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Short Communication

ISOLATION AND IDENTIFICATION OF ANTIOXIDANT CONSTITUENTS FROM EXTRACELLULAR METABOLIT ES OF *LACTOBACILLUS BREVIS*

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ABSTRACT

Oxidative stress is a metabolic condition characterized by excessive production of free radicals in the body. The condition leads to metabolic diseases including; aging, diabetes, atherosclerosis, myocardial infarction and cancer. Antioxidants are the mainstay to modulate the consequences of oxidative damage. Secondary metabolites from lactic acid producing bacteria possess antioxidant activity. The present study aimed at isolating and identifying antioxidant metabolites present in cell free supernatant (CFS) of broth culture of *Lactobacillus brevis* MG 000874. Thin layer chromatography (TLC) coupled bioautography of different fractions of methanol extract of CFS using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) model indicated antioxidant activity in ethyl acetate fraction. Fourier transform infrared (FTIR) spectroscopy of the isolated compound revealed the presence of aldehyde and alkyl functional groups. GC-MS analysis led to identification of four active compounds *viz.* a) L-proline, N-valeryl, hexyl ester b) Ergotaman-3',6',18-Trione,12'-Hydroxy-2'-Methyl-5'-(2-Methylpropyl) c) 2-Methylthiolane, S, S-Dioxide and d) Phenol, 3,5-Bis(1,1-Dimethylethyl). Findings of present study may be useful in developing antioxidant drugs from bacterial origin.

Keywords: *Lactobacillus brevis* MG 000874, antioxidant, cell free supernatant, TLC coupled bioautography, GCMS, FTIR.

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INTRODUCTION

Free radicals are the byproducts of metabolic processes and are highly reactive chemical species towards biomolecules present in the cellular environment such as proteins, lipids and DNA causing damage to the cells. Excessive production of free radicals is a metabolic condition known as oxidative stress which causes irreversible damage leading to metabolic diseases such as aging, diabetes, atherosclerosis, myocardial infarction and cancer (Firuzi *et al.* 2011; Amaretti *et al.* 2013; Wu *et al.* 2014). Preventive intervention includes use of antioxidant supplements which modulate the consequences of oxidative damage. Antioxidants may be of synthetic or natural origin; however, natural antioxidants have become more popular than the synthetic antioxidants due to less toxicity (Luo and Fang 2008).

Among natural sources, secondary metabolites of beneficial microbes such as lactic acid bacteria (LAB) have been reported to possess powerful antioxidant activity (Shen *et al.* 2011; Suzuki *et al.* 2013; Noureen *et al.* 2018). The chemical nature of the antioxidants from microbial origin is diverse and is species specific. For instance, the antioxidants separated from marine bacteria and identified through H-NMR as uric acid, indole, 3,4-dimethoxyphenol, and 3-hydroxyindolin-2-1 (Takao *et al.* 1994). In another study, three active antioxidant compounds from *Lactobacillus plantarum viz.* 3-(4-hydroxyphenyl) lactic acid, 3-phenyllactic acid and indole-3-lactic acid were identified by C-NMR (Suzuki *et al.* 2013).

Lactobacillus brevis is a Gram-positive rod-shaped bacterium. It is a microaerophilic, obligatory heterofermentative lactic acid bacterium (LAB). The bacterium is found in humans as normal flora in intestine, vagina and feces. In addition, *L. brevis* has been isolated from plants, cereal products, silage, fermented vegetables and meats, sewage, cow manure, cheese and milk. It is routinely used for the fermentation of food materials and as probiotic (Kunduhoglu and Hacioglu 2020). *L. brevis* MG000874 is a strain isolated from milk of camel and secondary metabolites of the strain have been reported to possess antioxidant activity (Noureen *et al.* 2018; Noureen *et al.* 2019). However, to the best of our knowledge, the chemical nature of the active antioxidants has not been reported. So, the aim of present study was to isolate extracellular antioxidant metabolites secreted by

L. brevis MG000874 and to investigate the chemical nature of the active components.

MATERIALS AND METHODS

Chemicals: Methanol (Sigma), N-hexane (Merck, Germany), chloroform (Daejung, Korea), ethyl acetate (Daejung, Korea), n-butanol (Daejung, Korea), De Man, Ragosa and Sharpe (MRS) agar and broth (Oxoid, England), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma Aldrich), thin layer chromatography plates (Merck, Germany) were purchased from local vendor. All other chemicals used were of analytical grade.

Strain culture: The bacterial strain *L. brevis* (accession no MG000874) deposited in the FCBP, Institute of Agriculture Sciences, University of the Punjab, Lahore, and was assigned the number FCBI-691. *L. brevis* MG000874 strain was grown in MRS broth (pH 6.5±0.2) and incubated at 37°C for 3-5 days in a shaking incubator (Irmeco GmbH, Germany). Culture broth was centrifuged at 6000×g for 10 min. at 4ºC (Refrigerated Centrifuge; Sigma, 2K15) to separate the cell free supernatant (Noureen *et al.* 2018). For purification of metabolites, the cell free supernatant (CFS) was extracted with solvents of different polarity *viz.* n-hexane, chloroform, ethyl acetate and n-butanol. Samples were concentrated at room temperature

Thin layer chromatography coupled bioautography: The presence of secondary metabolites possessing antioxidant property in the extracts was identified through thin layer chromatography (TLC) coupled bioautography using DPPH spray method (Nickavar *et al.* 2014). In brief, the resulting concentrated sample was loaded on silica gel coated plate and eluted it using mobile phase consisting of n-butanol, methanol and water (5:3:2). The developed plates were dried at 37°C for 30 min. to remove traces of solvents. The separated compounds on TLC were visualized under UV light (trans-illuminator, USA) and subsequently with the locating agent (0.1% DPPH) as yellow spots and their R_f values were recorded (Wang *et al.* 2013; De Carvalho *et al.* 2016). Ascorbic acid and gallic acid were used as standard antioxidants.

Preparatory TLC: Preparatory TLC was run on silica coated glass plates using same mobile phase consisting of n-butanol, methanol and water (5:3:2). The antioxidant fraction was scrapped off from TLC plates and sample was isolated from silica using methanol.

High performance liquid chromatography: The active fraction obtained by preparatory TLC was analyzed by HPLC using reverse phase C-18 column (Phenomenex) with UV detector set at 254 nm (Noureen *et al.* 2016). Isocratic Methanol and water (95:5) was used as mobile phase at a flow rate of 1 mL/min, the injection volume was 20 µL for a run time of 15 min.

Fourier Transform Infrared (FTIR) spectroscopy: The chemical nature of antioxidant fraction was determined using FTIR spectroscopy (Hazra *et al.* 2007). Agilent Cary 630 with diamond ATR and Micro-lab analysis software having NIST standard library of organic compounds was used to perform the matching analysis.

Gas Chromatography Mass Spectroscopy (GC-MS): GC-MS analysis of TLC separated extract was carried out by following the method of Hema *et al.* (2010). A Perkin-Elmer Clarus 500GC-MS with Elite1 fused silica capillary column (30 m long × 0.25 mm ID) and helium gas at constant flow rate 1 mL/min was used for chromatography. A sample of 1 µL was injected at injector temperature of 250°C and the ion-source temperature was set at 280°C. The oven was programmed from 110°C (isothermal for 2 min.) with step of 10°C/min to 200°C, then 5°C/min. to 280°C, ending with a 9 min. isothermal at 280°C. For GC-MS detection an EI system with ionizing energy of 70 eV with scan interval of 0.5 seconds and fragments from 45 to 450 Da. Total GC running time was 36 min. The components with retention time ranging from 13 to 35 min. were compared with NIST spectral library.

RESULTS AND DISCUSSION

TLC coupled bioautography of the ethyl acetate fraction (Figure 1) showed antioxidant components at different R_f values. However, maximum activity was exhibited by the band with R_f 0.70.

Figure 1. TLC coupled bioautography of gallic acid (1), ascorbic acid (2), MRS broth (3) and ethyl acetate fraction (4) of methanol extract of extracellular supernatant of *L. brevis* MG000874.

The active components were isolated from TLC and subjected to HPLC, FTIR and GCMS analyses. HPLC analysis (Figure 2) showed a prominent peak at retention time (R_t) 3.23 min. The FTIR spectra (Figure 3) of the active compound exhibited prominent bands at wave numbers 1730 cm^{-1} and 1250 cm^{-1} which correspond to aldehyde and alkyl functional groups, respectively. Previously, an aldehyde (cinnamaldehyde) from natural source (*Cinnamomum verum* bark) have been reported to possess antioxidant activity (Mathew and Abraham 2006; Wondrak *et al.* 2010).

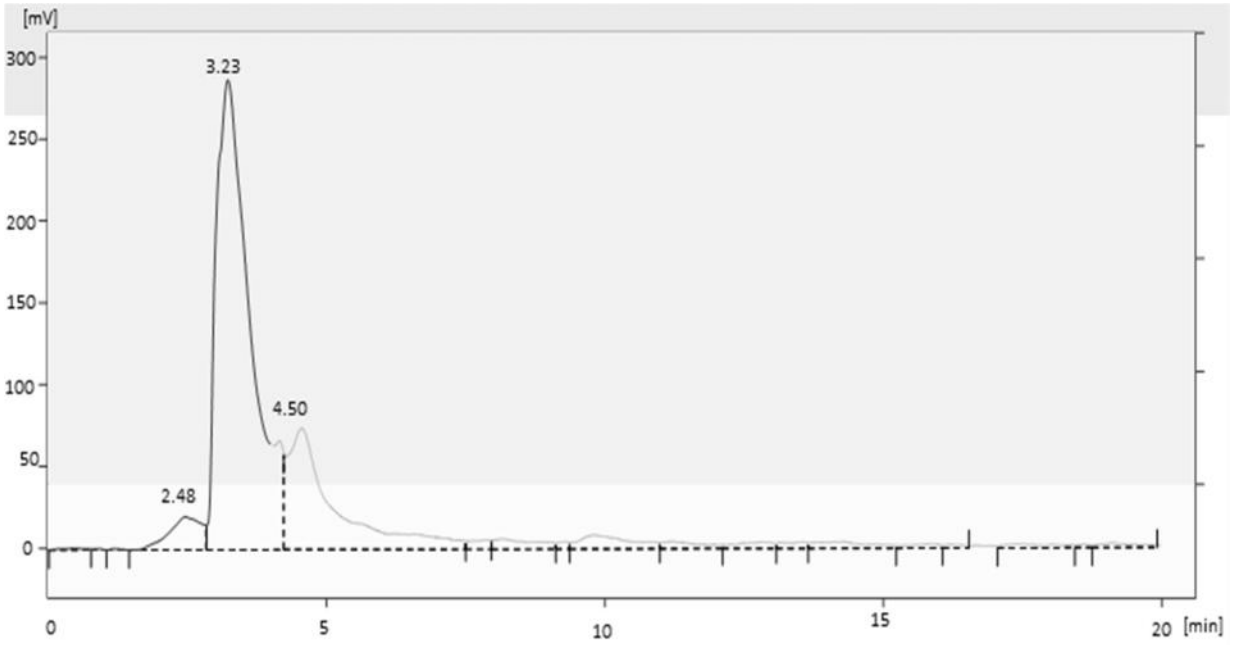

Figure 2. HPLC chromatogram of antioxidant compounds from *L. brevis* MG000874.

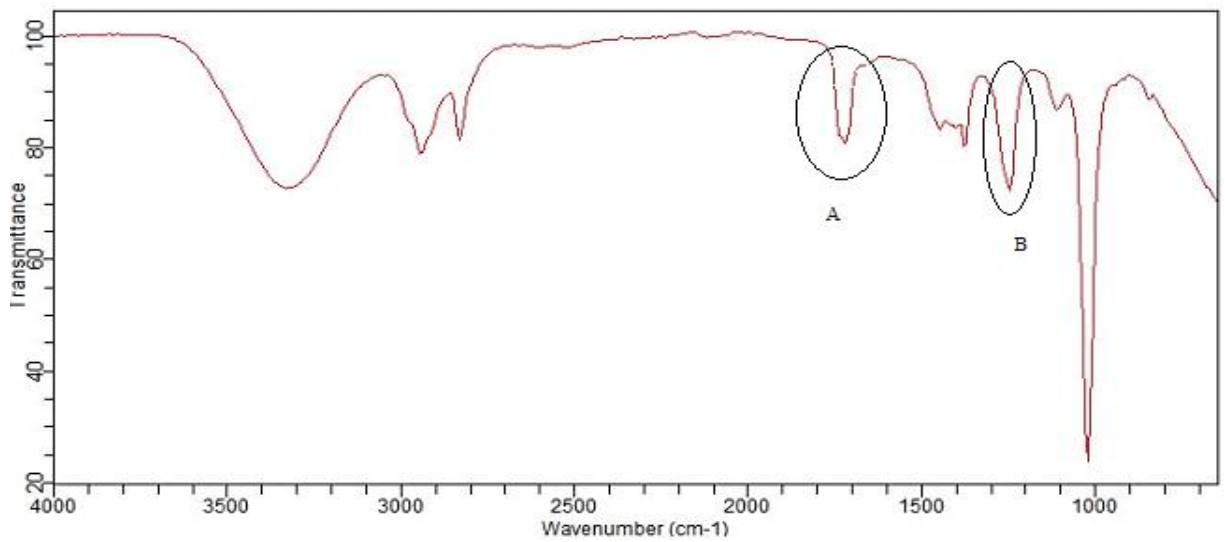

Figure 3. FTIR spectrum showing IR bands corresponding to different functional groups present in antioxidant constituents from *L. brevis* MG000874.

The results from gas chromatography mass spectrometry (GC-MS) spectra (Figure 4) were compared with NIST standard spectral library. Results indicated four compounds (Table 1) in the chromatogram *viz.* a) L-Proline, N-Valeryl, Hexyl Ester b) Ergotaman-3',6',18-Trione,12'-Hydroxy-2'-Methyl-5'-(2-Methylpropyl c) 2-Methylthiolane, S, S-Dioxide and d) Phenol, 3,5-Bis(1,1-Dimethylethyl). These compounds belonged to ester, alkaloid, heterocyclic and phenolic classes of organic compounds.

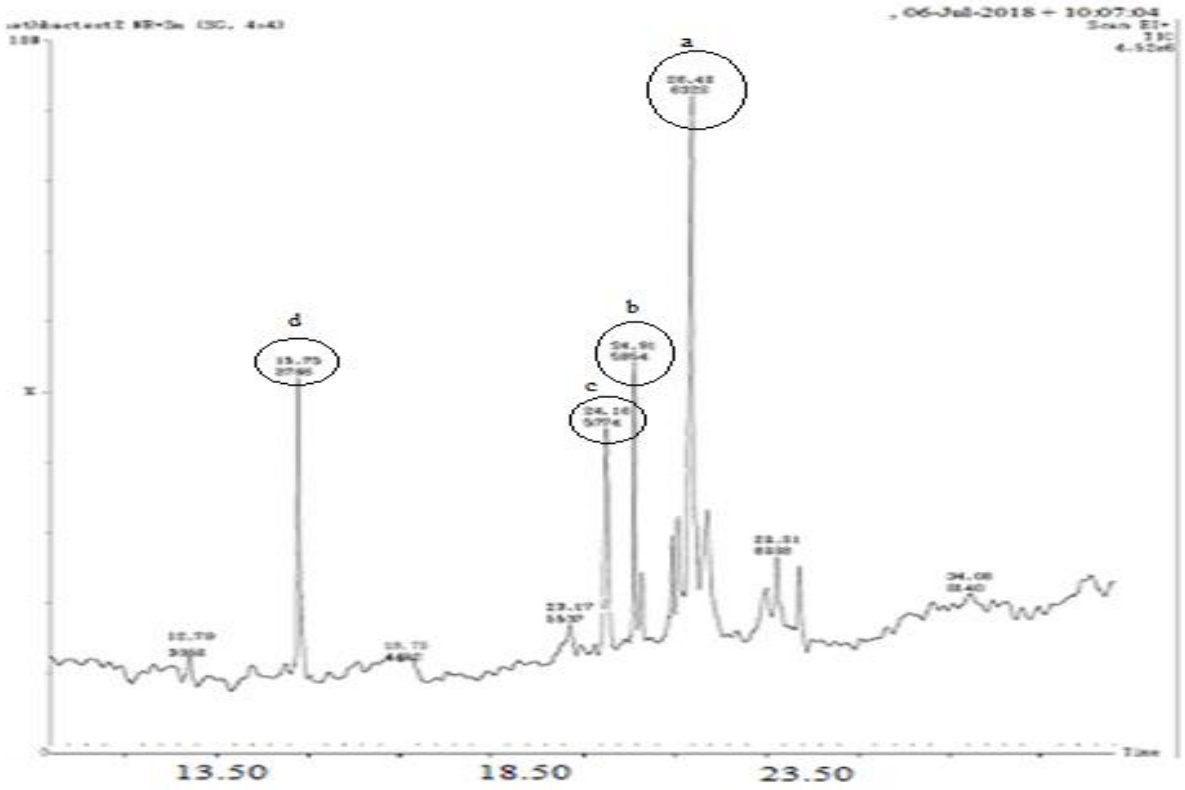

Figure 4. GC-MS chromatograph of antioxidant components from ethyl acetate fraction of methanol extract of extracellular supernatant of *L. brevis* MG000874

Table 1: The antioxidant compounds predicted from the GCMS analysis.

Sr.	R_t	Name of compound	Molecular formula	M.W	Peaks
A	31.6	L-Proline, N-Valeryl-, Hexyl Ester	$C_{16}H_{29}NO_3$	283.4	26.48
B	27.55	Ergotaman-3',6',18-Trione,12'-Hydroxy-2'-Methyl-5'-(2-Methylpropyl)	$C_{33}H_{55}N_5O_5$	581	24.91
C	26.2	2-Methylthiolane, S, S-Dioxide	$C_5H_{10}O_2S$	134	24.16
D	20.25	Phenol, 3, 5-Bis(1,1-Dimethylethyl)	$C_{12}H_{22}O$	206	15.75

An earlier study reported Ergotaman-3', 6', 18-Trione, 12'-Hydroxy-2'-Methyl-5'-(2-Methylpropyl and Phenol, 3, 5-Bis (1, 1-Dimethylethyl) as active antimicrobials from the *Streptomyces sp.* DOSMB-A107 (Baskaran *et al.* 2015). Similarly, cyclic dipeptides have been isolated from broth cultures of *L. brevis* which contained preservative and antifungal activities (Axel *et al.* 2014). This is the first report on the chemical nature of antioxidant compounds isolated from *L. brevis* MG000874. The *L. brevis* MG000874 produces antioxidant metabolites in extracellular supernatant including a) L-Proline, N-Valeryl, Hexyl Ester, b) Ergotaman-3',6',18-Trione,12'-Hydroxy-2'-Methyl-5'-(2-Methylpropyl)- c) 2-Methylthiolane, S, S-Dioxide and d) Phenol, 3,5-Bis(1,1-Dimethylethyl).

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