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Research Article

Improving the antioxidant and anticancer potential of *Cinnamomum cassia* via fermentation with *Lactobacillus plantarum*

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ABSTRACT

This study designed to investigate effect of fermentation by *Lactobacillus plantarum* on antioxidant and anticancer properties of *Cinnamomum cassia* aqueous solution. The optimum condition to produce high antioxidant activity was 10^7 CFU *L. plantarum*/10 g cinnamon at pH6 after 3 days of incubation at 35 °C. Fermented cinnamon showed an increase in ABTS, DPPH and H_2O_2 by 24.63, 58.31 and 60.27%, respectively over the control. Also, the total phenolic and flavonoid contents were increased, 8.15 to 11.40 mg GAE/g and 0.43 to 2.61 mg QE/g, respectively. The gallic acid, p-hydroxybenzoic acid, catechin and chlorogenic acid were increased by 37, 404, 11 and 98%, respectively. Also, anticancer activity was developed after fermentation. The increased antioxidant activity of fermented cinnamon could be attributed to the increase of some phenolics and flavonoids. Hence, cinnamon fermentation using L. *plantarum* is able to enhance its antioxidant and anticancer activities without producing toxic substances.

1. Introduction

Cinnamon is a multifaceted medicinal plant, commonly used around the world as a spice for food and also as a drink. It is obtained from the inner bark of trees belonging to the tropical genus *Cinnamomum* (family *Lauraceae*). The genus *Cinnamomum* includes *C. cassia* (the most famous species), *C. verum, C. zeylanicum*, and *C. burmannii* ; Wan et al., 2021; [1]). The medicinal importance of cinnamon includes its antioxidant, anesthetic, anti-ulcer, anti-bacterial, anti-allergic, anti-inflammatory, antipyretic, antitumor, and antidiabetic activity [2, 3]. Cinnamon volatile oils are highly active against many types of fungi, and its essential oils have been shown to be effective in inhibiting some multidrug-resistant bacteria [4,5]. The antibacterial activity of *Cinnamomum cassia* (*C. cassia*) essential oils was more effective than some commercial antibiotics such as ampicillin, chloramphenicol and streptomycin [4]. In the context of the medicinal significance of medicinal plant extracts, there is a close relationship between the antioxidant activity and the plant's content of phenols and flavonoids. Phenolic substances and flavonoids are effective electron donors due to the hydroxyl groups that directly contribute to free radical scavenging and antioxidant activity [6,7]. In a comparative study, Jang et al., [8] reported that *C. cassia* extract was more efficient as an antioxidant than both turmeric (*Curcuma longa*) and golden thread (*Coptidis rhizoma*), which is consistent with its higher total phenol content.

Many research trials have focused on using appropriate food processing technology to increase phenolic compounds to reduce or eliminate oxidative damage. Fermentation, enzymatic pretreatment, pulsed electric fields (PEF), ultrasound-assisted extraction (UAE), ultrasonic extraction, and solar drying have been reported as effective techniques in this regard [9,10]. Fermentation is an important biotechnological process used in ancient and modern times to improve the nutritional value and organoleptic properties of foods by converting organic compounds by the action of microbial bio-products [11]. Changes during fermentation can affect the digestibility and bioactivity of the food as well as the compositional alteration through changes in protein fractions [12,13]. Fermentation is a tool for increasing biologically active compounds and functional properties of nutritional and medicinal plants [14]. It can improve antioxidant activity by increasing the phenol content via fermentation and microbial hydrolysis. Additionally, fermentation can encourage the breakdown of plant cell walls which helps produce various antioxidant compounds [15]. However, the conversion of phenolic and biologically active compounds to less active forms is an undesirable process and should be considered when designing fermentation processes. Fermentation of food by probiotic bacteria has many benefits. It supports the dynamic balance of active bacteria in the host

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Abbreviations

C	cassia: Cinnamon (Cinnamomum Cassia)
L	plantarum: Lactobacillus plantarum
MRS	De Man, Rogosa and Sharpe medium
DPPH	2, 2-Diphenyl-1-picrylhydrazyl
ABTS	(2,20-azinobis(3-ethylbenzothiazoline-6-sulfonate diammonium)
GAE	Gallic acid equivalent
QE	Quercetin equivalent
TLC	Thin Layer Chromatography
HPLC	High Performance Liquid Chromatography
HCC	Hepatocellular carcinoma
Huh7	human hepatoma
Wi38	human lung fibroblast cell line
DMEM	Dulbecco's Modified Eagle Medium
LC50	Lethal concentration 50

body, enhances human immunity, and improves the intestinal flora [16]. In addition, it is able to cause some compositional changes in the fermented food (substrate). For instance, *Lactobacillus* has been demonstrated to cause biotransformation of polyphenols via releasing of aglycones from glycol-conjugated phenolics by various glycosyl hydrolases [17]. Some anti-inflammatory and anti-bacterial compounds were also produced as a result of fermentation of barley with the lactic acid bacterium L. *plantarum* [18]. Therefore, this study aimed to evaluate the ability of L. *plantarum* to enhance the antioxidant and anti-cancer activity of cinnamon (*C. cassia*). Factors expected to have an effect on antioxidant activity were also studied, in addition to studying the nature of the biologically active compounds resulting from fermentation.

2. Materials and methods

2.1. Materials, reagents and media

Cinnamon (*C. cassia*) powder was purchased from a local store in Egypt (Kirkland organic ground Saigon cinnamon, Vietnam). Lactobacilli De Man, Rogosa and Sharpe medium (MRS) (NEOGEN, NCM0079B) were obtained from sales company. All chemicals and reagents were the analytical grade and they purchased by lab suppliers from Sigma-Aldrich, VWR chemicals and Merk.

2.2. Microorganism and culture conditions

L. *plantarum* 4496 was obtained from Northern Regional Research Laboratory (NRRL), National Center for Agriculture Utilization Research, Peoria, Illinois, USA. The bacterium was maintained on Lactobacilli MRS agar slants and kept at 4°C until use. To prepare the starter culture, L. *plantarum* strain was cultured in 250-ml conical flasks containing 100 ml of MRS broth. The inoculum was incubated for 24 h at 35 °C on a rotary shaker at 140 rpm. After incubation, the cells biomass were harvested by centrifugation (5000 rpm for 10 min) and then re-suspended in a sufficient volume of sterile distilled water to obtain a cell density of 10^7 CFU/ml. The immediately obtained L. *plantarum* suspension was used as a fermentation initiator with a specified volume according to the scheme. Bacterial enumeration of the starter and fermented product(s) was performed on MRS agar plates after appropriate dilutions were performed.

2.3. Fermentation of cinnamon

Fermented cinnamon was prepared according to the method of Zhao et al., [18] with some minor modifications. In brief; 10 g of sterilized cinnamon powder was mixed with 70 ml of sterilized distilled water and then specific volume of L. *plantarum* inoculum was added. The fermentation mixture was incubated at 35±2 °C on a rotary shaker at 140 rpm. Some important fermentation factors were included to study their effect on antioxidant production, namely inoculum density, incubation period, and initial pH. The cell density of the L. *plantarum* inoculum was 10^2, 10^3, 10^5, 10^7, and 10^9 CFU/10 g cinnamon. While the incubation periods were 1, 2, 3, 4 and 5 days, and the initial pH was adjusted at values of 4, 5, 6, 7 and 8. The counts of L. *plantarum* in the fermented samples were enumerated by pour plate sandwich technique using MRS agar medium.

2.4. Extraction of fermented cinnamon

The aqueous phase obtained after the centrifugal removal of cells (5000 rpm for 10 min) and sediment of the cinnamon fermentation mixture was used as an 'extract'. For comparison, non-fermented cinnamon was subjected to a similar extraction process using only distilled water. The aqueous extracts of fermented and non-fermented cinnamon were freeze-dried at −50 °C for 48 h (Labconco freeze dryer, Console, U. S.A). Freeze-dried cinnamon extracts were analyzed for their total phenol and flavonoid content, antimicrobial activity, and anticancer activity. High performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) analyses also have been conducted for the lyophilized extracts.

2.5. Determination of antioxidant activity

The samples for determination of antioxidant activity were prepared by dissolving 0.2 mg of the lyophilized cinnamon extract in 1 ml of distilled water, in which 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), 2,20-azinobis(3-ethylbenzothiazoline-6-sulfonate diammonium (ABTS), and Hydroxyl radical scavenging activity (%) were measured.

2.5.1. DPPH radical scavenging assay

The antioxidant activity of cinnamon was measured using the DPPH method described by Darwesh et al. [19] with some modifications. Briefly, a portion of 0.1 ml of sample was added to 3.9 ml of freshly prepared DPPH solution (22 mg of DPPH in 50 ml methanol). The mixture was vortexed for 30 s then kept in a dark place at room temperature for 30 min. The decolorization of DPPH reaction mixture was measured at 515 nm using a UV–Vis spectrophotometer (JASCO serial NO. A114761798, JAPAN). The DPPH free radical scavenging activity (%) was calculated as the percentage of absorbance decrease.

2.5.2. ABTS radical scavenging assay

According to Darwesh et al. [19] with some modifications, an equal volume of 2.4 mmol/L potassium persulfate was mixed with 7 mM ABTS and then incubated at room temperature for 12–16 h in the dark. The $ABTS^+$ solution was diluted with distilled water to obtain initial absorbance of 0.70 ± 0.02 at 734 nm. A 0.25 ml of sample was added to 0.75 ml of distilled water and 1 ml of $ABTS^+$solution, and then incubated at room temperature for 7 min. The absorbance decrease was recorded at 734 nm and $ABTS^+$ scavenging effect (%) was calculated as the percentage of absorbance decrease.

2.5.3. Hydroxyl radical scavenging activity assay

Hydroxyl radical scavenging activity was determined by the method presented by Bhatti et al., [20] with some modifications. A volume of 0.3 ml sample was added to 0.9 ml of 50 mM phosphate buffer (pH= 7.4) and 1.8 ml of H_2O_2 solution (2 mM). The mixture was vortexed and kept at room temperature for 10 min. The absorbance decrease was measured at 230 nm and the scavenging activity (%) was calculated as the percentage of absorbance decrease.

2.6. Determination of total phenolic contents

The total phenolic content (TPC) was determined according to Khan et al. [21] and Darwesh et al. [19] with minor modifications. A 0.5 ml of cinnamon extract solution (0.2 mg lyophilized extract/ml DW) was mixed with 0.5 ml of 10% Folin-Ciocalteu's reagent diluted in 13 ml distilled water. After that, 2.5 ml of 7% Na_2CO_3 solution was added followed by mixing. The reaction mixture was incubated at room temperature for 2 h in the dark. The absorbance was measured at 760 nm. Total phenolic content was calculated by extrapolating a calibration line constructed with gallic acid stander solution. The total phenolic content was expressed as mg gallic acid equivalent per gram dry weight extract (mg gallic acid equivalent (GAE)/g lyophilized extract).

2.7. Determination of total flavonoid contents

The total flavonoids content was determined according to method described by Khan et al. [21] with some modifications. In brief, 0.5 ml of cinnamon extract solution (0.2 mg lyophilized extract/ml DW) was mixed with 2.5 ml of distilled water, 1 ml of potassium acetate (1 M) and 1 ml of 10% aluminum chloride. The total flavonoids content was estimated by extrapolating a calibration line constructed with quercetin solution. The absorbance of the reaction was measured at 415 nm using UV–Vis spectrophotometer. The total flavonoid content was stated in terms of quercetin equivalent (mg QE/g of lyophilized extract).

2.8. Evaluation of antimicrobial activity

The antimicrobial activity of the lyophilized aqueous cinnamon extract (0.2 g /ml DMSO) was determined using the agar well diffusion method reported by Sultan et al., [22] with some modifications. The tested microorganisms were Gram-negative bacteria (*E. coli* ATCC-25, 922 and *Salmonella typhi* ATCC-15,566), Gram-positive bacteria (*Listeria monocytogenes* ATCC-35,152, and *Staphylococcus aureus* ATCC-47, 077), and yeast (*Candida albicans* ATCC-10,231). All test strains were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The test strains were cultured in nutrient broth medium for 24 h after that an adequate volume (from each) was spread onto the surface of nutrient agar plates. The wells (7 mm diameter) were mined on the inoculated plates and 75 µl of the previous DMSO-cinnamon mixture was added to the well [23]. The plates were incubated at 35 ±2 °C for 16–24 h. Then, the clear zones have been measured. The antimicrobial activities calculated according to the obtained inhibition zones on agar plates.

2.9. Anticancer activity and cytotoxicity determination

Huh7 (human hepatoma) and Wi38 cells (human lung fibroblast cell line) were purchased from VACSERA (the Holding Company for Biological Products and Vaccines) at Giza governorate, Egypt and the procedures were done in Cairo University Research Park (CURP). The test was done according to neutral red uptake assay [24]. Trypsinized cells were sub-cultured into 25 cm^2 tissue culture flasks. A 5×10^5 cells were cultured in each flask containing 7 ml of complete Dulbecco's modified eagle medium (DMEM) supplemented with 1% antibiotic solution (100 U/ ml penicillin and 100 µg/ml streptomycin) and 10% of fetal bovine serum and incubated at 37±1 °C. All culture reagents were purchased from Lonza supplier in Egypt. Huh7 cells were divided as follow: Group 1 served as untreated control without any treatment, Group 2 cells inoculated with freeze dried fermented cinnamon extract at 100, 300, 500 and 700 µg/ml and Group 3 cells inoculated with freeze dried non-fermented cinnamon extract at 100, 300, 500 and 700 µg/ml. Also, Wi38 cells were divided as Huh7 cells. The cells were exposed to neutral red dye (4 mg/ml) in serum free DMEM after treatment periods for 3 h. The cells were washed with Phosphate-buffered saline (PBS) then de-stained using de-staining solution (50% EtOH and 5% glacial acetic acid in distilled water). The absorbance was measured at 540 nm using spectrophotometer. Each treatment was done in triplicates after 24 h of incubation.

2.10. Thin layer chromatography

Active components of lyophilized fermented and non-fermented cinnamon extracts were characterized by TLC. TLC Silica gel 60 F_{254} plates (Merck KGaA, Darmstadt, Germany) were prepared in system of ethyl acetate: Hexane at a ratio of 1:1 v/v using TLC paper covered with silica gel as stationary phase [25]. They were emerged in the working solvent system as mobile phase. The TLC plate was visualized under UV light (254 and 366 nm).

2.11. HPLC analysis

HPLC analysis was carried out according to Kim et al., [26] using Agilent Technologies 1100 series liquid chromatograph equipped with an auto sampler and a diode-array detector. The analytical column was an Eclipse XDB-C18 (150 × 4.6 µm; 5 µm) with a C18 guard column (Phenomenex, Torrance, CA). The mobile phase consisted of acetonitrile (solvent A) and 2% acetic acid in water (v/v) (solvent B). The flow rate was kept at 1 ml/min for a total run time of 60 min and the gradient program was as follows: 100% B to 85% B in 30 min, 85% B to 50% B in 20 min, 50% B to 0% B in 5 min and 0% B to 100% B in 5 min. The injection volume was 20 µl and peaks were monitored simultaneously at 280 nm and 320 nm for the benzoic acid and cinnamic acid derivatives, respectively as well as 360 nm for flavonoids. All samples were filtered through a 0.45 µm Acrodisc syringe filter (Gelman Laboratory, MI) before injection. Peaks were identified by congruent retention times and UV spectra and compared with those of the standards.

2.12. Statistical analysis

All analyses were performed in triplicate and data reported as the mean ± standard deviation (SD). Results were processed by One Way ANOVA: Analysis of variance using the statistical software of Statistix 8.1 (Analytical Software, Tallahassee, FL, USA) using LSD's test for pairwise comparison.

3. Results and discussion

Many microorganisms have ability to utilize raw plant materials in their growth and thus cause changes in the composition of these plant components to varying degrees. As a result of this fermentation, an increase or decrease in some chemical components of the factory raw materials may occur, or new ones may forms. A desirable change in some microbial fermentation processes is to increase the bioavailability of nutrients, produce antioxidant compounds, stimulate the functions of probiotics, and potentiate certain health-promoting bioactive compounds [27]. The present study aimed to enhance the antioxidant activity of cinnamon by fermentation with edible probiotic bacterium, L. *plantarum* 4496. The fermentation product(s) expected to be influenced by the number of bacteria that involved in fermentation, incubation period, and the initial pH of fermentation mixture. Hence the study focused on the influence of such parameters on the antioxidant activity of the common medicinal plant powder, cinnamon (*C. cassia*).

3.1. Effect of inoculum density

Fermentation begins with the right bacteria in sufficient numbers in the appropriate fermentation medium. Thus, the number of bacteria is a major factor in the quality of the fermentation process. However, bacteria density may be a double-edged sword. If the number of bacteria is limited, the fermentation process may take a long time or may not start at all [28]. On the contrary, if the number of bacteria is too high, it may

Table 1
Effect of different L. *plantarum* inoculum densities (as CFU) used for cinnamon fermentation on its antioxidant activity.

CFU/10 g cinnamon	ABTS radical scavenging activity %	DPPH radical scavenging activity %	Hydroxyl radical scavenging activity %
0.0	29.49^{bc}	64.86^{ab}	60.40^{ab}
10^2	24.80^{cd}	40.97^{d}	30.51^{cd}
10^3	31.52^{abc}	55.72^{c}	39.19^{c}
10^5	32.80^{ab}	60.35^{bc}	44.04^{bc}
10^7	37.39^{a}	70.01^{a}	67.47^{a}
10^9	20.32^{d}	58.54^{bc}	20.81^{d}

Where the data are the means of triplicate experiments,$^{a, b, c, d}$values in the same column with different superscript letters are significantly different ($P < 0.05$). ^{a} value is the highest antioxidant activity and ^{d} value is the lowest antioxidant activity in the same column.

cause bacteria to suppress each other through bacterial competition and bacteriocins, thus reducing fermentation efficiency and increasing economic cost [29].

In this study, five bacterial densities of L. *plantarum* were used to ferment cinnamon to select the best density in terms of producing high antioxidant compounds. The effect of Bacterial count on the antioxidant activity of fermented cinnamon was illustrated in Table 1. The highest antioxidant activity by the three methods was attributed to the fermented cinnamon with 10^7 CFU/10 g. The antioxidant activity was 37.39 and 29.49% for the fermented cinnamon with 10^7 CFU/10 g and non-fermented cinnamon, respectively by the ABTS method. Also it was 70.01 and 64.86% for the fermented cinnamon with 10^7 CFU/10 g and non-fermented cinnamon, respectively as DPPH indicator. Furthermore, it was 67.47 and 60.40% for the fermented cinnamon with 10^7 CFU/10 g and non-fermented cinnamon, respectively determined by H_2O_2 method. Obtained results indicates that the inoculum density of 10^7 CFU L. *plantarum* /10 g cinnamon represents the better choice for starting cinnamon fermentation in terms of antioxidant production.

3.2. Effect of fermentation time

The contact time between microbes and substrate is a critical factor for fermentation process. Therefore, it is very important to allow sufficient time for the fermentation process to obtain the required products [28]. In the fermentation of cinnamon by L. *plantarum*, it was important to determine the appropriate incubation time to maximize antioxidant production. To achieve this goal, five different incubation periods for cinnamon fermentation were applied, which are one, two, three, four, and five days. Aqueous extract samples of fermented cinnamon after different incubation period have been analyzed for its antioxidant activity and the results were illustrated in Fig. 1. In general, the antioxidant activity increased during the first three days of fermentation and decreased in the 4th and 5th incubation days. The free radical scavenging activity of three days fermented cinnamon was 29.57, 71.18 and 67.92% by ABTS, DPPH and H_2O_2 methods, respectively. A too-long fermentation time after the third day caused a further decrease in the antioxidant activity. It was shown that the third-day results by ABTS and H_2O_2 methods were significantly different among all fermentation times used. Nevertheless, by using the DPPH method, we found that the differences between the second, third and fourth days were not significant. The third-day fermentation time was chosen because of three reasons. First, according to the DPPH method, the third-day antioxidant activity was higher than the fourth day and it is economically better. Second, according to the DPPH method, the second-day result was not significantly different from the first day and also the third day was higher. Third, according to ABTS and H_2O_2 methods, the third-day antioxidant activity was significantly higher among all fermentation times used. Based on these results, the appropriate incubation time to maximize antioxidant activity through cinnamon fermentation is three days.

3.3. Effect of initial pH

The pH value of the fermentation medium directly affects the enzymatic system of the microbe and, consequently, its ability to grow and produce various byproducts [30]. In this study the effect of different pH values on the antioxidant production of cinnamon by fermentation with L. *plantarum* was examined. The studied initial pH values ranged between 4 and 8 and the antioxidant activities obtained after three days of incubation were recorded in Table 2. The ABTS radical scavenging activity of fermented cinnamon wasn't significantly affected by changing the initial pH value. However, the value of the antioxidant activity was slightly higher when the fermentation was started at pH 6 (which was the original pH value of fermentation mixture). Also, the DPPH radical scavenging activity method showed that the best antioxidant activity

Table 2
The effect of initial pH on the scavenging ability of cinnamon.

pH value	ABTS radical scavenging activity%	DPPH radical scavenging activity%	Hydroxyl radical scavenging activity%
4	38.26^{a}	68.55^{ab}	37.98^{bc}
5	42.70^{a}	71.20^{ab}	49.49^{ab}
6	45.32^{a}	77.06^{a}	55.35^{a}
7	40.67^{a}	76.38^{a}	36.57^{bc}
8	39.61^{a}	62.30^{b}	32.12^{c}

Where the data are the means of triplicate experiments ^{a,b,c} values in the same column with different superscript letters are significantly different ($P < 0.05$). ^{a} value is the highest antioxidant activity and ^{c} value is the lowest antioxidant activity in the same column.

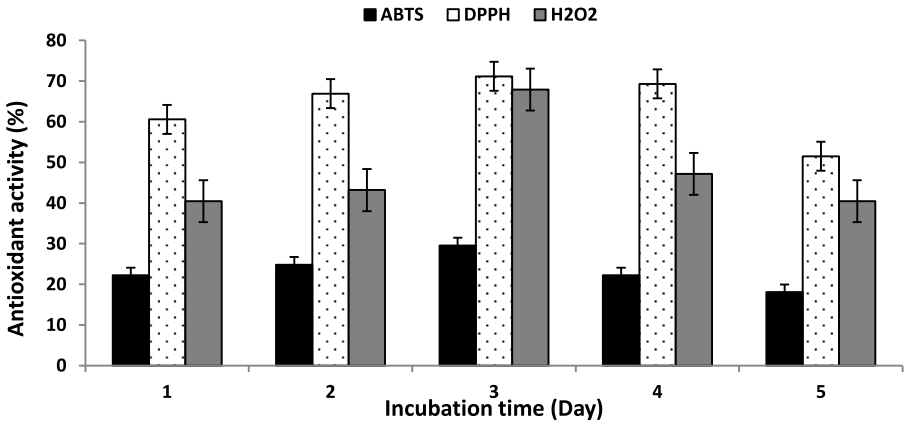


Fig. 1. The scavenging activity (%) of cinnamon during different fermentation periods by L. *plantarum*. Where the data are the average of triplicate treatments.

Table 3
Antioxidant activity, total phenols and flavonoids contents of lyophilized fermented and non-fermented cinnamon.

Antioxidant analysis	Non-fermented cinnamon	Fermented cinnamon
ABTS radical scavenging activity%	24.20[b]	30.16[a]
DPPH radical scavenging activity%	42.72[b]	67.63[a]
Hydroxyl radical scavenging activity%	21.52[b]	34.31[a]
Total phenolic content (mg GAE/g lyophilized extract)	8.15[b]	11.40[a]
Total flavonoids content (mg QE/g lyophilized extract)	0.43[b]	2.61[a]

Where the data are the means of triplicate experiments; Samples in the same row with different superscript letters are significantly different ($P < 0.05$).
[a] value is the highest antioxidant activity and.
[b] value is the lowest antioxidant activity in the same row.

was attributed to pH 6 and pH 7. The H_2O_2 scavenging activity evaluation showed that fermentation with pH 6 had the significant highest antioxidant activity. According to the three methods used to assess the antioxidant activity, it was found that the probiotic fermentation of cinnamon with a pH value of 6 gave the highest efficiency.

3.4. Optimized fermentation processes of cinnamon with L. plantarum

Previous results showed that the antioxidant production of cinnamon can be improved by optimizing the fermentation conditions. The optimized conditions include starting fermentation with L. *plantarum* density of 10^7 CFU/10 g cinnamon; initial pH 6; and incubation period of three days at 35 °C. Freeze-dried aqueous extracts of fermented (under optimal conditions), as well as non-fermented cinnamon, were characterized. The property includes antioxidant activities, total phenols and flavonoids, antimicrobial activity, anticancer and cytotoxic activities, TLC and HPLC analysis.

3.5. Antioxidant activities, total phenols and flavonoids

The antioxidant activities of lyophilized cinnamon extracts were evaluated as DPPH, ABTS, and H_2O_2 indicators. The fermented cinnamon significantly scavenged ABTS, DPPH and Hydroxyl radicals higher than non-fermented cinnamon (Table 3 &Fig. 2). The ABTS radical scavenging activity was 30.16 and 24.20% for fermented and non-fermented cinnamon, respectively. While the DPPH radical scavenging activity was recorded as 67.63 and 42.72% for lyophilized fermented and non-fermented cinnamon extracts, respectively. Furthermore, the H_2O_2 radical scavenging activity for fermented and non-fermented cinnamon was 34.31 and 21.52%, respectively.

The total phenol and flavonoid contents were evaluated and represented in **Table (3)**. Transformation of the cinnamon components by fermentation increased the total phenolic content from 8.15 to 11.40 mg/g of lyophilized cinnamon extract. The fermentation technique also increased cinnamon's total flavonoid content by 500%. Therefore, fermentation of cinnamon with L. *plantarum* enhanced antioxidants of cinnamon.

3.6. Antimicrobial activity

The antimicrobial activity of cinnamon is mainly attributed to the presence of cinnamaldehyde and eugenol which support the combat of many pathogens such as *P. fluorescens, Bacillus licheniformis* and *S. aureus* [31]. As the antimicrobial activity of cinnamon can be affected by various factors, it was therefore very important to evaluate this activity before and after fermentation with L. *plantarum*. The evaluation was performed against *E. coli* and *S. typhi* as Gram-negative bacteria, L. *monocytogenes* and *St. aureus* as Gram-positive bacteria, and *C. albicans*

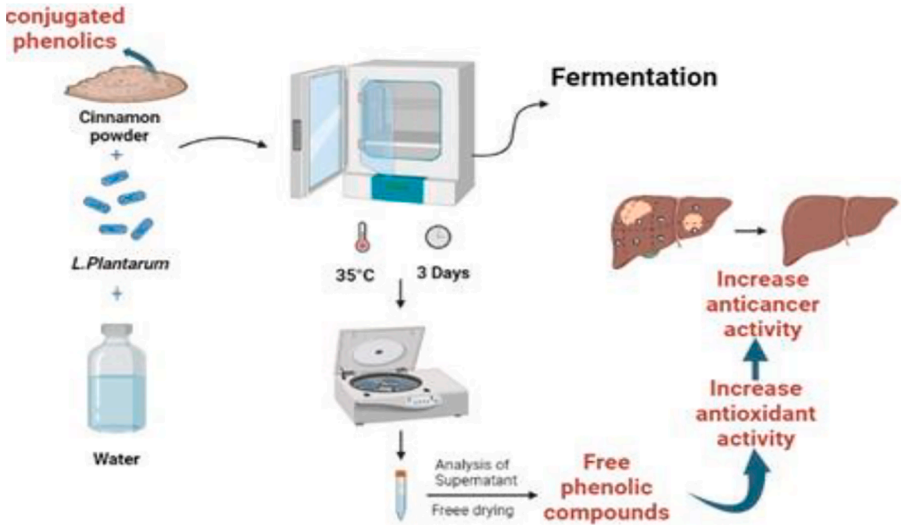
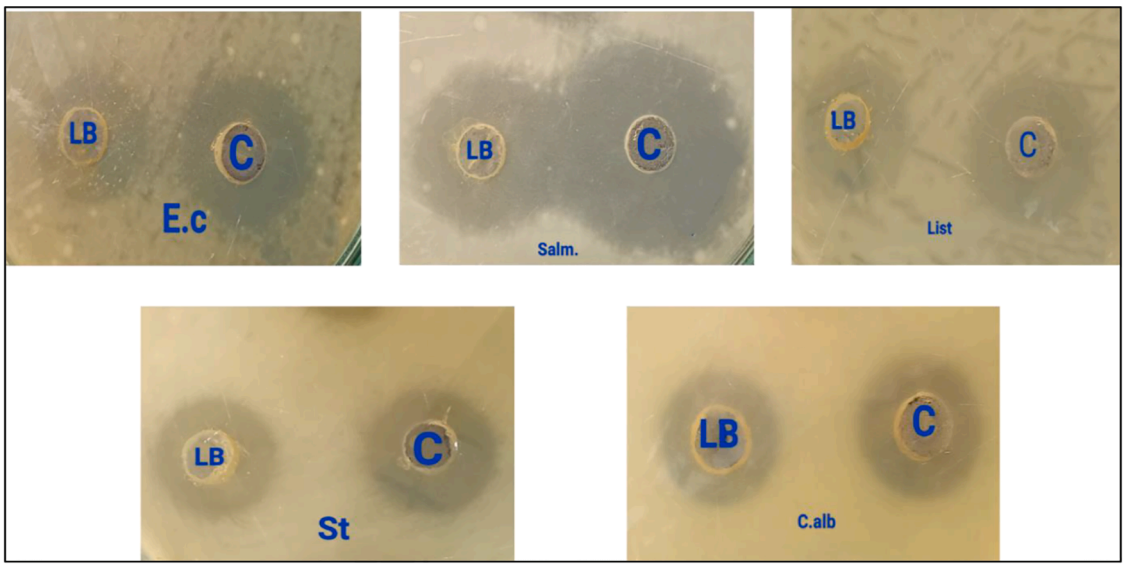


Fig. 2. Flowchart of increasing antioxidant activity of cinnamon by fermentation processes.

Table 4
Inhibition zone diameters of the lyophilized extracts of fermented and non-fermented cinnamon against different pathogenic microorganisms.

Pathogens	Inhibition zone of fermented cinnamon (mm)	Inhibition zone of non-fermented cinnamon (mm)
E. coli	23*	26
S. typhi	29	38
L. *monocytogenes*	24	26
S. aureus	23	23
C. albicans	20	21

* The data are the means of triplicate experiments, Where; LB, fermented sample by L. *plantarum*, C, non-fermented sample; E.C, *E. coli*; Salm, *S. typhi*; List, L. *monocytogenes*; St, *S. aureus* and C. alb., *C. albicans*.



Where; LB, fermented sample by *L. plantarum*, C, non-fermented sample; E.C, *E. coli*; Salm, *S. typhi*; List, *L. monocytogenes*; St, *S. aureus* and C. alb., *C. albicans*.

Fig. 3. Comparison between the inhibition zones of the lyophilized extracts of fermented and non-fermented cinnamon against different pathogenic microorganisms.

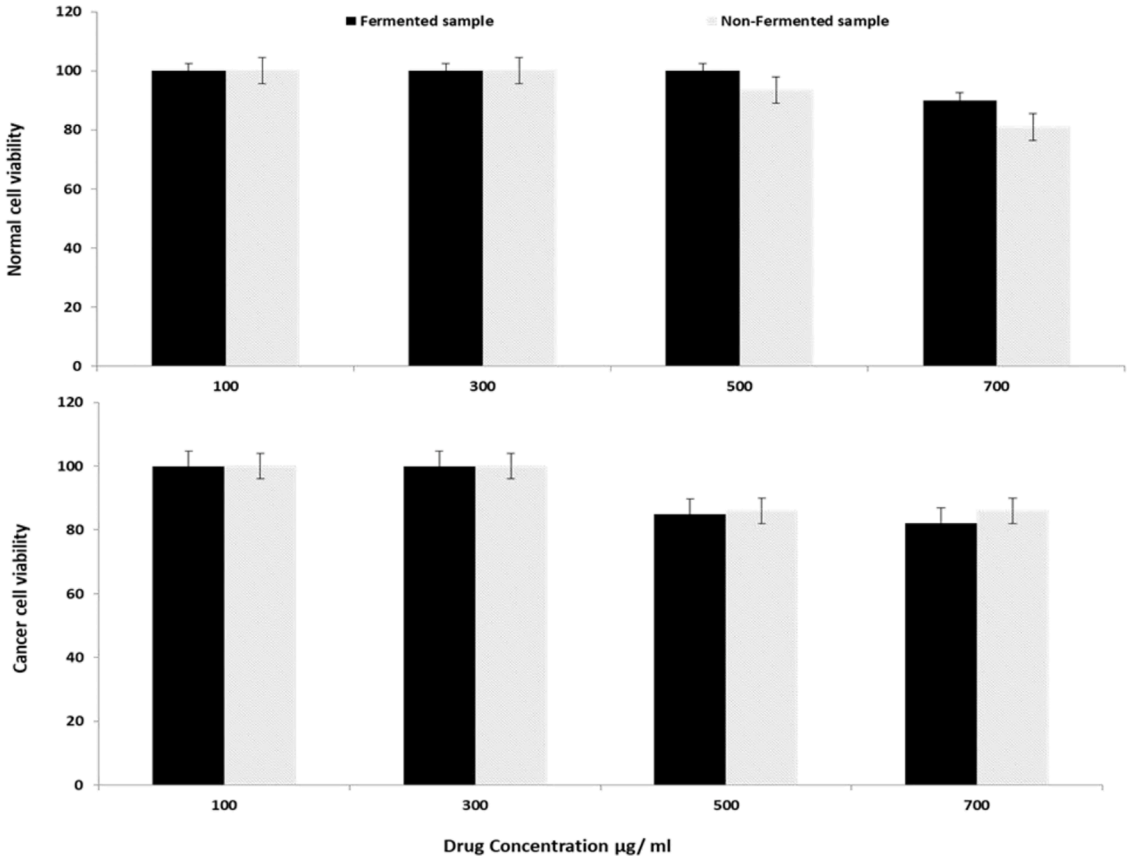


Fig. 4. Percentage of Normal and Cancer cell viability after treating with fermented and non-fermented cinnamon extracts. Where the data are the average of triplicate treatments.

as representative for yeast. The results on the agar plates gave inhibition zones with diameters ranging from 20–29 mm, including 7 mm of well diameter in case of lyofelized fermented cinnamon extract. In contrast, the unfermented cinnamon extract showed higher activity against all tested pathogens and gave inhibition zones ranging between 21 and 38 mm in diameters (Table 4 and Fig. 3). This decrease in the antimicrobial activities of the fermented cinnamon could be attributed to the degradative oxidation of cinnamaldehyde and eugenol during fermentation [32]. The decomposition of cinnamaldehyde leads to the production of cinnamic acid while eugenol results in ferulic acid and vanillin

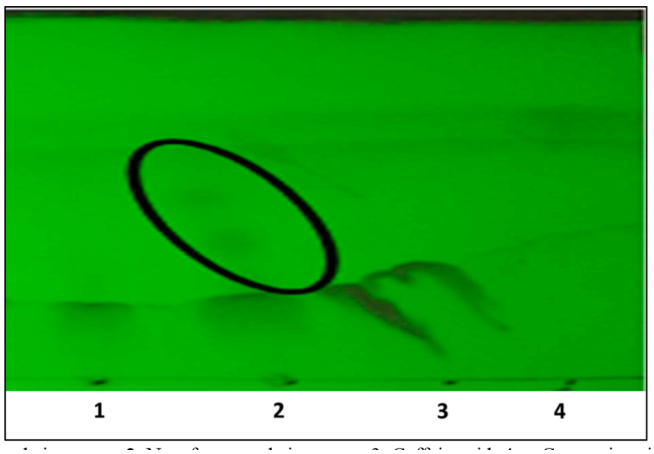


Fig. 5. TLC chromatogram of lyophilized fermented and non-fermented cinnamon extracts.

production which have lower antimicrobial activities (Tadasa et al., [32]; Surianti et al., 2018). However, the decrease in antimicrobial activity was not typically significant, which does not diminish the importance of the lyophilized extract of fermented cinnamon as an antimicrobial.

3.7. Anticancer and cytotoxicity activity

Cinnamon has a proven anticancer activity, but it is important to know whether this activity is decreased or increased due to fermentation using the probiotic bacterium L. *plantarum* [2]. Hepatocellular carcinoma (HCC) is a common liver cancer that represents "the fourth leading cause of cancer death globally" [33]. According to obtained results, the lyophilized extract of fermented cinnamon outperformed the non-fermented as an anticancer agent. The viability of cancer cells decreased with increasing concentration of the extract at concentrations of 500 and 700 µg/ml (Fig. 4). In addition, the Lethal concentration 50 (LC_{50}) was decreased by 18.75% (from 1.9 to 1.6 mg/ml of the lyophilized extract) in case of fermented cinnamon. **Figure (S3)** contains micrographs of cancer cells without treatment, treated with fermented cinnamon extract and treated with non-fermented cinnamon extract at a concentration of 700 µg/ml. According to the images examined, the cancer cells in the sample treated with fermented cinnamon extract were less crowded than the other two samples, confirming the presence of anti-cancer compounds. As is well known, anticancer drugs may cause side effects when taken by patients [34]. For that, the cytotoxicity effect of fermented and non-fermented cinnamon extracts was studied using Wi38 cells to ensure its safety as a drug for cancer. The test showed that the normal cell viability of fermented cinnamon is higher than that of non-fermented cinnamon, which means that it is better in terms of its cytotoxicity. Based on the toxicological and anticancer results, it can be confirmed that cinnamon fermentation with L. *plantarum* increases the production of anticancer agents without producing toxic substances.

3.8. TLC analysis

As a result of fermentation, some biologically active substances can be produced or disappeared from the fermentation medium. Hence, the thin-layer chromatography was used to compare the main components of cinnamon before and after fermentation. The analysis showed the disappearance of one of the compounds from the fermented cinnamon (shown in the black circle on the TLC panels in Fig. 5). This compound may be cinnamaldehyde which is an aromatic aldehyde compound in *C. cassia* [25]. The separation of cinnamaldehyde on silica gel was faster than cinnamic acids [35]. Also, the oxidation of aldehydes resulted in the production of carboxylic acids such as cinnamic acid [36]. Therefore, fermentation may oxidize cinnamaldehyde to cinnamic acid. Also, Thin-layer chromatography showed that both fermented and non-fermented cinnamon has caffeic acid and *p*-coumaric acid which are antioxidants [37,38]. TLC analysis is considered as an initial step for characterization which must be followed by another advanced analysis such as HPLC. Therefore, the fermented samples were subjected to HPLC analysis using several standard antioxidant compounds.

Where 1, Fermented cinnamon; 2, Non-fermented cinnamon; 3, Caffeic acid; 4, *p*-Coumaric acid

3.9. HPLC analysis

The purpose of HPLC analysis was to identify and quantify some bioactive compounds in fermented and non-fermented cinnamon extracts. Certain bioactive components were injected into HPLC as standards i.e. gallic acid, *p*-hydroxybenzoic acid, catechin chlorogenic acid, and protocatechuic acid. As a result of cinnamon fermentation by L. *plantarum* bacteria, gallic acid, p-hydroxybenzoic acid, catechin and chlorogenic acid were increased by 37, 404, 11 and 98%, respectively (Table 5 and Fig. 6). The increase of gallic acid may be attributed to the microbial hydrolysis of tannic acid [39,40]. Whereas, the increase in the antioxidants catechin percentage may be due to the transformation of epicatechin during the fermentation process [41,42]. Additionally, caffeic acid may join to quinic acid producing chlorogenic acid which is antioxidant [43,44]. Although, protocatechuic acid content was lowered, it may be esterified and the esterification of hydrophilic phenolic antioxidants increased their antioxidant activity [45].

4. Conclusion

Fermentation of cinnamon using L. *plantarum* is able to enhance its antioxidant and anticancer activities without producing toxic substances. The increased radical scavenging activity of fermented cinnamon aqueous extracts could be attributed to the increase of some

Table 5
Differentiation between some bioactive compounds in L. *plantarum* fermented and non-fermented cinnamon extracts.

Compound	Retention time (min)	L. *plantarum* fermented cinnamon (µg/g)	Non-fermented cinnamon (µg/g)	Conversion hypothesis	Activity of produced or increased compound	References
Gallic acid	4.183	309.90	225.48	Hydrolysis of tannic acid to form gallic acid	Antioxidant	[39,40]
Protocatechuic acid	6.753	221.31	520.47	It may be esterified	Protocatechuic esters has antioxidant activity higher than protocatechuic acid	[45]
p-hydroxybenzoic acid	9.937	73.89	14.65	Hydrolysis of benzoic acid to form *p*-hydroxybenzoic acid	Promotion the expression of antioxidant enzymes	[46]
Catechin	11.738	32.75	29.40	Conversion of epicatechin to form catechin	Antioxidant	[41,42]
Chlorogenic acid	12.872	17.46	8.80	Quinic acid and caffeic acid formed chlorogenic acid	Antioxidant	[43,44]

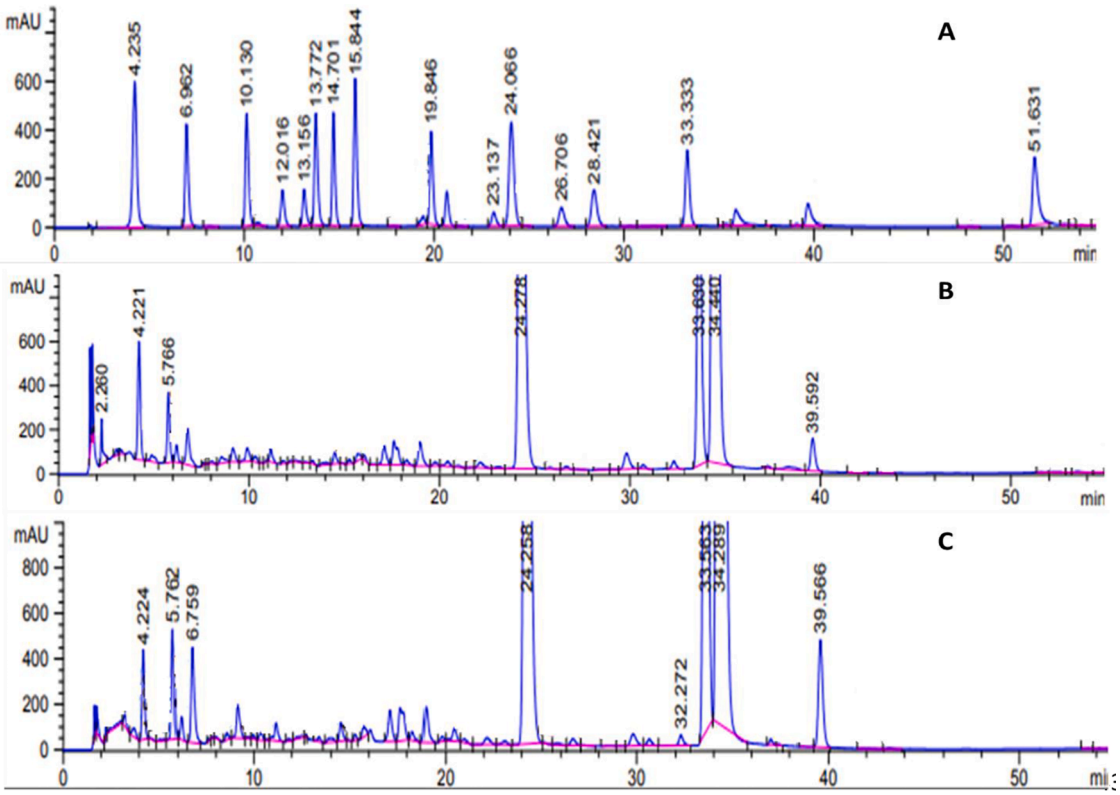


Fig. 6. Chromatogram of standards (a), non-fermented (b) and L. *plantarum* fermented cinnamon extract (c).

phenolic compounds and flavonoids. The obtained results demonstrate the importance of fermentation technique in improving the antioxidant and anticancer activities of many medicinal herbs.

Author contributions

All authors conceived and planned the study; A.S.E. and O.M.D. performed the methodology and data analysis. Y.-S.Z. contributed as supervisor. O.M.D., A.S.E. and Y.-S.Z. contributed for discussion of results and commented on the manuscript. O.M.D. and A.S.E. wrote and edited the first draft of manuscript. O.M.D., A.S.E. and Y.-S.Z. reviewed, read, revised and agreed to the final version of the manuscript.

Declaration of Competing Interest

Authors declare that there are no conflicts of interest.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.btre.2022.e00768.

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