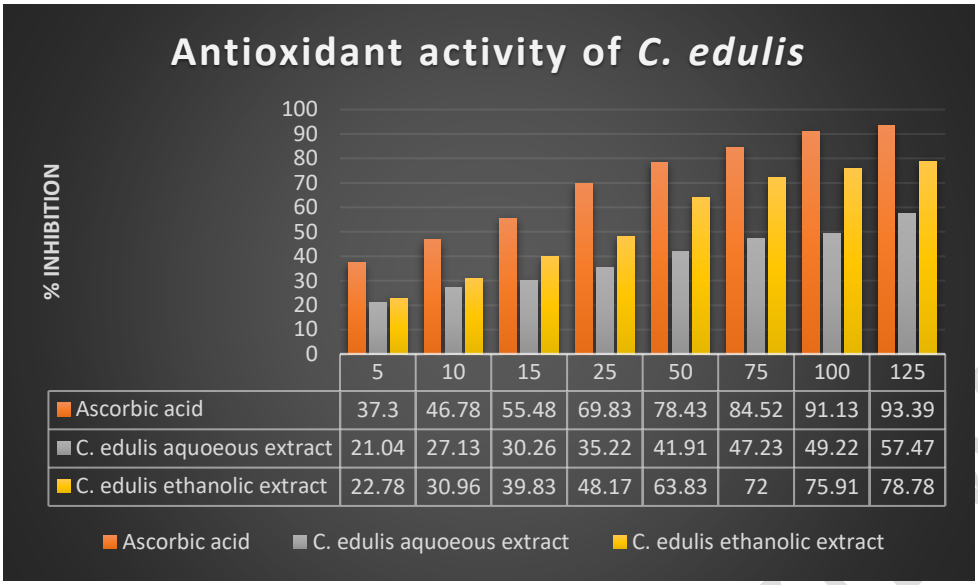

Figure 5: Antioxidant activity of C. edulis

We can therefore confirm that that *C. edulis* extracts are rich in various antioxidant compounds, which help mitigate oxidative stress, a contributing factor in diabetes complications[48]. Although not investigated in our study, anti-diabetic properties of *C. edulis* are well documented. In studies by Elfiky *et al*.,[42] oral administration of the ethanolic extract of *C. edulis* (2 g/kg body weight) significantly decreased blood glucose levels in diabetic adult male albino rats throughout the 3 h sampling period ($P < 0.05$). *C. edulis* (2 g/kg) had significant antidiabetic activity in comparison with the reference drugs metformin (500 mg/kg) and glibenclamide (3 mg/kg)[49].

3.3 Anti-inflammatory tests

Table 2: Anti-inflammatory activity of lyophilised C. edulis extracts

Concentration	% Inhibition	% Inhibition
µg/ml	***C. edulis* hydroethanolic extract**	**Diclofenac (standard)**
250	12± 0.08	39±0.84
500	18±0.03	65±1.78
1000	76±0.28	302±3.40
2000	265±1.76	760.12± 4.20
4000	386±1.34	Not tested
6000	488±3.56	Not tested
8000	716±3.54	Not tested

At related concentrations, the lyophilised extracts from the aerial parts of *C. edulis* exhibited anti-inflammatory effects that are approximately a third of the anti-inflammatory attributes of the standard Diclofenac (Table 2). The anti-inflammatory activity of *C. edulis* became comparable to the least dose of the positive control Diclofenac (250 µg/ml) used in this study at approximately around 750µg/ml. The anti-inflammatory effect of 2000 µg/ml of the positive control Diclofenac was approximately 6% greater than that for 8000 µg/ml lyophilised crude extracts of *C. edulis*. Much higher values were administered in previous studies using the carrageen induced oedema foot model, where the root extract of *C. edulis* inhibited acute oedema in a dose dependent manner from 30-300mg/kg, with a maximal inhibition of 53.8±8.2% attained over 4 h period (Woode et al., 2007). Ya'u *et al*[51] used up to 600mg/kg to achieve reduction in oedema. Although different methods are used here to represent inhibition of inflammation,

the value of 8000µg/ml recorded in our protein denaturation assay, represents significant anti-inflammatory potential of *C. edulis*, since the extract was used in its crude form. Activity is expected to increase further using bioactive fractions and isolated compounds. Phytosterols, triterpenoids such as lupeol and oleanolic acid, sesquiterpenes, and flavonoids such as rutin and kaempferol have been associated with anti-inflammatory activity of the *Carissa* genus[50]. Saponins which are normally found in abundance in the plant, have also shown anti-inflammatory activity in addition to anti-oxidant capacityjk[13]. Saponins were absent from our extract (Table 1), possibly as a result of geographical and environmental influences. However, our findings suggest that by targeting chronic inflammation pathways, *C. edulis* can potentially improve insulin sensitivity and reduce insulin resistance, in the management of DM.

3.4 Acute oral toxicity evaluation

Table 3: Acute oral toxicity study of C edulis behavioural Observations

Observed parameter	Dose of *C. edulis* in mg/kg body weight					
	250mg	**500mg**	**1000mg**	**2500mg**	**5000mg**	**Control**
Food intake	Normal	Normal	Normal	Normal	Normal	Normal
Water intake	Normal	Normal	Normal	Normal	Normal	Normal
Death	Alive	Alive	Alive	Alive	Alive	Alive
Breathing	Normal	Normal	Normal	Normal	Normal	Normal
Diarrhea	Not observed	Not observed	Not observed	Not observed	Not observed	Not observed
Urination	Normal	Normal	Normal	Normal	Normal	Normal
Skin colour	Normal	Normal	Normal	Normal	Normal	Normal
Drowsiness	Not observed	Not observed	Not observed	Not observed	Not observed	Not observed
Erection of Fur	Not observed	Not observed	Not observed	Not observed	Not observed	Not observed

The acute toxicity study was carried out as per OECD technical guideline 425. The observations, results and interpretation were done by a qualified veterinary expert. Our findings indicated that the extract at doses up to 5000 mg/kg body weight imparted neither visible signs of toxicity nor mortality in rats, suggesting its safety. No animals were withdrawn from the study for any reason during the observation period. Our results are in agreement with studies by Ya'u *et al*[51] where the LD50 of the *C. edulis* extract was estimated to be > 5000 mg/kg body weight. Our extracts were deemed non-toxic, based on the toxicity classification proposed by Loomis and Hayes[52], which categorise substances with LD50 values from 500 to 5000 mg/kg as slightly toxic and those with LD50 values 5000 to 15,000 mg/kg body weight are regarded as practically non-toxic. This therefore implies that the use of high extract concentrations to achieve the desired bioactivity effects will not pose any toxicity in *C. edulis* based treatments. This is in contrast to the purported toxicity arising from the presence of the poisonous triterpenoid, carissin, from which the genus *Carissa* derives its name. Carissin has been previously isolated from root and bark extracts of *C. edulis*, hence it may be absent, or present in insignificant quantities in the aerial plant parts used in this study. Further tests would be required to determine the actual phytocompounds represented by the classes shown in Table 1.

3.5 Rat weights observations

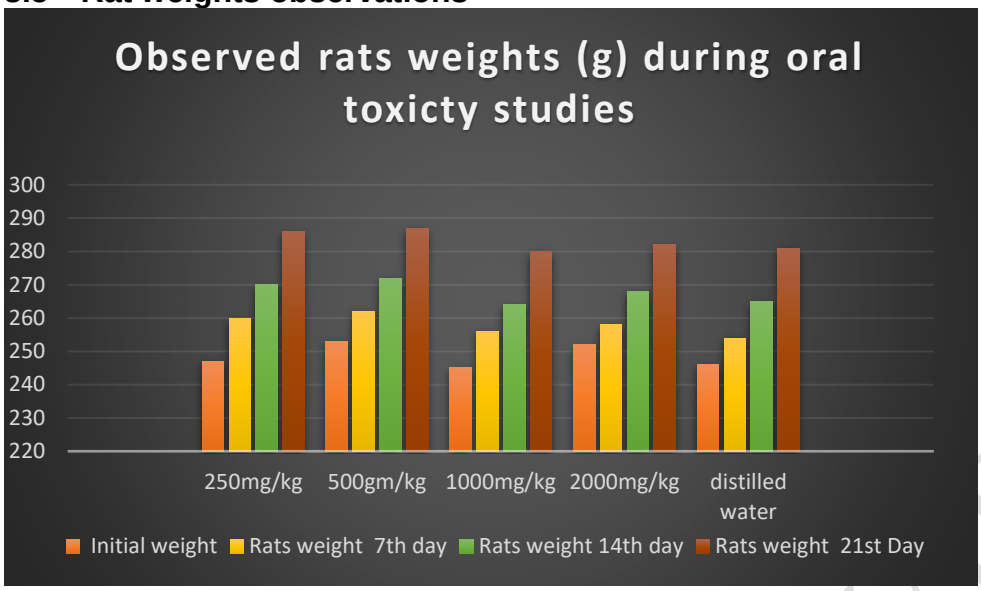

Figure 6: Observed rat weights during acute oral toxicity studies of C edulis

In toxicity evaluations, generally, unexpected fluctuations in body weight are a simple and sensitive reflection of toxicity after exposure of study animals to materials. Progressive Weight loss or gain of animals is usually indicative of stress, failure to feed or a response to observed or underlying adverse health conditions. In the present study, the lyophilised extracts did not significantly affect normal body weight growth during the study period suggesting that the extract did not alter rat growth at the concentrations investigated.

4 Conclusions

The aerial lyophilised hydro-ethanolic extracts of *C. edulis* were shown to possess considerable antioxidant and anti-inflammatory activities. The observed activities were attributable to the presence of secondary metabolites including lignins, phenolic compounds, flavonoids, and phytosterols. These contribute to the underlying mechanisms behind the plant's proven hypoglycaemic effects and therapeutic activities in DM. *C. edulis* was nontoxic at 5000mg/kg. Our biosafety and bioactivity studies therefore authenticate the use of *C. edulis* as a potential antidiabetic remedy in traditional medicine.

5 References

1. Zubairu M, Taila N, Rabiu KM, Zubairu A, Abdullahi JA. Effect of Methanolic Leaf Extract of Carissa Edulis on Alloxan Induced Diabetic Albino Rats. NIGERIAN ANNALS OF PURE AND APPLIED SCIENCES. 2022 Nov 15;5(2):353-8.
2. Fanta Yadang SA, Taiwe Sotoing G, Ngatcha Zouakeu KS, Khan MA, Agbor GA, Ur-Rahman N, Ngo Bum E. Quantification of bioactive compounds and evaluation of the antioxidant activity of Carissa edulis Valh (Apocynaceae) leaves. The Scientific World Journal. 2019;2019(1):7549620.
3. Musinguzi E, Kikafunda JK, Kiremire BT. Promoting indigenous wild edible fruits to complement roots and tuber crops in alleviating vitamin A deficiencies in Uganda.
4. Nedi T, Mekonnen N, Urga K. Diuretic effect of the crude extracts of Carissa edulis in rats. Journal of ethnopharmacology. 2004 Nov 1;95(1):57-61.
5. Inzucchi, S., Porte, D., Sherwin, R. and Baron, A. (2004). Diabetes Mellitus Manual.Mcgraw-Hill Professional Publishing
6. Yau JW, Rogers SL, Kawasaki R, Lamoureux EL, Kowalski JW, Bek T, Chen SJ, Dekker JM, Fletcher A, Grauslund J, Haffner S. Global prevalence and major risk factors of diabetic retinopathy. Diabetes care. 2012 Mar 1;35(3):556-64.
7. Oguntibeju OO. Type 2 diabetes mellitus, oxidative stress and inflammation: examining the links. International journal of physiology, pathophysiology and pharmacology. 2019;11(3):45.

8. Butkowski EG, Jelinek HF. Hyperglycaemia, oxidative stress and inflammatory markers. Redox Report. 2017 Nov 2;22(6):257-64.
9. Atangwho, I.J., Ebong P.E., Eyong E.U., Eteng M.U. and Uboh F.E. (2007).Vernonia Amygdalina Del: A Potential Prophylactic Anti-Diabetic Agent in Lipid Complication. Global Journal of Pure and Applied Science. 18(1): 103-106.
10. Donath MY, Shoelson SE. Type 2 diabetes as an inflammatory disease. Nature reviews immunology. 2011 Feb;11(2):98-107.
11. Böni-Schnetzler M, Boller S, Debray S, et al. Free fatty acids induce a proinflammatory response in islets via the abundantly expressed interleukin-1 receptor I. Endocrinology 2009;150:5218–29. https://doi.org/10.1210/en.2009-0543; PMID: 19819943.
12. Arafat HA, Katakam AK, Chipitsyna G, et al. Osteopontin protects the islets and beta-cells from interleukin-1 betamediated cytotoxicity through negative feedback regulation of nitric oxide. Endocrinology 2007;148:575–84. https://doi.org/10.1210/en.20060970;PMID:17110428
13. Maedler K, Oberholzer J, Bucher P, et al. Monounsaturated fatty acids prevent the deleterious effects of palmitate and high glucose on human pancreatic beta-cell turnover and function. Diabetes 2003;52:726–33. https://doi.org/10.2337/diabetes.52.3.726; PMID: 12606514
14. Niinistö S, Takkinen HM, Uusitalo L, Rautanen J, Vainio N, Ahonen S, Nevalainen J, Kenward MG, Lumia M, Simell O, Veijola R. Maternal intake of fatty acids and their food sources during lactation and the risk of preclinical and clinical type 1 diabetes in the offspring. Acta diabetologica. 2015 Aug;52:763-72.
15. Yashin A, Yashin Y, Xia X, Nemzer B. Antioxidant activity of spices and their impact on human health: A review. Antioxidants. 2017 Sep 15;6(3):70.
16. Caturano A, D'Angelo M, Mormone A, Russo V, Mollica MP, Salvatore T, Galiero R, Rinaldi L, Vetrano E, Marfella R, Monda M. Oxidative stress in type 2 diabetes: impacts from pathogenesis to lifestyle modifications. Current Issues in Molecular Biology. 2023 Aug 12;45(8):6651-66.
17. Vodošek Hojs N, Bevc S, Ekart R, Hojs R. Oxidative stress markers in chronic kidney disease with emphasis on diabetic nephropathy. Antioxidants. 2020 Sep 27;9(10):925.
18. Sagoo MK, Gnudi L. Diabetic nephropathy: is there a role for oxidative stress?. Free Radical Biology and Medicine. 2018 Feb 20;116:50-63.
19. Pieme CA, Tatangmo JA, Simo G, Biapa Nya PC, Ama Moor VJ, Moukette Moukette B, Tankeu Nzufo F, Njinkio Nono BL, Sobngwi E. Relationship between hyperglycemia, antioxidant capacity and some enzymatic and non-enzymatic antioxidants in African patients with type 2 diabetes. BMC Research Notes. 2017 Dec;10:1-7.
20. Haydinger CD, Oliver GF, Ashander LM, Smith JR. Oxidative stress and its regulation in diabetic retinopathy. Antioxidants. 2023 Aug 21;12(8):1649.
21. Ruan H, Miles PD, Ladd CM, et al. Profiling gene transcription in vivo reveals adipose tissue as an immediate target of tumor necrosis factor-alpha: implications for insulin resistance. Diabetes 2002;51:3176–88. https://doi.org/10.2337/diabetes.51.11.3176; PMID: 12401708.
22. Sell HC, Habich C, Eckel J. Adaptive immunity in obesity and insulin resistance. Nat Rev Endocrinol 2012;8:709–16. https://doi.org/10.1038/nrendo.2012.114; PMID: 22847239.
23. Atkinson MA, Eisenbarth GS. Type 1 diabetes: new perspectives on disease pathogenesis and treatment. The Lancet. 2001 Jul 21;358(9277):221-9.
24. Eizirik DL, Colli ML, Ortis F. The role of inflammation in insulitis and β-cell loss in type 1 diabetes. Nature Reviews Endocrinology. 2009 Apr;5(4):219-26.
25. Cappon G, Vettoretti M, Sparacino G, Facchinetti A, Kim MK, Ko SH, Kim BY, Kang ES, Noh J, Kim SK, Park SO. 2019 Clinical practice guidelines for type 2 diabetes mellitus in Korea. Diabetes & metabolism journal. 2019 Aug;43(4):398-406.
26. Tiwari BK, Pandey KB, Abidi AB, Rizvi SI. Markers of oxidative stress during diabetes mellitus. Journal of biomarkers. 2013;2013(1):378790.
27. Todd JA. Etiology of type 1 diabetes. Immunity. 2010 Apr 23;32(4):457-67.
28. Shoelson SE, Lee J, Goldfine AB. Inflammation and insulin resistance. The Journal of clinical investigation. 2006 Jul 3;116(7):1793-801.
29. Pieme CA, Tatangmo JA, Simo G, Biapa Nya PC, Ama Moor VJ, Moukette Moukette B, Tankeu Nzufo F, Njinkio Nono BL, Sobngwi E. Relationship between hyperglycemia, antioxidant capacity and some enzymatic and non-enzymatic antioxidants in African patients with type 2 diabetes. BMC Research Notes. 2017 Dec;10:1-7.
30. Tilg H, Hotamisligil GS. Nonalcoholic fatty liver disease: cytokine-adipokine interplay and regulation of insulin resistance. Gastroenterology. 2006 Sep 1;131(3):934-45.

31. Alemu M, Lulekal E, Asfaw Z, Warkineh B, Debella A, Abebe A, Degu S, Debebe E. Antibacterial activity and phytochemical screening of traditional medicinal plants most preferred for treating infectious diseases in Habru District, North Wollo Zone, Amhara Region, Ethiopia. PLoS One. 2024 Mar 5;19(3):e0300060. doi: 10.1371/journal.pone.0300060. PMID: 38442129; PMCID: PMC10914283.
32. Kebede T, Gadisa E, Tufa A. Antimicrobial activities evaluation and phytochemical screening of some selected medicinal plants: A possible alternative in the treatment of multidrug-resistant microbes. PLoS One. 2021 Mar 26;16(3):e0249253. doi: 10.1371/journal.pone.0249253. PMID: 33770121; PMCID: PMC7997013.
33. Jaafar HZ, Ibrahim MH, Karimi E. Phenolics and flavonoids compounds, phenylanine ammonia lyase and antioxidant activity responses to elevated CO_2 in Labisia pumila (Myrisinaceae). Molecules. 2012 May 25;17(6):6331-47. doi: 10.3390/molecules17066331. PMID: 22634843; PMCID: PMC6268359.
34. https://brainly.in/question/10471653
35. Hongzhen Lian, Danli Li, Xiaoxian Wu, Liching Tian, Determination of phenolic preservatives in gelatin and vacant capsules for medicine use by ion-suppression reversed-phase high performance liquid chromatography,Journal of Pharmaceutical and Biomedical Analysis,Volume 37, Issue 2,2005,Pages 369-372,ISSN 0731-7085,https://doi.org/10.1016/j.jpba.2004.11.001.
36. Rai S, Kafle A, Devkota HP, Bhattarai A. Characterization of saponins from the leaves and stem bark of Jatropha curcas L. for surface-active properties. Heliyon. 2023 Apr 28;9(5):e15807. doi: 10.1016/j.heliyon.2023.e15807. PMID: 37187903; PMCID: PMC10176063.
37. Pérez M, Dominguez-López I, Lamuela-Raventós RM. The Chemistry Behind the Folin-Ciocalteu Method for the Estimation of (Poly)phenol Content in Food: Total Phenolic Intake in a Mediterranean Dietary Pattern. J Agric Food Chem. 2023 Nov 22;71(46):17543-17553. doi: 10.1021/acs.jafc.3c04022. Epub 2023 Nov 10. PMID: 37948650; PMCID: PMC10682990.
38. Ramos RTM, Bezerra ICF, Ferreira MRA, Soares LAL. Spectrophotometric Quantification of Flavonoids in Herbal Material, Crude Extract, and Fractions from Leaves of *Eugenia uniflora* Linn. Pharmacognosy Res. 2017 Jul-Sep;9(3):253-260. doi: 10.4103/pr.pr_143_16. PMID: 28827966; PMCID: PMC5541481.
39. Chifamba, J, A J Addae, S Zengeni, M Pomerai, and N Kurebgaseka. 2024. "Lyophilised Aloe Excelsa Fractions, Photo-Protection and Actinic Damage Retardation Claims Substantiation". *Journal of Complementary and Alternative Medical Research* 25 (5):37-52. https://doi.org/10.9734/jocamr/2024/v25i5536.
40. Baliyan S, Mukherjee R, Priyadarshini A, Vibhuti A, Gupta A, Pandey RP, Chang CM. Determination of Antioxidants by DPPH Radical Scavenging Activity and Quantitative Phytochemical Analysis of *Ficus religiosa*. Molecules. 2022 Feb 16;27(4):1326. doi: 10.3390/molecules27041326. PMID: 35209118; PMCID: PMC8878429.
41. https://www.oecd-ilibrary.org/environment/test-no-425-acute-oral-toxicity-up-and-down-procedure_9789264071049-en
42. Elfiky F.K., Aboukaram M.A. and Afify E.A. (1996). Effect of Luffa aegyptiaca (seeds) and Carissa edulis (leaves) extracts on blood glucose level of normal and streptozotocin diabetic rats. Journal of Ethnopharmacol. 50:43–47. doi: 10.1016/0378-8741(95)01324- 5
43. Muruthi CW, Ngugi MP, Runo SM, Mwitari PG. *In Vitro* Antiproliferative Effects and Phytochemical Characterization of *Carissa edulis* ((Forssk) Vahl) and *Pappea capensis* (Eckyl and Zeyh) Extracts. J Evid Based Integr Med. 2023 Jan-Dec;28:2515690X231187711. doi: 10.1177/2515690X231187711. PMID: 37489007; PMCID: PMC10387709.
44. Hano C, Tungmunnithum D. Plant Polyphenols, More than Just Simple Natural Antioxidants: Oxidative Stress, Aging and Age-Related Diseases. Medicines (Basel). 2020 May 9;7(5):26. doi: 10.3390/medicines7050026. PMID: 32397520; PMCID: PMC7281114.
45. Dias MC, Pinto DCGA, Silva AMS. Plant Flavonoids: Chemical Characteristics and Biological Activity. Molecules. 2021 Sep 4;26(17):5377. doi: 10.3390/molecules26175377. PMID: 34500810; PMCID: PMC8434187
46. Gulcin, İ.; Alwasel, S.H. DPPH Radical Scavenging Assay. *Processes* **2023**, *11*, 2248. https://doi.org/10.3390/pr11082248
47. Nedi T, Mekonnen N, Urga K. Diuretic effect of the crude extracts of Carissa edulis in rats. J Ethnopharmacol. 2004 Nov;95(1):57-61. doi: 10.1016/j.jep.2004.06.017. PMID: 15374607.
48. Fanta Yadang, Sabine Adeline, Taiwe Sotoing, Germain, Ngatcha Zouakeu, Katoucha Sidoine, Khan, Muhammad Ahsan, Agbor, Gabriel Agbor, Ur-Rahman, Nisar, Ngo Bum, Elisabeth, Quantification of Bioactive Compounds and Evaluation of the Antioxidant

Activity of *Carissa edulis* Valh (Apocynaceae) Leaves, *The Scientific World Journal*, 2019, 7549620, 9 pages, 2019. https://doi.org/10.1155/2019/7549620
49. Elfakhrany A, Abo-Elsoud RAEA, Abd El Kareem HM, Samaka RM, Elfiky SR. Autophagy and Oxidative Balance Mediate the Effect of Carvedilol and Glibenclamide in a Rat Model of Renal Ischemia-Reperfusion Injury. Open Access Maced J Med Sci [Internet]. 2022 Feb. 5 [cited 2024 Sep. 20];10(A):1402-10. Available from: https://oamjms.eu/index.php/mjms/article/view/10125
50. Galipalli S, Patel NK, Prasanna K, Bhutani KK. *Nat. Prod. Res.* 2015;29:1670–1672. doi: 10.1080/14786419.2014.989846
51. J. Ya'u, B.A. Chindo, A.H. Yaro, S.E. Okhale, J.A. Anuka, I.M. Hussaini, Safety assessment of the standardized extract of Carissa edulis root bark in rats, Journal of Ethnopharmacology,Volume 147, Issue 3,2013,Pages 653-661,ISSN 378-8741,https://doi.org/10.1016/j.jep.2013.03.064
52. Loomis, T.A. and Hayes, A.W. (1996) Loomis's Essentials of Toxicology. 4th Edition, Academic Press, Cambridge,208-245. https://doi.org/10.1016/B978-012455625-6/50014-3