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Investigation of the antioxidant properties of *Persea americana* seed flour altered by the fermentation process with *Lactobacillus plantarum*

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RESEARCH ARTICLE

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ABSTRACT

This study aims to investigate the effect of *Lactobacillus plantarum* fermentation on the antioxidant potential of *Persea americana* seed flour. The half-maximal inhibitory concentration (IC_{50}) value of avocado seed flour after fermentation for 24, 48, and 72 h was compared with the unfermented avocado seed flour using the measurement of free radical scavenging activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH). Additionally, the change in pH value was measured to confirm the continuous fermentation process within the desired period. The results for the DPPH assay for unfermented, 24, 48, and 72 h fermented avocado seed flour were 61.5±0.71, 51.18±0.10, 46.00±0.21, 43.73±0.21 µg/mL, respectively, which indicated a significant increase ($p < 0.05$) of IC_{50} values of avocado seed flour with the fermentation. Furthermore, with the fermentation period, there was a significant decrease in the pH value for the 72-hour fermented sample (4.15±0.03) compared to the unfermented sample (6.81±0.04). These results supported avocado seed as an important by-product source for the further development of health-promoting products, by confirming the increased antioxidant capacity of avocado seed flour after fermentation.

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1. Introduction

The food industries are providing a wider range of food products to consumers. By-products and wastes generated by these industries cause many problems for the pollution of the environment [1]. At present, the development of new value-added products for commercial applications using discarded by-products and discarded materials is gaining much attention. The bioactive compounds found in waste and by-products are highly nutritional and bioactive, making their recovery important for use in the development of nutraceuticals and pharmaceuticals.

According to epidemiological studies, many phytonutrients found in fruits and vegetables can protect the human body against damage caused by reactive oxygen and nitrogen species [2]. Since some synthetic antioxidants could be toxic and involve high manufacturing costs but may not be as effective as natural antioxidants, it would be beneficial to identify natural and more economically viable antioxidants that could be included in foods. Many types of plants have been studied for their antioxidant properties, including oil, seeds, cereal crops, vegetables, fruits, leaves, roots, spices, and herbs [3].

Among various methods to analyze the antioxidant potential of different types of plants, the DPPH test is simple because it measures the mechanism between the antioxidant and the DPPH reagent and it is one of the most widely used methods [4]. It is based on the reduction of the DPPH molecule and causes its color change from violet to yellow-colored stable molecules via the process of transferring a hydrogen atom from the antioxidant. This test measures nonreduced violet DPPH molecules using a UV-Vis spectrometer at 515-520 nm to measure the antioxidant capacity of the sample as shown in Figure 1 [5-7].

Avocados, commonly known as *Persea americana* Mill., have been widely distributed in the tropical regions of the world for centuries. Nutritionally, it is a very healthy fruit. The smooth flavor of avocado has contributed to its popularity as an oleaginous fruit crop throughout the world. Aside from being fresh, it is also processed to create food, cosmetic, and pharmaceutical products to increase commercialization and provide more added value. Due to its nutritional and bioactive composition, the avocado (*P. americana*) fruit has various benefits [8]. Avocados are used for their pulp, but their seeds and skins are discarded as waste.

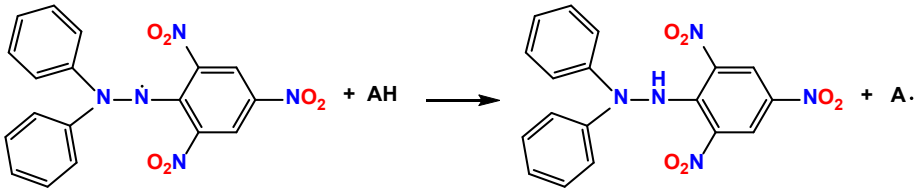

Figure 1. Reaction of DPPH radical and antioxidant. AH; an antioxidant donor molecule, A; a produced free radical.

The polyphenols present in these residues are powerful antioxidants and antimicrobials. In terms of avocado seeds, they represent 13-18% of the fruit and are a by-product that is not normally used. These are typically discarded along with the pulp during processing. Seed waste may pose a significant ecological problem [9]. The bioactive compounds that contain these compounds may be of interest to industry. It is reported to contain phytosterols, triterpenes, fatty acids, and two new abscisic acid glucosides. In addition to its antioxidant properties, avocado seeds have also been reported to have larvicidal, fungicidal, hypolipidemic, and recently amoebicidal and giardicidal properties. Among the various applications of *P. americana* for ethnomedicine is the treatment of diarrhea, dysentery, toothache, and intestinal parasites, as well as skincare and beauty. In vitro studies have shown that tocopherols from the fruit inhibit the growth of prostate cancer. Seeds are also a rich source of tannins and carotenoids [10].

The use of non-edible parts of fruits, such as seeds, is becoming a trending approach to mitigate the increase in food industry waste. Many of the bioactive compounds in fruits can be inactive when the underutilized parts are discarded. As a result, fermentation can produce bioactive substances with enhanced functionality, thus improving the quality of foods. Therefore, the use of enzymes and plant microorganisms can improve the quality of microbial fermentation. There are pros and cons to each of these production approaches [11]. Therefore, fermentation can transform raw seeds into value-added products through microbiological treatment. In this study, we tested the antioxidant potential of avocado seed flour using microbial fermentation to reveal perspectives on its potential of avocado seed flour utilizing microbial fermentation to reveal perspectives on its potential use as a therapeutic agent in the future.

2. Experimental

2.1. Collection and preparation of samples

For the experiment, ripe avocado fruits of a local variety were collected in June 2023 from a local supermarket in Sri Lanka. Avocado seeds can be collected by removing the fleshy part of the fruit. After cutting the seeds into 2 mm thick slices, the seeds were blanched with steam. A constant weight was obtained by drying the slices at 50 °C. The dried samples were packed in an airtight container for further analysis in the microbiology laboratory.

2.2. Preparation of the Lactobacillus plantarum starter culture

According to Reference [9], starter cultures were prepared from *Lactobacillus plantarum* with some modifications. Here, *L. plantarum* stock culture was grown in sterile DeMan, Rogasa, and Sharp (MRS) broth (10.0 mL) at 37 ° C for 24 hours. This MRS broth culture was further grown in sterile medium (50.0 mL) containing avocado seed flour (2.50 g), refined sugar (1.50 g), and skim milk (1.00 g), and then incubated at 37 °C for 24 hours.

2.3. Preparation of fermented and unfermented avocado seed flour

Using dried avocado seed chips (20.0 g) and sterile distilled water (200.0 mL), three samples were prepared. After sterilizing each flask at 120 °C for five minutes, the starter *plantarum* cultures were transferred to three flasks for fermentation at 37 °C for 24, 48, and 72 hours. In the following steps, we washed avocado chips with sterile distilled water and soaked them for 15 minutes in a salt solution (10%, 500 mL). After that, rinse it three times with sterile distilled water to remove salt residues. Over 2-3 days, the fermented avocado seed samples were oven dried at 55 °C. Each fermented avocado seed sample was oven dried at 55 °C for 2-3 days. The dry chips were ground in a blender and sieved to an 8 mm mesh size. The unfermented avocado seed flour is prepared by the same procedure without adding the starter culture to carry out fermentation [12].

2.4. Determination of antioxidant potential

The antioxidant potential of the avocado seed flour (ASF) samples was determined using the measurement of 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity [11]. Firstly, a suitable solvent that can be used for avocado seed extraction was found by observing the solubility of ASF in each solvent. Methanol was found to be the suitable solvent for avocado seed to perform the DPPH assay. The antioxidant activity of the methanol extract of ASF was determined using a 96-well microtiter plate according to the method described in reference [13] with slight modifications. A stock solution of the sample and the standard butylated hydroxyl-toluene (BHT) (1000 µg/mL) was prepared. A series of BHT standards with concentrations of 1000, 500, 125, 62.5, and 31.25 µg/mL were prepared. Methanolic DPPH solution (0.25 µg/mL, 40.00 µL) was added to each sample solution and the standard solution (160 µL) in the well and incubated for 30 minutes at room temperature in dark conditions. A microtiter plate reader was used to measure the absorbance of each well at 520 nm. On the basis of the equation below, the percentage inhibition was calculated.

$$\% \text{ Inhibition} = (A_{control} - A_{sample}/A_{control}) \times 100 \quad (1)$$

The calculated % inhibition corresponding to each sample in Table 1 was used to plot the graph (Figure 2) with the respective concentrations of the sample standards to obtain the IC_{50}

2.5. Determination of the pH change

The pH value of each sample (Unfermented, 24, 48, and 72 h fermented) was determined using a pH meter, calculated in triplicate and average values (Table 2).

Table 1. % Inhibition avocado seed flour and fermented avocado seed flour (24, 48, and 72 h).

Sample concentration (µg/mL)	% Inhibition			
	Unfermented sample	Fermented sample		
		24 h	48 h	72 h
500	68.89	92.21	89.19	89.90
250	58.00	76.56	74.67	77.67
125	55.12	65.01	60.31	62.31
62.5	49.98	52.23	53.56	52.89
31.25	47.01	37.89	41.22	40.22

Table 2. IC_{50} value and pH of avocado seed flour and fermented avocado seed flour (24, 48, and 72 h).

Parameters	Unfermented	Fermented		
		24 h	48 h	72 h
IC_{50}	61.5±0.71	51.18±0.10	46.00±0.21	43.73±0.21
pH	6.81±0.04	5.99±0.06	4.87±0.05	4.15±0.03

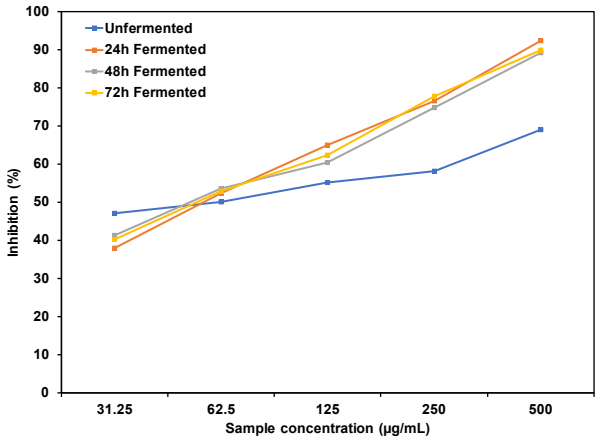

Figure 2. Variation of antioxidant activity after fermentation by *L. plantarum* compared to the unfermented control sample (n = 3). Results are represented as mean±standard deviation.

2.6. Statistical analysis

Data obtained in triplicate were carried out using the one-way analysis of variance (ANOVA) technique. Tukey's multiple comparison test was used to identify the means that differ significantly at $p < 0.05$. Results were expressed as means±SEM.

3. Results and discussion

Figure 2 shows the antioxidant capacity of fermented and unfermented ASF in different incubation periods. Antioxidants can be defined as reductants, and inactivation of oxidizing agents by reductants can be explained as a type of redox reaction. Avocado seeds contain considerably higher amounts of phenols, flavonoids, and paraanthocyanin, making them a good source of antioxidants obtained [13]. According to the results obtained for the antioxidant capacity of ASF corresponding to IC_{50} (the concentration of antioxidant required to give 50% inhibition of the probe in the antioxidant assay). Hence, the lower IC_{50} value denotes the high antioxidant activity of a given food source. IC_{50} values decreased in the order 0 h fermented flour (61.5±0.71) > 24 h fermented flour (51.18±0.10) > 48 h fermented flour (46.00±0.21) > 72 h fermented seed flour (43.73±0.21) (Table 2). According to the reference study [11], it has also shown an increase in the antioxidant activity of tomato seeds with *L. plantarum*-mediated fermentation. More importantly, a significant increase in antioxidant potential was observed for fermented seed flours for 72 h. Therefore, 72 h fermented ASF has the highest antioxidant potential. These results show a significant effect of microbial fermentation on the antioxidant potential of avocado seed flour. According to the previous study [12] on the application of microbial fermentation using *L. plantarum* on jackfruit seed (*Artocarpus heterophyllus*) seeds, it has clearly proven the positive impact of microbial fermentation on the antioxidant potential of A. *heterophyllus* seeds, and how the antioxidant potential of A. *heterophyllus* seeds has increased significantly with fermentation time. Phytochemicals in plant materials are mainly responsible for their antioxidant properties. As phytonutrients are important for human health, they are functionally essential for human consumption [14]. The increase in antioxidant potential is possibly due to the synthesis of bioactive peptides during fermentation by *L. plantarum*. Bioactive peptides are often inactive in the parent proteins and can be generated through the hydrolysis of different protein sources using a wide variety of approaches, mainly via fermentation using proteolytic microbes such as lactic acid bacteria (LAB) [14]. Figure 3 shows how the pH value of each sample changed with fermentation time. According to the results obtained, there is a significant decrease in the pH value of all fermented samples (24, 48, and 72 h) compared to the unfermented sample. When comparing all fermented samples, pH values of 24, 48, and 72 h fermented samples were 5.99±0.12, 4.87±0.05, and 4.15±0.13, respectively (Table 2). By the significant decline in pH value, it can be confirmed that the production of acids by the microbial fermentation process confirms the continuous process of microbial fermentation during the period of 72 h. This result also agrees with almost all lactic acid bacteria-mediated and also natural fermentation according to previous studies [15]. This may be due to the production of lactic acid by *L. plantarum* in the process of synthesis of energy for their metabolism [15]. Figure 3 shows how the calculated IC_{50} values decrease in order 0 h fermented flour (61.5±0.71) > 24 h fermented flour (51.18±0.10) > 48 h fermented flour (46.00±0.21) > 72 h fermented seed flour (43.73±0.21).

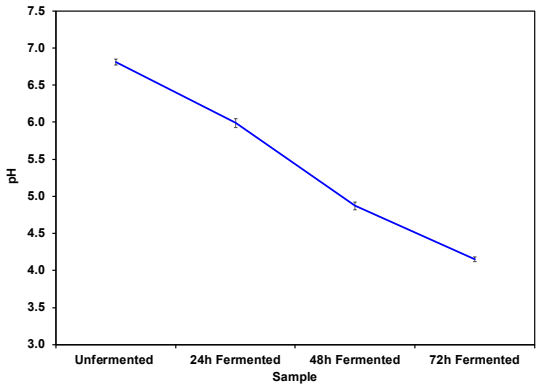

Figure 3. Variation of the pH value after fermentation by *L. plantarum* compared to the unfermented control sample (n = 3). Results are represented as mean ± standard deviation.

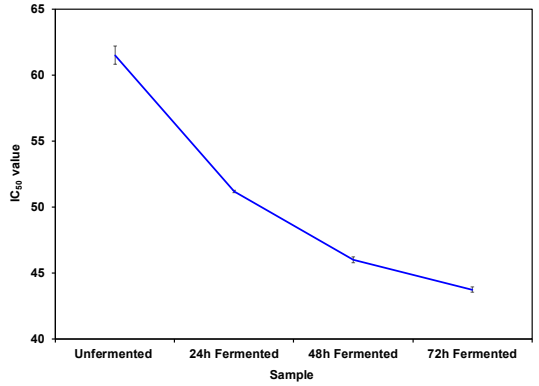

Figure 4. Variation in IC_{50} values after fermentation by *L. plantarum* compared to the unfermented control sample (n = 3). Results are represented as mean±standard deviation.

4. Conclusions

In conclusion, this study has indicated how microbial fermentation affects the potential health beneficial effects of avocado seed flour, such as antioxidant activities. In particular, the potential of avocado seed flour to effectively scavenge various free radicals increased with the fermentation period compared to the unfermented sample. These promising pharmaceutical properties indicate the value of avocado seeds. However, further studies that reveal these promising results can be translated into clinically or nutritionally useful agents are necessary.

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Disclosure statement

Conflict of interest: The authors declare that they have no conflict of interest.
Ethical approval: All ethical guidelines have been adhered.
Sample availability: Samples of the compounds are available from the author.

CRediT authorship contribution statement

Conceptualization: Dilushi Sureka Ruwan Kumari Polegodage, Methodology: Dilushi Sureka Ruwan Kumari Polegodage, Investigation: Dilushi Sureka Ruwan Kumari Polegodage, Data Curation: Madushan Dhammika Gunarathna, Writing - Original Draft: Dilushi Sureka Ruwan Kumari Polegodage, Writing - Review and Editing: Madushan Dhammika Gunarathna, Supervision: Ranjani Amarakoon.

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