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EVALUATION OF METHANOLIC EXTRACT OF *CHYTRANTHUS MACROBOTRYS* SEED (CMS) FOR ANTIMICROBIAL, *α*-GLUCOSIDASE AND *α*-AMYLASE INHIBITORY ACTIVITIES

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Keywords:

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ABSTRACT: *Chytranthus macrobotrys* (Sapindaceae) is one of the notable African medicinal plants with traditional history but lack scientific data. This report was aimed to evaluate *C. macrobotrys* seed (CMS) methanol extract for its phytochemical composition, antimicrobial activity, *α*-glucosidase, and *α*-amylase inhibitory activities. Methanolic extract of *C. macrobotrys* seed was investigated for its chemical composition, antimicrobial activity, α-glucosidase, and α-amylase inhibitory activities. The results revealed the presence of flavonoid (18.61 ± 1.88 mg/100g), phenol (28.71 ± 2.95 mg/100g), tannin (17.29 ± 1.84 mg/100g), terpene (587.07 ± 3.63 mg/100g), sterol (111.54 ± 3.85 mg/100g) and saponin (761.78 ± 1.35 mg/100g). The extract using agar diffusion method showed antimicrobial activity against *Staphylococcus aureus, Proteus vulgaris, Pseudomonas aureuginosa, Streptococcus pneumonia, Bacillus cereus, and Micrococcus luteus* with a diameter of inhibition zones from 10-20 mm. A weak antimicrobial activity was observed against *Escherichia coli* and *Shigella sp.* while no activity was observed against *Klebsiella pneumonia* and *Salmonella typhi*. The CMS showed *α*-glucosidase and α-amylase inhibitory activity at the concentration range of 10-100 µg/ml. The highest % inhibition was observed at 100 µg/ml (41.61 ± 1.85) and (37.90 ± 1.89) respectively. The study nominated CMS as a possible alternative natural remedy in the treatment of microbial infections and diabetes. Therefore, elucidating the active components, mechanistic mode of actions and further subjection of CMS for an *in-vivo* study are required.

INTRODUCTION: Mankind has depended on medicinal plants for its healing values since time immemorial. For thousands of years, a large number of modern drugs have been isolated from natural sources either directly or as a synthetic analog. There is an increased exploration of plant-based traditional medicine in health care resulting to about 80% of the world's population depending solely on traditional medicines for the treatment of disease ailments within their vicinity [1].

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The high cost of drug prescription in the maintenance of personal health and wellbeing as well as in the bioprospecting of new plant-derived drugs has increased the interest in medicinal plants as a re-emerging health aid [2].

The development of drugs and chemotherapeutics from medicinal plants as well as the cultural and traditional acceptability of herbal remedies is owed to the increased dependence on the use of these plants in the industrialized societies [3]. The broad biological and pharmacological activities demonstrated by phytochemicals in medicinal plants might have been responsible for their use as drugs for the treatment of diseases in mankind [4]. Phytochemicals present in fruits, vegetables, grains, and other plant foods have played a major role in the reduction of risk of major chronic diseases.

These bioactive non-nutrient plant compounds in plants have shown remarkable potentials and tremendous benefits in accelerating medicinal activities [5]. The medicinal activities of these compounds in their leaves, stems, barks, fruits, seeds, roots, and flowers are caused by the presence of the phytoconstituents secondary metabolites (such as terpenoids, alkaloids, tannin, steroids, glycosides, phenolics, saponins and flavonoids) present either in the whole or plant parts [6, 7].

Sapindaceae belongs to the family of flowering plants, and they are highly valued in Africa for their varying degree of traditional uses, high nutritional and medicinal values. There are about 136 genera and 2000 species existing as trees, shrubs and tendril-bearing vines from temperate to tropical regions [8]. Some of the traditional uses of the plants belonging to this family include in the treatment of diarrhea, dysentery, ulcer, boils, pain, wound healing and skin diseases related problems [8, 9, 10].

Various pharmacological activities such as; antioxidant, antidiabetic, antimigraine, anti-ulcerogenic, antimalarial, anti-microbial and anti-inflammatory activities of these family have been widely reported [9, 11, 12] while notable secondary metabolites such as flavonoids, triterpenes, tannins, and saponins have been isolated from the family [13, 12]. Sapindaceae has been found to produce a special group of secondary metabolites called cyanolipids [14]. *Chytranthus macrobotrys* (Gilg) Exell & Mendonca, native to Africa, belonging to the family Sapindaceae is a forest shrub (small tree) up to 35ft high, sparingly branched, racemes on old wood, pendulous, with erect brownish flowers. The leaves are imparipinnate (simple tendrils are present), circinately coiled and borne on the peduncles.

The fruits are with a terminal style, lobed, 3-8 rifled flowers zygomorphic. The petals are usually four (4), the ovary is 3-8 celled, inflorescence is borne on the thick main stem or sometimes on the ground [15]. *C. macrobotrys* can be found in Cameroon, Ghana, Congo, Ivory Coast, Angola, and Nigeria. The seeds have been found to be edible [16]. To date, there is no scientific report on the chemical constituents and the biological activity of *C. macrobotrys*. This study is therefore to assess the methanolic extract of *C. macrobotrys* seed for its phytochemical constituents, antimicrobial activity, *α*-glucosidase, and α-amylase inhibitory activities.

MATERIALS AND METHODS:

Plant Collection: The plant material was collected from a farm in Igbara-odo (7.5038° N, 5.0625° E), Ekiti State, Nigeria in May 2016. The plant was identified and authenticated by Mr. Omotayo, A.O. of the Department of Plant Science and Biotechnology, Ekiti State University, Ado-Ekiti, Ekiti State, Nigeria with a voucher number UHAE.EPH 2:002.

Plant Preparation and Extraction: The fresh fruits from the plant were opened up and the seeds removed and dried at room temperature for one month until constant weight was recorded. The dried seed sample was pulverized using mortar and pestle, and finally with an electric blender (Marlex). 15 g of the powdered plant material was extracted with methanol in a stoppered glass container for 72 h. The resultant mixture was vacuum filtered with Whatman no. 1 filter paper. The filtrate was concentrated to dryness using rotary evaporator at 40 °C to yield (33.4 g) of the methanol extract of *C. macrobotrys*.

Phytochemical Analysis: The phytochemical analysis of the methanol extract of *C. macrobotrys* was performed using the methods [17, 18, 19] with some modifications. The experiment was carried out in triplicate.

Test for Flavonoids: Methanol extract of *C. macrobotrys* (1 g) was dissolved with 5 mL of distilled water in a test tube, and 2 mL of 10 % (w/v) sodium hydroxide (NaOH) solution was added. The formation of a yellow colored solution indicates the presence of flavonoids [18].

Test for Saponins: The method previously described [18] was adopted. The methanol extract of *C. macrobotrys* (1 g) of the extract was dissolved with 5 mL of distilled water in a test tube and shaken vigorously for 1 min. The formation of a persistent honeycomb indicates the presence of saponins.

Test for Alkaloids: This assay was performed using the method previously described [17]. 1 g of the

extract was dissolved with 5 mL distilled water in a test tube, and 10 mL of 1 % HCl was added followed by the addition of two (2) drops of Wagner's reagent (solution of iodine in potassium iodide). The formation of amorphous or crystalline precipitates or colored precipitate indicates the presence of alkaloids.

Test for Sterols (Lieberman-Burchard Test): Test for sterols was carried out using method described [19]. The methanol extract of *C. macrobotrys* (1 g) was dissolved with 5 mL distilled water in a test tube and 1 mL of acetic anhydride was added and shaken followed by addition of 1 mL of concentrated sulphuric acid down the wall of the test tube to form a layer underneath. The formation of a reddish-violet color at the lower layer indicates the presence of sterols.

Test for Terpenes (Salkowski's Test): 1 g of the methanol extract of *C. macrobotrys* was dissolved with 5 mL distilled water in a test tube, and 2 mL of concentrated sulphuric acid was carefully added so that the sulphuric acid formed a lower layer. A reddish-brown color at the interface indicates the presence of a steroidal ring [17].

Test for Tannins: Test for tannins was carried out using the previous method documented [18]. 1 g of the methanol extract of *C. macrobotrys* was dissolved with 5 mL distilled water in a test tube, and 2 drops of 10% ferric chloride were added. Instant formation of blue-black colored solution indicates the presence of tannins.

Test for Cardiac Glycosides: Keller-Kiliani's test for the presence of glycosides previously described method was adopted [17]. 1 g of the methanol extract of *C. macrobotrys* was dissolved with 5 mL distilled water in a test tube and 2 mL of glacial acetic acid containing 1 drop of 10% ferric chloride solution was added with 1 mL concentrated sulphuric acid added down the wall of the test tube to form a layer underneath. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides, indicative of cardiac glycosides.

Quantitative Determination of Phytochemicals:

Estimation of Total Phenolic Content by Folin-Ciocalteau Method: The total polyphenol content in the methanol extract of *C. macrobotrys* was determined using the Folin-Ciocalteau's phenol reagent with gallic acid as the standard according to the method described [20]. The reagent measures the total reducing capacity of a sample, not just the level of phenolic compounds. Briefly, in 1.5 mL Eppendorf tubes, the sample was diluted five-folds in 10% methanol and appropriate concentration of standards made according to the standard operating procedure (SOP). On a 96-well assay plate, 25 µL diluted sample/standards were pipetted in triplicates. This was followed by the addition of 125 µL of diluted Folin-Ciocalteau reagent to all the wells. After 5 min incubation at room temperature, 100 µL of 7.5 % Na_2CO_3 was added to all wells and left again at room temperature for 2 h before reading the plate in a microplate ELIZA reader (Multiskan Spectrum by Thermo Electron Corporation, MA, USA). Gallic acid was used as a standard for constructing a calibration curve.

Estimation of Total Flavonoid Content by Aluminium Chloride Method: Total flavonoid content is determined by a colorimetric method previously described [21]. Appropriate dilutions of methanol extract of *C. macrobotrys* is allowed to react with sodium nitrite, followed by a flavonoid-aluminum complex formation using aluminum chloride. The absorbance at 510 nm is immediately measured and compared to that of catechin standard. In this assay, in 1.5 mL Eppendorf tubes, a five-fold diluted methanol extract of *C. macrobotrys* in 10% methanol was made, followed by suitable dilutions of Catechin standard to get appropriate concentrations according to SOP. On a 96-well assay plate, 50 µL diluted sample/standards were pipetted in triplicates. This was immediately followed by the addition of 42 µL of distilled water to all the wells. Mixed, then add 60 µL of sodium nitrite ($NaNO_2$) to each well and mixed. After 10 min of incubation at room temperature, 60 µL of aluminum chloride ($AlCl_3$) was added to each well and then mixed again. After 5 min of incubation at room temperature, 60 µL of sodium hydroxide (NaOH) was added to each well, mixed and read the plate in a microplate ELIZA reader (Multiskan Spectrum by Thermo Electron Corporation, MA, USA) at 510 nm.

Estimation of Tannins by Modified Prussian Blue Method: Previously described method [22] was adopted to quantify the amount of tannin in the

methanol extract of *C. macrobotrys*. 100 µL of the methanol extract of *C. macrobotrys* and 6.9 mL distilled water was added, 1 mL of 0.008M potassium ferric cyanide, 1 mL of 0.2M ferric chloride in 0.1M HCl were mixed. The absorbance of the blue color formed was measured at 700 nm. Tannic acid was used as a standard for the calibration curve.

Estimation of Sterols by Liberman-Burchard Method: Liberman-Burchard method was used for the quantification of the total sterol in the methanol extract of *C. macrobotrys*. Chloroform was added to 100 µL of the methanol extract of *C. macrobotrys* to make the volume up to 5 mL in a test tube. 2 mL of Liberman-Burchard reagent (0.5 mL of concentrated sulphuric acid in 10 mL of acetic anhydride) was added and properly mixed. The test tube was covered with black paper and kept under dark for 15 min. The green color complex formed was measured at 640 nm. Cholesterol was used as a standard for constructing the calibration curve.

Determination of *α*-glucosidase Inhibitory Activity: The inhibitory effect of the methanolic extract of the seeds of *C. macrobotrys* on α-glucosidase activity was determined according to the chromogenic method described [23] with slight modifications. 5 Units of the α-glucosidase enzyme were pre-incubated with 20 µg/mL of the methanolic extract of the seeds of *C. macrobotrys* for 15 min. Para-nitrophenyl glucopyranoside (PNPG) (3 mM) dissolved in 20 mM phosphate buffer; pH 6.9 was added to start the reaction. The reaction mixture was further incubated at 37 °C for 20 min and stopped by addition of 2 mL of 0.1 M Na_2CO_3. The *α*-glucosidase activity was determined by measuring the yellow colored p-nitrophenol released from PNPG at 400 nm. Each test was performed in triplicate, and the mean absorption was used to calculate percentage *α*-glucosidase inhibition.

$$\% \ \alpha\text{-glucosidase inhibition} = A_0 - A_1 \times 100 / A_0$$

Where A_0 = Absorbance of the control (blank without extract) and A_1 = Absorbance in the presence of the extract.

Determination of *α*-amylase Inhibitory Activity: *In-vitro* amylase inhibition was determined [24] with slight modifications. 100 µL of the methanol extract of *C. macrobotrys* was allowed to react with 200 µL of α-amylase enzyme (Hi-media Rm 638) and 100 µL of 2 mM of phosphate buffer (pH 6.9). After 20 min incubation, 100 µL of 1% starch solution was added. The same was performed for the controls where 200 µL of the enzyme was replaced by the buffer. After incubation for 5 min, 500µL of dinitrosalicylic acid reagent was added to both control and extract. They were kept in a boiling water bath for 5 min. The absorbance was recorded at 540 nm, and the percentage inhibition of *α*-amylase enzyme was calculated as:

$$A_0 - A_1 \times 100 / A_0$$

Where A_0 = Absorbance of the control (blank without extract) and A_1 = Absorbance in the presence of the extract. The IC_{50} value was determined graphically. Acarbose was used as the reference α-amylase inhibitor. All tests were carried out in triplicate.

Determination of Antimicrobial Activity: The potency of the methanolic extract of *C. macrobotrys* as an inhibitory agent to microbial growth was determined using a modified method of Agar well diffusion [24]. Ten (10) strains of bacteria including six (6) Gram-negative isolates; *Escherichia coli, Proteus vulgaris, Klebsiella pneumonia, Salmonella typhi, Shigella sp.* and *Pseudomonas aeruginosa* and four (4) strains of Gram-positive; *Staphylococcus aureus, Bacillus cereus, Micrococcus luteus* and *Streptococcus pneumonia* were subjected to susceptible test in the course of this research. These organisms were obtained from the culture bank of the Microbiology laboratory of the Department of Microbiology, Ekiti State University, Ado-Ekiti.

The methanol extract of *C. macrobotrys* was reconstituted with dimethyl sulphoxide (DMSO) to make a stock solution and was sterilized by filtration using 0.45 µM aqua membrane nylon filter disk (Becton, Dickinson Company). Reconstituted and filtered extract serving as the stock solution was stored in the freezer of a regular refrigerator (Haier Thermocool, Deluxe series HRF-350N) set at 4 °C. 20 mL of sterile Mueller-Hinton agar (MHA) was poured and allowed to set before 100 µL of a bacterial suspension with standard density for each bacterial strain and spread

on the surfaces using a sterile swab. With the aid of a 6 mm diameter cork borer, five cups were bored separately at equidistance on the agar. The borer was sterilized before and after every use. Each cup was filled with 0.2 mL of the extract at different concentrations. The plates were kept on the working bench at room temperature for 1 h to allow extract diffusion before being taken for incubation at 37 °C for 24 h. Zone of inhibition was measured to determine the effectiveness of the extract against each organism and the results were expressed in millimeters.

Statistical Analysis: Statistical analysis was performed using GraphPad Prism 5.0 statistical package (GraphPad Software, USA). All the results were expressed as mean ± SEM for triplicate determinations.

RESULTS:

Phytochemical Analysis: The results of the phytochemical test of the extract of *C. macrobotrys* presented in **Table 1** showed that seven major classes of secondary metabolites were detected namely: terpenes, sterols, tannins, saponins, phenols, flavonoids, and proteins. Alkaloids and cardiac glycosides were not detected in the extract. The quantification of the phytochemicals in *C. macrobotrys* seeds is presented in **Table 2**.

The result showed that the seed is a rich source of phytochemicals as it was found to contain flavonoid (18.61 ± 1.88 mg/100g), phenol (28.71 ± 2.95 mg/100g), tannin (17.29 ± 1.84 mg/100g), terpene (587.07 ± 3.63 mg/100g), sterol (111.54 ± 3.85 mg/100g) and saponin (761.78 ± 1.35 mg/100g).

TABLE 1: PHYTOCHEMICAL ANALYSIS OF THE SEEDS OF *C. MACROBOTRYS*

Phytochemicals	Result
Phenols	+
Flavonoids	+
Terpenes	++
Tannins	+
Sterols	++
Saponins	+++
Alkaloids	-
Glycosides	-
Proteins	++

+ = slightly present, ++ = present, +++ = highly present

The percent *α*-amylase and *α*-glucosidase inhibition of the methanolic extract of *C. macrobotrys* were plotted as a function of concentrations in comparison with acarbose as shown in **Fig. 1** and **2**. The results revealed that the methanolic seed extract of *C. macrobotrys* inhibited *α*-amylase and α-glucosidase enzyme *in-vitro*. At the concentration used in this assay (10-100 µg/ml), the % inhibition ranged from (8.34 ± 0.58 - 37.88 ± 1.89) and (21.79 ± 1.04 - 41.61 ± 1.85) for *α*-amylase and *α*-glucosidase inhibition respectively. The highest % inhibition for both enzymes was recorded at the highest dose. The present study indicates that *C. macrobotrys* could be useful in the management of postprandial hyperglycemia.

TABLE 2: QUANTIFICATION OF PHYTOCHEMICALS IN THE METHANOLIC EXTRACT OF THE SEEDS OF *C. MACROBOTRYS*

Phytochemicals	Concentration (mg/100g)
Phenolics	28.71 ± 2.95
Flavonoids	18.61 ± 1.88
Saponins	761.8 ± 1.35
Terpenes	587.1 ± 1.35
Tannins	111.5 ± 1.85
Polyphenols	119.73 ± 7.35

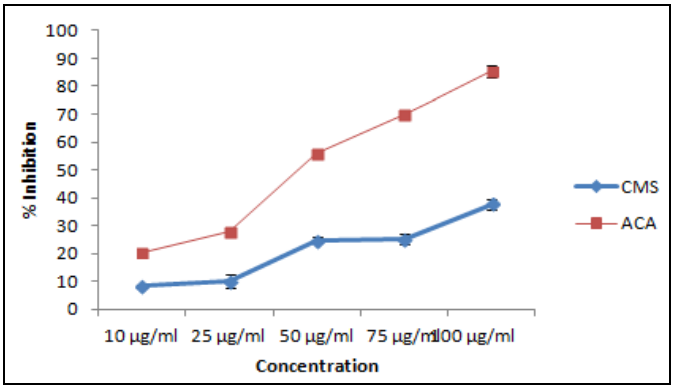

FIG. 1: % INHIBITION OF A-AMYLASE ENZYME BY METHANOLIC EXTRACT OF *C. MACROBOTRYS* AND REFERENCE A-AMYLASE INHIBITOR, ACARBOSE

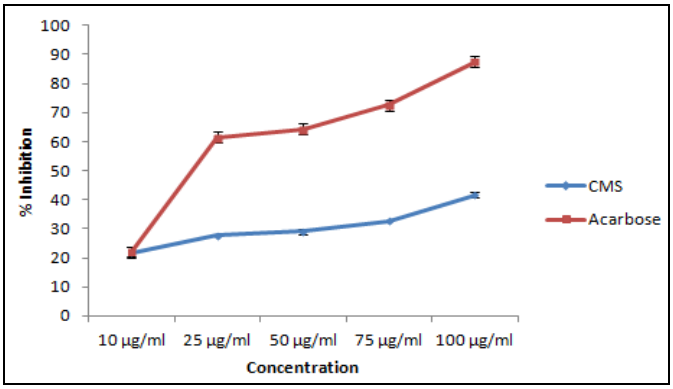

FIG. 2: % INHIBITION OF A-GLUCOSIDASE ENZYME BY METHANOLIC EXTRACT OF *C. MACROBOTRYS* AND REFERENCE A- GLUCOSIDASE INHIBITOR, ACARBOSE

Values are expressed as mean ± SEM, n=3

Antimicrobial Study: The extract as shown in **Table 3** was found to be active against *S. aureus, P. vulgaris, P. aureuginosa, S. pneumonia, B. cereus* and *M. luteus*. The extract also showed weak activity against *E. coli* and *Shigella sp.* while no activity was shown against *K. pneumonia* and *S. typhi.* The antimicrobial activity result from this study suggests that the seeds of *C. macrobotrys* may be used for the treatment of respiratory tract and opportunistic infections.

TABLE 3: ZONE OF INHIBITION (MM) OF THE METHANOLIC EXTRACT OF *C. MACROBOTRYS* SEEDS

Conc. (mg/ml)	1000	500	250	125	62.5
S. aureus (Gram +ve)	10	11	12	10	14
P. vulgaris (Gram –ve)	14	11	16	15	12
P. aureuginosa (Gram –ve)	11	12	12	15	11
K. pseumonia (Gram –ve)	-	-	-	-	-
E. coli (Gram –ve)	-	14	-	11	10
S. pneumonia (Gram +ve)	17	13	13	13	12
Shigella sp. (Gram +ve)	-	-	12	11	-
B. cereus (Gram +ve)	19	12	13	12	12
M. luteus (Gram +ve)	11	11	18	12	14
S. typhi (Gram –ve)	-	-	-	-	-

- = no activity

DISCUSSION: Notable phytochemicals are present in *C. macrobotrys* seeds according to **Table 1** and **2** in an appreciable amount. Some of these secondary metabolites present in significant amount include saponins, polyphenols, and tannins as 761.8 ± 1.35; 119.73 ± 7.35; 111.5 ± 1.85 mg/100g respectively. Flavonoids and saponins have been reported to possess antioxidants, hepatoprotective and anti-inflammatory activities and are therefore employed as antimicrobial, anticancer and antiallergic remediating agents. Flavonoids and other polyphenols have been scientifically documented for their major protective roles in liver diseases, cataracts and cardiovascular diseases [26] Condensed tannins (Proanthocyanidins) are the most abundant tannins, and these are the gallotannins and ellagitannins. Procyanidins are particularly abundant in the human diet and are responsible for the sensation of astringency (drying and puckering of the oral mucosa) by interacting with salivary proteins and found potent to act as α-amylase inhibitors [27]. Numerous biological and pharmacological activities have been exhibited by the condensed tannins which are of interest in human and veterinary medicine such as inhibition of lipid oxidation, antioxidant, anti-viral, the mutagenicity of carcinogens and tumor promotion [28].

Plant sterols (phytosterols) which chemically resemble cholesterol can block the absorption of dietary and endogenously derived cholesterol from the gut. They are not synthesized by the human body and are minimally absorbed by the human intestine. All vegetable foods contain bioactive components, phytosterols which resemble cholesterol invertebrates in both functions and structure. More than 200 different types of phytosterols have been reported in plants in which the most abundant are *β*-sitosterol, campesterol and stigmasterol [29]. The main function of phytosterols and phytostanols is to inhibit the uptake of dietary and endogenously produced cholesterol from the gut. Investigation of the dichloromethane extract of *Strongylodon macrobotrys* revealed the presence of phytosterols such as stigmasterol and *β*-sitosterol [30]. Terpenes like the mono-sesqui- and triterpenes have been reported to exhibit various biological activities in animals and microorganisms some of which include anti-inflammatory, anti-microbial and hormonal activities [18].

Diabetic patients have great potential risk to a varying degree of serious health-related problems including cardiovascular diseases. Hence, concomitant maintaining blood glucose level and preventing glucose oxidation is an effective strategy to treat diabetes thereby delaying its complications [31]. In this study, the methanolic extract of *C. macrobotrys seeds* was investigated on inhibition of α-amylase and α-glucosidase enzyme in an *in-vitro* system. The results of **Fig. 1** and **2** indicated that the extract possessed moderate inhibition when compared to the control (Acarbose). The extract also demonstrated antimicrobial activity against some gram-positive

and negative bacteria strains. The study shows that the plant may be useful in the treatment of diabetes and microbial infections. Therefore, elucidating the phytochemistry and mechanistic mode of actions are required.

CONCLUSION: The seed of *C. macrobotrys* is rich in phytochemicals most of which may qualify it for ethnomedicinal uses for the management of coughs, tuberculosis, and malaria. The presence of some secondary metabolites like flavonoids, saponins, tannins, phenols, terpenes, and sterols, all of which have been reported in the literature to exhibit physiological activities in man, animals and microorganisms, suggests that the seeds of *C. macrobotrys* may be used as a potent drug. The results of the *α*-glucosidase and α-amylase inhibitory activity of the methanolic extract of the seeds of *C. macrobotrys* showed appreciable α-glucosidase and α-amylase inhibitory activities.

This might be as a result of the bioactive compounds such as polyphenols present in the extract. The literature shows a clear link between polyphenols and antidiabetic activity of herbal extracts. To the best of our knowledge, this is the first report on the phytochemical test, antimicrobial study as well as *α*-glucosidase and α-amylase inhibitory activities of the seeds of *C. macrobotrys.*

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CONFLICT OF INTEREST: The authors declare that there is no conflict of interest concerning this study.

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