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Research Article

Phytochemical content, antioxidant and antidiabetic activities variations in pre- and post-harvest conditions for the *Cnidoscolus chayamansa* McVaugh leaf

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Abstract

Cnidoscolus chayamansa, widely utilized in Nigeria for its antioxidant and antidiabetic properties, was investigated to determine its phytochemical profile, antioxidant potential, and antidiabetic effects at various growth stages, with a particular emphasis on the pre-flowering stage. The study examined how post-harvest practices affect the concentrations of bioactive compounds and their regulatory effects on critical glycolytic enzymes, including hexokinase, glucose-6-phosphatase, and fructose-1,6-bisphosphatase, in alloxan-induced diabetic rat models. The total phenolic and flavonoid contents were evaluated using Folin-Ciocalteu (FC) and aluminium chloride colorimetry assays respectively, while the antioxidant was evaluated using the DPPH, ABTS, FRAP and NO assays. *In vivo* antidiabetic activity was evaluated using alloxan-induced rats' model, while the α-amylase and α-glucosidase inhibitory assays were carried out *in vitro* assays. Additionally, biochemical marker such as fasting blood sugar level was also assessed. The results showed that antioxidant activity, assessed through DPPH had an IC_{50} value of 4.02 ± 0.01 µg/mL), ABTS (IC_{50} = 8.12 ± 0.01 µg/mL), FRAP (225.4 ± 5.2 µmol Fe^{2+}/g), and NO scavenging assays (IC_{50} = 12.3 ± 2.7 µg/mL), indicated robust free radical neutralization. The pre-flowering stage had the highest phenolic (1685.12 ± 2.14 mg GAE/g) and flavonoid contents (306.02 ± 2.01 mg QE/g), correlating with enhanced glucose metabolism through increased hexokinase activity (85.12% relative to control) and decreased glucose-6-phosphatase (45%) and fructose-1,6-bisphosphatase activities (50%). *In vivo* studies showed a significant reduction in fasting blood glucose levels (from 18.4 ± 0.7 mmol/L to 5.8 ± 0.5 mmol/L) and improved glucose tolerance in diabetic rats treated with pre-flowering extracts. Post-harvest handling, including drying and storage, reduced bioactive compound stability by 20-30%, suggesting the pre-flowering stage is optimal for therapeutic application.

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Cnidoscolus chayamansa, pre-flowering stage, antioxidant activity, antidiabetic potential, post-harvest stability.

1. Introduction

Oxidative damage caused by free radicals can lead to chronic diseases like atherosclerosis, cancer, diabetes, and ageing. While free radicals can come from the environment, they can also be produced by physiological and biochemical processes in the body. These reactive oxygen species can harm proteins and lipids, making antioxidants essential for their ability to slow down or inhibit the oxidation process [1]. Research suggests that the synthetic antioxidants commonly found in food may pose certain health

risks. As a result, there's a growing recommendation to turn to natural antioxidants derived from plants. These plant-based sources like vegetables, fruits, herbs, and spices are rich in beneficial compounds, including phenolic compounds, vitamins, and terpenoids, which all contribute to their antioxidant properties. Embracing these natural options not only supports health but also enhances the flavors of our food [2]. Consequently, there has been significant research on natural antioxidants sourced from plants due to their potential nutritional and therapeutic benefits.

Diabetes mellitus (DM) is a severe and chronic metabolic disorder characterized by high blood glucose levels. The number of individuals with type II. DM is rapidly increasing worldwide, with approximately 180 million people currently affected, according to a report by the World Health Organization. DM can also lead to serious complications like retinopathy and cataracts [3–5]. One method for managing diabetes is to delay the absorption of glucose by inhibiting carbohydrate-hydrolyzing enzymes, specifically α-glucosidase in the digestive system. There have been studies on plant-derived α-glucosidase inhibitors and their effects on postprandial blood glucose levels. By inhibiting α-glucosidase, the digestion of carbohydrates is delayed [6]. As a result, α-glucosidase inhibitors hold great promise as therapeutic agents for the treatment of type II DM and hyperglycemia. Acarbose is currently the most used α-glucosidase inhibitor; however, it can cause gastrointestinal side effects [7]. Plants are considered valuable sources of medicinal compounds, and many currently available drugs have been derived from plants. Ethnobotanical reports suggest that around 1000 medicinal plants may have anti-diabetic potential. Consequently, there has been significant interest in screening α-glucosidase inhibitors from plants in recent years.

The stages and seasons of medicinal plants' collection can have a significant impact on the composition of phytochemicals in medicinal plants [8]. Phytochemicals are secondary metabolites that play a crucial role in the survival and protection of plants. These bioactive compounds have been used for decades in traditional medicine and continue to be popular today. However, various environmental factors, such as soil type, humidity, and seasonal variations as well as collection stages (pre- and post-harvest) can influence the phytochemical compositions and biological activities of plants [8]. Therefore, studying the effects of these factors on plant phytochemicals and biological activities can provide valuable insights into the optimal stage to collect plants for maximum phytochemical contents and biological activities. One such plant with variations in phytochemical contents and biological activities in Nigeria is *Cnidoscolus chayamansa*.

Cnidoscolus chayamansa is a small tree or shrub that can reach a height of approximately 3 meters. Its leaves are green and divided into 3 to 5 lobes, measuring up to 15 cm in length. This plant produces separate male and female flowers, which are found on the same long stalked cluster of flowers. The flowers are white and around 1 cm wide. The fruit of this plant is a seed pod. The name of the genus, *Cnidosculos*, comes from the Greek words "*knide*" and "*skolos*," meaning "nettle" and "thorn," respectively, referring to the stinging hairs on the plant. The species name, chayamansa, is derived from the Mayan word "*chaay*," meaning "plant," and the Latin word "*mansa*," meaning house. In terms of ethnobotany, the leaves and stems are edible. However, it is important to cook the younger leaves and stems thoroughly before consumption because eating them raw can be poisonous. The plant has medicinal properties and can be used to treat various diseases, including anaemia, diabetes, cancer, inflammation, high cholesterol, liver, and heart protection. Because of its effectiveness in preventing illnesses, it has gained the nickname "hospital too far." The plant is native to Mexico but can also be found in Egypt, Central Africa, Nigeria, and other regions with moderate rainfall and well-drained soils [9,10].

This study aimed to assess the levels of phytochemicals, antioxidants, and anti-diabetic properties in the leaves of *C. chayamansa*. These leaves were collected both before and after harvesting for comparison.

2. Materials and methods

2.1 Chemicals and reagents

Alloxan-monohydrate, Folin-Ciocalteu, methanol, α-amylase, acarbose, α-glucosidase, and other necessary

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substances were obtained from Sigma-Aldrich (St Louis Mo, USA) unless mentioned otherwise. Every chemical and reagent used in the experiment was of analytical grade.

2.2 Collection, identification, and preparation of plant material

The leaves of *C. chayamansa* were freshly collected from different growth stages, including early vegetative (EVS) and pre-flowering stages (PFS), flowering (FS), and maturity stages (MS). These leaves were obtained from a forest in Fori, Maiduguri, in April and August 2024, with permission from the farm owner. Dr. C.A. Ukwubile, a taxonomist from the Department of Pharmacognosy at the University of Maiduguri, Nigeria, identified the plant. A voucher specimen with the number UMM/FPH/EUB/003 was deposited in the departmental herbarium. The taxonomic status of the plant was further confirmed using the website http://www.worldfloraonline.org. The studies followed local and national ethical regulations and guidelines regarding the usage of medicinal plants. The leaves were then shade-dried for two weeks at a temperature of 25 °C and the same storage conditions in the laboratory. They were ground into a fine powder using a Philips blender (1000W METAL HR3573/91, China)

2.3 Extraction of plant materials

Exactly 1000 g of powdered leaves were each extracted with absolute methanol (100% v/v) for 48 h using cold maceration technique and concentrated in a vacuum in a rotary evaporator (ThermoFisher Scientific, England) to obtain the dark green colour leaf methanol extract weighing 125 g each (% yield was 12.5 each). The extracts were accurately labelled and stored in the refrigerator at 4 °C for further usage.

2.4 Phytochemical screening

Standard qualitative protocols, adapted from previous research, were used to conduct a phytochemical screening of the various leaf extracts obtained from *C. chayamansa*. The aim was to determine the presence or absence of specific metabolites, including alkaloids, flavonoids, saponins, tannins, anthraquinones, proteins, phytosterols/ steroids, triterpenoids, fats/oils, and cardiac glycosides [11].

2.4.1 Determination of total phenolic content (TPC)

To determine the amount of phenolic compounds present, the Folin-Ciocalteu method was employed [12]. This involved adding 200 µL of the various extract concentrations (ranging from 15-240 µg/mL) to test tubes containing 2 mL of sodium carbonate. After 2 minutes, 200 µL of Folin-Ciocalteu reagent was added, and the mixtures were thoroughly mixed and then incubated in a water bath at 64.7 °C for 30 minutes. The absorbance of the samples was measured at 760 nm. Similarly, a standard solution of gallic acid, prepared in the same manner as the extract solutions, was used. The total phenolic content was determined using the same procedure as the extracts but with the standard solution instead. The total phenolic content (TPC) was calculated using the following formula derived from the gallic acid calibration curve:

$$TPC = C \times V/m \quad (i)$$

Where TPC denotes total phenolic content, C denotes the concentration of extract, V denotes the volume of solvent used and m denotes the mass of extract. The results were expressed in mg GAE/g.

2.4.2 Determination of total flavonoid content (TFC)

The total flavonoid content of the extracts was determined using the $AlCl_3$ colorimetry assay. Stock solutions of the extracts were prepared at concentrations ranging from 15-240 µg/mL. A volume of 1 mL of extract was added to clean test tubes, followed by the addition of 3 mL of distilled water and 0.3 mL of 5% $NaNO_2$. After 5 minutes, 0.3 mL of 10% $AlCl_3$ and 2 mL of 1 M NaOH were added. The volume of the solution in the test tube was then diluted to 10 mL with distilled water. The absorbance of the solution was measured at 510 nm. Quercetin was used as a reference compound, prepared by dissolving 4 mg in 1 mL of methanol. Various concentrations of the quercetin solution were prepared, and the total flavonoid content of the extracts was determined using the same method described earlier [12].

2.4.3 GC-MS analysis of non-volatile oils composition

The GC-MS analyses were conducted using a 7890A GC-MS apparatus (Agilent Technologies, USA) with an HP-5MS capillary column and a mass-selective detector. The carrier gas used was helium, flowing at a rate of 0.8 mL/min. The oven temperature was

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gradually increased from 50 to 240 °C at a rate of 2 °C/min. The injector temperature was maintained at 250 °C. The quadrupole and source temperatures were set at 150 °C and 230 °C, respectively. The mass scan ranged from 50 to 550 m/z with an electron energy of 70 eV. The non-volatile oil components were identified by comparing their retention times with those of known standards present in the lab's collection and by matching their retention indices with values reported in the literature using a series of n-alkanes (ranging from C9 to C24). To further confirm the identification, the mass spectra of the components were compared with those stored in the NIST08 and W8N08 libraries [13].

2.5 In vitro antioxidant determination

2.5.1 DPPH radical scavenging activity

The method described by Ukwubile et al. [14], was followed to evaluate the scavenging effect of the extracts on 2, 2-diphenyl-1-picrylhydrazyl (DPPH). In summary, 1 mL of the extract was added at concentrations ranging from 15 to 240 µg/mL to a solution of 0.4 mM DPPH prepared in methanol. The mixture was kept in the dark for 30 minutes before measuring the absorbance at 517 nm. This process was repeated three times. Ascorbic acid was used as a reference. The percentage of DPPH discolouration was then calculated from the formula below:

$$\% \ RSA = (absC - absE/absC)\ 100 \quad (ii)$$

Where %RSA denotes the percentage radical scavenging activity, absC denotes the mean absorbance of the control, and absE denotes the mean absorbance of extracts.

2.5.2 ABTS radical scavenging activity

To conduct the ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6)-sulfonic acid) assay, we followed the method provided by [15]. In this procedure, we combined 200 µL of ABTS solution with 50 µL of the extracts, which ranged in concentration from 15 to 240 µg/mL. The mixture was then incubated in a dark room at room temperature for 20 minutes. Subsequently, we measured the absorbance at a wavelength of 734 nm using a UV-vis spectrophotometer microplate reader (ThermoFisher Scientific, UK), comparing it to an equal amount of ABTS solution. For reference, ascorbic acid was used, and the results were calculated from the formula below:

$$\% \ RSA = (absC - absE/absC)\ 100 \quad (iii)$$

Where % RSA denotes the percentage radical scavenging activity, absC denotes the mean absorbance of the control, and absE denotes the mean absorbance of extracts.

2.5.3 FRAP radical scavenging activity

The FRAP (Ferric reducing ability of plasma or Ferric ion reducing antioxidant power) assay was performed according to Heckmann et al. [16]. Briefly, we combined 25 µL of the extracts (in concentrations ranging from 0 to 2 mg/mL) with 175 µL of FRAP working solution, which consisted of 300 mM acetate buffer (pH 3.6), 20 mM ferric chloride, and 10 mM TPTZ (2,4,6-tri(2-pyridyl)-S-triazine) dissolved in 40 mM HCl. The three solutions were mixed in a 10:1:1 ratio. The mixture was then incubated in a dark room at 37°C for 30 minutes, after which the absorbance was measured at a wavelength of 593 nm using a UV-VIS microplate reader with a blank consisting of the FRAP solution. Trolox was used as a reference, and the results were expressed as TEAC (mg TE/100 g dry weight).

2.5.4 Nitric oxide (NO) radical scavenging activity

The method described by Mohammed et al. [17] was used to assess the ability of the extracts to scavenge radicals. To do this, varying concentrations (15-240 µg/mL) of extracts and a reference sample were prepared. Then, 2.5 mL of 10 mM sodium nitroprusside in PBS was added to 0.5 mL of the different concentrations of extracts and the reference. The mixture was incubated for 150 minutes at 25 °C. After the incubation period, a 0.5 mL sample was taken and combined with 0.5 mL of Griess reagent, which contains sulphanilamide (1% w/v), H_3PO_4 (2% v/v), and naphthyl ethylenediamine dihydrochloride (0.1% w/v). Another 2 mL sample of sodium nitroprusside in PBS was used as the reference. The NO scavenging radical activities of the extracts and gallic acid were then calculated and expressed as percentages.

2.5.5 Evaluation of total antioxidant capacity (TAC)

The TAC was assessed using methods that were previously described by Letaief et al. [18]. To perform the evaluation, we introduced 1 mL of a reagent composed of 28 mM sodium phosphate, 4 mM

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ammonium molybdate, and 0.6 M sulfuric acid, alongside 100 µL (0.1 mL) of extracts at different concentrations, into test tubes. These test tubes were subsequently sealed using aluminium foil and placed in a water bath at a temperature of 90 °C for 90 minutes for incubation. Afterwards, the samples were cooled down, and the absorbance at 695 nm was measured using a UV-vis spectrophotometer (ThermoFisher Scientific, UK).

2.5.6 Evaluation of extract's reducing ability

To evaluate the reducing ability of the extracts, we followed the procedure outlined by Akpinar et al. [19] First, we mixed 2.5 mL of the powdered sample with 2.5 mL of 200 mM sodium phosphate buffer with a pH of 6.7 and 2.5 mL of 1% potassium ferricyanide. The mixture was then incubated at 50 °C for 30 minutes. Next, we added 2.5 mL of 10% tricarboxylic acid (JoeChem Ltd, Nigeria) to stop the reaction, and the mixture was centrifuged at 2000×g for 15 minutes. After that, we added 5 mL of the supernatant, an equal volume of water, and 1 mL of 0.1% $FeCl_3$. The same procedure was repeated using ascorbic acid as the reference sample. The absorbance of the reaction was measured at 700 nm with a UV-vis spectrophotometer, and the reducing ability was calculated and expressed as an ascorbic acid equivalent.

2.6 In vitro antidiabetic studies

2.6.1 Evaluation of α-amylase inhibitory activity

The inhibitory activity of α-amylase in the extracts was assessed according to Boulfia et al.'s method [20]. Extracts at concentrations ranging from 15 to 240 µg/mL were mixed with 2 U m/L porcine pancreatic amylase and incubated in phosphate buffer (pH 6.7) for 20 minutes at 37 °C. Next, starch (1%) in the same phosphate buffer was added and the mixture was kept at 37 °C for 1 h. The colour reagent, which included dinitro salicylic acid (1%), phenol (about 0.2%), Na_2SO_3 (0.05%), and sodium hydroxide (1%), was then added, followed by heating for 10 minutes at 100 °C. After cooling to around 25 °C using a cold-water bath, the absorbance of the mixture was measured at 540 nm using a spectrophotometer. Acarbose was used as a standard for comparison. The inhibitory activity was calculated and reported as a percentage.

2.6.2 Evaluation of α-glucosidase inhibitory activity

To determine the inhibitory activity of α-glucosidase by the extracts, a method provided by Boulfia et al.[20], was followed. First, 1 mg of α-glucosidase was dissolved in a phosphate buffer (100 mL) with a pH of 6.7 and containing 200 mg of bovine serum albumin. The mixture included 10 µL of the sample at concentrations ranging from 15 to 240 µg/mL. This mixture was then combined with 490 µL of phosphate buffer with a pH of 6.7 and 250 µL of 5 mM p-nitrophenyl α-d-glucopyranoside. The mixture was pre-incubated for 15 minutes at 37 °C, after which 2000 µL of Na_2CO_3 (200 mM) was added to stop the reaction. The activity of α-glucosidase was estimated by measuring the absorbance at 400 nm using a spectrophotometer. The positive control used for comparison was acarbose, an α-glucosidase inhibitor.

2.7 In vivo antioxidant and antidiabetic evaluations

2.7.1 Experimental animals

The study used thirty-six (36) opposite-sex Wistar rats purchased from PJ Rats Farm Ltd., Nigeria, with weights between 100 and 150 g. They were housed in individual aluminium cages at 25 °C under a photoperiod of 12:12 h light-dark cycle, with an air humidity being kept conspicuously at the level of 55%. The animals were fed animal feed and had free access to water (Wilson Feeds, Nig., Ltd.). Animals were allowed 2 weeks to acclimatize before the study commencement.

2.7.2 Acute oral toxicity assessment

The acute effects of the extract were evaluated by the short-term test OECD method for toxicity determination. Five rats were given 5000 mg/kg b.w. of extract orally and observed for manifestations of signs, symptoms, or death during the first four hours; then daily observation continued for up to one week. Due to the lack of any deaths, a terminal dose (LD_{50}) could not be obtained as it was >5000 mg/kg b.w. [21].

2.7.3 Induction of diabetes mellitus (DM)

Rats were given DM induction using the technique outlined by Ojo et al. [5]. Wistar rats (n = 36) weighing between 100 and 150 g were fasted for 12 hours without food and were only given water. The rats' blood sugar level was measured and recorded during their fast before alloxan-monohydrate was given to them. To establish insulin resistance, 30 rats were then given a single injection (I.P.) of 65 mg/kg of alloxan

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dissolved in 5% a glucose solution and normal saline. To confirm that diabetes mellitus had been induced in each DM-induced rat, we measured the fasting blood sugar level after 72 hours. Diabetic animals were those with a fasting blood sugar (FBS) level of more than 250 mg/dL.

2.7.3.1 Grouping of animals

The thirty-six (36) rats were divided into groups of six, each with six rats, using a random selection process. The divisions were:

Group I: Normal rats who received 0.2 mL of distilled water orally, daily for 28 days,

Group II: Diabetic rats treated with 0.2 mL of distilled water orally, daily for 28 days,

Group III: Diabetic rats who received 20 mg/kg b.w. of glibenclamide orally, daily for 28 days,

Group IV: Diabetic rats who received 100 mg/kg b.w. of extract orally, daily for 28 days,

Group V: Diabetic rats who received 200 mg/kg b.w. of extract orally, daily for 28 days,

Group VI: Diabetic rats who received 400 mg/kg b.w. of extract orally, daily for 28 days.

2.7.3.2 Harvesting of rat's organs and sample analysis

Rats were euthanized by using halothane at the end of the 28-day study. The liver and pancreas were then collected, homogenized in 0.1 M cold phosphate buffer, and stored at −4 °C; as described before [11]. The homogenate was centrifuged at 2000×g for 15 min to obtain a clear supernatant that can be used for assessment of various oxidative stress biomarkers relatively simply as mentioned below: Blood was also collected and left for 1 h allowed to centrifuge (15 min at 2000×g) the clear solution. We used this blood to measure the chosen biochemical parameters.

2.7.3.3 Evaluation of biochemical parameters

Insulin resistance and β-cell functions: The evaluation of insulin resistance based on the homeostasis model assessment (HOMA-IR) was done using the method developed by Wilson and Islam. Similarly, the estimation of the β-cell score (HOMA-β) using the homeostasis model assessment was performed with the equations used by Wilson and Islam [22].

Oxidative stress biomarkers: The glutathione (GSH) levels, glutathione peroxidase (GPX), catalase (CAT), and superoxide dismutase (SOD) activities, as well as malondialdehyde (MDA) levels, were measured in the supernatants obtained from the liver and kidneys [23].

Activities of glycolytic enzymes and glycogen levels: The enzyme activities of hexokinase, glucose-6-phosphatase (G6Pase), and fructose 1,6-bisphosphatase were determined using the liver supernatant. The liver glycogen levels were estimated using the method described by [5].

2.7.3.4 Ethical consideration and approval

The ethical guidelines for the care and use of animals in research set aside by a manual prepared for animal management used (and investigated) as a laboratory, were honoured: All experimental rats were assigned to this work. Furthermore, this work received ethical approval from the PJ Rat Farms ethics committee in line with ARRIVE guidelines.

2.8 Statistical analysis

The data was analyzed using one-way ANOVA, using SPSS statistical software version 23. The results are presented as means ± SD (n = 3). A significance level of $p < 0.05$ was used to determine statistical significance, followed by Dunnett's post-hoc test.

3. Results

3.1 Phytochemical analysis

The results of the phytochemical analysis indicated the existence of various compounds such as alkaloids, flavonoids, saponins, phenols, anthraquinones, cardiac glycosides, triterpenoids, and proteins. However, these metabolites were predominantly found at the pre and flowering stages of leaf development, in contrast to the early vegetative and maturity stages (Table 1).

3.2 Total phenolic content (TPC) and total flavonoid content (TFC)

Table 2 displays the variations in total phenolic and flavonoid content during different stages of development. The total phenolic content (TPC) ranged from 26.08 ± 0.02 to 1685.12 ± 2.14 mg GAE/100 g dry weight (DW). The highest TPC value was observed in the pre-flowering stage (PFS) at 1685.12 ± 2.14 mg GAE/g dry weight (DW). The total flavonoid content (TFC) ranged from 18.24 ± 0.01 to 306.02 ± 0.01 mg QE/100 g DW, with the PFS exhibiting the highest number of flavonoids followed by the flowering stage (FS).

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Table 1. Phytochemical content of *C. chayamansa* leaf extract at various developmental stages

Phytochemical	Tests	Pre-harvest		Post-harvest	
		EVS	PFS	FS	MS
Alkaloids	Dragendorff	-	+	++	++
Flavonoids	NaOH	+	++	+	-
Saponins	Frothing	-	++	+	-
Tannins	FeCl3	-	++	-	-
Anthraquinones					
Bontrager		+	-	-	-
Triterpenoids	Liebermann's	+	++	++	+
Phytosteroids	Salkowski's	-	++	+	-
Fats/oils	Sudan III	-	+	++	+
Cardiac glycoside					
Keller-Kiliani's		+	++	++	-
Proteins	Ninhydrin	-	+	+	++

+: moderately present, ++: largely present, -: absent, EVS: early vegetative stage, PFS: pre-flowering stage, FS: flowering stage, MS: maturity stage.

Table 2. Total phenolic and flavonoid contents of *C. chayamansa* leaf extract at various developmental stages

Developmental stage	TPC (mg GAE /g)	TFC (mg QE/g)
Early vegetative (EVS)	26.08 ± 0.02	18.24 ± 0.01
Pre-flowering (PFS)	1685.12 ± 2.14	306.02 ± 2.01
Flowering (FS)	462.01 ± 1.04	86.16 ± 1.02
Maturity (MS)	56.08 ± 0.01	28.01 ± 1.01

3.3 GC-MS analysis of volatile oil components of C. chayamansa leaf extract

The GC-MS analysis of *C. chayamansa* leaf extract at the pre-flowering stage (PFS), produced a chromatogram that showed sixteen peaks, which corresponded to sixteen volatile oil compounds with their retention times (RT) mainly unsaturated fatty acid volatile oils as listed in Table 3. These retention times were matched with the mass spectra in the NIST library. The result further showed that there are more compounds at the pre-flowering stages than at any other developmental stage (Fig. 1).

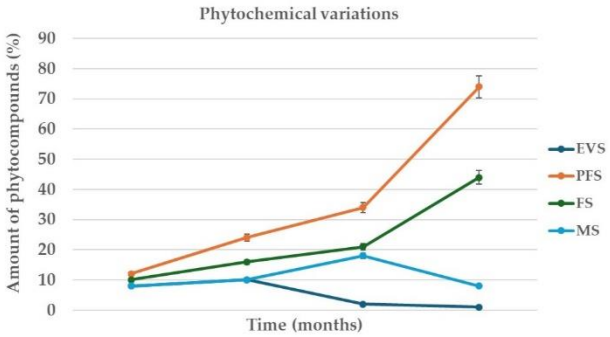

Figure 1. Variations in phytocompounds at various developmental stages of *C. chayamansa* leaf. EVS: early vegetative stage, PFS: pre-flowering stage, FS: flowering stage, and MS: maturity stage.

3.4 In vitro antioxidant activity

3.4.1 DPPH, ABTS, FRAP and NO inhibitory activities

The IC_{50} values represent the concentration of plant extracts needed to scavenge 50% of the free radicals. In Fig.2a, the values for radical scavenging activities of the plant extracts against DPPH and ABTS radicals are shown in Fig. 2b. Lower IC_{50} values indicate stronger antioxidant activity, while higher IC_{50} values indicate weaker antioxidant activities. The range of antioxidant activities for the plant extracts at various developmental stages (EVS, PFS, FS and MS) varied from 4.02 to 18.11 µg/mL for DPPH assay and 8.12 to 34.16 µg/mL for ABTS assay. These results were compared to the well-known antioxidant standards ascorbic acid and Trolox respectively. The plant leaf collected at the pre-flowering stage (PFS) displayed stronger antioxidant activities compared to ascorbic acid (22.02 µg/mL) and Trolox (28.04 µg/mL) in the DPPH assay. These findings align with the results obtained from qualitative phytochemical screening previously reported (Table 1).

Similarly, Fig. 2c shows the ferric-reducing capacities of the tested *C. chayamansa* extracts at various

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Table 3. Bioactive volatile oil compounds from *C. chayamansa* leaf methanol extract at the pre-flowering stage (PFS) using GC-MS apparatus.

Compound identified	Pk #	RT (min)	Area (%)	Mol. weight (g/mol)
2-Methyl-1-diisopropylsilyloxypropane	1.	4.95	1.36	307.61
1-Propanol, 3-[(2-hydroxyethyl) thiol	2.	4.99	0.50	136.21
9,11-Octadecadiynoic acid, 8-hydroxy-ME	3.	11.32	0.56	280.4
Neophytadiene	4.	12.19	8.92	278.5
Cyclohex, 2-butyl-1,1,3-trimethyl	5.	12.31	0.45	182.35
9,12-Tetradecadien-1-ol, (Z, E)-	6.	12.41	1.20	210.36
6-methyl-Hept-5-en-2-one	7.	12.56	2.96	126.20
3-Nitroso-2-phenyl-5-oxazolidinone	8.	12.86	0.48	282.29
2,6- Dodecadien-1-al	9.	13.05	12.46	180.29
9,12-Octadecadienoic acid, E	10.	13.28	16.22	294.00
11,14-Heptadecatetraene-Z,Z	11.	13.96	3.78	232.41
1,6-Hexanediol	12.	14.83	0.55	118.18
Eicosanal	13.	15.23	11.24	296.53
2-Methylhexacosane	14.	15.78	12.01	380.72
Palmitoleic acid	15.	17.01	10.11	254.41
9,12,15-Octadecatrienoic acid, ME (Z,Z,Z)	16.	17.24	2.12	278.40

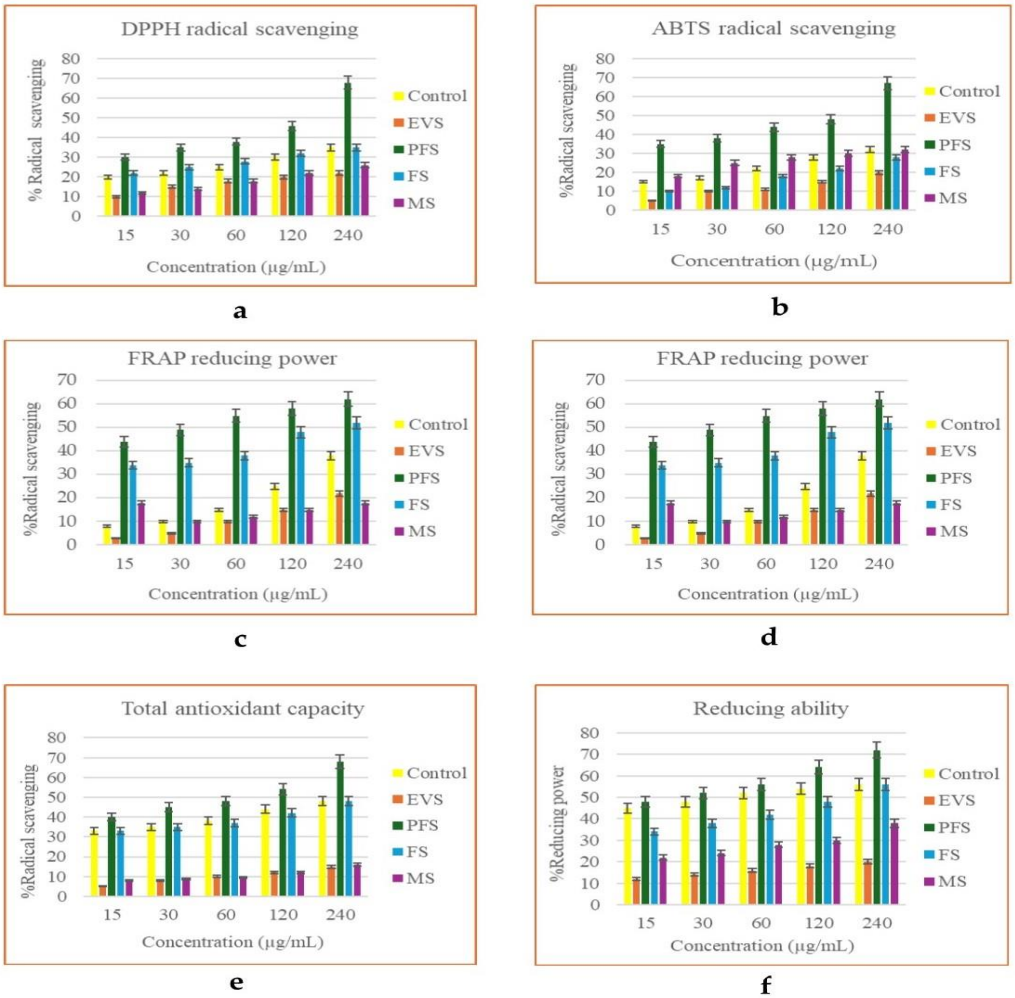

Figure 2. Antioxidant activity of *C. chayamansa* leaf extracts at various concentrations against the controls. Results are mean ± SD (n = 3). EVS: early vegetative stage, PFS: pre-flowering stage, FS: flowering stage, and MS: maturity stage.

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developmental stages (EVS, PFS, FS and MS). The standard ascorbic acid exhibited a ferric reducing capacity of 75.08 ± 1.16% at a concentration of 240 µg/mL. Among the extracts, the extract from PFS displayed the highest activity with a ferric reducing capacity of 68.82 ± 1.22% at 240 µg/mL. This was followed by the FS extract (54.01 ± 1.24%) and MS extract (34.22 ± 1.11%). Similarly, the methanol extract from the PFS exhibited a nitric oxide (NO) (Fig. 2d), reducing the capacity of 66.02 ± 0.44%, followed by the methanol extract from MS with a value of 62.12 ± 1.15%. Further, results in Fig. 2 e and f, showed that the plant leaves collected in the PFS also displayed the highest total antioxidant capacity (TAC) and reducing power against scavenging radicals.

3.5 In vitro antidiabetic evaluation

3.5.1 Inhibition of α-amylase and α-glucosidase

The IC_{50} values of all the extracts are shown in Table 4, which shows that the extract collected at the pre-flowering stage (PFS) had the highest α-amylase and α-glucosidase inhibitory activities with an IC_{50} of 8.44 ± 0.01 and 12.02 ± 0.01 µg/mL respectively.

The difference in these values was statistically significant ($p<0.05$) with acarbose as standard, which had an α-amylase and α-glucosidase inhibitory activities (IC_{50}) of 6.12 ± 0.01 and 10.31 ± 1.01 µg/mL respectively. The FS extract had the second highest α-amylase and α-glucosidase inhibitory abilities compared with other extracts (EVS and MS) with an IC_{50} of 32.02 ± 1.01 and 26.04 ± 1.02 µg/mL respectively.

3.6 In vivo antidiabetic study

3.6.1 Effect of C. chayamansa leaf extracts on fasting blood levels in rats

The effects of the therapies on fasting blood glucose levels (measured weekly) are shown in Fig. 3. In comparison to the negative control group, within the first week, reduction reached 56.13% due to *C. chayamansa* leaves polyphenols at a dose of 200 mg/kg and 66% for a dose of 400 vs. 72% for glibenclamide ($p < 0.05$). After that, hyperglycemia was reduced, and when compared to the negative diabetic control, the fasting blood sugar level was considerably maintained, decreasing from 450 mg/dL on the 7th day for PFS to 98 mg/dL on the 28th day. In the penultimate week, the extract's (PFS) antidiabetic effectiveness exceeded that of glibenclamide and returned to normal glycemic values (normal rats had a glycemia of 50-135 mg/dL).

Table 4. Inhibitory effects of *C. chayamansa* leaf extracts at various developmental stages against α-amylase and α-glucosidase activities.

Extracts and standard	α-Amylase (IC50)	α-Glucosidase (IC50)
Early vegetative (EVS)	56.12 ± 2.04	66.04 ± 2.10
Pre-flowering (PFS)	8.44 ± 0.01*	12.02 ± 0.01*
Flowering (FS)	32.02 ± 1.01*	26.04 ± 1.02*
Maturity (MS)	46.11 ± 2.01	42.10 ± 1.01
Acarbose (standard)	6.12 ± 0.01*	10.31 ± 2.01*

Results are mean ± SD (n = 3). IC_{50} was expressed in µg/mL. *Statistically significant at $p<0.05$ using one-way ANOVA followed by Dunnett's post hoc test.

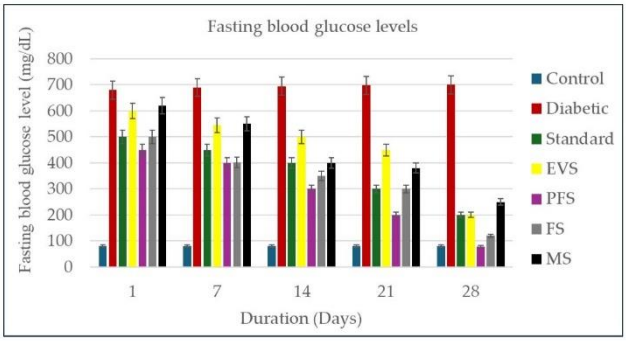

Figure 3. Effects of *C. chayamansa* leaf extracts on fasting blood glucose levels in alloxan-induced diabetic rats during the experimental period of 28 days. Results are shown as mean ± SD (n = 6 rats) and are statistically significant at $p < 0.05$ when compared to the diabetic control group. EVS: early vegetative stage, PFS: pre-flowering stage, FS: flowering stage, and MS: maturity stage.

3.6.2 Changes in body weights of induced rats in 28 days of study

Fig. 4 shows the bodyweight growth of the several experimental rat groups. Compared to the normal group, the untreated diabetes group showed a consistent decrease in body weight. Conversely, the groups that received 4 weeks of treatment with extract from PFS demonstrated a statistically significant ($p<0.05$) increase when compared to the diabetic control group. For 4 weeks, diabetic rats received oral administration of high dose (400 mg/kg b.w.) PFS leaf methanol extract and standard drug, glibenclamide enhanced their body weights and controlled their hyperglycemic status.

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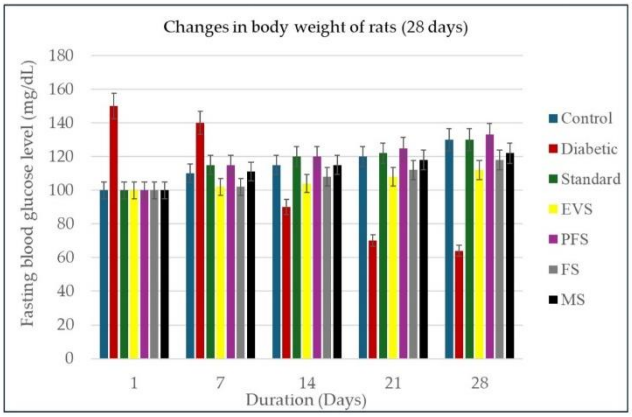

Figure 4. Effects of various doses of *C. chayamansa* and standard drug on the body weight of alloxan-induced diabetic rats in a 28-day study. Results are shown as mean ± SD (n = 6 rats) and are statistically significant at *p < 0.05 when compared to the diabetic control group. EVS: early vegetative stage, PFS: pre-flowering stage, FS: flowering stage, and MS: maturity stage.

3.6.3 Effects of C. chayamansa leaf extracts on biochemical parameters of induced Wistar rats in 28 days antidiabetic study

3.6.3.1 Effects on Serum Insulin Resistance and β-cell Functions

As indicated in Table 5, no notable differences were observed among the groups concerning insulin resistance (HOMA_IR) and insulin sensitivity (QUICKI). However, β-cell function (HOMA_β) significantly declined ($p < 0.05$) in diabetic rats compared to the normal controls. The administration of PFS extract at a dose of 100 mg/kg notably improved HOMA_β levels compared to the diabetic controls. Pearson correlation analysis was used to examine the relationship between insulin resistance and factors like basal blood glucose (Basal_Glu) and β-cell function (Fig. 5a and b). Variance calculations revealed a strong correlation between insulin resistance and basal blood glucose ($r^2 = 0.960$; $p < 0.01$), while a weaker correlation was found between insulin resistance and pancreatic β-cell function ($r^2 = 0.893$; $p = 0.277$).

3.6.3.2 Effects on oxidative stress biomarkers

The results presented in Fig. 6 indicate that no significant differences were observed in catalase (CAT) and superoxide (SOD) levels in the liver and kidneys across the groups. Diabetic rats induced by alloxan

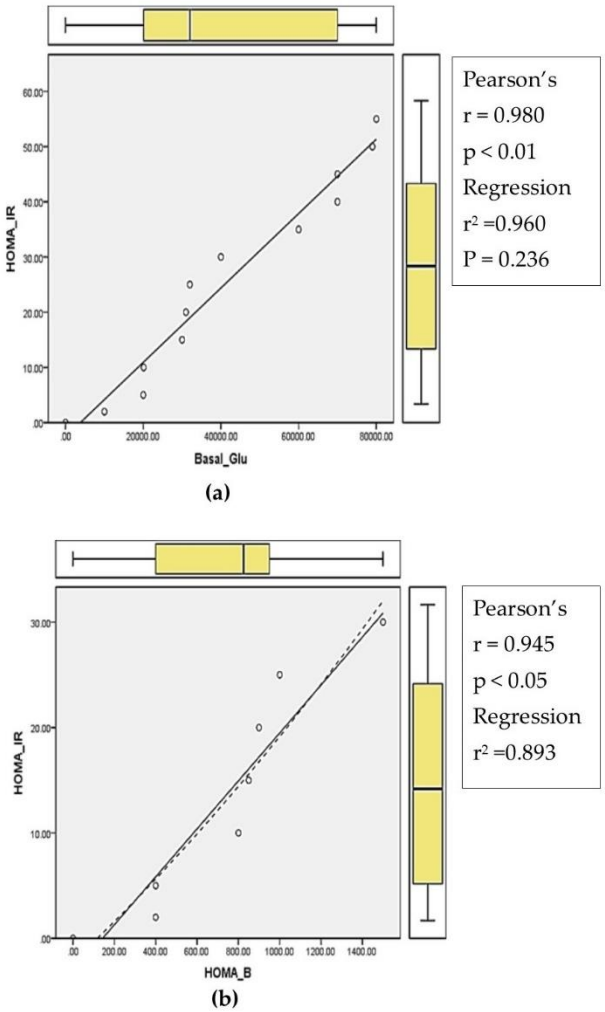

Figure 5. Pearson's correlation between HOMA_IR and basal blood glucose, Basal_Glu (**a**), and between HOMA_IR and HOMA_B (**b**).

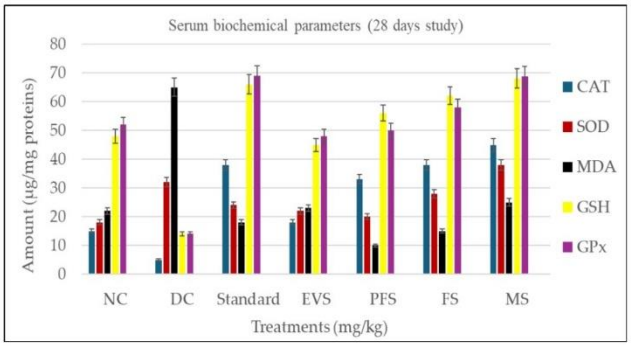

Figure 6. Effects of various doses of *C. chayamansa* and standard drug on the biochemical markers of alloxan-induced diabetic rats in a 28-day study. Results are shown as mean ± SD (n = 6 rats) and are statistically significant at *p < 0.05 (one-way ANOVA followed by Dunnett's post hoc test) when compared to the diabetic control group. EVS: early vegetative stage, PFS: pre-flowering stage, FS: flowering stage, and MS: maturity stage.

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Table 5. Effect of *C. chayamansa* leaf extracts and glibenclamide on insulin levels, insulin resistance and β-cell functions.

Extracts	NC	DC	GLIB.	PF	FS	MS
Insulin	22.01± 1.01	26.01 ± 1.03*	28.04 ± 2.10*	12.02 ± 0.01*	46.06 ± 4.16	36.01 ± 2.02
HOMA_IR	5.01 ± 0.01	14.11 ± 1.01*	6.04 ± 0.01*	3.22 ± 0.01*	8.44 ± 0.01*	6.02 ± 0.01
HOMA_β	712.1 ± 12.01	38.02 ± 2.10*	252.01 ± 4.12	258.14 ± 1.12*	438.22 ± 4.16	205 ± 2.16
QUICKI	0.20 ± 0.01	0.30 ± 0.01*	0.29 ± 0.01*	0.31 ± 0.01*	0.32 ± 2.10	0.33 ± 0.01

Results are mean ± SD (n = 6 rats). Insulin was expressed in µUI/mL. *Statistically significant at $p<0.05$ using one-way ANOVA followed by Dunnett's post hoc test. NC: normal control group, DC: diabetic control, GLIB: glibenclamide, PFS: pre-flowering stage, FS: flowering stage, and MS: maturity stage.

showed a marked increase in MDA concentrations and a significant reduction in GSH and GPx levels in both the liver and kidneys. Untreated diabetic rats had significantly higher MDA, CAT, and SOD concentrations in both organs compared to the normal control and the PFS extract groups. Treatment with PFS extract at 400 mg/kg body weight and glibenclamide significantly reduced MDA, CAT, and SOD levels compared to the diabetic control, with these reductions being more pronounced in the kidneys than in the liver. Additionally, GSH and GPx levels were significantly lower ($p < 0.05$) in the diabetic control group compared to normal controls. The extracts from *C. chayamansa* leaves at different stages (EVS, PFS, FS, and MS) significantly ($p < 0.05$) reduced oxidative stress markers in a dose-dependent manner (100-400 mg/kg b.w.), especially in the kidneys.

3.6.3.3 Effects on glycolytic enzymes' activities and glycogen level

The activities of carbohydrate enzymes in the liver and kidney are shown in Figs. 7a and 7b, respectively. In diabetic control rats, hexokinase activity in the tissue significantly decreased. However, treatment with glibenclamide and PFS extract of *C. chayamansa* led to a significant increase ($p < 0.05$) in hexokinase activity. The activities of the gluconeogenic enzymes, glucose-6-phosphatase, and fructose-1,6-bisphosphatase, were notably elevated in the tissues of diabetic control rats. The PFS extract's treatment significantly ($p < 0.05$) reduced the activity of both glucose-6-phosphatase and fructose-1,6-bisphosphatase in a dose-dependent fashion. There were also significant reductions in these gluconeogenic enzymes in rats treated with the standard drug, glibenclamide and FS extract. However, these reductions were not greater than those from the PFS extract.

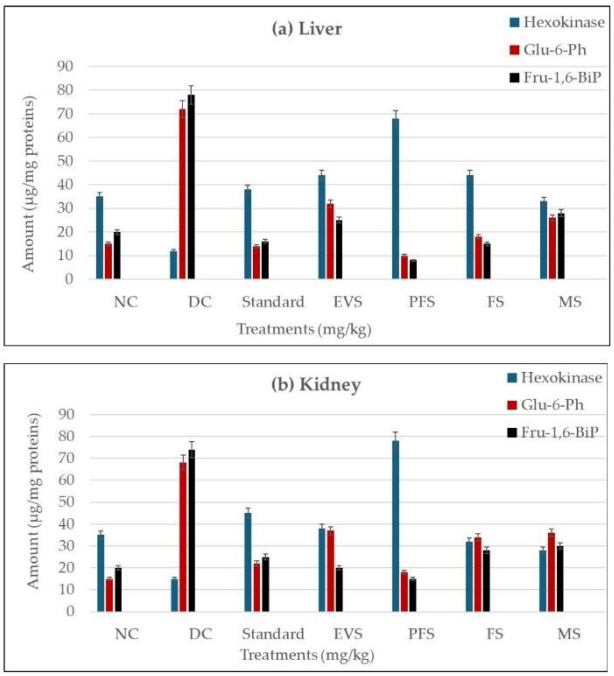

Figure 7. Effects of *C. chayamansa* extracts on hexokinase, glucose-6-phosphatase (Glu-6-Ph), and fructose-1, 6-bisphosphatase (Fru-1,6-BiP) in liver and kidneys of control and experimental rats in 28 days study. Results are shown as mean ± SD (n = 6 rats) and are statistically significant at *$p < 0.05$ (one-way ANOVA followed by Dunnett's post hoc test) when compared to the diabetic control group. EVS: early vegetative stage, PFS: pre-flowering stage, FS: flowering stage, and MS: maturity stage.

4. Discussion

Phytochemicals, particularly phenolic compounds, are naturally occurring bioactive compounds in plants, playing a vital role in their defence mechanisms and human health. Phenolics, a broad class of secondary metabolites, include flavonoids, phenolic acids, tannins, and other compounds, known for their antioxidant, anti-inflammatory, and antidiabetic properties [24]. This research discussion examines the abundance of these compounds in *C. chayamansa* leaf methanol extracts during the

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preharvest conditions (early vegetative state (EVS) and pre-flowering stage (PFS)), and post-harvest conditions (flowering stage (FS) and maturity stage (MS)), and their correlations with enhanced antioxidant and antidiabetic activities. Plants undergo various physiological changes during their lifecycle, and the accumulation of phytochemicals is strongly influenced by these changes [8]. In this present study, the pre-flowering stage (PFS), has shown a heightened concentration of bioactive compounds than other stages of development, including phenolics, due to the plant's natural defence mechanisms. This is because the PFS is characterized by rapid growth, energy storage, and the production of secondary metabolites for protection against environmental stressors such as UV light, herbivores, and pathogens [25].

Similarly, the accumulation of phenolics during the PFS may be attributed to the plant's need to prepare for reproductive processes. Many plants synthesize these compounds in greater quantities during early growth stages to ensure survival until they can produce seeds [26]. Phenolic compounds are key antioxidants, and their increased presence during PFS than other stages (EVS, FS and MS) is likely due to the heightened metabolic activities and oxidative stress the plant undergoes as it transitions into flowering [24]. For instance, flavonoids and tannins, two major classes of phenolics were present in high concentrations during the PFS in this study. These compounds not only serve as antioxidants but also have a role in regulating plant growth and protecting against biotic stress. Flavonoids, particularly quercetin and kaempferol, have been reported to reach peak concentrations during this pre-flowering stage [27].

The methanol leaf extracts of *C. chayamansa* were characterized by the GC-MS in this study. Our study showed that PFS extracts had the highest number and diversity of volatile oil compounds, particularly fatty acids, monoterpenes and sesquiterpenes, which may be linked to the plant's defensive strategy during early development. The rich chemical profile during this stage (PFS) than other stages suggest a peak in bioactive compound production that could maximize the medicinal efficacy of the plant when harvested before flowering.

This finding is significant for both traditional medicine and industrial applications, where the timing of harvest can be optimized to extract the highest yield of bioactive compounds. The identification of specific compounds such as 9,12-octadecadienoic acid, eicosanal, palmitoleic acid, and neophytadiene highlights the potential for further research into the specific biological activities of these volatiles concerning the health benefits attributed to *C. chayamansa*. In contrast, the decline in both the number and concentration of volatile compounds during the EVS, FS and MS aligns with the plant's shift in metabolic priorities. This suggests that while volatile oils are present throughout the plant's growth cycle, the pre-flowering stage (PFS) offers the most chemically diverse profile, providing insight into the optimal harvesting time for pharmacological use. From our study, the GC-MS analysis revealed significant variations in the volatile oil composition of *C. chayamansa* leaves across different growth stages, with the PFS extract having the richest chemical diversity. These findings underscore the importance of selecting the appropriate growth stage for harvesting when targeting the extraction of bioactive compounds for medicinal purposes.

Furthermore, antioxidants are crucial for scavenging free radicals and reactive oxygen species (ROS), which cause oxidative damage to cellular components [24]. Phenolics, particularly in pre-flowering plant extracts, exhibit strong antioxidant activity due to their ability to donate hydrogen atoms or electrons and stabilize free radicals. In this study, the increased levels of phenolic compounds in *C. chayamansa* leaf extract directly contribute to the enhanced antioxidant potential of pre-flowering stage extracts than other stages. Our study has shown that there was a significant ($p < 0.05$) difference in the antioxidant activity of the PFS extract when compared to the control ascorbic acid. Studies have demonstrated that extracts from plants harvested during this stage (PFS) exhibit significantly higher total antioxidant capacity, measured by assays such as DPPH (2,2-diphenyl-1-picrylhydrazyl), FRAP (ferric reducing antioxidant power), and ABTS (2,2'-casino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid)).

DPPH is a stable free radical that exhibits a deep violet colour. In the presence of an antioxidant, DPPH

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accepts an electron or a hydrogen atom, leading to a colour change from violet to yellow, which can be quantified spectrophotometrically [28]. Our study showed that extracts collected at the various stages show varying antioxidant activity due to differences in the concentration of phenolic compounds, flavonoids, and other antioxidants. These compounds can neutralize free radicals and reduce oxidative stress, with the PFS often exhibiting significant antioxidant potential due to the high biosynthesis of secondary metabolites [2]. Also, ABTS is converted to its radical form (ABTS+), which has a blue-green colour. In this study, antioxidants present in the *C. chayamansa* extracts reduce ABTS+, causing a decolourization that can be measured spectrophotometrically [15]. From our study, the extract collected at the PFS contains high levels of antioxidants, such as polyphenols and flavonoids, leading to significant ABTS+ reduction. Furthermore, the FRAP assay measures the ability of antioxidants to reduce ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) at a low pH, leading to the formation of a blue-coloured complex that can be quantified spectrophotometrically [29]. The study showed that the PFS extracts exhibited strong reducing power, indicating high antioxidant activity when compared to the ascorbic acid standard. This is likely due to the high concentration of reducing agents like phenolic acids and flavonoids, which are synthesized at this stage of plant development. Similarly, nitric oxide (NO) is a reactive free radical that can contribute to oxidative stress and inflammatory processes. In this assay, sodium nitroprusside generates NO, which reacts with oxygen to form nitrite [30]. From our study, the PFS extract exhibits significant NO scavenging activity. High levels of phenolic compounds, terpenoids, and other antioxidants during this stage can neutralize nitric oxide, thereby reducing its harmful effects.

The analysis of antioxidant activities using tests like DPPH, ABTS, FRAP, and NO radical scavenging showed a strong link between the phytochemical content of Cnidoscolus chayamansa across various growth stages. Interestingly, during the pre-flowering stage, the plant had the highest amounts of total phenolic content (TPC) and total flavonoid content (TFC), which greatly boosted its antioxidant abilities. The IC_{50} values found in the DPPH and ABTS tests indicated impressive free radical scavenging activity, which matched the findings of the FRAP test that highlighted its strong capacity to reduce ferric ions. This connection illustrates how essential phenolic and flavonoid compounds are in stabilizing and neutralizing harmful free radicals, thereby strengthening the plant's antioxidant defenses. Additionally, the NO scavenging activity, which serves as a marker for anti-inflammatory potential, supported the results from the other tests, emphasizing the combined benefits of these bioactive compounds.

It has been reported that phenolic compounds act through various mechanisms to exert their antioxidant effects, including neutralizing ROS, chelating metal ions, and inhibiting oxidative enzymes [31]. For example, flavonoids can scavenge hydroxyl radicals, superoxide anions, and singlet oxygen, reducing oxidative stress in cells [32]. These mechanisms are more pronounced in extracts from the pre-flowering stage due to the higher availability of bioactive compounds. The strong correlation between phenolic content and antioxidant activity suggests that the pre-flowering stage is optimal for harvesting plant extracts with potent antioxidant properties.

Diabetes, particularly type 2 diabetes, is characterized by insulin resistance and hyperglycemia [15]. The management of diabetes often involves enhancing insulin sensitivity, reducing oxidative stress, and inhibiting carbohydrate-hydrolyzing enzymes like α-amylase and α-glucosidase [33]. Plant extracts rich in phenolics are well-documented for their antidiabetic activities. Our study has shown that *C. chayamansa* leaves collected at the EVS, PFS, FS and MS showed variations in antidiabetic and antioxidant activities due to the presence of different metabolites. For instance, phenolic compounds, particularly flavonoids and phenolic acids, have been shown to inhibit the activities of α-amylase and α-glucosidase, enzymes responsible for the breakdown of carbohydrates into glucose [20]. This inhibition leads to a reduction in postprandial blood glucose levels. The PFS extract, due to its higher phenolic content, exhibits stronger inhibitory effects on these enzymes compared to extracts harvested at other growth

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stages. For example, previous studies on *Momordica charantia* (bitter melon) and *Camellia sinensis* (green tea) show that pre-flowering extracts can significantly reduce blood glucose levels in diabetic models [34]. Similarly, phenolic-rich extracts from the PFS have also been found to improve insulin sensitivity by modulating the activity of key proteins involved in glucose uptake, such as the GLUT-4 transporter in adipose and muscle tissues [35]. Flavonoids, particularly quercetin and catechins, enhance insulin receptor signalling pathways, thereby improving glucose uptake and lowering blood glucose levels. This mechanism must have been used by the extract in this current study. This finding was statistically significant ($p < 0.05$) when compared to the standard drug glibenclamide and the diabetic control rats' group. In addition, oxidative stress plays a critical role in the pathogenesis of diabetes, leading to complications such as neuropathy, nephropathy, and cardiovascular diseases [36]. The high antioxidant capacity of pre-flowering plant extracts contributes to their antidiabetic effects by reducing oxidative damage in pancreatic β-cells and protecting tissues (liver and kidneys) from the deleterious effects of hyperglycemia. The potent antioxidant activity of phenolics helps mitigate the oxidative stress associated with diabetes, which is crucial in preventing the progression of the disease and its complications in rats in groups III to VI. It is evident from our study that the antioxidant and antidiabetic activities of the *C. chayamansa* leaf extracts follow a similar pattern, with pre-flowering extracts showing superior bioactivities. Extracts harvested during the flowering and post-flowering stages, while still bioactive, tend to be less potent due to the reduced concentration of key bioactive compounds [14]. These findings highlight the importance of understanding the optimal harvest times for maximizing the therapeutic potential of plant-based products, particularly in the context of managing oxidative stress-related diseases like diabetes.

The high phenolic and flavonoid content observed in the pre-flowering stage was directly linked to the enhanced antioxidant activity, which, in turn, supported the plant's antidiabetic potential. Phenolic compounds are known to mitigate oxidative stress by donating hydrogen atoms to free radicals, thereby preventing cellular damage. This antioxidant mechanism plays a vital role in protecting pancreatic β-cells, which are particularly susceptible to oxidative damage in diabetic conditions. In the antidiabetic activity assays, the pre-flowering extracts significantly improved glucose metabolism, as evidenced by increased hexokinase activity and reduced glucose-6-phosphatase and fructose-1,6-bisphosphatase activities. These effects are attributed to the modulatory action of the phenolic and flavonoid compounds, which inhibit key enzymes involved in gluconeogenesis and enhance glycolysis. Additionally, the α-glucosidase inhibitory activity demonstrated by the extracts suggests that these compounds delay carbohydrate breakdown, thereby reducing postprandial glucose spikes. Post-harvest handling influenced the stability of these bioactive compounds, with drying and storage reducing phenolic and flavonoid content. This reduction was reflected in decreased antioxidant and antidiabetic activity, reinforcing the importance of processing conditions in preserving the therapeutic efficacy of the plant. These findings establish a clear relationship between the antioxidant activities and the analyzed compounds, highlighting their dual role in mitigating oxidative stress and regulating glucose metabolism, thereby contributing to the plant's antidiabetic effects.

To further validate our understanding of the current study, the effects of *C. chayamansa* leaf extracts at different stages of development were assessed on insulin resistance and beta-cell function. Insulin resistance occurs when the body's cells do not respond effectively to insulin, leading to elevated blood glucose levels [22]. Plants with high antioxidant and anti-inflammatory properties, like *C. chayamansa*, may help improve insulin sensitivity by reducing oxidative stress and inflammation, which are key factors in the development of insulin resistance. On the other hand, beta-cells in the pancreas are responsible for the production and secretion of insulin [37]. In diabetes, especially type 2 diabetes, these cells become dysfunctional due to prolonged stress from high blood sugar and insulin resistance [22]. Plant compounds with regenerative or protective effects on pancreatic cells are valuable in preserving or restoring beta-cell functions. Our study showed that the PFS extract contains high levels of bioactive compounds,

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such as flavonoids, alkaloids, and antioxidants, which are crucial for therapeutic effects. Thus, the leaves at this stage contain higher concentrations of active compounds that help in reducing insulin resistance by modulating glucose uptake in peripheral tissues and enhancing insulin signalling pathways [22]. Studies on similar plants suggest that extracts obtained at the PFS can improve insulin sensitivity, possibly due to the presence of higher polyphenolic and flavonoid contents [24]. Similarly, our study showed that, in rats with induced insulin resistance by alloxan administration, *C. chayamansa* extract from the PFS helped in lowering fasting blood glucose levels, reducing oxidative stress markers, which led to improved insulin sensitivity, and modulating liver enzymes responsible for glucose metabolism, resulting in better glycemic control as seen from the study. Moreover, the bioactive compounds in the PFS might have protected the beta cells from oxidative damage, thus preserving their insulin-secreting capacity, promoted the regeneration of beta cells, which is critical in diabetes management and improved insulin secretion in response to glucose, thus aiding in better regulation of blood sugar levels. In addition, bioactive compound levels decrease after flowering, leading to a reduced antidiabetic effect as seen from the study. The antioxidant capacity after flowering stage (FS) was lower, making it less effective in reducing insulin resistance and protecting beta cells. Besides, the study has shown that *C. chayamansa* leaf extracts at the PFS is rich in antioxidants, which could reduce oxidative stress in pancreatic cells and peripheral tissues [15]. This could also alleviate insulin resistance and support the regeneration or survival of beta cells.

To further confirm the in vivo therapeutic roles of *C. chayamansa* leaf extracts at various stages of development, we evaluated the effects on oxidative stress markers such as catalase (CAT), superoxide dismutase (SOD), malondialdehyde (MDA), glutathione (GSH), and glutathione peroxidase (GPX) in the liver and kidney of alloxan-induced diabetic rats. The administration of *C. chayamansa* leaf extract at doses of 100, 200 and 400 mg/kg b.w., particularly during the PFS, the high concentration of phytochemicals enhanced the activities of these enzymes. The result was significant ($p < 0.05$) compared to the normal control group. These metabolites are known to scavenge free radicals and reduce oxidative stress [38], thus increasing the activity of CAT, SOD, and GPX in the liver and kidneys of rats. From the study, an increase in SOD, CAT, and GPX levels in both the liver and kidneys indicates an enhanced antioxidant defence mechanism in response to the phytochemicals in the leaf extract. Glutathione (GSH) is a vital intracellular antioxidant, and it works as a substrate for GPX in reducing peroxides. When oxidative stress is high, GSH gets converted to its oxidized form (GSSG) [38]. From the study, *C. chayamansa* leaf extracts showed increased GSH levels due to their antioxidant properties, reducing oxidative stress and protecting cells from lipid peroxidation. An increase in GSH levels means a greater ability to neutralize reactive oxygen species (ROS) in the liver and kidney cells. Therefore, the elevated GSH levels in both the liver and kidneys in the study, indicate that the extract (PFS) has a strong reducing capacity that helps combat oxidative stress. Furthermore, MDA is a by-product of lipid peroxidation and serves as a marker for oxidative damage to cellular membranes. High MDA levels indicate elevated oxidative stress and damage [5]. In the study, *C. chayamansa* leaf extract, collected at the PFS, inhibits lipid peroxidation by scavenging free radicals, thereby reducing MDA levels in liver and kidney tissues. A decrease in MDA levels, suggests reduced oxidative damage to lipids in the liver and kidneys due to the protective effects of the extract. Flavonoids and phenolics are potent antioxidants present in *C. chayamansa* leaves, particularly in PFS from this study. They neutralize free radicals and upregulate the expression of antioxidant enzymes. Thus, *C. chayamansa* leaf extracts, particularly when collected during the pre-flowering period, are enhanced the antioxidant defence system in the liver and kidneys of Wistar rats by increasing the activity of antioxidant enzymes (SOD, CAT, and GPX), boosting GSH levels, and reducing oxidative damage as indicated by lower MDA levels. These effects make *C. chayamansa* a promising candidate for protecting against oxidative stress-related conditions.

Finally, the *C. chayamansa* leaf extracts collected at different stages of growth, especially at the PFS, are of

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particular interest due to their higher concentration of bioactive compounds such as polyphenols, flavonoids, and alkaloids. These compounds could influence glycolytic enzymes, which are crucial in glucose metabolism. For example, hexokinase is responsible for phosphorylating glucose to form glucose-6-phosphate, the first step in glycolysis [5]. In diabetic conditions, the activity of hexokinase is often reduced, leading to impaired glucose metabolism. The study showed that the PFS extract potentially enhanced hexokinase activity due to its antioxidant and antidiabetic properties. This could result in improved glucose uptake and utilization, thus helping to restore normal glycolytic function in induced diabetic rats. Similarly, glucose-6-phosphatase catalyzes the final step of gluconeogenesis and glycogenolysis, converting glucose-6-phosphate into free glucose, thereby increasing blood glucose levels [39]. In diabetes, there is often an increase in glucose-6-phosphatase activity, contributing to hyperglycemia. From our study, *C. chayamansa* leaf PFS extract downregulated glucose-6-phosphatase activity, thus reducing excessive glucose production and release into the bloodstream. This is beneficial in controlling hyperglycemia in diabetic rats. In addition, fructose-1,6-bisphosphatase plays a critical role in gluconeogenesis, converting fructose-1,6-bisphosphate to fructose-6-phosphate [40]. Increased activity of this enzyme in diabetes leads to enhanced gluconeogenesis and contributes to high blood sugar levels [40]. The extract (PFS) from *C. chayamansa* inhibited fructose-1,6-bisphosphatase activity, thereby reducing gluconeogenesis. This inhibition could be attributed to the presence of bioactive compounds that interfere with the enzyme's activity, ultimately contributing to improved blood glucose control.

5. Conclusions

This study reveals important differences in the nutritional and medicinal properties of *Cnidoscolus chayamansa* (Chaya) leaves at various growth stages and after harvest. Notably, the pre-flowering stage stands out, containing the highest levels of beneficial compounds like phenolics, flavonoids, and alkaloids, which are key to its strong antioxidant and antidiabetic effects. These pre-flowering leaves have enhanced antioxidant capabilities, as shown by tests that measure their ability to combat free radicals. They also exhibit significant antidiabetic effects by hindering enzymes that contribute to blood sugar spikes after meals. However, the study notes that post-harvest practices, such as drying and storage, can reduce the stability of these beneficial compounds and diminish the leaves' medicinal properties. In conclusion, our study showed that harvesting *C. chayamansa* leaves at the pre-flowering stage maximizes their phytochemical richness, antioxidant capacity, and antidiabetic potential, making this stage the most suitable for therapeutic applications. Future research should focus on elucidating the molecular mechanisms underlying these effects and exploring the development of nutraceuticals or pharmaceuticals utilizing *C. chayamansa* extracts, potentially enhancing the management of oxidative stress-related diseases and diabetes.

Abbreviations

ANOVA	Analysis of variance
GAE	Gallic acid equivalent
GC-MS	Gas chromatography-mass spectrometry
HOMA-IR	Homeostasis Model Assessment Insulin Resistance
LD50	Lethal dose
OECD	Organization
RSA	Radical scavenging activity
UV	Ultra-violet
QE	Quercetin equipment

Ethics of approval and consent to participate

The animals used in this study were approved by the Animals Research and Ethical Committee of PJ Rats Farm Ltd, Nigeria guidelines which prohibit harsh treatment of animals with approval number PJR/RAT-CLE/0240/2024.

Authors' contributions

Conceptualization, methodology, data collection and analysis, C.A.U.; Study design, C.A.U., H.B.Y.; Data interpretation and visualization, C.A.U., M.O.A.; Writing and submitting manuscript: C.A.U., H.B.Y.; Editing and approval of final draft, C.A.U., H,B,Y.,

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Availability of data and materials

All data will be made available on request according to the journal policy.

Conflicts of interest

We have none to declare.

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