Biotechnology Reports 38 (2023) e00799

Contents lists available at ScienceDirect

Biotechnology Reports

journal homepage: www.elsevier.com/locate/btre

Research Article

Extraction and characterization of bioactive secondary metabolites from lactic acid bacteria and evaluating their antifungal and antiaflatoxigenic activity

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ARTICLE INFO

Keywords:
Aflatoxins
Antifungal activity
Bioactive secondary metabolites
Brine shrimp bioassay
Lactic acid bacteria
Mouse bioassay

ABSTRACT

Aflatoxins are toxic carcinogens and mutagens formed by some moulds, specifically *Aspergillus* spp. Therefore, this study aimed to extract and identify bioactive secondary metabolites from *Lactobacillus* species, to evaluate their efficacy in reducing fungal growth and aflatoxin production and to investigate their toxicity. The bioactive secondary metabolites of *Lactobacillus* species showed variable degrees of antifungal activity, whereas *L. rhamnosus* ethyl acetate extract No. 5 exhibited the highest antifungal activity and, thus, was selected for further identification studies. Data revealed that *L. rhamnosus* ethyl acetate extract No. 5 produced various organic acids, volatile organic compounds and polyphenols, displayed antifungal activity against *A. flavus*, and triggered morphological changes in fungal conidiophores and conidiospores. *L. rhamnosus* ethyl acetate extract No. 5 at a 9 mg/mL concentration reduced AFB_1 production by 99.98%. When the effect of *L. rhamnosus* ethyl acetate extract No. 5 on brine shrimp mortality was studied, the extract attained a 100% mortality at a concentration of 400 μg/mL, with an IC_{50} of 230 μg/mL. Meanwhile, a mouse bioassay was performed to assess the toxicity of *L. rhamnosus* ethyl acetate extract No. 5, whereas there were no harmful effects or symptoms in mice injected with *L. rhamnosus* ethyl acetate extract at concentrations of 1, 3, 5, 7, and 9 mg/kg body weight.

1. Introduction

Fungi are crucial microorganisms in deteriorating food, food products, and feed [1,2], whereas spoiling fungus and their mycotoxins contaminate more than 25% of agricultural raw materials globally [3]. Aside from the economic loss, the presence of fungi in foodstuffs may result in the formation of mycotoxins, which are among the most concerning components of human health [4]. Mycotoxins were named among the 10 top hazards in food in 2019 by the Rapid Alert System for Food and Feed [5]. Aflatoxins (AFs) are hazardous secondary metabolites produced by *Aspergillus* species, particularly *Aspergillus flavus* and *Aspergillus parasiticus* [6]. Aflatoxin B_1 (AFB_1) is the most toxic and commonly occurring among AFs and has been categorized as a group I human carcinogen by the International Agency for Research on Cancer [7].

Numerous physical and chemical treatments have been employed to reduce AFs contamination in crops and its toxicity below the acceptable limit [8]. Although various physical and chemical detoxification strategies have been evaluated, none have met the required safety and effectiveness standards [9] due to the nutritional loss of processed foods and difficulty removing toxic compound residues. Therefore, cost-effective methods to detoxify mycotoxin-contaminated grains and foods are urgently needed to minimize potential farmer losses and consumer toxicity [10]; therefore, biological control is considered a more environmentally friendly and safer method [10,11]. Scientific evidence suggests that microorganisms such as yeast, bacteria, and fungi can mitigate mycotoxins by preventing their presence in animal feed and food from animal sources [12]. Lactic acid bacteria (LAB) are a group of Gram-positive organisms, and they have been identified as Generally Regarded as Safe (GRAS) and Qualified Presumption of Safety (QPS) by the American Food and Drug Administration (FDA) and the European Food Safety Authority (EFSA), respectively [13,14]. As a result, LAB is approved for use as probiotics and bio preservatives in food [15]. The LAB cultures isolated from native fermented food items with probiotic properties and mycotoxin binding are extremely useful in decontaminating mycotoxins in food [16]. *Lactococcus lactis (L. lactis), L. bulgaricus,*

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https://doi.org/10.1016/j.btre.2023.e00799
Received 29 December 2022; Received in revised form 27 April 2023; Accepted 2 May 2023
Available online 3 May 2023

L. acidophilus plantarum, and *L. rhamnosus* strains isolated from the dairy, silage, and food industries significantly suppressed mycotoxin bioactivity [17].

Many LAB metabolites, including organic acids, carboxylic acids, phenolic acids, cyclic dipeptides, hydrogen peroxide, reuterin, several proteinaceous compounds, and various antifungal compounds, have been reviewed for their role in inhibiting a wide range of fungal species [18,19]. Recently, antifungal metabolites from LAB strains have been used to inhibit fungal growth in a variety of fermented and non-fermented products, which include yoghurt [20,21], cheese [22], cereal-based products [23,24], soybeans [25], and fresh fruits and vegetables [26]. Recently, many novel antifungal metabolites of LAB have been reported, including 10-hydroxy-12-octadecenoic acid (10-HOE), 13-hydroxy-9-octadecenoic acid [27], benzene acetic acid, 2-propenyl ester [28], phenolic antioxidants (such as 2,4 di-tert-butyl phenol) [29,30], 2-hydroxy-(4-methylthio) butanoic acid, 2-hydroxy-3-methylbutanoic [31]. Therefore, this study aimed to extract and identify bioactive secondary metabolites from *Lactobacillus* species, evaluate their efficacy in reducing fungal growth and aflatoxin production, and investigate their toxicity.

2. Materials and methods

2.1. Chemicals

Potato dextrose agar and De Man Rogosa Sharpe broth were purchased from Neogen (Lansing, MI 48,912, USA). Sodium sulphate anhydrous and yeast extract were purchased from Loba Chemie (Mumbai 400 005, India). Ethyl acetate HPLC grade was purchased from Merck KGaA (Darmstadt, Germany), whereas dimethyl sulfoxide (DMSO) was purchased from Research lab fine chem industries (Mumbai, 400 002, India).

2.2. Microorganisms

Fungal isolates; *A. parasiticus, A. niger, A. ochraceus; Fusarium oxysporum, Fusarium* spp., and *Penicillium* spp. were isolated from various grains in Egypt [32]. The aflatoxin-producing *Aspergillus flavus* used in this study was isolated from maize samples in Egypt [33], and the gene sequence was deposited in the GenBank database as; *A. flavus* AAM2020 (Accession No. OP942201).

2.3. Lactic acid bacteria

Lactobacillus plantarum subspecies *plantarum* DSM 20,174 and *Lactobacillus rhamnosus* ATCC 7469 were obtained from the Microbiological Resources Centre (MIRCEN), Faculty of Agriculture, Ain Shams University. *Lactobacillus gasseri* LA39 was kindly donated by Dr T. Saito, Faculty of Biological Resource Science, Tohoku University, Japan.

2.4. Extraction of bacterial bioactive secondary metabolites

The effect of different media on the production of bioactive secondary metabolites from three *Lactobacillus* species (*L. gasseri, L. plantarum, L. rhamnosus*) was studied. The *Lactobacillus* species were grown on rice medium (100 g rice in 100 mL distilled water) and rice yeast medium (100 g rice, 0.5 g yeast extract, in 100 mL distilled water). The rice media were incubated at 35 °C for 7 days in a static condition. On the other hand, *Lactobacillus* species were grown on De Man Rogosa Sharpe broth. The broth media were incubated at 35 °C, under shaking at 120 rpm for 3 days [34]. After incubation, the broth media were centrifuged at 10,000 rpm for 10 min. (Thermo Fisher Scientific, USA) at 4 °C to collect the cell-free supernatant, which was later filtered through sterilized 0.22 μm pore-size filters (Millex-GS, Millipore, USA).

Ethyl acetate was added at 1:1 (v/v) to the cell-free supernatant and the rice media, which was shaken vigorously for a few min. The ethyl acetate mixtures were poured into separating funnels and allowed to stand until organic and aqueous phases were separated, and the organic phase was collected and passed over anhydrous sodium sulphate. This process was repeated three times, and the organic phase was evaporated to dryness using a rotary evaporator (Heidolph Instruments GmbH & Co. KG, Germany) to yield ethyl acetate extracts [35]. Nine ethyl acetate extracts were prepared, and extracts No. 1, 2, and 3 were extracted from *L. plantarum* and No. 4, 5, and 6 were extracted from *L. rhamnosus*. On the other hand, extracts No. 7, 8, and 9 were extracted from *L. gasseri* (Table 1).

2.5. Antifungal activity of bacterial bioactive secondary metabolites

The antifungal activity of ethyl acetate extracts was determined using an agar well diffusion assay according to the method described by Salman and Al-Haddad [36]. Potato dextrose agar media was placed on Petri dishes, and different fungal isolates (100 μL, 10^6 spores/mL) were spread uniformly over the agar surface with a sterile cotton swab. Wells were cut using a sterile cork borer, and then 100 μL of ethyl acetate extracts at a concentration of 5 mg/mL DMSO were added to each well. The plates were incubated at 28 °C and observed after 48 h for inhibition zones (mm) to determine the antifungal activities.

2.6. Determination of organic acids in bacterial ethyl acetate extracts

The *L. rhamnosus* ethyl acetate extract No. 5 was mixed well by vortex, filtered through a 0.22 μm syringe filter (Millex-GS, Millipore, USA), and injected in an HPLC system as described by Bukhari et al. [37]. An Inert Sustain was used for HPLC analysis. The Eclipse AQ-C18 HP column (4.6 mm x 150 mm i.d., 3 μm) was used for the separation. The mobile phase was 0.005 N sulfuric acid. The mobile phase was designed in a linear gradient for flow rate in the following order: 0–4.5 min. (0.8 mL/min.); 4.5–4.7 min. (1 mL/min.); 4.7–4.71 min. (1 mL/min.); 4.71–8.8 min. (1.2 mL/min.); 8.8–9 min. (1.3 mL/min.); 9–23 min. (1.3 mL/min.); 23–25 min. (0.8 mL/min.). At 210 nm, the Diode Array Detector (DAD) was monitored. For each of the sample solutions, the injection volume was 5 μL. The temperature in the column was kept constant at 55 °C.

2.7. Determination of volatile organic compounds in bacterial ethyl acetate extracts

A qualitative and quantitative evaluation of volatile organic compounds in *L. rhamnosus* ethyl acetate extract No. 5 was conducted using the gas chromatography-mass spectrometer technique (GC–MS) (Agilent Technologies, USA). The GC–MS system included a gas chromatograph (7890B) and a mass spectrometer detector (5977A). The HP-5MS column (30 m x 0.25 mm internal diameter and 0.25 m film thickness) was installed in the GC.

Hydrogen was used as the carrier gas in the analyses, with a 1 mL/min flow rate at a splitless injection volume of 1 μL and the following temperature program: 50 °C for 1 min.; 5 °C/min rise to 100 °C and hold for 0 min.; 10 °C/min rise to 300 °C and hold for 5 min. The injector and

Table 1
Bacterial ethyl acetate extracts used in this study

Bacterial isolates	Ethyl acetate extracts No.	Type of media
Lactobacillus plantarum	1	De Man Rogosa Sharpe broth
	2	Rice medium
	3	Rice yeast extract medium
Lactobacillus rhamnosus	4	De Man Rogosa Sharpe broth
	5	Rice medium
	6	Rice yeast extract medium
Lactobacillus gasseri	7	De Man Rogosa Sharpe broth
	8	Rice medium
	9	Rice yeast extract medium

detector were kept at 250 and 260 °C, respectively. Electron ionization (EI) at 70 eV yielded mass spectra with a spectral range of *m/z* 50–550 with a 6 min solvent delay. Different constituents were identified by comparing the spectrum fragmentation pattern to those stored in the Wiley and NIST Mass Spectral Library data.

2.8. Determination of polyphenols in bacterial ethyl acetate extracts

The *L. rhamnosus* ethyl acetate extract No. 5 was analysed with liquid chromatography-electrospray ionization–tandem mass spectrometry (LC-ESI-MS/MS). Exion LC AC system for separation and a SCIEX Triple Quad 5500+ MS/MS system with electrospray ionization (ESI) was used for the detection. ZORBAX SB-C18 Column (4.6 × 100 mm, 1.8 μm) (Agilent Technologies, USA) was used for the separation. The mobile phases comprised two eluents: A (0.1% formic acid in water) and B (acetonitrile). The mobile phase was programmed as follows: 2% B from 0 to 1 min., 2% B from 1 to 21 min., 60% B from 21 to 25 min., and 2% B from 25 to 28 min. The injection volume was 3 μL, and the flow rate was 0.8 mL/min. Positive and negative ionization modes were used in the same run for MRM analysis of the selected polyphenols, using the following parameters: Ion spray voltage: 4500 and - 4500 for positive and negative modes, respectively; curtain gas: 25 kPa; source temperature: 400 °C; ion source gases 1 and 2 were 55 kPa with a de-clustering potential of 50; collision energy: 25; collision energy spread: 10.

2.9. Evaluation of the effect of bacterial bioactive secondary metabolites on the microstructure of A. flavus using a scanning electron microscope (SEM)

This study was conducted to determine the effect of *L. rhamnosus* ethyl acetate extract No. 5 on *A. flavus* microstructure. Twenty-five milliliters of molten potato dextrose agar were transferred to Petri plates and allowed to solidify. The fungal spore suspension of *A. flavus* (100 μL, 10^6spores/mL) was then spread uniformly over the agar surface, after which wells were cut using a sterile cork borer, then 100 μL of ethyl acetate extract at a concentration of 5 mg/mL DMSO were added to each well.

The plates were incubated at 28 °C for 5 days. Mycelium segments (1 cm^2) were cut and placed in vials containing 3% glutaraldehyde in 0.05 M phosphate buffer (pH 6.8) at room temperature, as described by Mims [38] and Gong et al. [39]. Chemical fixation followed using ethanol series ending with absolute ethanol. Samples were dried in liquid carbon dioxide, and fungal cultures were placed in a freeze-dryer.

The samples were placed in Petri dishes, and a vial cap containing 4% osmium tetraoxide in water was placed in an unoccupied part of the plate. Segments were then coated with 20 – 30 nm of 60:40 gold palladium. All samples were viewed in an electron probe micro-analyser scanning electron microscope (SEM; Quanta FEG 205, FEI Company, Hillsboro, OR, USA) operating at 20.00 kV.

2.10. Antifungal and antiaflatoxigenic activity of bioactive secondary metabolites

The effect of *L. rhamnosus* ethyl acetate extract No. 5 on mycelial dry weight (MDW) and AFs production was determined according to Roshan et al. [40] with some modifications (by using potato dextrose broth instead of sucrose malt-yeast extract- broth). The requisite volumes of ethyl acetate extract were added to different flasks containing 20 mL potato dextrose broth to achieve final concentrations of 1, 3, 5, 7, and 9 mg/mL of growth medium. The *A. flavus* spore suspension (100 μL, 10^6 spores/mL) from a 7-day-old culture of the aflatoxigenic isolate was incorporated into each flask. Cultures were incubated at 28 °C for 10 days. After incubation, the culture medium was filtered (Whatman No. 1), and the mycelial dry weight was washed with water and dried using a hot air oven (110 °C, 12 h). The filtrate was extracted with 20 mL chloroform twice in a separating funnel, and the extract was passed through anhydrous sodium sulphate and evaporated to dryness. Aflatoxins were determined using HPLC. The percentage of inhibition was calculated using the following equation:

$$Percentage\ of\ inhibition(\%) = 1 - \frac{Treatment}{Control} x100$$

2.11. Toxicity tests

2.11.1. Brine shrimp lethality bioassay

The brine shrimp lethality bioassay was performed to estimate in vivo lethality of *L. rhamnosus* ethyl acetate extract No. 5 in a simple zoological organism, using nauplii of the brine shrimp *Artemia salina*. Artificial seawater was prepared by mixing 27 g of commercially available salt mixture with 900 mL of distilled water. *Artemia salina* eggs were added to a small commercial tank containing artificial seawater for nauplii hatching and incubated under a halogen lamp, providing direct light and warmth for 48 h.

Twenty mg of the ethyl acetate extract No. 5 were dissolved in 2 mL ethyl acetate, and then various concentrations (50, 100, 200, 400, 600, 800, and 1000 μg/mL) were prepared. The tubes were allowed to evaporate to dryness. After that, 4.5 mL of artificial seawater was added to each tube, and ten nauplii were counted macroscopically in the stem of the graduated Pasteur pipette against a lighted background and transferred to test tubes. After adding the nauplii, the final volume in each tube was adjusted to 5 mL with artificial seawater. Twenty-four h later, the number of surviving nauplii in each tube was counted macroscopically by two independent counters and recorded [41,42]. The test was conducted with three replicates for each treatment and ten nauplii per replicate.

The LC_{50} values were determined with 95% confidence intervals by analysing the data and analysed according to the Reed-Muench method. The Reed-Muench method assumes that an animal that survived a given dose would have survived any lower dose and that an animal that died with a specific dose would have also died at any other higher dose. It could be concluded that information from any group can be added to the other groups' range of doses tested [43,44].

2.11.2. Mouse bioassay

Swiss Albino mice weighing 20±2 g were obtained from the Animal House Colony in the National Research Centre, Egypt. Mice were fed a regular lab diet in an artificially lit, temperature-controlled room without any other chemical contamination. The mice were divided into five groups (3 mice/group) and housed in filter-top polycarbonate cages. All animals were treated humanely following the rules of the National Research Center's Animal Care and Use Committee, Egypt.

Animals within treatment groups were maintained on their respective diets for 24 h as follows: group 1: untreated control; groups 2, 3, 4, and 5 were treated intraperitoneally with 1 mL of *L. rhamnosus* ethyl acetate extract No. 5 at a concentration of 3, 5, 7, and 9 mg/kg body weight respectively [45]. During the 24 h, mice were observed for signs of toxicity and abnormal behaviour [46].

3. Results

3.1. Antifungal activity of bacterial bioactive secondary metabolites

The ethyl acetate extracts of the three lactic acid bacteria (*L. plantarum, L. rhamnosus*, and *L. gasseri*) were used to investigate the antifungal activity against different fungal species (Table 2). Results showed that bacterial ethyl acetate extracts demonstrated variable degrees of antifungal activity. Data also revealed that *L. rhamnosus* ethyl acetate extract exhibited the highest antifungal activity against *A. flavus, A. parasiticus, A. niger, A. ochraceus*, recording a zone inhibition of 17.00, 10.00, 17.67 and 18.00 mm, respectively. Data revealed that ethyl acetate extracts from LAB grown on rice medium showed higher antifungal

Table 2
Antifungal activity of bioactive secondary metabolites using zone inhibition (mm).

Fungal species	Bioactive secondary metabolites								
	1	2	3	4	5	6	7	8	9
Aspergillus flavus	7.33±6.66	12.33±11.24	10.00±10.00	14.00±5.29	17.00±3.61	8.67±7.51	15.67±2.08	13.00±2.00	10.00±10.00
Aspergillus parasiticus	7.33±6.43	14.33±1.15	13.67±5.13	9.67±8.39	10.00±10.00	12.67±15.53	5.00±8.66	9.00±7.94	ND
Aspergillus niger	12.00±0.00	12.33±0.58	12.33±1.53	11.33±0.58	17.67±4.04	11.67±10.41	12.00±2.00	13.00±0.00	9.00±7.94
Aspergillus ochraceus	ND	18.00±2.00	10.33±8.96	9.67±8.39	18.00±6.56	11.67±10.41	ND	ND	7.33±6.43
Fusarium oxysporum	8.67±7.57	ND	6.00±10.39	ND	ND	ND	ND	ND	ND
***Fusarium* spp.**	14.33±12.50	ND	6.00±10.39	8.00±13.86	10.00±9.17	16.00±1.73	5.00±8.66	ND	11.00±9.64
***Penicillium* spp.**	15.33±4.16	12.67±2.31	8.33±7.23	12.67±0.58	14.33±1.15	12.67±2.31	13.00±1.00	12.33±2.51	12.67±2.08

Results are mean ± SD ($n = 3$); ND: Not detected.
Ethyl acetate extracts 1, 2, and 3: *L. plantarum*; Ethyl acetate extracts 4, 5, and 6: *L. rhamnosus*; Ethyl acetate extracts 7, 8, and 9: *L. gasseri*.
Ethyl acetate extracts 1, 4, and 7: De Man Rogosa Sharpe broth, Ethyl acetate extracts 2, 5, and 8: Rice medium; Crude extracts 3, 6, and 9: Rice yeast medium.

activity than other media types. Therefore, due to its highest antifungal activity, *L. rhamnosus* ethyl acetate extract was selected for further analysis. A flow chart explaining the selection of the extract that exhibited high antifungal activity for further studies is shown in Fig. 1.

3.2. Organic acids production

Seven different organic acids were detected in *L. rhamnosus* ethyl acetate extract (Table 3, Figure S1), whereas lactic acid and butyric acid were detected at a concentration of 64,859.93 and 13,795.18 mg/mL, respectively. Acetic and formic acid were also detected at 8921.53 and 5158.44 mg/mL concentrations, respectively. Meanwhile, citric acid was identified at a lower concentration, recording 720.78 mg/mL.

3.3. Volatile organic compounds in bacterial ethyl acetate extracts

The chemical analysis of *L. rhamnosus* ethyl acetate extract indicated four volatile organic compounds, representing 100% (Tables 4, Figure S2). The major components in the *L. rhamnosus* ethyl acetate extract were 9, 12-octadecadienoic acid (Z, Z) (79.66%) (Figure S3), followed by n-hexadecanoic acid (17.64%) (Figure S4). Meanwhile, beta-sitosterol (1.87%) and tetradecanoic acid (0.84%) were detected at a lower percentage.

Table 3
Organic acid profile of *L. rhamnosus* ethyl acetate extract No. 5 with their respective area and concentration.

Peak No.	Area (%)	Concentration (mg/mL)	Organic acid
1	131.88	5158.44	Formic acid
2	1555.21	64,859.93	Lactic acid
3	152.73	8921.53	Acetic acid
4	26.81	720.78	Citric acid
5	21.60	1141.34	Succinic acid
6	16.23	1534.97	Propionic acid
7	133.84	13,795.18	Butyric Acid

3.4. Polyphenols in bacterial ethyl acetate extracts

Twelve phenolic acids have been detected by LC-MS (Table 5, Figure S5) in *L. rhamnosus* ethyl acetate extract, whereas ferulic acid was the most abundant at a concentration of 614.17 μg/g. Vanillin, coumaric acid, and caffeic acid were also detected at high concentrations, recording 267.42, 210.76, and 172.39 μg/g, respectively. On the other hand, gallic acid (0.72 μg/g), ellagic acid (0.33 μg/g), and quercetin (0.20 μg/g) were also detected but at lower concentrations. The *L. rhamnosus* ethyl acetate extract reflected a distinguished flavonoid content, with five compounds (Table 5), whereas naringenin, daidzein, and catechin were detected at concentrations of 104.64, 75.74, and

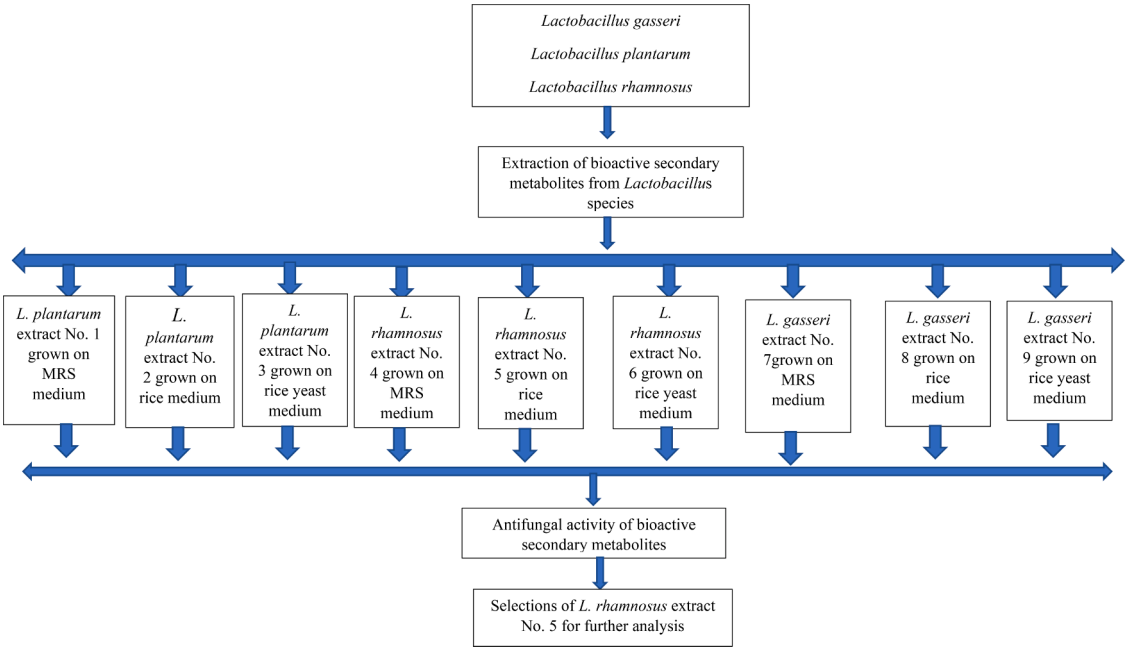

Fig. 1. Flow chart showing the selection of suitable extract that showed high antifungal activity for further analysis.

Table 4
Volatile organic compounds of *L. rhamnosus* ethyl acetate extract No. 5.

Peak No.	RT	Area sum (%)	Chemical formula	Name
1	23.422	0.84	$C_{14}H_{28}O_2$	Tetradecanoic acid
2	25.897	17.64	$C_{16}H_{32}O_2$	n-Hexadecanoic acid
3	27.879	79.66	$C_{18}H_{32}O_2$	9,12-octadecadienoic acid (Z,Z)[1]
4	35.215	1.87	$C_{29}H_{50}O$	beta-Sitosterol

RT: Retention time.
[1]Antiviral; The activity of the chemical compounds is obtained from the National Center for Biotechnology Information.

Table 5
Contents of phenolic acids and flavonoids detected in *L. rhamnosus* ethyl acetate extract No. 5.

Compounds	Phenolic acid (μg/g)	Compounds	Flavonoids (μg/g)
Gallic acid	0.72	Catechin	30.47
Caffeic acid[1,2,3,4]	172.39	Daidzein	75.74
Coumaric acid	210.76	Naringenin[1]	104.64
Vanillin[1]	267.42	Luteolin	0.86
Quercetin	0.20	Rutin	0.05
Ellagic acid	0.33	–	–
3.4-Dihydroxybenzoic acid[1]	82.50	–	–
Cinnamic acid[1]	22.36	–	–
Ferulic acid[1,2]	614.17	–	–
Syringic acid[1]	26.79	–	–

[1]Antioxidant; 2: Anticancer; 3: Antiviral; 4: Antibacterial
The activity of the chemical compounds is obtained from the National Center for Biotechnology Information.

30.47 μg/g. On the other hand, luteolin (0.86 μg/g) and rutin (0.05 μg/g) were detected in relatively low concentrations.

3.5. Scanning electron microscope (SEM)

The effect of *L. rhamnosus* bioactive secondary metabolites on *A. flavus* structure using a scanning electron microscope (SEM) was determined. Fig. 2 demonstrates typical conidia and conidiospores of untreated *A. flavus*. Data in Fig. 3 revealed the effect of *L. rhamnosus* ethyl acetate extract on *A. flavus*, which caused the loss of some fungal spores and the deformation of conidiophores and spores, as well as the fusion.

3.6. Effect of bioactive secondary metabolites on fungal growth and aflatoxin production

3.6.1. Antifungal activity

The effect of different concentrations of *L. rhamnosus* ethyl acetate extract on the fungal growth of *A. flavus* after 10 days of incubation was studied (Table 6). Results revealed that different concentrations of *L. rhamnosus* ethyl acetate extract showed variable degrees of antifungal activity. On the other hand, data indicated that *L. rhamnosus* ethyl acetate extract at 9 mg/mL concentrations reduced fungal growth by 32.27%. It was also noticed that fungal growth inhibition increased by increasing ethyl acetate extract concentration.

3.6.2. Antiaflatoxigenic activity

The effect of different concentrations of *L. rhamnosus* ethyl acetate extract on aflatoxin production was investigated (Table 6). Results revealed that *L. rhamnosus* ethyl acetate extract at a concentration of 1 mg/mL reduced AFB_1 production by 80.93%, whereas the reductions increased by increasing the ethyl acetate extract concentration. Adding *L. rhamnosus* ethyl acetate extract at a 9 mg/mL concentration reduced AFB_1 production by 99.98%. On the other hand, AFB_2, AFG_1, and AFG_2 production were reduced by 92.10%, 75.36%, and 67.19%, respectively, by *L. rhamnosus* ethyl acetate extract at a 1 mg/mL concentration. On using *L. rhamnosus* ethyl acetate extract at 3, 5, and 7 mg/mL concentrations, AFB_2, AFG_1, and AFG_2 production were prevented entirely. Moreover, *L. rhamnosus* ethyl acetate extract at 9 mg/mL concentration increased AFB_2 and AFG_2 production, whereas AFG_1 was utterly prevented.

3.7. Toxicity tests

3.7.1. Brine shrimp lethality bioassay

The mortality percentage values of the brine shrimp lethality bioassay obtained for the *L. rhamnosus* ethyl acetate extract were presented in Fig. 4. The toxicity test revealed a 100% percentage of mortality for larvae, which was recorded at a concentration of 400 μg/mL. The LC_{50} value was established based on the death of *A. salina* larvae

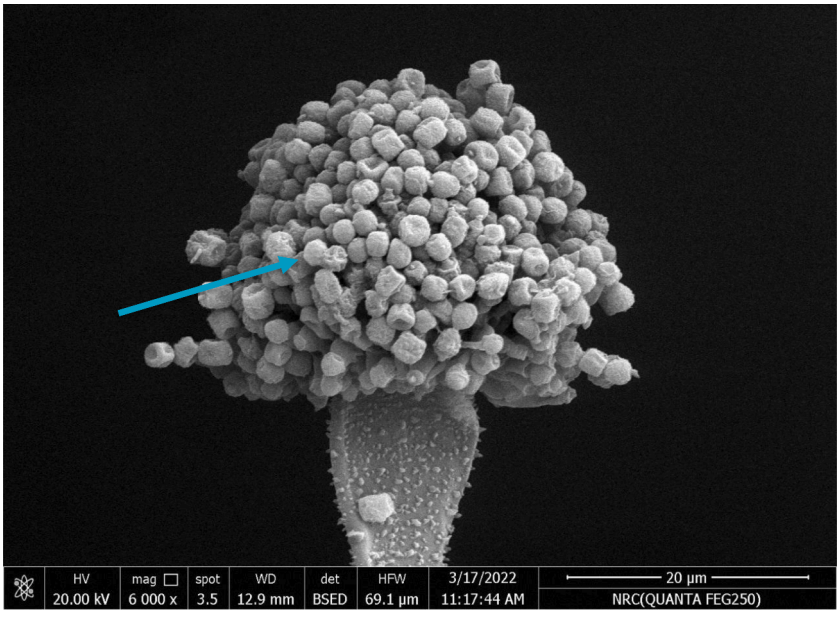

Fig. 2. Scanning electron microscope of untreated *A. flavus* (control) showing typical conidia and conidiospores.

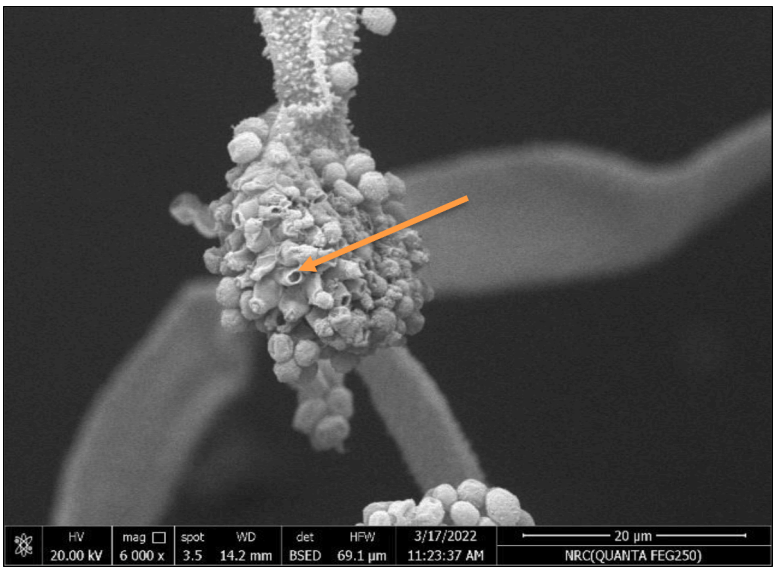

Fig. 3. Scanning electron microscopes of *A. flavus* after treatment with *L. rhamnosus* ethyl acetate extract No. 5 showing loss and mutation of fungal conidiospores.

Table 6
Effect of L. *rhamnosus* ethyl acetate No. 5 on *A. flavus* growth and aflatoxin production.

Treatment	Concentration (mg/mL)	Dry mycelium weight mg/50 mL medium	%*	Aflatoxin production (ng/g) AFB_1	%*	AFB_2	%*	AFG_1	%*	AFG_2	%*
Control	–	137.3		1,935,723.75		21,505.4		3421.84		6456.91	
Ethyl acetate extract	1	133.8	2.55%	369,091.51	80.93%	1698.30	92.10%	843.30	75.36%	2118.43	67.19%
No. 5	3	128.5	6.41%	519.32	99.97%	ND	100%	ND	100%	ND	100%
	5	127.7	6.99%	195.99	99.99%	ND	100%	ND	100%	ND	100%
	7	124.4	9.40%	496.69	99.97%	ND	100%	ND	100%	ND	100%
	9	93	32.27%	443.29	99.98%	43.69	99.80%	ND	100%	4.31	99.93%

Values of dry mycelium weight were obtained by weighing mycelium mats; *Percentage of inhibition; ND: Not detected.

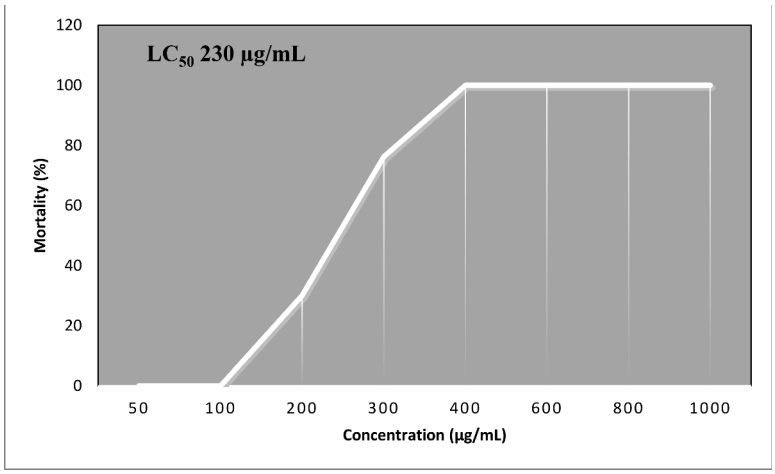

Fig. 4. Effects of *L. rhamnosus* ethyl acetate extract No. 5 on brine shrimp mortality.

caused by the bacterial ethyl acetate extracts. The *L. rhamnosus* ethyl acetate extract recorded an LC_{50} of 230 µg/mL.

3.7.2. Mouse bioassay test

The results showed no harmful effects or symptoms in mice injected with *L. rhamnosus* ethyl acetate extract at concentrations of 1, 3, 5, 7, and 9 mg/kg body weight (Table 7). Throughout the experiment, no deaths or apparent clinical symptoms were observed in mice receiving the ethyl acetate extract. Throughout the investigation, a physical assessment of the treated rats revealed no signs of toxicity or behavioural abnormalities.

4. Discussion

The antifungal activity of LAB ethyl acetate extract was studied, and results showed that *L. rhamnosus* ethyl acetate extract exhibited higher antifungal activity against various fungal species. The antifungal activities of *L. rhamnosus* ethyl acetate extracts could be due to the presence

Table 7
Mouse bioassay of different concentrations of *L. rhamnosus* ethyl acetate extract No. 5.

Ethyl acetate extract concentration (mg/kg body weight)	Number of mice	The volume of extract injected intraperitoneal (mL)	Observations
Control	3	–	Alive
1	3	1	Alive
3	3	1	Alive
5	3	1	Alive
7	3	1	Alive
9	3	1	Alive

of organic acids in the ethyl acetate extracts. Various studies have proved that organic acids such as acetic acid, lactic acid, and phenyl lactic acid are significant contributors to antifungal activities [47,48]. Furthermore, citric acid and lactic acid are thought to have an antifungal effect by affecting membrane structure, reducing intracellular pH, impeding active transport, and interfering with various metabolic activities [49]. Also, the antifungal activity of the *L. rhamnosus* ethyl acetate extract could be due to the volatile organic compound 9, 12-octadecadienoic acid (Z, Z), which was detected in the extract [37, 50].

It was reported that secondary metabolite production by bacteria is frequently linked to and regulated by primary metabolism and that the composition and quantities of the media contents are directly related to the metabolic capacity of the generating organism and have a significant impact on the production of bioactive compounds. Other parameters influencing secondary metabolite formation include medium volume, oxygen transfer rate, temperature, starting pH of the medium, and incubation period [51]. In a different study, Elleuch et al. [52] reported that adding five carbohydrate sources to the medium did not alter the bioactive molecule production.

Bioactive secondary metabolites were studied to determine the presence of different organic acids using the HPLC approach, which quantified various organic acids in *L. rhamnosus* ethyl acetate extract No. 5. Seven different organic acids were detected in *L. rhamnosus* ethyl acetate extract No. 5, whereas lactic acid and butyric acid were detected at higher concentrations recording 64,859.93 and 13,795.18 mg/mL, respectively. In agreement, Zalán et al. [53] reported that *L. rhamnosus* VT1 produced lactic acid in the broth medium.

The most often used method for identifying volatile organic compounds (VOCs) found in bioactive secondary metabolites is gas chromatography-mass spectrometry (GC–MS). A total of four volatile organic compounds were identified from *L. rhamnosus* ethyl acetate extract No. 5. In a similar study, Gallegos et al. [54] reported that four volatile organic compounds were emitted from *L. casei* and *L. paracasei* subsp. *paracasei*. Although these volatile organic compounds were completely different from those produced by *L. rhamnosus* ethyl acetate extract, the differences in the volatile organic compounds could be due to previous authors who measured the volatile organic compounds in the lactic acid bacteria cell-free supernatant. In contrast, we extracted these metabolites from cell-free supernatants with ethyl acetate in this study.

Polyphenols are the most abundant natural antioxidant, flavonoids, hydrolyzable and condensed tannins, phenolic acids, stilbenes, lignans, and phenolic aldehydes [55]. The LC-MS detected twelve phenolic acids in *L. rhamnosus* ethyl acetate extract No. 5, whereas ferulic acid was the most abundant. The *L. rhamnosus* ethyl acetate extract No. 5 reflected a distinguished content of flavonoids, with the detection of naringenin, daidzein, catechin, luteolin, and rutin. Similarly, Liu et al. [56] found that *L. rhamnosus* L08 fermentation of apple pomace significantly increased the contents of ferulic acid.

Because of their antioxidant properties, phenolic compounds have received much attention in recent years; additionally, these compounds have been reported to be potential candidates in lowering cardiovascular diseases [57] and anti-carcinogenic activities, antiallergenic, anti-atherogenic, anti-inflammatory, antimicrobial, and antithrombotic effects [58]. Phenolics are gaining popularity in the food sector because they slow the oxidative breakdown of lipids, improving food quality and nutritional content [59,60].

In the setting of developing antibiotic resistance, there is a need for active chemicals that inhibit disease propagation, infection, and virulence. The food production industry also relies heavily on developing novel food preservatives that are less damaging to human and environmental health. This prospective sector of microbial VOC application has yet to be investigated [61].

In studying the effect of *L. rhamnosus* ethyl acetate extract No. 5 on the microstructure of *A. flavus*, it could be pointed out that the antifungal activity of this extract may be due to the severe damage to the fungal membranes and cell walls, which led to the morphological deformations, collapse and deterioration of the conidia and hyphae. Altered morphological changes in conidiophores and conidiophores of *A. flavus* were profound. Similar observations were reported by Muhialdin et al. [62], who demonstrated that peptide in fraction 7 of *L. plantarum* caused apparent damage at the tip of *A. flavus* MD3 mycelia. The authors added that the observed morphological changes of fungal hyphae suggested that the peptides present in fraction 7 were more likely to cause the cell wall lysis for the targeted fungi.

The effect of different concentrations of *L. rhamnosus* ethyl acetate extract No. 5 on the fungal growth of *A. flavus* and aflatoxin production after 10 days of incubation was studied. Results revealed that *L. rhamnosus* ethyl acetate extract No. 5 reduced fungal growth. Olonisakin et al. [63] indicated that *L. plantarum, L. delbrueckii*, and *L. fermentum* inhibited the growth of *A. flavus*. Similar results were reported by Parappilly et al. [64], who revealed that all three methanolic extracts of the isolates (*L. plantarum* AL1, *L. fermentum* AL2, and *Weissella confusa* AL3) showed very high inhibitory activity towards aflatoxigenic *A. flavus* MTCC 2798. The antifungal action of *L. rhamnosus* ethyl acetate extract No. 5 could be attributed to the presence of different metabolites such as organic acids [53], volatile organic compounds [54], phenolic compounds and bioactive peptides [65].

Results revealed that *L. rhamnosus* ethyl acetate extract No. 5 at a 9 mg/mL concentration reduced AFB_1 production by 99.98%, ultimately preventing AFB_2, AFG_1, and AFG_2 production. In experimental animals, Hathout et al. [66] reported that treatment with *L. casei* and *L. reuteri* significantly improved all biochemical parameters and histological pictures of the liver in rats fed AFs contaminated diet.

The brine shrimp lethality bioassay has been widely used as a preliminary screening of crude extracts to assess their toxicity to brine shrimps, which could potentially indicate the potential cytotoxic qualities of the test materials [67]. The brine shrimp lethality bioassay was initially employed as a simple test to determine toxicity; nevertheless, it is not a specific test. The brine shrimp lethality bioassay is a quick, inexpensive, and straightforward method for assessing the biological activities of the extracts and is considered an initial screening method used to assess bioactive compounds or compounds considered suitable as anticancer drugs. The *L. rhamnosus* ethyl acetate extract No. 5 recorded an LC_{50} of 230 μg/mL. The current investigation revealed that the degree of mortality of *A. salina* larvae was proportional to the concentration of the ethyl acetate extracts. This could be because the higher the concentration, the more potent bioactive contents can be produced.

In agreement, Audi et al. [68] indicated that the LC_{50} value of the *Aspergillus niger* crude extract HR-ASN was 3.165 μg/mL, while the LC_{50} values of the pure components HR_{66}, HR_{94}, and HR_{102} were 4.094, 5.52, and 2.295 μg/mL, respectively. Similarly, Nainangu et al. [69] reported that the fractions of the cyanobacteria *Oscillatoria* sp. SSCM01 exhibited an LC_{50} value of 51.63 and 59.43 μg/mL for fractions FIV and FV, respectively. In a different study, Adebayo-Tayo et al. [70] found that toxicity of the silver nanoparticles containing methanol extract of *Oscillatoria* sp. was insignificant with an LC_{50} of 2630.3 μg/mL. On the

other hand, The brine shrimp lethality test was utilized to assess the toxicity of the extracted coconut drinks fermented by *Lactococcus lactis*, and the extracts were shown to induce low brine shrimp mortalities, with LC_{50} ranging from 4800.49 to 9261.30 µg/mL [71].

The mouse bioassay technique was used to assess the toxicity of *L. rhamnosus* ethyl acetate extract No. 5, whereas mice were injected with different concentrations of *L. rhamnosus* ethyl acetate extract No. 5. Results showed no harmful effects or abnormal behaviour signs, and no death was observed. Similarly, the toxicity of pyocyanin isolated from *Pseudomonas* MCCB102 was investigated using mouse bioassay, and no harmful effects or symptoms were seen in mice injected with pyocyanin at concentrations of 50, 100, 250, 500, and 750 g/mL [46]. Mulatu et al. [72] revealed that oral administration of methanolic extracts and spore suspensions of each *Trichoderma* species to female Swiss albino mice for 14 days revealed no symptoms of toxicity or death. Thus it is crucial to standardize toxicity testing on model species, such as mouse bioassays, that can be used to determine the safety status of new products, components, and chemicals that could be used as a food source or added to food.

5. Conclusion

The bioactive secondary metabolites extracted from *L. rhamnosus* contained organic acids, volatile organic compounds and polyphenols and exhibited antifungal and antiaflatoxigenic activity. These bioactive secondary metabolites induced toxicity against brine shrimp lethality test and showed no harmful effects or signs of abnormal behaviour in mice. This study is considered the first time to report the ability of *L. rhamnosus* ethyl acetate extract to reduce and prevent aflatoxin production.

CRediT authorship contribution statement

Aya Abdel-Nasser: Conceptualization, Methodology, Writing-original draft; **Amal Hathout**: Supervision, Methodology, Writing-review & editing; **Ahmed Badr**: Supervision; **Olfat Barakat**: Supervision, Writing-review & editing; **Hayam Fathy**: Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

We wish to thank Prof. Mosad A. Ghareeb, Medicinal Chemistry Department, Theodor Billharz Research Institute, for his valuable help in the brine shrimp lethality bioassay.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.btre.2023.e00799.

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