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Biochemical Effects of Fermentation on Selected Phytochemicals, Enzymes and Antioxidant Activities in The Under-Utilized Seeds of *Chrysophyllum albidum* Linn and *Terminalia catappa* Linn

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ABSTRACT

Chrysophyllum albidum Linn and *Terminalia catappa* Linn are both tropical plants. Fruits from these plants are eaten because of their edibility and delicious taste, and the seeds being under-utilized are mostly discarded. As the quest for food security increases, food processing methods that can improve under-utilized seeds for edibility are needed. This study's aim was to examine the outcome of fermentation on the phytochemical and antinutrient constituents of these seeds. Their *in vitro* antioxidant and enzyme activities were also studied. Tannin, phytate, alkaloid and oxalate levels decreased significantly in the two seeds after fermentation ($p<0.05$). Fermented extracts from the seeds significantly decreased ($p<0.05$) in their ability to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. *C. albidum* seeds significantly increased ($P<0.05$) in ferric reducing assay property (FRAP) only, *T. catappa* seeds however showed a significant decrease ($P<0.05$) in TAC and FRAP at the end of fermentation. A remarkable reduction ($P<0.05$) was detected in the α-amylase activity of the fermented seeds of *C. albidum*, while a significant elevation ($P<0.05$) in the α-amylase activity of the fermented *T. catappa* seeds manifested. Lipase activity increased significantly ($P<0.05$) in the fermented seeds of both species. Significant elevation ($P<0.05$) of protease activity also manifested in the fermented *C. albidum* seeds. The distinct reduction in antinutrient status of the seeds, coupled with the increased digestive enzymes activities, was shown during fermentation, thus indicating fermentation as a tool to enhance the edibility and health outcomes of these under-utilized seeds.

Keywords: *Chrysophyllum albidum*, *Terminalia catappa*, fermentation, antinutrient reduction, digestive enzymes, antioxidant

Introduction

For ages now, the fermentation of foods has been a human-initiated food processing tool. Fermented foods are popular for reasons such as enhanced nutritional qualities, functionalities, digestibility, palatability, shelf life, safety, and sensory properties.[1] Fermentation keeps gaining popularity due to its ability to increase the availability of vitamins, as well as other biomolecular compounds in foods.[2] Fermentation of foods is the process of making foods through desired microbial growth, enzymatic and metabolic transformation of food component.[3] It is one of the oldest methods used in food processing. It is a technique that has been widely employed in reducing antinutrients present in food substances. In a study by Babalola and Giwa.[4] a general decrease in the total antinutrients present in soybean was noticed after fermentation for a day. Ojokoh *et al*.[5] studied the outcome of fermentation on the anti-nutritional constituents of cowpea and breadfruit flour, a remarkable decrease in the oxalate, hydrogen cyanide, and phytate contents of the flours was observed.

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It has also been reported of fermentation to decrease the phytate content of chickpea by 45 % and increase the protein content by 13 %.[6] In another study by Adeyemo *et al*.,[7] the outcome of the fermentation of sorghum on tannin, phytic acid, protease inhibitor, and trypsin inhibitor at 0, 72, and 120 hours were assessed, the results showed a significant reduction of tannin (72 %), phytic acid (60 %), protease inhibitor (30 %), and trypsin inhibitor (69 %) at 120 hours. Different fermentation techniques have been used to produce healthy food products from poisonous crops like *Carica papaya* seeds and *Adenanthera pavonine*.[8,9,10] Some studies have also reported that fermentation led to the increase in the bioavailability and concentration of amino acids, minerals and vitamins.[11]

Chrysophylum albidium Linn Sapotaceae, widely known as African star apple is an African forest fruit tree species.[12] Fruits from these plants are sweet and edible, and usually consumed as desired. On the other hand, the seeds from this fruit are poorly utilized, and there is limited information on its usefulness. The seeds because of their poor utilization, have however gained the interest of researchers over the years. Omeje *et al*[13] reported these seeds as one of the un-exploited sources of Phyto-oil that could serve as an alternative to oil from conventional plants such as coconut, oil palm, cotton seeds, soybeans, groundnuts, sunflower, and rape seeds. Nutrition wise, *C. albidum* seeds have been reported to have high levels of energy, however, the presence of antinutrients such as phytate, oxalate, saponin, and tannin in the seeds have hindered humans and animals from benefitting nutritionally from them.[14] *C. albidium* seeds have been investigated and reported to be an alternative to starch from conventional sources such as sago starch, corn starches, cassava starch, potato starch, maize starch, bean starch, and glutinous rice starch. They have also been utilized in non-food and food applications.[15]

Terminalia catappa Linn Combretaceae, commonly called 'fruit' in Nigeria, is a tropical plant known popularly as tropical, Indian, country or sea almond.[16] *T. catappa* tree is commonly grown for ornamental purposes, and serves as shade tree in cities, towns, villages, public places, and residential quarters in Nigeria. The fruits from this tree get ripened and fall to the floor, consumers pick them up and eat up the sweet fleshy part, while the hard endocarp that contains the seed is discarded afterwards. The tiny size of this seed coupled with the difficulty of its extraction from the hard endocarp are probable reasons for its under-exploitation. These seeds have however gained research interest over the years.[16, 17, 18] The other plant parts such as leaves, trunk, fruit shell, root, and bark are used medicinally, and for some other non-food purposes.[19,20] *T. catappa* seeds have been reportedly rich in protein including the amino acids; histidine, valine, threonine, tryptophan, tyrosine, and isoleucine.[21] In an assessment of the vitamin constituents of these seeds, there were numerous displays of essential vitamins namely; ß-carotene, thiamine(vitamin B_1), niacin (vitamin B_3), pyridoxine(vitamin B_6), folic acid (vitamin B_9) and tocopherol (vitamin E).[22] A study on the sensory, functional, and physicochemical, properties of tapioca (a cassava product) with *T. catappa* seed flour blend, gave the conclusion, that fortification of cassava starch with 10 % *T. catappa* seed flour has high commercial potential.[21] Despite availing reports of studies conducted on *C. albidum* and *T. catappa*, some of which have been highlighted above, there is dearth information on the improvement of *C. albidum* and *T. catappa* seeds with fermentation, to promote their potential as alternative sources of food. Globally, food prices keep rising dramatically, due to inflation in the prices of other commodities. Hunger and undernourishment are problems faced by most developing countries in the world today. Hence, to tackle the problem of undernutrition, while considering the purchase power of much of the population, it is essential to investigate the improvement of nutrient levels in alternative sources of foods by fermentation. This study assessed the outcome of fermentation on the phytochemical, antinutrient composition, and antioxidant status of the poorly utilized seeds of the plants; *C. albidum* and *T. catappa*. In addition to the studies mentioned above, studies on the activities of the digestive enzymes; alpha amylase, protease, and lipase, obtained from these seeds before and after fermentation showed the power fermentation in obtaining useful products, such as enzymes from poorly or underutilized seeds.

Materials and Methods

Equipment

Telstar Cryodos-50 freeze dryer, Freezer (-80 ºC) by Thermo Scientific limited (model: 8602, serial/no: 8^{22}518-180), pH meter (model:pHep, serial no: HI96107, Italy), UV-Visible spectrophotometer by Thermo Scientific limited (model:GEN10S UV-Vis, serial no: 2L5V095205), and Refrigerated centrifuge (model: CR21G, serial no: S2025709).
Methanol (Sigma Aldrich, United States of America), absolute ethanol (Sigma Aldrich, United States of America), potato starch (Sigma Aldrich, United States of America), 2,2-diphenyl-1-picrylhydrazyl (Sigma Aldrich, United States of America), glycerol tributyrate (Merck Schuchardt, Germany), gum acacia (Mallinckrodt Baker, United States of America).

Collection and Identification of Seeds

Collection of the *C. albidum* seeds was done March 2020, and identification was done using the methods outlined by Odutayo *et al.*[23] The *C. albidum* fruits for obtainment of the seeds were purchased at Afobaje market, Ota, Ogun State, Nigeria (popularly referred to as Oja Ota). The market is located at an approximate distance of 6.6841°N, 3.2153°E. The voucher number used for identification and verification was 112774, a FHI number issued by FRIN in Ibadan, Oyo State, Nigeria.
The *T. catappa* seeds were collected August, 2020, and identified, using the methods described by Odutayo *et al.*[24] The seeds were sourced from *T. catappa* fruits collected from the viscinity of the University of Ibadan, Oyo State, situated on the geographical axis 7.4443º N, 3.8995º E. Identification and verification of these seeds were also done at the Forest Research Institute of Nigeria (FRIN), Ibadan, with voucher number, FHI: 112775.

Processing of seeds

The *C. albidium* and *T. catappa* seeds were de-hulled manually. The *T. catappa* seeds were ground immediately after de-hulling while the *C. albidium* seeds were dried in the oven at 40 ºC till they were crunchy and crispy enough to be grinded. This process was completed within a 24 hr period. The seeds were then ground using an electric blender. (Nakai Japan, Patent no: 013219812).[23,24]

Preparation of the Fermented and Unfermented Seeds Extracts

This was done by applying the methods outlined by Odutayo *et al.*[24] Both extracts were made by steeping the ground seeds in distilled water at 1:3 w/v, the mixtures were stirred, and stood for 24 hr at a temperature range of (25 to 28) ºC. After 24 hr, filtration paper (Whatmann no. 1) was used for filtering each of the mixtures. Filtrates were extracted from both seeds and divided into I and II. The portion I was fermented prior to analysis, while the portion II was not fermented prior to analysis. The first part (I) filtrate was placed in an air-tight vessel and made to undergo anaerobic fermentation for 72 hr at ambient temperature before being placed in the freezer (-20 ºC) The second part (II) filtrate was immediately placed in the freezer, at -20 ºC for freezing, so as to avoid fermentation. The filtrates I and II were then removed from the freezer and placed in a freeze dryer (Telstar cryodos-50) where sublimation of the aqueous solvent occured till the dried extract was left. The dried extracts from filtrates I and II respectively, were classified as as the fermented and unfermented extracts.

Qualitative phytochemical analysis

Initial phytochemical analysis of extracts was conducted to confirm the presence of flavonoids, glycosides, terpenoids, tannins, alkaloids, tri-Terpenoids, phenol, and steroids. These tests were carried out in accordance with the established protocols described by Varadharajan *et al*.[25]

Quantitative phytochemical determination

Both extracts were subject to quantitative phytochemical analysis to determine their total phenolic, flavonoid, alkaloid, tannin, oxalate and phytate contents. Methods used for the analyses are described as follows:

Examination of total phenolic content

Total phenolic content of both extracts was determined using the methodology outlined by Hammerschmidt and Pratt.[26] 1 g of plant extracts was dissolved in distilled water (100 ml) to yield 10 mg/ml extract. Aliquots (0.2 ml) from each test solution were added to 1 ml (10 % Folin-Cocalteu) and 0.8ml (7.5% sodium carbonate solution), with thorough mixing. Mixtures were incubated for 1 hr at 25 ºC. Their absorbance values were determined at 760 nm with the aid of a UV-Visible spectrophotometer (GEN10S UV-Vis, 2L5V095205). A standard curve was established for gallic acid at a concentration of 0.2 to 1 mg/ml. The phenolic content of the extracts was determined from the calibration curve generated for gallic acid. Total phenolic content present in extracts was calculated as mg gallic acid equivalent (GAE) per g of extract. The basic principle behind this method is based on an oxidation / reduction reaction (equations 1 and 2), in which a molybdotungstate reagent is used to oxidize phenolic compounds, resulting in a colored product with a wavelength max of 745 to 7765 nm.[27]

$$Na_2WO_4/Na_2MoO_4 \longrightarrow (Phenol - MoW_{11}O_{40})^{-4} \text{ --------- equation 1}$$

$$Mo(VI)(yellow) + e^- \longrightarrow Mo(V)\ (blue) \text{ ---------- equation 2}$$

Estimation of total flavonoid content

The total flavonoid content of the extracts was determined in accordance with the established method of Zhishen *et al.*[28] An aliquot 0.1 ml of 10 mg/ml test extract was withdrawn into a 10 ml volumetric flask, with addition of distilled water to make up the 5 ml volume. Addition of 5 % $NaNO_2$ (0.3 ml) solution followed thereafter. 5 min

later, 10 % $AlCl_2$ (3ml) was added. 6 min later, 1 M Sodium (2 ml) was added, and absorbance readings were taken at wavelength of 510 nm. A standard curve was prepared using different rutin concentrations (0.2 mg/ml to1 mg/ml) to estimate flavonoid content in the extracts. The total amount of flavonoids present in the extracts was expressed as mg of rutin equivalent (RE)/g of extract.

Estimation of total alkaloid content

Quantitative determinations of alkaloid status of extracts were conducted in accordance with the method of Harborne.[29] 200 ml (10% acetic acid) in ethanol was added to 250 ml (2.5 g) of each extract and left to stand for 4 hr. Each suspension was then reduced to a quarter of the initial volume by concentrating it on water bath. The solution was thereafter precipitated on a dropwise basis with the addition of a concentrated ammonium hydroxide for total precipitation. After 3 hr mixture sedimentation on standing, the supernatants were disposed, and the precipitates were washed with 0.1 M ammonia hydroxide (20 ml). Filtration followed with the use of filter paper (whatmann no 1). The residue was dried in an oven. Weighing followed with the use of an electronic weighing balance. The alkaloid percentage was calculated as follows:

% Alkaloid = (Alkaloid weight/Sample weight) × 100

The principle behind this is the quantitative extraction of the ion pair complex by the organic solvent, this is the combination of the acid and salt ion resulting from the reaction of the alkaloids and hydrogen ions under acidic conditions.

Total tannin content determination

The extracts were analyzed for their tannin content using the established protocol outlined by Ci and Indira.[30]. 1 mg/ml of each sample extract was placed in an aliquot of 0.1 ml in a 10ml volumetric flask that contained distilled water (7.5ml), Folin-Ciocalteu phenol reagent (0.5ml), and 35 % sodium carbonate solution (1 ml). The mixture was afterward made up to 10 ml with distilled water, vigorously shaken, and allowed to stand at room temperature for a duration of 30 min. Tannic acid reference standard solutions (0.02 to 0.1 mg/ml) were made as previously described previously and used to prepare the tannin standard curve. The absorbance values of the standard and test solutions were read at 700 nm using a uv/visible spectrophotometer (GEN10S UV-Vis, 2L5V095205). Following this, the tannin composition of the extracts was estimated from the standard curve previously prepared for tannic acid. The tannin composition of the extracts was reported as mg tannic acid equivalent (TAE)/g of extract.

Estimation of oxalic acid content

The oxalic acid component of the extracts was estimated using the established protocol described by Soetan.[31] Oxalate extraction was carried out by weighing one gram of each extract which was afterward steeped in distilled water (100 ml). Each suspension was allowed to stand for 3 hr, followed by filtration through a double layered filter paper. 20, 40, 60, 80, and 100 ppm standard solution of oxalic acid were made, and the readings were taken at an absorbance of 420 nm. The absorbance values of filtrate from each of the extracts were also determined with the aid of the spectrophotometer (GEN10S UV-Vis, 2L5V095205). Oxalate content in each extract was expressed as mg Oxalic acid equivalent (OAE)/g of extract.

Phytate estimation

Phytate content in the extracts was determined according to the method of Maga.[32] 1 g of each sample was weighed. 50 ml of 2 % concentrated hydrochloric acid was used in soaking each sample and made to stand for 3 hr. After the 3 hr of standing, they were filtered through a double layer of filter paper (Whatmann no 1). 25 ml of each filtrate was dispensed in a 250 ml beaker, afterwards, 53.5 ml of distilled water was added to give proper acidity. For each filterate, an aliquot (5 ml) of 0.3% ammonium thiocyanate solution was added as an indicator, and the filterate was titrated with a solution of Iron (III) chloride (0.00495 g/ml). The end point was a light shade of brownish yellow, which lasted for five minutes. The phytate content of each extract was presented in milligrams per gram (mg/g) of extract, using the calculations shown below:

Phytate (mg/g) = titre value x 0.00495 x 1000
Where:
titre value = obtained titre value from the experiment
0.00495 = solution of iron(iii)chloride solution (g/ml)
1000 = factor for conversion of g/ml to mg/ml

Assessment of in vitro antioxidant activity

Assessment of *in vitro* antioxidant activity of the extracts were carried out by the determination of their 2, 2-Diphenyl-1-picrylhydrazyl radical scavenging activity (DPPH), Feric reducing assay property (FRAP) and Total antioxidant capacity (TAC). The methods for the analyses are described below:

Assay for 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The effectiveness of the extracts in scavenging DPPH radicals was tested by the method described by Sakat *et al*.[33] For each test extract, an aliquot (0.5 ml) of 0.2 to 1 mg/ml of the extract dissolved in methanol was mixed with 0.5 ml of 100 mM methanolic solution of DPPH. Incubation of the reaction mixtures in the dark was done for a duration of 30 min at room temperature. The absorbance values were thereafter measured at a wavelength of 517 nm. Ascorbic acid was the standard used, while for the control, an aliquot of 0.5 ml of methanol replaced the test extract. The DPPH scavenging activity (percentage inhibition) was reported as follows:

% Inhibition = (Control Absorbance – Sample Absorbance)/Control Absorbance] x 100

When an antioxidant compound reacts with DPPH, the compound donates hydrogen ions and becomes reduced thereafter (equations 3 and 4), this leads to a colour shift in the DPPH solution from deep violet to light-yellow.

$$DPPH^{\cdot} + AH \longrightarrow DPPH - H + A^{\cdot} \quad \text{equation ---------- 3}$$

$$DPPH^{\cdot} + R^{\cdot} \longrightarrow DPPH - R \quad \text{equation ---------- 4}$$

Ferric reducing antioxidant power (FRAP) Assay

The reducing strength of each extract was determined in accordance with the methodology outlined by Sharma *et al*.[34] An aliquot (1 ml) of the extract (1 mg/ml) was added to an aliquot (2.5 ml) of 0.2 M, pH 6.6 phosphate buffer and 2.5 ml potassium ferricyanide (1%) and mixed thoroughly. The reaction mixture was then incubated for 20 min at a temperature of 50 ºC. An aliquot of 10 % trichloroacetic acid (2.5 ml) was added to the solution and centrifugated for 10 min. A reference standard of ascorbic acid with a concentrations range of 0.2 to 1 mg/ml was prepared and treated as described above. An aliquot of 2.5 ml of each reaction mixture was combined with distilled water (2.5 ml) and 0.5 ml of $FeCl_3$ (0.1 %). All solutions were spectrophotometrically read at 700 nm using uv/visible spectrophotometer, and the absorbance was expressed as mg ascorbic acid equivalent per g of extract (mg AAE/g). The principle of ferric reducing power assay is that reducing-potent substances react with ferricyanide (Fe^{3+}), which in turn forms potassium ferrocyanide (Fe^{2+}) that forms a ferric-ferrous complex that can be measured at an absorbance of 700nm, after reaction with ferric chloride.[35]

Total antioxidant capacity (TAC)

Total antioxidant capacity assay was carried out as described by Sharma *et al*.[34] Aliquots (0.3 ml) of each extract were combined with 3 ml of freshly made reagent solution (0.6 M Sulfuric acid), 28 mM sodium phosphate, and 4 mM ammonium molybdate (1:1:1). A reference standard of ascorbic acid having a concentration range of (0.2 to 1) mg/ml was prepared and treated as described above. The reaction mix was capped and heated at a temperature 95 ºC for 90 min. The absorbance of the product against a blank (0.3 ml of methanol) was determined at a wavelength of 695 nm after cooling to a room temperature. The total antioxidant capacity of the extracts was reported as mg AAE/g (mg Ascorbic Acid Equivalent per g) of extract.

Assay of digestive enzymes activities from the unfermented and fermented seeds

Extraction of protease, lipase and amylase from the unfermented and fermented seeds was done in accordance with the established protocols described by Omafuvbe *et al*.[36] Extraction of these enzymes from the unfermented seeds was done using a mortar and pestle, 10 g of the ground seeds were pulverized in 100 ml of the appropriate extracting buffer, the suspensions were afterwards centrifuged at 5000 rpm in a high-speed refrigerated centrifuge (CR21G, S2025709) at 5 ºC for 30 min. The supernatants were removed for storage in the -80 ºC freezer before the subsequent enzyme assays. To extract the enzymes from the fermented seeds, 10 g of ground seeds were steeped in 100 ml distilled water and left to ferment anaerobically for 72 hr, the pH and titratable acidity of the fermenting mesh before and after fermentation were determined. After fermentation, 5 g of the fermented seeds were withdrawn from the fermentation mesh and transferred into 50 ml of the appropriate buffer, using a mortar and pestle, the seeds were thoroughly macerated in the buffer, and the suspensions were also centrifuged at 5000rpm in a high-speed refrigerated centrifuge (CR21G, S2025709) at 5 ºC for 30 min. The supernatants were removed for storage in the -80 ºC freezer before the subsequent enzyme assays.

pH and titratable acidity

The methodology employed in these analyses was derived from that of Volmer *et al*.[37] with minor modifications. The pre-fermentation and post-fermentation pH of the fermentation meshes were measured using a pre-standardized pH meter (PHEP H196107), which was standardized prior to its usage with standard solutions having pH values of 4, 7, and 9. To obtain the values for titratable acidity, 200 ml of boiled and cooled distilled water was added to 500 ml of Erlenmeyer's flask, followed by 1 ml phenolphthalein indicator. The water was titrated to a pink end point with a concentration of 0.1 N NaOH. 5 ml of the samples were released into a conical flask, and they were titrated with 0.1 N NaOH to the same end point. The volume of NaOH used during titration was recorded thereafter. The titratable acidity (TA) of the sample was calculated as shown below.

TA of sample (0.1 g/ml) = (V x N)/ v

Where:

V = Solution of NaOH for titration (ml)

N = NaOH Normality used for titration (0.1 N)

v = Volume of sample (5 ml)

Titratable acidity determinations are based on the principle that a solution with higher hydrogen ions concentration will remain acidic, while a solution with higher hydroxide ions concentration will remain alkaline and vice-versa. The titratable acidity of a base, such as NaOH, is determined by the extent to which it neutralizes the acids, such as lactic acid and phosphoric acid, in a given volume of liquid, thereby determining the number of free hydrogen ions and the number of hydrogen ions bound to weak acids that can interact with the strong base to be neutralized.

Quantification of protein in the seeds samples

In accordance with Lowry et al.,[38] serial dilution of a stock solution containing BSA (1 mg/ml) was used for the preparation of a range of BSA concentrations (0.05, 0.1, 0.2, 0.4, 0.6, 0.8, and 1 mg/ml). 0.2 ml each of protein solution from the various concentrations were dispensed into test tubes by pipetting, with 2 ml of alkaline copper sulfate reagent added. The solutions were then mixed and kept in the incubator for a duration of 10 minutes at 25 ºC. To each tube, 0.2 ml Folin Ciocalteau solution was added and their incubation was carried out in the dark at 25 ºC for a 30 min duration. The absorbance of the solutions were thereafter read on the UV/visible spectrophotometer (Evolution 60s, 2R1R235201) at 660 nm. A calibration curve of absorbance against protein concentration was obtained and used in the determination of the protein concentration in the seeds samples.

Determination of alpha-amylase activity

The extracting buffer for α-amylase was 0.1 M potassium phosphate buffer, pH 6.0. To assess the alpha-amylase activity, the protocol outlined by Ho and Yin-Sze.[39] was employed. 1 % (0.01 g/ml) starch was used as substrate. The reaction mixture containing aliquots of 0.5 ml of the enzyme, 0.25 ml of the substrate, and 0.25 ml of 0.4 M Tris HCl buffer, pH 9 was incubated at 37ºC for 30 min, the enzyme reaction was stopped by the application of an ice bath, and an aliquot of 3 ml of 3,5-dinitro salicylic acid (DNSA) was added and heated for 5 min in a 100 ºC water bath, the reaction was stopped afterwards by using an ice bath, and the absorbance was read at 550 nm. For the control, the enzyme was replaced with distilled water, while for the blank, enzyme and substrate were replaced with distilled water. A standard curve was generated for maltose and used as a reference for the estimation of the α-amylase activities of the different enzyme extracts. 1 mg/ml maltose solution was made and used thereafter as a stock solution. Five accurate dilutions ranging from (0.2 to 1.0) mg/ml were made from the standard stock solution, 3 ml 3,5-dinitro salicylic acid (DNSA) was dispensed into 1 ml of each dilution and heated for a duration of 5 min in a 100 ºC water bath, the reaction was halted by applying an ice bath, and the absorbance readings were done at a wavelength of 550 nm. Distilled water was used to replace maltose and served as the blank. One unit of activity for amylase was given as follows:

(Maltose released in Milligrams)/(Incubation time in min) x Enzyme in reaction mixture (ml)

Specific activity of amylase (U/mg protein) was calculated by dividing the activity of amylase in the respective plant samples by the amount of protein in their corresponding plant samples. This method works on the principle that alpha-amylase activity is quantified in a colorimetric manner with the aid of DNSA reagent. Here, the amylase activity converts the starch into maltose. Maltose, on the other hand, is released from the starch and is determined by the amount of 3,5-dinitrosalicylic acid that is reduced.

Determination of protease activity

Assay for protease activity was performed according to the established protocols outlined by Ho and Yin-Sze.[39] 0.6 g of casein dissolved in 100 ml of 0.4 M Tris-HCl buffer, pH 9 served as a substrate for the reaction. Each mixture for the reaction, which consisted of 0.25 ml of enzyme and 0.25 ml of substrate was kept in the incubator at a temperature of 37 º C for 30 min. The enzymic reaction was brought to a halt by the addition of 0.5 ml (5 g/100ml) trichloroacetic acid. To the reaction mixtures, an aliquot (1 ml) of Bradford's reagent was added and allowed to react under room temperature for 5 min. The absorbance readings were taken at 595 nm. For the control, distilled water replaced the enzyme, while the substrate and enzyme were substituted with distilled water for the blank. To measure the protein content of the reaction, a tryptophan standard curve was constructed. To obtain 5 correct dilutions of 0.2 mg/ml to 1mg/ml tryptophan solution, a 1mg/ml tryptophan solution was first prepared and used as stock. An aliquot (1 ml) of Bradford's reagent was added to 1 ml of the dilutions and allowed to react under room temperature for 5 min. The absorbance readings were taken thereafter at 595 nm.

One unit of protease activity was reported as:

(Protease released in Milligrams)/(Incubation time in min) x Enzyme in reaction mixture (ml)

Specific activity of protease (U/mg protein) was calculated by dividing the activity of protease in the respective plant samples by the amount of protein in their corresponding plant samples. The principle involved here is based on the application of casein as substrate, the test enzyme (protease) digests the casein, and brings about the liberation of the amino acid tryptophan alongside other amino acids and peptide fragments.

Determination of lipase activity

Lipase activity was determined in accordance with the methodology of Yong and Wood[40] using glycerol tributyrate as substrate. The fine substrate emulsion was prepared by mixing together with an electric blender the following; 20 ml glycerol Tributyrate, 0.08 g Sodium Taurocholate, 0.44 g Calcium Chloride in 20 ml of water, 2 g Gum Acacia (for emulsion stabilization), and 120 ml (0.1 M Acetate Buffer pH 5.0). Aliquot (0.5 ml) of the enzyme solution was added to 1 ml of the substrate emulsion solution, and incubated at a temperature of 40 °C for 1 hr. The reaction halted, following the addition of 8 ml absolute

ethanol. The resultant mix was then titrated with 0.02 N NaOH, using phenolphtalein (0.1 g/50 ml absolute ethanol + water (5 ml)) as indicator. To prepare the blanks, 8 ml of absolute ethanol was added prior to addition of the enzyme solution. Differences between the titre figures of the blank and reaction mixtures was an indication of the alkali quantity needed for neutralization of the free butyric acid. The Lipase activity was reported using the formula shown below:

Lipase activity (U/min) = $(V_N - V_0)$ x N NaOH x 1000 / V_E x t
Where: V_N = NaOH used for titration of sample (ml)
V_0 = NaOH used for titration of blank (ml)
V_E = Volume of enzyme (ml)
T = Incubation time expressed in min
N NaOH = NaOH Normality
Specific activity of lipase (U/mg protein) was expressed by the division of lipase activity in the respective plant samples by the amount of protein in their corresponding plant samples. This method revolves round the titrimetric quantification of the liberated fatty acids from triacylglycerols through lipase catalyzed hydrolysis. It involves the incubation of sample, and alkaline titration of free fatty acids at the end-point.

Statistical analysis
One way analysis of variance (ANOVA), combined with Tukey's test for comparing multiple means ($P < 0.05$) was employed for the statistical analyses of all the results shown in this study. The results are reported as mean ± standard error of the mean (SEM) of readings done in triplicates. The statistical analysis software used was GraphPad prism 8.0.2 (GraphPad Inc., San Diego California USA).

Results and Discussion

The results obtained from the qualitative phytochemical determination indicated the presence of tannin, flavonoid, alkaloid, and phenol in all the extracts, namely the unfermented extract of *C. albidium* (UFCA), the fermented extract of *C. albidium* (FCA), the unfermented extract of *T. catappa* (UFTC) and fermented extract of *T. catappa* (FTC). The comparative phytochemical values between the extracts of each fermented and unfermented seed of *C. albidium* and *T. catappa* (Table i), revealed that the reduction in the tannin, alkaloid, oxalate, and phytate levels in UFCA, FCA, UFTC, and FTC was significant at ($P < 0.05$). The reduced values of flavonoid and phenol were also significant ($P < 0.05$) in UFTC and FTC and insignificant at ($P > 0.05$) in UFCA and FCA. The phytochemicals: flavonoid, phenol, tannin, alkaloid, oxalate, and phytate in both the unfermented and the fermented seeds (Table 1) have been known to possess antinutritional activities, hence the need for a suitable method like fermentation for their reduction before consumption. These general reductions in the antinutrient levels expressed in both the FCA and FTC agree with previous findings on soybean and sorghum. [4,6,41] This also agrees with a similar report in which fermentation was used to reduce the levels of antinutrients present in several legume seeds.[42] The general reductions in the antinutrients levels could be a result of the production of degrading enzymes such as tannase, oxalate oxidase, phytase, etc. released by fermenting microorganisms during the process of fermentation.[43] The results from the 2,2-diphenyl-1-picrylhydrazyl scavenging activity of extracts are presented in Figure 1. Ascorbic acid (AA), UFCA, FCA, UFTC, and FTC showed increasing percentage DPPH scavenging ability in a manner dependent on concentration, with AA exhibiting the highest scavenging activity. The free radical scavenging activity of the extracts were not as high as that of ascorbic acid (Figure 1). The free radical scavenging activities of both fermented extracts were also lower than those of their unfermented counterparts. The low free radical scavenging activities could thus imply that these seeds possess low phenolic and flavonoid contents (Table 1). This agrees with the discovery of Boonsong *et al*.[44] which stated that "the free radical scavenging activity of extracts from mushroom is dependent on the concentration of phenolics and flavonoids in the extracts." Flavonoids and phenolics have the hydrogen-donating capacity to scavenge DPPH radicals, thus the reason for their direct relationship. Water-soluble polyphenols such as oligomers of flavan-3-ols and flavan-3, 4-diols found in foods and beverages have been reported to show antioxidant properties.[45,46] Their antinutritional properties impair the digestion of nutrients,[46] hence, there is need to process foods to strike a safe balance between the antioxidant and antinutrient properties of the phenolic constituents. The reduction in the phenolic contents of the fermented extracts agrees with the reduction in the phenolic contents of some corn genotypes after their subjection to fermentation.[47] The comparative values of the total antioxidant capacity (TAC) and ferric reducing assay property (FRAP) in UFCA; FCA and UFTC; FTC (Table 2), showed a remarkable elevation for FRAP in FCA, and a significant ($P < 0.05$) reduction in FTC. Significant reduction ($P < 0.05$) in TAC was found in only FTC during the fermentation.

Table 1: Quantitative phytochemical analysis of extracts from the *C. albidium* and *T. catappa* seeds

Phytochemical	UFCA	FCA	UFTC	FTC
Phenol mg(GAE)/g	3.664 ± 0.027^a	3.452 ± 0.000^b	3.214 ± 0.046^c	3.003 ± 0.026^d
Flavonoid mg(RE)/g	1.162 ± 0.016^a	1.139 ± 0.008^a	0.756 ± 0.004^b	0.696 ± 0.010^c
Tannin mg(TAE)/g	0.139 ± 0.001^a	0.136 ± 0.003^a	0.103 ± 0.002^b	0.087 ± 0.002^c
Alkaloid (%)	21.85 ± 0.058^a	18.01 ± 0.003^b	24.51 ± 0.001^c	20.61 ± 0.058^d
Oxalate mg(OAE)/g	5.020 ± 0.005^a	2.708 ± 0.002^b	5.447 ± 0.003^c	3.903 ± 0.004^d
Phytate (mg/g)	9.405 ± 0.025^a	4.455 ± 0.015^b	5.445 ± 0.015^a	1.485 ± 0.050^d

Values are expressed as mean ± Standard error of the means (n = 3). Superscripts with different alphabets shows significantly different ($P < 0.05$) values. UFCA: Unfermented *C. albidium*, FCA: Fermented *C. albidium*, UFTC: Unfermented *T. catappa*, FTC: Fermented *T. catapp*, GAE: Gallic acid equivalent, RE: Rutin equivalent, TAE: Tannic acid equivalent, OAE: Oxalic acid equivalent.

Table 2: Ferric reducing assay property (FRAP) and total antioxidant capacity (TAC) of extracts from the *C. albidum* and *T. catappa* seeds

Antioxidant analysis	UFCA	FCA	UFTC	FTC
Frap mg(AAE)/g	0.009 ± 0.001^a	0.017 ± 0.001^b	0.013 ± 0.001^c	0.004 ± 0.000^d
TAC mg(AAE)/g	0.029 ± 0.000^a	0.020 ± 0.005^a	0.156 ± 0.005^c	0.100 ± 0.000^d

Values are expressed as mean ± Standard error of the means (n = 3). Superscripts with different alphabets shows significantly different ($P < 0.05$) values. UFCA: Unfermented *C. albidium*, FCA: Fermented *C. albidium*, UFTC: Unfermented *T. catappa*, FTC: Fermented *T. catappa*

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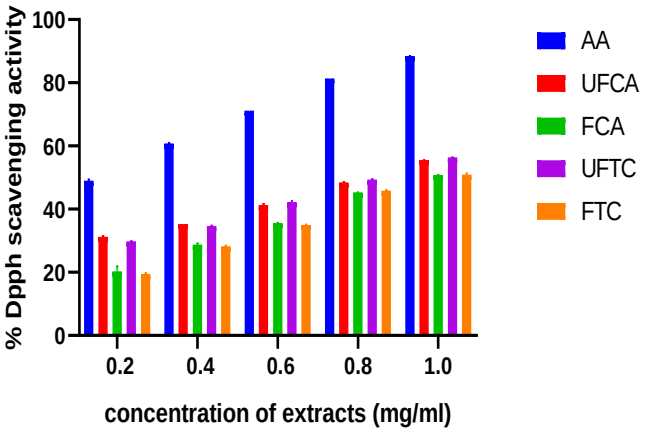

Figure 1: DPPH scavenging activities of extracts from the UFCA, FCA, UFTC, and FTC seeds
AA, UFCA, FCA, UFTC, and FTC showed increasing percentage DPPH scavenging ability in a concentration-dependent manner, with AA exhibiting the highest scavenging activity. AA: Ascorbic acid, UFCA: Unfermented *C. albidium*, FCA: Fermented *C. albidium*, UFTC: Unfermented *T. catappa*, FTC: Fermented *T. catappa.* AA(IC_{50}) = 0.195 ± 0.018[a] mg/ml, UFCA(IC_{50}) = 0.850 ± 0.065[b] mg/ml, FCA(IC_{50}) = 0.960 ± 0.015[c] mg/ml, UFTC(IC_{50}) = 0.824 ± 0.070[d] mg/ml, FTC(IC_{50}) = 0.951 ± 0.018[e] mg/ml. IC_{50} values indicated are the concentration of the samples that scavenged 50 % of the DPPH radical.). Superscripts with different alphabets shows significantly different (P < 0.05) values.

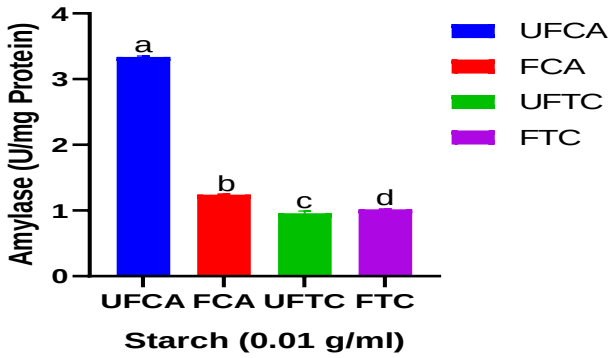

Figure 2: Alpha-amylase activities expressed by UFCA, FCA, UFTC, and FTC seeds
Values are expressed as mean ± Standard error of the means (n = 3). Superscripts with different alphabets shows significantly different (P < 0.05) values. UFCA: Unfermented *C. albidium*, FCA: Fermented *C. albidium*, UFTC: Unfermented *T. catappa*, FTC: Fermented *T. catappa*

Table 3: pH and titratable acidity of the unfermented and fermented C. *albidum* and *T. catappa* seeds

Treatment	Ph	Titratable acidity (0.1 g/ml)
UFCA	7.3	0.012 ± 0.002[a]
FCA	8.3	0.008 ± 0.001[b]
UFTC	7.8 [24]	0.006 ± 0.001[c]
FTC	7.5 [24]	0.005 ± 0.001[c]

Values are expressed as mean ± Standard error of the means (n = 3). Superscripts with different alphabets shows significantly different (P < 0.05) values. UFCA: Unfermented *C. albidium*, FCA: Fermented *C. albidium*, UFTC: Unfermented *T. catappa*, FTC: Fermented *T. catappa*
In the FRAP and TAC determination of the extracts (Table 2), FRAP value was slightly elevated in the fermented *C. albidum* seed extract. This may have resulted from the activities of molecules with antioxidant properties that were produced during the process of fermentation. This suggests that *C. albidum* seed might possess useful compounds that could have been catabolized to synthesise these compounds.[48] The reduction in FRAP and TAC observed in the fermented *T. catappa* seeds extract further suggests the correlation between the flavonoids and phenols contents of the extract and the antioxidant status as earlier discussed. This also agrees with what has been earlier reported of *Nauclea latifolia* leaf decoction by Iheagwam *et al*.[49] In their study, the DPPH radical-scavenging, ferric reduction and total antioxidant capacity of the extract was in strong correlation with the total phenolic content. The insignificant reduction observed in the TAC of the fermented *C. albidum* seed extract may have possibly occurred from the metal ion chelating capability and reactive oxygen species scavenging capability of fermenting microorganisms[50] involved in the fermentation, thus replenishing the reduction from the antioxidant capacity. This reduction of the antioxidant capacity may have occurred from the reduction of the flavonoid and phenolic content of the extract.
The pH and titratable acidity of the *C. albidum* and *T. catappa* seeds prior to and at the conclusion of fermentation are presented in Table 3. An elevation in the pH values, and a reduction in the titratable acidity values of the *C. albidum* seeds were detected at the end of fermentation. For *T. catappa* seeds, a reduction was seen for both pH and titratable acidity after fermentation. As presented in Table iii, the rise in the pH of the fermenting medium of the *C. albidum* seeds from 7.3 to 8.3 following fermentation is presumed to be a result of proteolysis and the release of ammonia from the utilized amino acids. [51] This could also depict that the fermentation of *C. albidum* seeds is an alkaline fermentation. This release of ammonia has been reported by several studies to be responsible for the ammoniacal odour characteristic of most vegetable protein fermentation.[52,53,54] On the other hand, a decrease from a pH of 7.8 to 7.5 was observed after the fermentation of the *T. catappa* seeds. Majority of bacteria are favored by reactions that are near neutrality while few are favored by alkaline reaction.[55] Acidic pH inhibits most microorganisms and is a major determinant of the types of microorganisms that grow in a fermenting medium.[55] The pH range of 7.8 to 7.5 observed in *T. catappa* seeds is an indication that the fermentation of this seed occurred at reactions near neutrality. In the case of the titratable acidity with both seeds, a decrease was observed at the end of fermentation in *T. catappa* seeds, while the observed decrease for *C. albidum* seeds was significant, the decrease observed for the *T. catappa* seeds was insignificant; this could be a correlation with their close pH values (Table 3). The decrease in titratable acidity during fermentation is common to protein foods, and similar cases have been reported.[36,55,56,57]. The activities of the digestive enzymes; alpha-amylase, protease, and lipase expressed by the unfermented and fermented of *C. albidium* and *T. catappa* seeds are compared and presented in Figures 2 - 5. UFCA significantly (P < 0.05) had the highest α-amylase activity; 3.338 ± 0.007 U/mg protein, followed by FCA; 1.241 ± 0.006 U/mg protein, FTC; 1.018 ± 0.002 U/mg protein, and UFTC; 0.959 ± 0.019 U/mg protein. The protease activity (0.909 ± 0.031 U/mg protein) in FCA was significantly higher (P < 0.05) than that of UFCA (0.341 ± 0.001 U/mg protein) while there was no significant difference between the protease activities in UFTC (0.436 ± 0.006 U/mg protein) and FTC (0.384 ± 0.002 U/mg protein). Lipase activity in FCA was significantly the highest (P < 0.05) with a value of 4.363 ± 0.128 U/mg protein, followed by FTC; 2.860 ± 0.229 U/mg protein. The lipase activities from FCA and FTC were significantly higher (P < 0.05) than that from UFCA and UFTC respectively. A slightly significant increase was observed for the α-amylase activity of the fermented *T. catappa* seeds, on the other hand, the α-amylase activity in the unfermented *C. albidum* seeds was higher than that of the fermented *C. albidum* seeds and the significantly highest among the four extracts (Figure 2). In addition, the high amylase activity is an indication that *C. albidum* seed in its raw form possesses endogenous α-amylase. The drop in the α-amylase activity of the fermented *C. albidum* seeds was rather surprising, as different studies have recorded increased enzyme activities with fermentation. As fermenting microorganisms break down the carbon-containing compounds in the substrates, these enzymes are secreted.[58] However, the pH value of 8.3 from the fermented *C. albidum* seeds that was obtained at the conclusion

of the fermentation could be a possibility for the decrease in the α-amylase activity extracted from the fermented *C. albidum* seeds, and also, considering the optimum pH for α-amylase to be 7, this is an indication that the extraction of α-amylase from *C. albidum* seeds may not be favored by alkaline fermentation. The activity of protease expressed by the fermented *C. albidum* seeds was remarkably higher than that extracted from its unfermented counterpart. This therefore implies secretion of protease by fermenting microorganisms during the period of fermentation. The protease activities in both the fermented and unfermented *T. catappa* seeds were not significantly different from each other. The near neutral pH values of 7.5 and 7.8 in the fermented and unfermented seeds respectively, and the insignificant difference in the titratable acidity values of both could be responsible for this non-significant change in the protease activities of both seeds, and this could imply that they are directly related to the protease activity in the seed. The activities of lipase expressed by the fermented seeds were far higher than that of their unfermented counterparts. Considering both seeds to be oil-rich seeds, the very low lipase activities in the raw seeds were rather surprising but not unique to them as similar findings have been reported for the fermentation of some other oil-rich seeds such as soy-bean, telfairia, and melon seeds. [36,59,60].The increase in the activities of lipase observed after fermentation may therefore indicate that fermenting microorganisms involved in the fermentation process possessed good lipase secreting ability. Both seeds however showed promissory prospects for obtaining lipase industrially, through fermentation enhanced biotransformation. In our previous study, the elimination of the aldehyde compounds from *C. albidum* seeds, and production of compounds of interest revealed from the Gas Chromatography/Mass Spectrometry (GC/MS) analyses of both seeds after fermentation, [24,61] gives credibility to fermentation, as a tool to process affordable and alternative sources of food in the tackling malnutrition irrespective of population and purchase power.

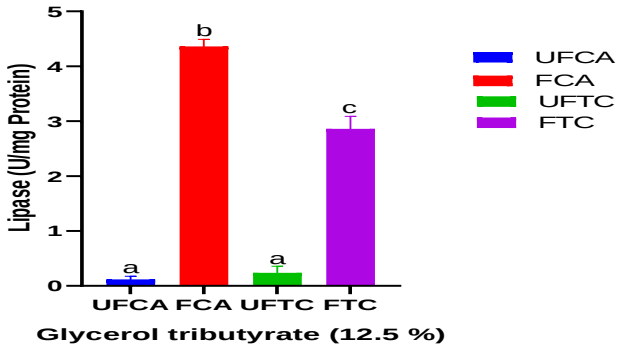

Figure 3: Lipase activities expressed by UFCA, FCA, UFTC, and FTC seeds

Values are expressed as mean ± Standard error of the means (n = 3). Superscripts with different alphabets shows significantly different (P < 0.05) values. UFCA: Unfermented *C. albidium*, FCA: Fermented *C. albidium*, UFTC: Unfermented *T. catappa*, FTC: Fermented *T. catappa*

Conclusion

This study revealed the potential use of fermentation to refine the seeds of *C. albidum* and *T. catappa*. The distinct reduction in antinutrient status of the seeds, coupled with the increase in activities of digestive enzymes, manifested during this fermentation. The benefits of fermentation in the improvement of edibility and health outcome of alternative sources of foods as seen in this study should not be underrated. For subsequent studies, we recommend the application of High-Performance Liquid Chromatography (HPLC) and Oxygen Radical Absorbance Capacity (ORAC) to further ascertain the phytochemical and antioxidant properties of the seeds.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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