Antioxidants and antigenotoxic properties of Rhizopus oligosporus fermented cassava (Manihot esculenta Crantz)

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Research Article

Keywords: Cassava, fermentation, Rhizopus oligosporus, Antioxidants, Antigenotoxicity

Posted Date: April 4th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1513723/v1

Abstract

The antioxidant and antigenotoxic effects of solid state fermented cassava (Manihot esculenta Crantz) using Rhizopus oligosporus were investigated. Solid state fermentation was carried out for 72 hours at room temperature under acidic and basic conditions. Total phenolic content were significantly ($p<0.05$) higher for peeled and unpeeled cassava when compared to fermented forms at pH 7 and 5, respectively. Similarly, the total flavonoid contents of peeled and unpeeled cassava at pH 4 and 7 were significantly ($p<0.05$) higher than in fermented cassava. The Allium cepa assay was used to assess the antigenotoxic effects of unfermented and fermented peeled and unpeeled cassava. The fermented extracts did not induce chromosomal type aberrations in the treated cells. The present study thus showed that R. oligosporus has the ability to breakdown cassava and considerably increase the antioxidant properties in fermented cassava peeled and unpeeled, which may serve as natural antioxidants in industrial broiler chickens.

1. Introduction

Recently, the poultry industry has made immense efforts in obtaining alternative energy sources for its livestock production (Morgan & Choct 2016; Abomohra et al., 2020; Egbune et al., 2021b). Maize has been the main energy source used in poultry feeds due to its palatability, high-energy value and the presence of pigments and essential fatty acids. However, due to the demand for maize for biofuel production, food for humans among others, maize price has risen dramatically in recent years. The increases in the cost of traditional raw materials used for poultry feeds have driven the quest for alternative feed resources at a reduced production cost. According to Tonukari *et al*. (2015), cassava is a carbohydrate-rich staple crop, with potential to totally replace maize as an energy source in poultry diets.

Cassava (*Manihot esculenta* Crantz) is a popular tuber crop in Nigeria, with an estimated annual yield of 45 million tonnes (Ehebha & Eguaoje 2018). Tonukari *et al*. (2015) reported that cassava-based animal feed has the potential to enhance feed production while cutting feed costs in both commercial and subsistence agricultural settings. However, research has shown that cassava's widespread usage as a primary feed component in animal feeding regimens is limited due to the presence of deadly cyanogenic compounds as well as high fibre and ash levels found in particular fractions and cultivars (Asaoluet al., 2012; Lukuyuet al., 2014). Thus, to optimize its use in chicken feed formulations, cassava's nutritional value must be increased through appropriate processing techniques.

Solid state fermentation (SSF) is a microbiological approach used to improve the nutritional value of animal feed (Okhonlaye & Foluke 2016). Studies have shown that fermentation improves micronutrient bioavailability and aids in the breakdown of antinutritional compounds (Morgan & Choct 2016). It has also been employed to give functional properties that may be beneficial to broiler chickens (Sugiharto & Ranjitkar 2018).

Synthetic antioxidants are commonly utilized as feed additives in an effort to improve chicken feed quality, particularly during heat stress and during vaccination (Sugiharto, 2019; Gouda et al., 2020). Antioxidants can shield the body from the negative effects of oxidative stress. Excessive usage of synthetic antioxidants, on the other hand, may be carcinogenic and/or mutagenic to consumers (Fellenber & Speisky 2006). As a result, nutritionists are looking for natural antioxidants in industrial broiler chickens.

Antioxidants are prevalent in the filamentous fungus Grifola frondosa, Monascus purpureus, Pleurotus spp., Lentinula edodes, and Trametes versicolor, according to Smith *et al*. (2015). Similarly, Sugiharto *et al*. (2016) found *Acremonium charticola*, *Rhizopus oryzae*, and *Chaetomorpha crassa* as promising natural antioxidant sources. Other filamentous fungi with antioxidant capacities include *Aspergillus* PR78 and PR66 (Arora & Chandra 2010), *Chaetomium sp., Cladosporium sp., Torula sp., Phoma sp.* (Huang et al., 2007), and *Mycelia sterilia* (Moon et al., 2006; Huang et al., 2007).

Rhizopus oligosporus is a filamentous fungi that has been employed less often (Egbune et al., 2022). It generates no harmful substances, is easy to culture, and lacks pathogenic potential (Sugiharto, 2019; Aganbiet al., 2020). It grows quickly at 34-45°C and is used to produce food and feed. The current work intends to assess the antioxidant and antigenotoxic properties of solid-state fermented cassava (*Manihot esculenta* Crantz) utilizing the fungus *R. oligosporus*.

2. Materials And Methods

2.1 Plant material and Starter Organism

Cassava roots, both peeled and unpeeled, were collected from Sapele, Songhai Delta Amukpe, Nigeria. Skins were removed from tubers after thorough washing to achieve tubers without peels. Peeled and unpeeled tubers were cut into bits separately and dried to a uniform weight. The dried materials were milled and kept (37 °C) until additional assays were performed. The strains of *R. oligosporus* (produced by Aneka Fermentasi Industri. PT - Ragi dalam BANDUNG) were obtained from the Harmony Path Limited, Sapele Delta State. Solid state fermentation was carried out in petri plates for 72 hours at room temperature under acidic and basic conditions (pH 3 - 9) using 50 mM phosphate and citrate buffers.

2.2 Substrate Preparation for Solid State Fermentation

One gram (1 g) of *R. oligosporus* (1.4 x 10^2 CFU) (The fungus's colony forming unit (CFU) per gram was calculated using the method published by Ofuya and Nwajiuba (1990), was homogenized in 10 ml of prepared citrate and phosphate buffers ranging from pH 3 - 9 in seven different petri dishes which were labeled according to the corresponding pH. In the homogenization phase, 10 g of the ground peeled and unpeeled cassava roots were utilized; the they were allowed to ferment for 72 hours at 25 ^oC. An unfermented control (containing dried and grounded peeled and unpeeled cassava, devoid of any presence of molds; with buffer only, and without any cells) was prepared alongside the test samples. Following fermentation, 6 g of the mixture was removed from each of the petri dishes at the various pH levels; 40 ml of distilled water was added prior to homogenization using mortar and pestle; and 10 ml of homogenate was centrifuged at 3500 rpm for 10 minutes to get supernatant. The supernatant served as the crude extract or sample for the different tests, which were performed in triplicate.

2.3 Determination of antioxidant activities inhibition of 2,2-diphenyl-1- (DPPH) radical of peeled and unpeeled cassava tubers

The antioxidant activities of peeled and unpeeled cassava tubers were evaluated using the DPPH Assay. This was computed using the method provided by Hatano *et al.* (1988). A 2.8 ml methanolic solution of DPPH radical (6 × 10−5 mol/l) was put to 0.3 ml of extract. To get consistent absorption readings, the mixture was rapidly agitated and placed in the dim for 60 minutes. The absorbance at 517 nm was utilized to calculate the DPPH radical's reduction. Ascorbic acid was utilized as a control. The radical scavenging activity was calculated by the formula:

%RSA = ((ADPPH – AS)/ ADPPH) x 100.

Where %RSA = % DPPH discolouration; A absorbance of DPPH solution, and AS absorbance of the solution after a certain amount of sample was added

2.4 Determination of Total Phenol Content

This was done in line with the protocol outlined by Singleton and Rossi (1965). One milliliter of Folin C reagent was added to one milliliter of material. After 3 minutes, 1 ml of saturated Na2CO3 solution was added, followed by 10 ml of distilled water. For 90 minutes, the reaction mixture was maintained in the dark. At 725 nm, the absorbance was measured. Catechin was used as standard.

2.5 Determination of Total Flavonoid Contents

Colorimetric determination of total flavonoid contentswas done using the method of Jia *et al.* (1999). 250 µl of extract was combined with 1.25 ml of distilled water and 75 µl of 5% NaNO2. After 5 minutes, 150 l of 10% AlCl3.H2O, 500 µl of 1 M NaOH, and 275 µl of distilled water were added. The solution was thoroughly mixed, and the mixture's color intensity was measured at 510 nm. Catechin served as the standard.

2.6 Determination of Antigenotoxic activities

2.6.1 Preparation of Extracts

400g of fermented peeled and unpeeled cassava flour was measured at room temperature and placed in a basin with 120cl of valve water. This was left to saturate for 5 minutes before being agitated and the extract pressed and sieved through a cheese cloth. The leftover particles were thrown away.

2.6.2 *Allium cepa* Assay

In Sapele, Delta State, Nigeria, onion bulbs (*Allium cepa* L., 2n=16) of average size (15-22 mm diameter) were purchased locally. After six weeks of sun drying, to uncover the nascent meristematic tissues, with a fine razor blade, the dry roots at the base of the onion bulbs were meticulously scraped out. To keep the primodial cells from dehydrating, the bulbs were immersed in newly produced purified water. Taking into consideration the quantity of bulbs in the population are essentially slow or poor growers, each test sample and control received seven duplicate bulbs, and the best five bulbs were examined concurrently (Rank & Nielsen1993). Blotting paper was used to dry the bulbs.

To determine root growth inhibition, newly obtained stock extracts were watered into five concentrations of 20, 10, 5, 2.5, and 1%. For each concentration of each extract and the control, seven onion bulbs were used (tap water). For 72 hours in the dark, the bases of each bulb were hung on the extracts in 100mL beakers. The test extracts were refreshed on a regular basis. After the exposure time, the roots of the five onion bulbs that grew the quickest at each concentration were detached with forceps and their lengths (in cm) were measured using a metre rule. The percentage root growth inhibition in comparison to the negative control and the EC50 (effective concentration at which root growth equals 50% of the controls) for each extract were computed using weighted averages for each concentration and the control (Fiskesjo, 1985). The effect of each sample on the morphology of growing roots was also studied.

To test chromosomal aberration induction, 5 onion bulbs were suspended for 48 hours on 10, 5, 2.5, and 1 percent (v/v) concentrations of each extract and the control. After 48 hours, the root tips of these bulbs were stored in a solution of ethanol:glacial acetic acid (3:1, v/v). These were hydrolyzed in 1N HCl for five minutes at 60^{o}C before being rinsed in distilled water. After pressing two root tips onto each slide for 10 minutes, they were coloured with acetocarmine and cover slips were carefully attached to exclude air bubbles. Grant (1982) suggested using clear fingernail paint to seal the cover slips to the slides. This is done to keep the preparation from drying out as a result of the heat generated by the microscope (Sharma, 1983).

Six slides were created for each concentration and the control, with five (at 1000 cells per slide) examined for chromosomal aberration induction at 1000 magnification. The mitotic index was calculated by dividing the total number of cells by the number of cells identified per 1000. (Fiskesjo, 1985; Fiskesjo, 1997) The proportion of abnormal cells was calculated by dividing the total number of cells examined at each concentration of each extract by the number of abnormal cells (Bakare et al., 2000).

2.7 Statistical Analysis

All statistical analyses were performed using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). Values were reported as Mean ± Standard deviation and the experimental results were analyzed using analysis of variance (ANOVA) and also a Fischer test of least significance (LSD) was carried out to compare the various group means. The results were considered significant at p-values of less than 0.05, that is, at 95% confidence level ($p< 0.05$).

3. Results

Figure 1 depicts the capacity of 2,2-diphenyl-1-picrylhydrazyl to scavenge free radicals (DPPH) in peeled and unpeeled cassava tubers. The findings indicated a substantial ($p<0.05$) increase in DPPH free radical-scavenging capacity from (17.81±1.38) and (44.40±5.27) (%) in peeled and unpeeled cassava to (24.40±0.46) (%) in fermented peeled at pH 8 and (56.91±6.72) (%) in fermented unpeeled at pH 6.

Figure 2 depicts the phenolic content of fermented peeled and unpeeled cassava. The phenol content of fermented peeled and unpeeled cassava increased significantly ($p<0.05$) as compared to the experimental control. The total phenolic content increased from (20.4±0.6) and (20.4±1.1) (µg/ml) in peeled and unpeeled cassava to (72.4±1.5) (µg/ml) in fermented peeled cassava at pH 7 and (48.0±1.1) (µg/ml) in fermented unpeeled cassava at pH 5. A rise in phenolic content was also seen at different pH levels.

The total flavonoid level of fermented peeled and unpeeled cassava is shown in Figure 3. Total flavonoid concentration increased significantly ($p<0.05$) from (7.8±0.9) and (9.8±0.3) (µg/ml) in peeled and unpeeled cassava to (25.7±1.4) (µg/ml) in fermented peeled at pH 6 and (26.2±0.7) (µg/ml) in fermented unpeeled at pH 7. At various pH levels, fermentation resulted in a rise in total flavonoid.

The results of the influence of unfermented and fermented peeled and unpeeled cassava extracts on the growth of *A. cepa* are shown in Table 1. The highest root outshoot was obtained in the control, which was free of any morphological abnormalities. They were stable, uncomplicated, and white in hue. Root development was greatest at the 1% (percent) absorption of the unpeeled fermented cassava extract (3.75±0.21). Alternatively, with 10% unfermented peeled cassava extract (0.2±0.03), the average root size was the lowest (0.2±0.03). Obstruction of root development was concentration dependant and mathematically significant ($P <0.05$) at verified levels, with no progress at any extracts at a concentration of 20%. The EC50 values for unfermented, fermented, peeled, and unpeeled cassava extracts were (2.50, 3.10, 3.50, 4.0) percent (percent).

Table 2. displays the results of the microscopic inspection. There was no chromosomal abnormality in the fermented extracts, and the mitotic index (MI) in the control was 40% (percent). There was an absorption-dependent decrease in all concentrations of the unfermented extracts compared to the fermented extracts when equated to the control mitotic index (MI) of 40. The lowest MI of 9 was found for 10% unfermented peeled cassava extract. Unfermented extracts completely produced disorder of the mitotic spindle abnormalities, which were significant ($p <0.05$) as compared to fermented extracts.

4. Discussion

Studies have shown that antioxidants are important in diets for a variety of reasons, including extending food shelf life, promoting good health, and preventing sickness in consumers, notably animals and humans (Atta et al., 2018). Polyphenolic substances have been associated to antioxidant, anti-cancer, and antibacterial activities (Sumazian et al., 2010; Tonukari et al., 2016). This necessitated the assessment of the DPPH free radical-scavenging capacity, total phenolic content, and total flavonoid content inherent in fermented peeled and unpeeled cassava, This may give insight into the antioxidant capacity of the anticipated livestock feed composition in comparison to the unfermented version.

The findings of this investigation revealed that fermented peeled and unpeeled cassava had a substantial increase in DPPH free radical-scavenging capability, total phenolic content and total flavonoid content when compared to the unfermented sample. The findings are

consistent with previous reports, implying that solid-state fermentation (SSF) is an exciting technique for increasing the antioxidant potentials of fermented peeled and upeeled cassava due to their high polyphenolic content, which is necessary for the sequestration of reactive oxygen species (ROS) produced by the lipid peroxidation process (Nuri et al., 2010; Chiunghui et al., 2010; Francilene et al., 2011; Egbune et al., 2021a). This shows that the inherent polyphenolic chemicals in pulverized fermented peeled and unpeeled cassava in the presence of R. oligosporus strain may function as proton donors to ROS, terminating the lipid peroxidation process through the creation of more stable and less reactive molecules.

Table 1 displays the antigenotoxic activity of solid state fermented peeled and unpeeled cassava. This shows the effects of unfermented and fermented peeled and unpeeled cassava extracts on the growth of *A. cepa*. The highest root outshoot was obtained in the control, which was free of any morphological abnormalities. They were stable, uncomplicated, and white in hue. Root development was greatest at the 1% (percent) absorption of the unpeeled fermented cassava extract (3.75±0.21). Alternatively, with 10% unfermented peeled cassava extract (0.2±0.03), the average root size was the lowest (0.2±0.03). Obstruction of root development was concentration dependant and mathematically significant ($P<0.05$) at verified levels, with no progress at any extracts at a concentration of 20%. The EC50 values for unfermented, fermented, peeled, and unpeeled cassava extracts were (2.50, 3.10, 3.50, 4.0) percent (percent). The antigenotoxic effects of fermented peeled and unpeeled cassava extracts on *A. cepa* were investigated in this study. Based on the EC50 values of fermented peeled and unpeeled cassava extracts, the effect of fermented peeled and unpeeled cassava extracts on *A. cepa* root progress revealed that there was a concentration drop in root progress inhibition. These data suggest that the trials were lethal; unpeeled cassava extracts had the most inhibitory and mitodepressive effects of any extract tested. This might be due to a cynogenic component found in garri and all poorly fermented cassava meals (Coursey, 1973). When there is root progress reluctance, the number of separate cells in A. cepa is always reduced (Fiskesjo, 1997; Bakare & Wale-Adeyemo2004). The presence of heavy metals in the extracts might be to blame for the suppression of root development in *A. cepa*.

Table 2 displays the results of the microscopic inspection. There was no chromosomal abnormality in the fermented extracts, and the mitotic index (MI) in the control was 40% (percent). There was an absorption-dependent decrease in all concentrations of the unfermented extracts compared to the fermented extracts when related to the control. The lowest MI of 9 was found for 10% unfermented peeled cassava extract. Unfermented extracts completely produced disorder of the mitotic spindle abnormalities, which were significant ($p<0.05$) as compared to fermented extracts. Chromosomal abnormalities are changes in chromosome configuration caused by a disruption or exchange of chromosome structure. Among the oddities identified in the unfermented cassava extract were the beginnings of gluey chromosomes, bridges, and the rupture of spindle fibers in onion root cells at various stages of mitotic division. When chromosomal abnormalities occurred in *A. cepa*, certain developmental limitations were virtually invariably present (Fiskesjo, 1997). The majorities of these anomalies are harmful and can cause genetic defects, whether somatic or inherited (Swierenga et al., 1991).

Conclusion

Cassava might theoretically be substituted quantitatively for maize in chicken meals up to 50% without affecting bird performance; however, it must first be processed using processes such as drying, boiling, and fermentation. Solid state-fermentation has become a popular method in broiler industry to improve the nutritional contents of feed. Solid state fermentation has also been used to provide functional qualities that may benefit broiler chickens. The presence of filamentous fungus in the solid state fermentation process improves the health and growth performance of broilers. Filamentous fungus has the potential to operate as fermentation starters, antioxidant sources, and enzyme makers. Results obtained show that *R. oligosporus* has the ability to breakdown cassava and efficiently elevate antioxidant properties in peeled and unpeeled fermented cassava. The results also indicate that the fermented unpeeled extracts tested exhibited inhibitory, mitodepressive, and turbagenic effects on A. cepa root formation, cell division, and chromosomal behavior.

Declarations

Author Contribution

Egoamaka O. Egbune: Resource investigation, formal analysis and manuscript drafting. Theresa Ezedom: Resource investigation, formal analysis and manuscript drafting. Akpovwehwee A. Anigboro: Supervision, methodology, investigation and manuscript drafting. Eferhire Aganbi: Supervision, methodology, investigation and manuscript drafting. Alex-Ifo Amata: Conceptualization, supervision and editing. Nyerhovwo J. Tonukari: Conceptualization, methodology, supervision and editing.

Data Availability

The experimental data would be made available upon reasonable request.

Code Availability

Microsoft® Excel® 2010 program was used to analyze and graphically depict the experimental data gathered in this investigation.

Ethical Approval

This study received no ethical approval, however all experimental methods were carried out in accordance with international norms.

Consent to Participate

All authors showed absolute commitment to participate in this study from conceptualization through experimentation to final draft of manuscript.

Consent for Publication

The authors involved in this study are aware of this contribution and have given their agreement for it to be published.

Conflict of Interest

The authors declare no competing interests.

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Tables

Table 1. 0 Root growth of *A. cepa* full-grown in unfermented and fermented peeled and unpeeled cassava

Conc. (%)	Unfermented peeled cassava			Unfermented unpeeled cassava			Fermented peeled cassava			Fermented unpeeled cassava		
	Mean root length±S.D.	RG (%) of control	95% CL	Mean root length±S.D.	RG (%) of control	95% CL	Mean root length±S.D.	RG (%) of control	95% CL	Mean root length±S.D.	RG (%) of control	95% CL
0	4.9 ± 0.17	-	0.22	4.9 ± 0.17	-	0.26	4.9 ± 0.17	-	0.27	4.9 ± 0.17	-	0.13
1	1.5 ± 0.12	71.09*	0.38	2.0 ± 0.22	79.09*	0.35	3.75±0.21	88.7*	0.31	3.75±0.21	90.7*	0.51
2.5	0.5 ± 0.04	69.65*	0.34	0.8 ± 0.09	71.02*	0.44	2.13±0.17	85.1*	0.34	2.13±0.17	87.3*	0.24
5	0.3 ± 0.03	46.62*	0.42	0.4 ± 0.11	56.92*	0.51	2.04±0.14	42.9*	0.42	2.04±0.14	62.4*	0.21
10	0.2 ± 0.03	30.51*	0.58	0.3 ± 0.03	40.51*	0.51	1.56±0.08	30.6*	0.58	1.56±0.08	56.1*	0.41
20	There is no root development			There is no root development			There is no root development			There is no root development		
EC 50	2.50%			3.10%			3.50%			4.00%		

The root growth percentage (RG) of the control is expressed as a percentage of the control's root growth.

95 percent CL: Confidence limit of 95 percent.

* P<0.05, amount of influence of root progression blockage compared to control.

Table 2. The effects of unfermented and fermented peeled and unpeeled cassava extracts on *A. cepa* cells were studied cytologically

Conc. (%)	Unfermented peeled cassava			Unfermented unpeeled cassava			Fermented peeled cassava			Fermented unpeeled cassava		
	No. of dividing cells	Mitotic index	% of aberrant cells	No. of dividing cells	Mitotic index	% of aberrant cells	No. of dividing cells	Mitotic index	% of aberrant cells	No. of dividing cells	Mitotic index	% of aberrant cells
0	207	40	-	207	40	-	207	40	-	207	40	-
1	101	19	0.11	105	20	0.11	201	39	0.14	203	40	0.06
2.5	63	14	-	73	14	-	198	38	-	196	39	0.12
5	31	11	-	32	11	-	133	37	-	190	37	0.14
10	41	9	0.02	41	9	0.02	142	21	0.04	176	36	0.16
20	40	3	0.1	45	5	0.1	150	10	0.1	160	10	0.02

*5000 cells (5 slides) for each absorption of the control and each emission.

Figures

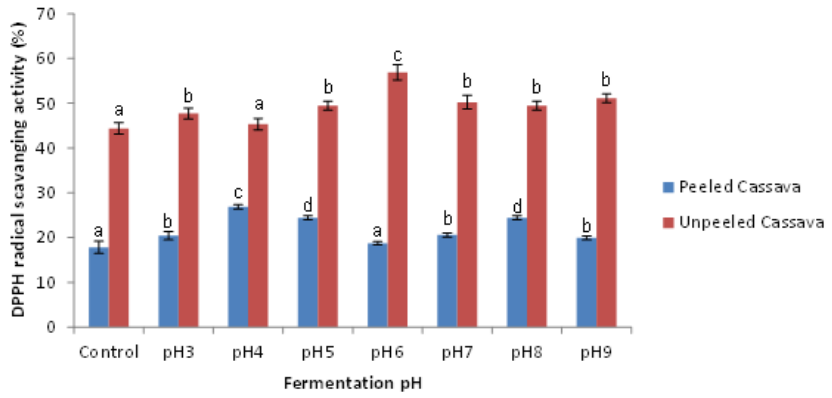

Figure 1

Antioxidant activities inhibition of 2,2-diphenyl-1-prycrilhydrazil (DPPH) radical of peeled and unpeeled cassava tubers. At p<0.05, the labeling of each bar with a distinct letter resulted in a significant difference.

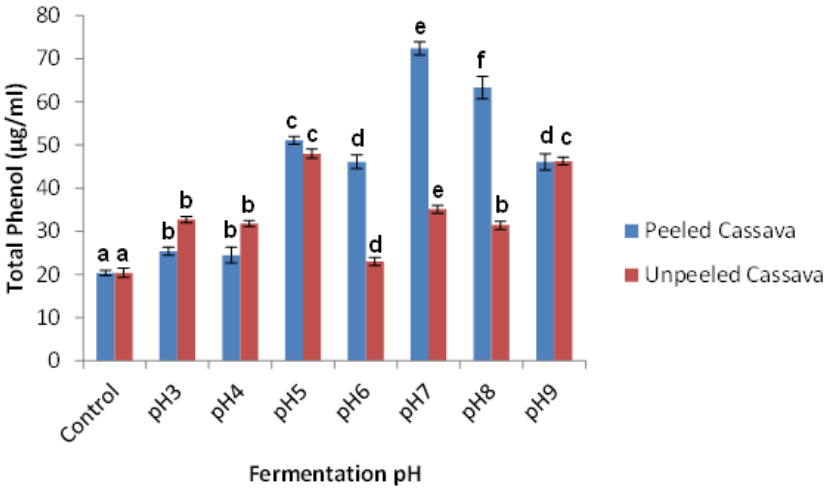

Figure 2

Total phenolic content in the *R. oligosporus* fermented peeled and unpeeled cassava tubers. At p<0.05, the labeling of each bar with a distinct letter resulted in a significant difference.

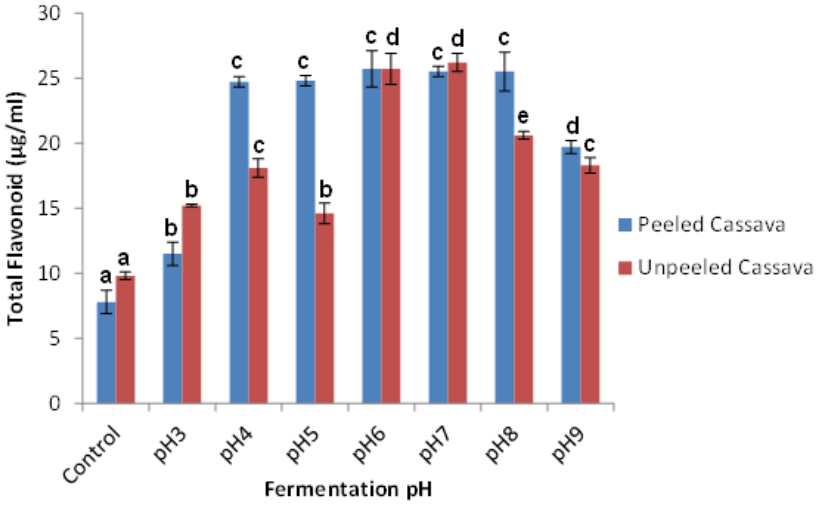

Figure 3

Total flavonoid content in the *R. oligosporus* fermented peeled and unpeeled cassava tubers. At p<0.05, the labeling of each bar with a distinct letter resulted in a significant difference.