In Vitro Investigation of Potential Anti-Diabetic Mechanisms of Chromolaena Odorata Methanol Extract

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Abstract
Diabetes mellitus is one of the most common endocrine diseases characterized by hyperglycemia due to absolute or relative deficiency of insulin. Chromolaena odorata (CO) has been reported to exhibit normoglycemic potential, however, the mechanisms of action of this plant has not been comprehensively investigated. In this study, the methanolic extract of leaves of Chromolaena odorata was investigated for its antioxidant activity; inhibition of carbolytic enzyme activity and potential to improve glucose uptake in yeast cells. The extraction was carried out by maceration in methanol. The phytochemicals were determined by qualitative chemical tests and spectrophotometric methods. Alpha-amylase and Alpha-glucosidase inhibition was determined in vitro by using the dinitrosalicylic acid and ρ-nitrophenyl-α-D-glucopyranose methods respectively. Theantioxidant activity was monitored by radical scavenging and reducing power assays while the glucose uptake was monitored in yeast cell using glucose oxidase method. The phytochemistry analysis revealed the presence of high level of polyphenols (124.67 mg gallic acid eqv/g of CO, 48.5 mg rutin eqv/g of CO and 72.3 mg tannic acid eqv/g of CO, for total phenolics, flavonoids and tannin respectively). The extract exhibited a moderate inhibition of α-amylase ($IC_{50:}$ 1.858 mg/ml) and higher inhibition of α-glucosidase (IC_{50}: 0.653 mg/ml). The extract also showed a moderate capacity to enhance the uptake of glucose by yeast cells, scavenge DPPH and hydroxyl radicals and high reducing power. In conclusion, the study showed that methanolic extract of Chromolaena odorata leaves has the potential to ameliorate hyperglycemic condition and this may be due to a combination of multiple mechanisms of amelioration of oxidative stress, improved uptake of glucose and reduction of postprandial sugar. Further work should be carried out to further characterize this extract.

Keywords: Diabetes, carbolytic enzymes, uptake of glucose, antioxidant

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Introduction

Diabetes mellitus is a metabolic disorder affecting carbohydrates, proteins, and lipids, with an increasing incidence worldwide (Hameed *et al.*, 2015). Despite significant advances in treatment modalities and prevention strategies, the prevalence of this disease has increased exponentially in the last decade (Wu *et al.*, 2014). Currently, the most effective drugs available for treating diabetes mellitus are insulin and synthetic hypoglycemic agents. However, their use is limited by high cost, side effects, and poor management of long-term complications (Bahamani *et al*., 2014). Consequently, there has been a growing interest in exploring plants and natural products as antidiabetic agents, due to their rich and diverse phytochemical content, low cost, and fewer side effects (Ekor, 2014). Given the rich diversity of chemicals in plants, it is logical to hypothesize that they may provide treatment options with multimodal antidiabetic mechanisms of action.

Chromolaena odorata (CO) is a weedy scrambling perennial herb that is native to Central and South America but has spread throughout tropical and subtropical regions (Omokhu *et al.*, 2016). It is a traditional medicinal plant widely used for its wound-healing properties to treat wounds, burns, and skin infections. Additionally, it has been shown to possess anticancer, anti-hepatotoxic, anti-inflammatory, antimicrobial, and antioxidant properties (Sirinthipaporn et al., 2017), as well as antidiabetic potential (Omonije *et al.*, 2019; Adedapo *et al.*, 2016; Onkaramurthy *et al.*, 2012). Previous studies have demonstrated that alcohol extracts of the leaves and roots of the plant significantly reduced blood glucose levels in diabetic Wistar rat models. However, studies investigating the mechanisms underlying the amelioration of hyperglycemia and improvement of other pathophysiological outcomes are still limited. This study aims to investigate the potential carbolytic enzyme inhibition, antioxidant, and glucose uptake enhancement effects of the methanol extract of *Chromolaena odorata*.

Materials and Method

Enzymes such as amylase and glucosidase and their substrates were purchased from Sigma-Aldrich (China). The Randox Glucose (GLUC-PAP) kit purchased from Randox Laboratories Ltd, UK. All other reagents used were of analytical grade and were purchased from Loba Chemie (India)

Plant materials

Chromolaena odorata leaves were obtained from a botanical garden in University of Abuja, Campus, Federal Capital Territory, Abuja, Nigeria in the month of June, 2021. Specimen of the leaves were identified at the Institute of Forestry Research of Ibadan, Nigeria, with the voucher number of FHI 109494 deposited at the Herbarium.

Preparation of plant extracts

The crude extract and solvent fractions were prepared as described by (Teke *et al* 2012) with little modifications. The leaves were shade dried and coarsely powdered using a blender. A portion (500 g) of the ground leaves was macerated in 2.5 L of methanol (95%) for 72hours in an airtight glass cylinder. The extract was filtered with Whatman No.1 filter paper and concentrated in vacuo at 50 ^{o}C using a rotary evaporator. The extract and solvent fractions were stored separately in well labeled airtight dark bottles at 4 ^{o}C until it was time to use.

The extract was subjected to phytochemical analysis using the methods outlined by Sofowora (1982), Harbourne (1984), and Saeed et al. (2012). The extract was first prepared in methanol at a concentration of 1mg/ml.

To detect alkaloids, a 3 ml solution of the extract was mixed with 1ml of 1%v/v HCl in different test tubes. After heating the mixture for 20 minutes, it was cooled and filtered, and the filtrate was used for further testing. Two drops of Mayer's reagent were added to 1ml of the extract-filtrate in one test tube, and a creamy precipitate indicated the presence of alkaloids. In another test tube, two drops of Wagner's reagent were added to 1ml of the extract-filtrate, and a reddish-brown precipitate was observed if alkaloids were present.

For detecting tannins, a volume of 1ml of freshly prepared 10%w/v KOH was added to 1ml of the extract in different test tubes. The appearance of a white precipitate indicated the presence of tannins.

To test for phenolics, two drops of 5% $FeCl_3$ were added to 1ml of the extract in different test tubes. If the solution turned greenish, it indicated the presence of phenolics.
To detect the presence of glycosides in the extract, a volume of 10ml of 50 v/v% H_2SO_4 was added to 1ml of the extract. The mixture was heated in boiling water for 15 minutes, and then 10 ml of Fehling's solution was added, and the mixture was boiled. If a brick-red precipitate was observed, it indicated the presence of glycosides in the extract.
Saponins were detected using two different tests: the frothing test and the emulsion test. For the frothing test, a volume of 2 ml of the extract in a test tube was vigorously shaken for 2 minutes. Frothing indicated the presence of saponins. For the emulsion test, five drops of olive oil were added to 3 ml of the extract in a test tube and vigorously shaken. A stable emulsion formed indicated the presence of saponins.

The presence of flavonoids was detected by adding a volume of 1ml of 10w/v% NaOH to 3ml of the extracts in different test tubes. A yellow colouration indicated the presence of flavonoids.
The Salkowsti test was used to detect steroids. Five drops of concentrated H_2SO_4 were added to 1ml of the extract, and a red colouration indicated the presence of steroids.
To detect phlobatannins, an aliquot of 1ml of each of the extract was added to 1% HCl. A red precipitate indicated the presence of phlobatannins.

Triterpenes were detected by adding five drops of acetic anhydride to 1ml of the extract each in a different test-tube. A drop of concentrated H_2SO_4 was then added and steamed for 1 hour and subsequently neutralized with NaOH followed by the addition of chloroform. A blue-green colour indicated the presence of triterpenes.
Carotenoids were detected by extracting a gram of each sample with 10ml of chloroform in a test tube with vigorous shaking. The resulting mixture was filtered, and 85%v/v sulphuric acid was added. A blue colour at the interface indicated the presence of carotenoids.2.4.2 Estimation of

Total phenolic content
The total phenolic content was determined using a spectrophotometer according to the method described by Saeed *et al*. (2012) with slight modifications. To a 10ml test tube containing 0.1ml of extract prepared in methanol (in triplicates), 5.0ml of distilled water and 0.5ml of (10% v/v) Folin-Ciocalteau's phenol reagent prepared in water were added sequentially and shaken. After 5 minutes, 0.5ml of 2%w/v Na_2CO_3 solution was added and mixed thoroughly. The mixture was kept in the dark for 30 min at room temperature, after which the absorbance was read against a blank (all reagents with methanol substituting the extract) at 765 nm. The standard curve for total phenolics was made using catechol standard solution (0 to 100 mg/ml) following the procedure described above for the extract. The total phenolic were determined from the calibration curve and expressed as milligrams of catechol equivalents per g of dried crude extracts or fraction

Estimation of total flavonoid content
Total flavonoid content was determined by the method described by Saeed *et al.* (2012) with slight modifications. To a 10ml test tube containing 0.3ml of extract prepared in methanol (in triplicates), 3.4ml of 30v/v% methanol, 0.15ml of $NaNO_2$ (0.5M) and 0.15ml of Aluminum chloride [$AlCl_3.6H_2O$ (0.3 M)] were added sequentially. After 5 minutes, 1ml of NaOH (1 M) was added. The solution was shaken to mix well and the absorbance of the resulting solutions

were measured against the blank reagent (all reagents with methanol substituting extract) at 506 nm. The standard curve for total flavonoids was made using rutin standard solution (0 to 100 mg/ml) following the same procedure as earlier described for the extracts. The total flavonoids were determined from the calibration curve and expressed as milligrams of rutin equivalents per g of dried crude extracts or fractions.

Determination of tannin content

The tannins were determined by the Folin - Ciocalteu method according to Singh *et al*. (2014) with slight modifications. To a 10ml test tube containing 0.1 ml of the sample extract prepared in methanol (in triplicates), 7.5ml of distilled water and 0.5ml of Folin-Ciocalteu phenol reagent were added and shaken, after 5mins, 1ml of 2 % Na_2CO_3 solution was added and mixed thoroughly. The mixture was kept in the dark at room temperature for 30 minutes to develop colour. The absorbance for the test and standard solutions was measured against a blank (all reagents with methanol substituting the extract) at 725nm. The standard curve for tannin was made using tannic acid standard solution (0 to 100 mg/ml) following the procedure described above for the extracts. The tannin content was expressed as milligrams of (Tannic acid equivalence) TAE/g of dried crude extract or fractions.

2.5 α-Amylase and α-glucosidase inhibition assay

The effect of the crude extract and its fractions on α-amylase and α-glucosidase activity was determined according to the method described by Kazeem *et al.* (2013) with modifications.

2.5.1 α-amylase

To 250 µl of each extract concentration in a test-tube (0-360 ug/ml), the following was added sequentially: buffered α-amylase (250 µl, 0.05 mg/ml), Starch (250 µl, 1%w/v). The reaction mixture was incubated for 10 min at 25^{o}C. DNSA (500µl) was added subsequently and then boiled for 5mins. It was then cooled and diluted with 5ml of dH_2O. The control was prepared in the same manner as the test samples with distill water replacing the extract. The absorbance of each test-tube content was taken at 540nm and the percentage inhibition calculated as follows;

% Inhibition= $\frac{Ac-At}{Ac} x\ 100$, where A_c and A_t are the absorbance of the control and test respectively.

α-glucosidase

To 50µl of each extract concentration in a test-tube (0-40ug/ml) the following were added sequentially: buffered α-glucosidase (100µl, 1.0U/ml) and incubated at 37^{o}C for 10 minutes, then pNPG(50µl, 3.0mM) and incubated at 37^{o}C,for 20 minutes then Na_2CO_3 (5%w/v), cooled to 25^{o}C and lastly 5ml H_2O was added and vortexed. The absorbance of the resulting yellow p-nitrophenol from the different test-tube was taken at 405nm and the percentage inhibition calculated as follows;

% Inhibition=$\frac{Ac-At}{Ac} x\ 100$, where A_c and A_t are the absorbance of control and test respectively

The concentration of the extracts resulting in 50% inhibition of the enzyme activity (IC_{50}) was determined graphically

Determination of *in vitro* antioxidant activity of the extract

The *in vitro* antioxidant activity of the methanol crude extract of *Chromolaena odorata* were determined according to the methods described in Saeed *et al*. (2012)

2.6.1 DPPH (2, 2-diphenyl-1-picrylhydrazyl) Scavenging assay (DSA)

The free radical scavenging activity of the fractions was measured *in vitro* by 2,2′- diphenyl-1-picrylhydrazyl (DPPH) with slight modifications. Briefly, DPPH stock solution (0.1mM) was prepared by dissolving 4 mg of DPPH in 100 ml methanol and stored at 20°C until required. The working solution was obtained by diluting the DPPH solution with methanol to attain an absorbance of about 1.2 ± 0.09 at 517 nm using the spectrophotometer. A 3ml aliquot of this solution was mixed with 100µl of the various concentrations (0 - 100 µg/ml) of the extract. The reaction mixture was shaken well and incubated in the dark for 30 min at room temperature. The absorbance was taken at 517 nm. The control was prepared as above without any sample.

The % scavenging activity was determined by the following equation:

DPPH scavenging activity (%) = [(control absorbance−sample absorbance) / (control absorbance)] ×100

Hydroxyl Radical Scavenging Assay (HSA)

The superoxide scavenging activity of the extract was determined according to the method described in Saeed *et al* (2012). Briefly, The reaction mixture contained; 500 µl of 2-deoxyribose (2.8 mM) in phosphate buffer (50 mM, pH 7.4), 200 µl of premixed ferric chloride (100 mM) and EDTA (100 mM) solution (1:1; v/v), 100 µl of H_2O_2 (200 mM) with 100µl graded concentrations (0-250µg/ml) of the extracts or without the extract for control. The reaction was triggered by adding 10µl of 300mM ascorbate and incubated for 1hour at 37°C. A 0.5ml aliquot of the reaction mixture was taken and added to 1 ml of TCA (2.8% w/v aqueous solution), then 1ml of 1%w/v aqueous TBA was added to the reaction mixture. The mixture was heated for 15 min on a boiling water bath (100^{o}C). The mixture was cooled and the absorbance at 532 nm was taken against a blank (the same solution but without the test solution). The hydroxyl scavenging activity of the extracts was calculated as follows:

Scavenging activity (%) = [1− (absorbance of sample/absorbance of control)] ×100

Reducing Power Assay (RPA)

The reducing power was based on Fe (III) to Fe (II) transformation in the presence of the test samples that can be monitored by measuring the formation of Perl's Prussian blue at 700 nm as described in Saeed *et al.* (2012). Briefly, various concentrations (0-200µg/ml) of the extract/fractions (2 ml) were prepared and mixed with 2 ml of phosphate buffer (0.2 M, pH 6.6) and 2 ml of potassium ferricyanide (10 mg/ml). The mixture was incubated at 50°C for 20 min followed by the addition of 2 ml of trichloroacetic acid (100 mg/l). The mixture was centrifuged at 3000 RPM for 10 min to collect the supernatant of the solution. A volume of 2ml from each of the mixture earlier mentioned was mixed with 2 ml of distilled water and 0.8ml of 0.1% (w/v) fresh ferric chloride. After 10 min reaction, the absorbance was measured at 700 nm.

Glucose Uptake Assay

The assay was performed according to the method of Cirillo (1962) . In brief, commercial baker's yeast was dissolved in distilled water to prepare 1% w/v suspension. The suspension was kept for 3 hours at room temperature (28◦C). The yeast cells-suspension was centrifuged at 4200 rpm for 5 minutes. The working suspension was obtained by further diluting the obtained clear supernatant (1 part by volume of the supernatant plus 9 parts by volume of distilled water). One

(1) ml of the various concentrations of the extract (1-5mg/ml) was incubated with 10mM of 1mL of glucose solution for 10 min at 37∘C. To initiate the reaction, 200 μl of working yeast suspension was added to the mixture of glucose and extract, vortexed, and incubated for another 60 minutes at 37∘C. After incubation, the tubes were centrifuged for 5 minutes at 3800 rpm and glucose was determined by the glucose oxidase method (Randox glucose kit) as described in the manufacturer's manual. Glucose uptake was calculated by the formula:

% Glucose uptake $= \frac{Ac - At}{Ac} x\ 100$, where A_c and A_t are the absorbance of control and test respectively, where control is the solution having all reagents except the test sample. Metformin was used as a standard drug.

Result

Phytochemical

The phytochemistry of the methanol extract of *Chromolaena odorata* (CO) is presented in Table 1 and Figure 1. The result showed the presence of alkaloids, tannins, phenolics, glycosides, saponins, flavonoids, steroids, triterpenes and carotenoids. Quatitatively, a significantly high phenolics and polyphenolics composition (124.67 mg GAE/g, 48.5 mg RE/g and 72.3 mg TAE/g of sample) was observed in the extract.

Table 1: Phytochemical profile of methanol extract of *Chromolaena odorata*

No	Active substance	Outcome
1.	Alkaloids	
	a. Wagner Reagent	+
	b. Mayer Reagent	+
2.	Tannin	+
3.	Phenolics	+
4.	Glycosides	+
5.	Saponins: frothing test	-
	Emulsion test	-
6.	Flavonoids	+
7.	Steroids: Salkowsti test	+
8.	Phlobatannins	-
9.	Triterpenes	+
10.	Carotenoids	+

+ present, - not present

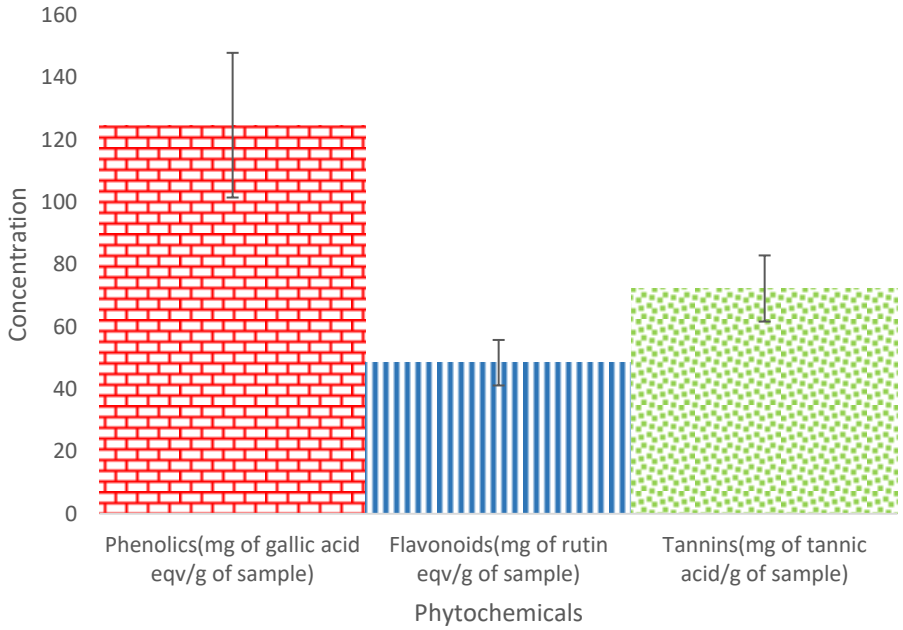

Figure 1: Polyphenolic content of crude methanol extract of *Chromolaena odorata* (CO). Values are mean ± SD of triplicate determination

Carbolytic Enzyme Inhibition

The effect of CO crude extract on carbolytic enzymes are presented in Figures 2 and 3. The result showed the crude extract (CO) showed a moderate inhibition of amylase and glucosidase. However, the observed inhibition of amylase by CO was significantly ($p<0.05$) lower than that of the standard drug acarbose.

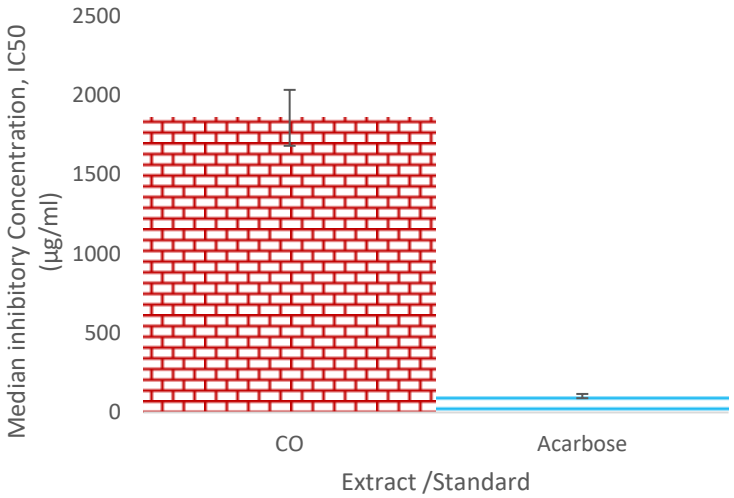

Figure 2: Alpha-amylase inhibition of crude methanol extract of *Chromolaena odorata* (CO) expressed in terms of IC50. Values are mean ± SD of triplicate determination.

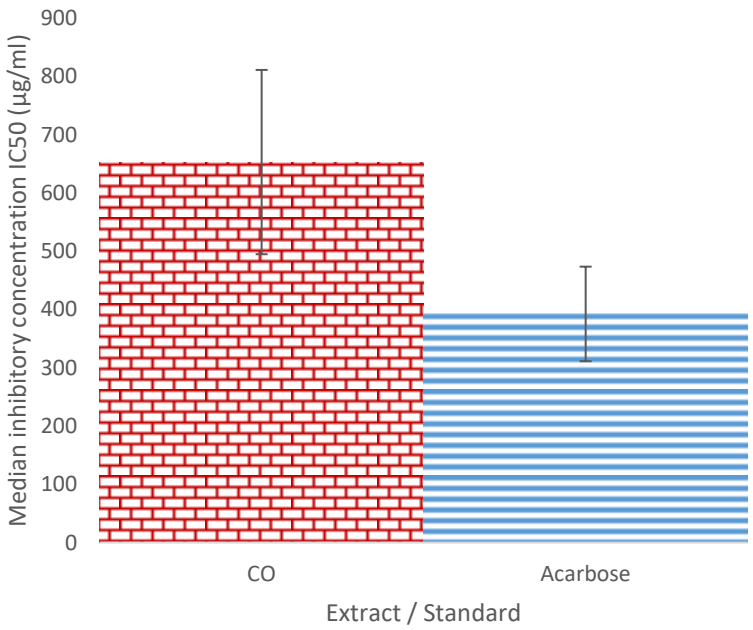

Figure 3: Alpha-glucosidase inhibition of crude methanol extract of *Chromolaena odorata* (CO) expressed in terms of IC50. Values are mean ± SD of triplicate determination.

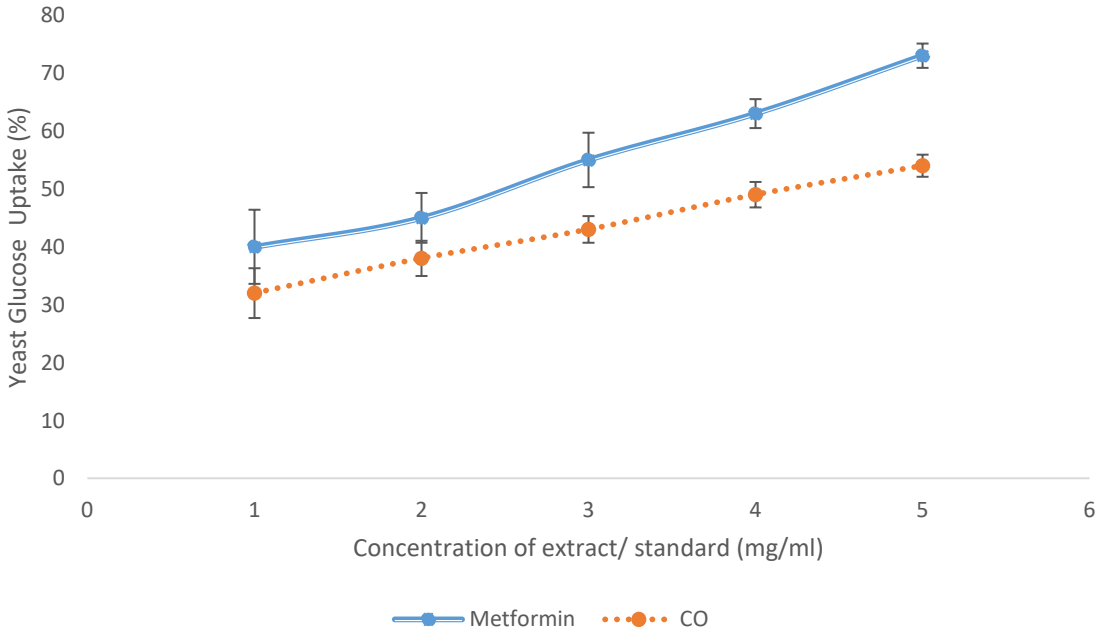

Figure 4: Effect of crude methanol extract of *Chromolaena odorata* (CO) on yeast glucose uptake. Values are mean ± SD of triplicate determination

Glucose uptake assay in yeast model

The effect of methanol crude extract of *Chromolaena odorata* (CO) leaf on glucose uptake in yeast cell model is presented in Figure 4. The result showed that the crude extract exhibited a dose dependent enhancement of glucose uptake in yeast cell model. This compared hugely with the effect of the standard drug, metformin. There was no significant difference between the observed action of the standard drug and the crude extract below the concentration of 3mg/ml.

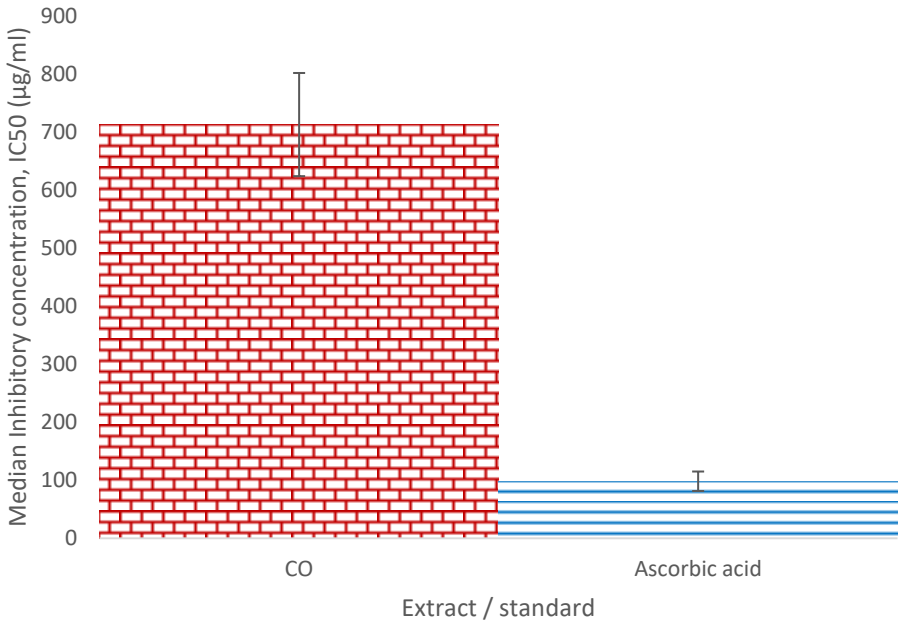

Figure 5: Free radical (DPPH) scavenging activity of crude methanol extract of *Chromolaena odorata* (CO) expressed in terms of IC50. Values are mean ± SD of triplicate determination

Antioxidant activities

The antioxidant activity (DPPH, OH radical scavenging and reducing power) of the crude extract of *Chromolaena odorata* is presented in Figures 5, 6 and 7. The result showed that the extract moderate capacity to scavenged DPPH and hydroxyl radicals relative to the reference standard ascorbic acid. However, the extract and the standard showed a comparable reducing power.

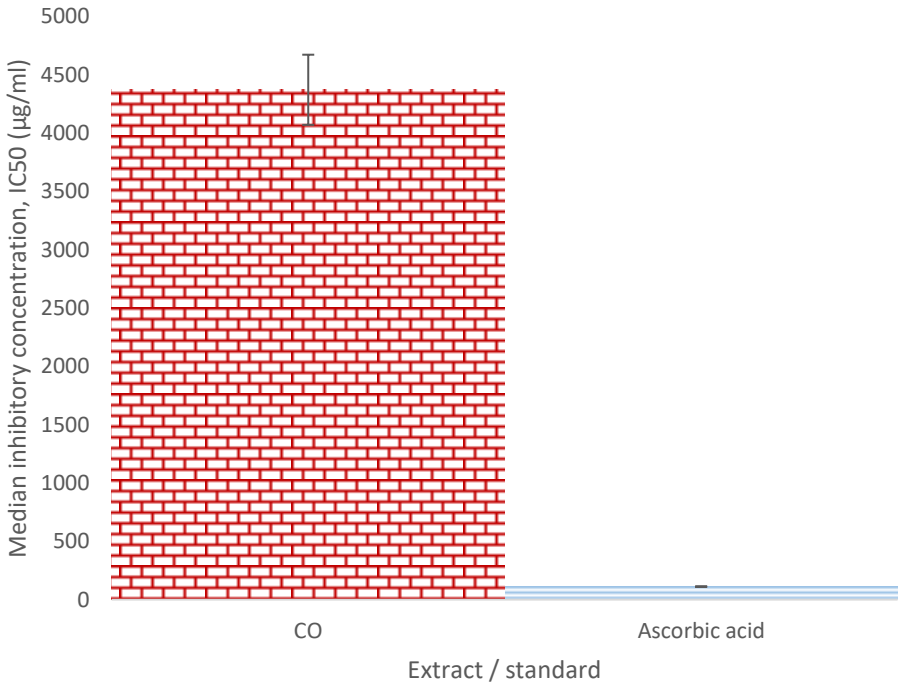

Figure 6: Free radical (Hydroxyl) scavenging activity of crude methanol extract of *Chromolaena odorata* (CO) expressed in terms of IC50. Values are mean ± SD of triplicate determination

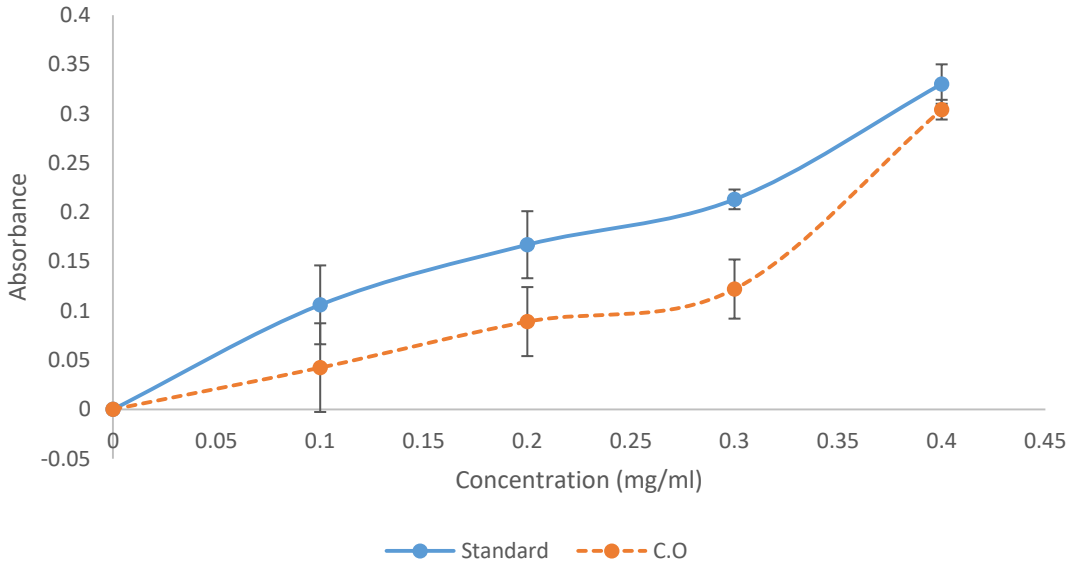

Figure 7: The reducing power of crude methanol extract of *Chromolaena odorata* expressed in terms of absorbance. Values are mean ± SD of triplicate determination.

Discussion

The phytochemical analysis of the methanol extract of *Chromolaena odorata* revealed the presence of a variety of phytochemicals, ranging from polar to nonpolar classes. This is attributable to the nature of the extracting solvent. Methanol has a polarity index that confers a composite polarity characteristic on it, making it capable of extracting both polar and nonpolar compounds (Abarca-Vargas et al., 2016). These classes of secondary metabolites contribute to the plant's ascribed medicinal benefits. A high composition of polyphenolics was also observed, with total phenolics having the highest level. This is in line with the reports of Abarca-Vargas et al. (2016) and Widyawati et al. (2014) and Ghasemzadeh et al. (2016), who demonstrated in different studies that methanol solvent significantly extracts polyphenolics. The very high level of total phenolics relative to other polyphenolics (flavonoids and tannins) may be due to the fact that the Folin method for determination of total phenolics is not highly specific; it detects any class of phenolic compound regardless (Singleton et al., 1965).

The effect of the CO extract on carbohydrate enzyme (amylase and glucosidase) activities showed that the extract is a more potent inhibitor of alpha glucosidase. A concentration of 693.33µg/ml inhibited the maximal glucosidase activity, while about three-fold higher concentration (1858.00 µg/ml) of the extract is required to achieve the same inhibitory effect in alpha amylase. This is similar to the value reported for the methanol extract of the root of *Chromolaena odorata* by Omonije et al. (2019). This pattern of inhibition suggests that the extract may not present with the gastrointestinal side effects associated with the use of acarbose, which is a more potent alpha amylase inhibitor (Oboh et al., 2012).

Oxidative stress plays a major role in the deterioration and complications of diabetes mellitus. The use of antioxidants to improve treatment outcomes in the management of diabetes is well documented (Johansen et al., 2005; Hill, 2008). Most synthetic antidiabetic drugs currently in use have not been reported to be strongly antioxidative; however, most of the antioxidative outcomes achieved with their use may have stemmed from their ability to control hyperglycemia, which is pivotal to oxidative stress of diabetes (Ononamadu et al., 2019). The CO extract in this study revealed a moderate potential to scavenge free radicals (DPPH and hydroxyl) and reducing

power that compared with the reference standard, ascorbic acid. This may somewhat complement the reported antidiabetic activity of extracts of this plant.

The rich chemical diversity of plants gives plant extracts the relative advantage of exhibiting a multimodal mechanism of action with respect to a particular bioactivity. In addition to the antioxidant and carbolytic enzyme inhibition potential, this study investigated the effect of the CO extract on glucose uptake. The extract demonstrated a significant potential to enhance glucose uptake in a yeast cell model. This observed effect was comparable to that of metformin - a standard drug known to act by stimulating glucose uptake by peripheral tissues.

Overall, these mechanistic findings may be connected to the rich polyphenolic phytochemistry of this plant. Previous studies have demonstrated that phenolic compounds and phenolic-rich extracts inhibit carbolytic enzymes (Oladunni and Ashafa, 2018; Ononamadu et al., 2019), ameliorate oxidative stress in diabetic conditions (Zeid et al., 2017; Marella, 2017), and also enhance glucose uptake via AMPK activation (Nagano et al., 2015; Azmi et al., 2018; Ooi et al., 2018).

Conclusion

From the results of the present study, it can be deduced that the methanolic extract of leaves of *Chromolaena odorata* possesses antidiabetic and antioxidant properties as demonstrated by the *in vitro* and *ex vivo* assays. Furthermore, the study shows that the hypoglycemic effect is mediated by one or synergy of the following mechanisms: Amelioration of oxidative stress via reduction of oxidants and marginal scavenging of free radicals, enhancement of glucose uptake in cells, and inhibiting the activities of the enteric enzymes such as α-amylase, α-glucosidase. However, further work is required to explore more mechanisms and as well characterize the plant for potent lead antidiabetic compounds.

Conflicts of interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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