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Antimicrobial activity screening of isolated flavonoids from *Azadirachta indica* leaves

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Abstract: The antimicrobial activities of two flavonoids, namely genistein 7-*O*--glucoside (**1**) and (–)-epi-catechin (**2**), isolated from *Azadirachta indica* A. Juss (neem) leaves, were evaluated against five fungal species, *viz*: *Alternaria alternata* (Fr.) Keissler, *Aspergillus fumigatus* Fresenius, *Aspergillus niger* van Tieghem, *Macrophomina phaseolina* (Tassi) Goid. and *Penicillium citrii*, and four bacterial species, *viz*. *Lactobacillus* sp., *Escherichia coli*, *Azospirillium lipoferum* and *Bacillus* sp. Six concentrations, *viz*. 100, 300, 500, 700, 900 and 1000 ppm of each of the two flavonoids were employed using malt extract agar medium. All the concentrations of both the test compounds significantly suppressed fungal as well as bacterial growth. The highest concentration (1000 ppm) of both fractions **1** and **2** reduced the growth of the different test fungal species by 83–99 % and 82–95 %, respectively. Compound **1** was highly effective against *Lactobacillus* sp., against which its various concentrations reduced the bacterial growth by 52–99.8 %. Compound **2** was highly effective against *A. lipoferum* and *Bacillus* sp., resulting in 94–100 % and 73–99% reduction in bacterial growth, respectively.

Keywords: antibacterial; antifungal; *Azadirachta indica*; flavonoids; leaves; neem.

INTRODUCTION

Azadirachta indica, a tree of the family Meliaceae, is known as a versatile source of components with bioactive properties. It has great medicinal importance and its chemical constituents possess anti-inflammatory, anti-oxidant, antipyretic, analgesic, immunostimulant, diuretic, hypoglycaemic, cardiovascular, antimicrobial, antiviral, antimalarial and anthelmintic activities.[1–4] Various types of chemical compounds, such as diterpenoids, triterpenoids, polyphenolics, sul-

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phurous, and polyacetate derivatives have hitherto been isolated from differrent parts of this tree.[5,6]

Flavonoids are a major class of oxygen-containing heterocyclic natural products that are widespread in green plants.[7] Generally, they are found as plant pigments in a broad range of fruits and vegetables.[8] These are C_{15} compounds composed of two aromatic rings linked through three carbon bridges with a carbonyl function located at one end of the bridge. Flavonoids were recognized to have a protective effect in plants towards microbial invasion by plant pathogens.[9,10] Flavonoid-rich plant extracts have been used for centuries to treat human disease.[11] Isolated flavonoids were shown to possess a host of important biological activities, including antifungal[12,13] and antibacterial activities.[14] The present study was aimed at investigating the antifungal and antibacterial activities of two flavonoids, isolated from *A. indica* leaves.

EXPERIMENTAL

General procedure

All the reagents and the solvents used in the present study were procured from E. Merck Germany, Fluka Switzerland, BDH Chemicals England and Sigma–Aldrich Chemicals Co., USA. All the employed solvents were of analytical grade. For column chromatography, silica gel 60 (Merck 230–400 mesh) and for TLC silica gel 60F254 (Merck,) were used. Melting points were determined by the sealed capillary method with the help of a Gallenkamp melting point apparatus.

The infrared spectra of the flavonoids in the solid state in KBr discs were recorded on FTIR Shimadzu 4200 spectrophotometer. ^{1}H- and ^{13}C-NMR in $CDCl_3$ and D_2O for compounds **1** and **2,** respectively, were recorded on a Bruker 14.1 T NMR spectrometer, operating at a frequency of 600 MHz. DEPT experiments were performed using polarization transfer pulses of 90 and 135°. The EI-MS spectra were measured with a JEOL JMS-AX 505 HAD mass spectrometer at an anionization voltage of 70 eV.

Procurement of microorganisms

Five fungal strains, namely, *Alternaria alternata*, *Aspergillus fumigatus*, *A. niger*, *Macrophomina phaseolina* and *Penicillium citrii*, and four bacterial species, *viz*. *Lactobacillus* sp., *Escherichia coli*, *Azospirillium lipoferum* and *Bacillus* sp. were procured from the Fungal Culture Bank of the Institute of Mycology and Plant Pathology, University of the Punjab, Lahore, Pakistan.

Extraction and isolation

Fresh leaves of *A. indica* (neem) were collected from the University of the Punjab, Quaid-e-Azam Campus, Lahore, Pakistan, washed with distilled water and air-dried. The leaves (500 g) were soaked in CH_3OH (1 L) for 15 min, to remove chlorophyll, and filtered. The leaves were then cut into small pieces, blended with methanol (750 mL), centrifuged at 2000 rpm for 5 min and the supernatant was evaporated under vacuum at 50 °C. The gummy material (50 mL) thus obtained was diluted with water (1:1), whereby precipitation occurred. These precipitates were removed by filtration, purified by preparative TLC (EtOAc:MeOH, 4:1), and recrystallized with $CHCl_3$:MeOH (98:2), to afford compound **1** (175 mg). To the filtrate, *n*-

-butanol was added (1:1) and the butanol extract was purified by silica gel column chromatography using the solvent system MeOH:$CHCl_3$:H_2O (3:1:1) to yield compound **2** (202 mg).

Antifungal bioassay

The antifungal activities of flavonoids **1** and **2** were evaluated by the poisoned medium technique.[15] Two percent malt extract agar medium was prepared by autoclaving at 121 °C for 30 min. Weighed quantities of compounds **1** and **2** (6, 16, 30, 42, 54 and 60 mg) were dissolved in 0.5 mL of sterilized distilled water, and added to flasks containing 59.5 mL of malt extract agar medium, when still molten, to obtain final concentrations of 100, 300, 500, 700, 900 and 1000 ppm, respectively. The control received the same quantity of distilled water. Twenty millilitres of each medium was poured in each 90 mm-diameter sterilized Petri dishes and the medium was allowed to solidify. Mycelia discs of 5 mm-diameter were prepared from the tips of 5–7 day-old cultures of the five test fungal species with the help of a sterilized cork borer and placed in the centre of each Petri plate. Each treatment was replicated three times. The plates were incubated at 25±2 °C for 7 days. Fungal growth was measured by averaging the three diameters taken at right angles for each colony.

Antibacterial bioassay

The antibacterial activities of the isolated flavonoids were assessed following the produced of Kanwal *et al.*[16] LBA broth medium was autoclaved at 121 °C and cooled to room temperature. Ten millilitres of LBA broth was added to 20 mL culture tubes. To achieve final concentrations of 100, 300, 500, 700, 900 and 1000 ppm, 1, 3, 5, 7, 9 and 10 mg, respectively, of the two flavonoids were added to the LBA broth medium in the culture tubes. The control did not receive the flavonoid solutions. One drop of suspension of each bacterial species was added to each culture tubes. Each treatment was replicated three times. The tubes were incubated at 37 °C for 24 h. Subsequently, the optical density of each suspension was recorded at 630 nm using a spectrophotometer. The greater the optical density of the suspension, the lower was the activity of the compound.

Statistical analysis

All the data were analyzed by analysis of the variance followed by the Duncan multiple range test ($P \leq 0.05$) to separate the treatment means.[17]

RESULTS AND DISCUSSION

Identification of the isolated compounds

*Compound **1***. Greenish powder; m.p. 165–167 °C (decomposed, uncorrected); IR (KBr, cm^{-1}): 3310, 2970, 2940, 1670, 1650, 1620, 1600, 1450, 1300, 1270, 880. ^{1}H-NMR (600 MHz, $CDCl_3$, δ / ppm): 8.12, (1H, *s*, H-2), 6.64 (1H, *d*, *J* = 2.2 Hz, H-6), 6.44 (1H, *d*, *J* = 2.2 Hz, H-8), 7.43 (1H, *dd*, *J* = 8.6 Hz, 2.6 Hz, H-2'), 6.91 (1H, *dd*, *J* = 8.3 Hz, 2.6 Hz, H-3', H-5'), 7.37 (1H, *dd*, *J* = 8.3 Hz, 2.6 Hz, H-6'), 5.00 (1H, d, *J* = 7.5 Hz, H-1"), 3.42–3.55 (2H, *m*, H-2", H-5"), 3.93 (1H, *dd*, *J* = 2.2 Hz, 12.0 Hz, H-6a"), 3.73 (1H, *dd*, *J* = 5.0 Hz, 2.2 Hz, H-6b"). ^{13}C-NMR (150 MHz, $CDCl_3$, δ / ppm): 154.8 (C-2), 122.6 (C-3), 180.3 C-4), 161.5 (C-5), 99.4 (C-6), 162.6 (C-7), 94.5 (C-8), 157.1 (C-9), 106.1 (C-10), 132.0 (C-1'), 130.1 (C-2', C-6'), 115.6 (C-3', C-5'), 162.3 (C-4'), 100.3 (C-1"),

73.4 (C-2”), 76.6 (C-3”), 69.4 (C-4”), 77.2 (C-5”), 60.7 (C-6”). EI-MS (*m*/*z*): 352 (M^+), 305, 245, 184, 170, 153, 125, 97, 108, 107. UV(MeOH, λ_{max} / nm): 270, 221.

*Compound **2***. Off-white powder, m.p. 241–245 °C (uncorrected). IR (KBr, cm^{-1}): 3331, 2923, 1650 1240, 1070, 880. ^{1}H-NMR: (600 MHz, CD_3OD, δ / / ppm): 4.87 (1H, *d*, *J* = 2.4 Hz, H-2), 3.98 (1H, *m*, H-3), 2.85 (1H, *dd*, *J* = 5.4 Hz, 16.2 Hz, H-4α), 2.51 (1H, *dd*, *J* = 4.2 Hz, 16.2 Hz, H-4β), 5.88 (1H, *d*, *J* = = 2.1 Hz, H-8), 6.03 (1H, *d*, *J* = 2.1 Hz, H-6), 6.89 (1H, *d*, *J* = 1.7 Hz, H-2’), 6.77 (1H, *d*, *J* = 7.4 Hz, H-5’), 6.73 (1H, *dd*, *J* = 7.4 Hz, 1.7 Hz, H-6’). ^{13}C-NMR (150 Hz, CD_3OD, δ / ppm): 74.8 (C-2), 71.8 (C-3), 31.4 (C-4), 160.1 (C-5), 96.8 (C-6), 156.1 (C-7), 96.5 (C-8), 155.3 (C-9), 104.0 (C-10), 132.1 (C-1’), 110.1 (C--2’), 145.5 (C-3’) 146.4 (C-4’), 116.0 (C-5’) 115.2 (C-6’). EI-MS (*m*/*z*): 290 (M^+), 245, 227, 170, 153, 126. UV (MeOH, λ_{max} / nm): 280, 212. $[\alpha]^{25}$: 14.9°.

The molecular formula of compound **1** was deduced from elemental analysis results and the EI-MS mass spectrum, which exhibited a (M^+) at *m*/*z* 352. Bands at 3310 (OH), 1670 (C=O) and 1650 and 1600 cm^{-1} (phenyl group) were observed in the IR spectrum. The ^{1}H-NMR spectrum showed a singlet at δ 8.12 ppm (H-2) and δ 154.8 ppm (C-2) characteristic for the isoflavone skeleton.[18] This was further supported by the UV spectrum with λ_{max} at 270 nm. The ^{1}H--NMR data indicated doublets of doublets (*J* = 8.6 and 2.6 Hz) at 7.43 (H-2’), 6.91 (H-3’ and H-5’) and 7.37 (H-6’), showing the presence of 4’–OH on ring B of the isoflavonoid. The OH at C-7 was not free as UV with NaOAc failed to produce any bathochromic shift.[19] The proton NMR resonance of an anomeric carbon at 5.00 (1H, *d*, *J* = 7.5 Hz, H-1”) suggests the glucose moiety is in the β-configuration. The ^{13}C-NMR upfield signals of C-8 and C-6 and the downfield resonance of C-7 indicate that the glucose moiety was attached to that of C-7. These data suggested compound **1** was (genistein 7-*O*-glucoside). This compound was previously isolated from *Trifolium subterraneum* with a feeding deterrent activity for red-legged earth mite (*Halotydeus destructor*).[20]

Compound **2** was positive to butanol/HCl and vanillin/HCl reagents. The UV spectrum (MeOH) showed absorbance maxima at 280 and 212 nm. Its EI-MS *m*/*z* of 313 $(Na+M)^+$ indicated a monomeric unit of *m*/*z* of 290. Bands at 3331, 2923 and 1650 cm^{-1} were observed in the IR spectrum. The ^{1}H-NMR data suggested that the compound was epicatechin, which was further supported by ^{13}C-NMR signals, especially at 74.8 (C-2) and 71.8 (C-3).[21] This compound was previously isolated from *Adansonia digitata*.[22]

Antifungal activity

Analysis of the variance showed that there was a highly significant difference ($P \leq 0.001$) in the antifungal activity of the two tested flavonoids (*F*). Similarly, the effect of concentration (*c*) as well as the response of various fungal

species (*S*) was also significant. The interactive effects of *F*×*S*, *F*×*c*, *S*×*c* and *F*×*S*×*c* were also significant (Table I).

TABLE I. Analysis of variance for the effect of different concentrations of two flavonoids isolated from neem, against different fungal species

Sources of variation	*df*	*SS*	*MS*	*F* values[a]
Treatments	69	128318	1860	529
Flavonoids (*F*)	1	203	203	58
Fungal species (*S*)	4	3273	818	233
Conc. (*c*)	6	122594	20432	5816
F×*S*	4	659	165	47
F×*c*	6	107	17.9	5
S×*c*	24	862	35.9	10
F×*S*×*c*	24	621	25.9	7
Error	139	492	3.5	–
Total	209	128809	–	–

[a]Significant at $P \leq 0.001$

In general, at all the tested concentrations, both flavonoids significantly reduced the growth of all the five test fungal species. There was a gradual reduction in fungal colony diameter as the concentration of the compounds was increased from 100 to 1000 ppm. Compound **1** was most effective against *A. fumigatus*, where the highest tested concentration of this compound significantly reduced the fungal colony diameter by 95 %. The highest concentration of this compound also resulted in a 91, 85, 82 and 94 % reduction of the colony diameter of *A. alternata*, *A. niger*, *M. phaseolina* and *P. citrii*, respectively (Fig. 1). Compound **2** was highly effective against *P. citrii*, *A. alternata* and *A. fumigatus*, where its highest concentration resulted in significant reductions of 94, 90 and 95 % in the fungal colony diameters, respectively. *A. niger* and *M. phaseolina* were found comparatively less susceptible to **2**, where the highest tested concentration of this compound resulted in only a 78 and 80 % suppression of the colony diameters, respectively.

Antibacterial activity

Analysis of variance showed that there was a highly significant difference ($P \leq 0.001$) in the antibacterial activities of the two tested flavonoids (*F*). Similarly, the effects of concentration (*c*) as well as the response of the various bacterial species (*B*) were also significant. The interactive effects of *F*×*B*, *F*×*c*, *B*×*c* and *F*×*B*×*c* were also significant (Table II).

In general, all the tested concentrations of both isolated flavonoids significantly suppressed the growth of all the four-targeted bacterial species. However, the activities of the two flavonoids varied with the bacterial species involved (Fig. 2).

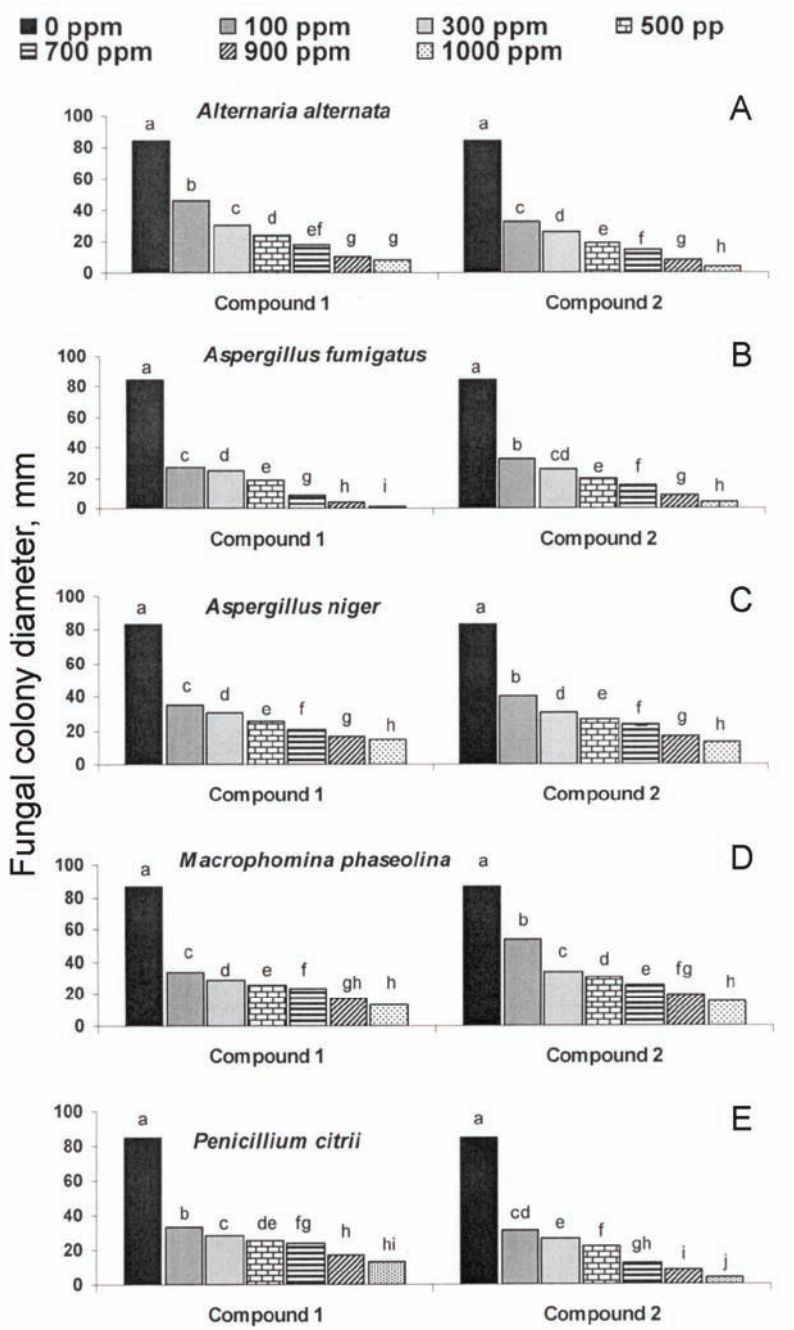

Fig. 1. Effect of different concentrations of the two flavonoids on the *in vitro* growth of fungi. Bars with different letters show significant difference ($P \leq 0.05$), as determined by the Duncan multiple range test. Compound **1**: genistein 7-*O*-glucoside, compound **2**: (–)-epi-catechin.

TABLE II. Analysis of variance for the effect of different concentrations of two flavonoids isolated from neem, against four bacterial species

Sources of variation	*df*	*SS*	*MS*	*F* values[a]
Treatments	55	52	0.93	4627
Flavonoids (*F*)	1	1.88	1.87	9256
Bacterial species (*B*)	3	5.35	1.78	8802
Conc. (*c*)	6	38.43	6.40	31593
F×*B*	3	2.85	0.95	4694
F×*c*	6	1.14	0.19	940
B×*c*	18	0.74	0.04	202
F×*B*×*c*	18	1.21	0.067	330
Error	112	0.023	0.0002	–
Total	167	52	–	–

[a]Significant at $P \leq 0.001$

Compound **1** was highly effective against *Lactobacillus* sp., where its various concentrations (100–1000 ppm) reduced the bacterial growth by 52–99.8 % (Fig. 2A). This compound was comparatively less effective against the other bacterial species, resulting in 40–92 %, 31–95 % and 39–91 % reduction in the growth of *E. coli*, *A. lipoferum* and *Bacillus* sp., respectively (Figs. 2B–2D). Compound **2** exhibited comparatively higher antibacterial activities compared to those of compound **1**. *A. lipoferum* was the bacterial species most susceptible to compound **2**, where 900 and 1000 ppm completely arrested bacterial growth (Fig. 2C). This compound was also highly toxic to *Bacillus* sp., where its different concentrations suppressed bacterial growth by 73–99% (Fig. 2D). Compound **2** was found to be comparatively less effective against *Lactobacillus* sp. and *E. coli*, where it suppressed bacterial growth by 59–96 % and 45–83 %, respectively (Figs. 2A and 2B). Recently, similar antibacterial activities were reported for other flavonoids isolated from different plant species.[14,23,24] Reports of activity in the field of antibacterial flavonoid research are widely conflicting, probably owing to inter- and intra-assay variation in the susceptibility testing.[11] Various antibacterial mechanisms of action of different flavonoids have were proposed, including inhibition of nucleic acid synthesis,[25] inhibition of cytoplasmic membrane function,[26] and inhibition of energy metabolism.[27]

From the results of the present study, it can be concluded that both the tested neem flavonoids possess antifungal as well as antibacterial activities. The antimicrobial activities of these natural compounds may be further enhanced by some structural modifications to use these compounds as commercial pesticides, especially against *M. phaseolina*. This soil-borne phytopathogenic fungal species attacks about 400 plant species, including such crops as soybean, sunflower, maize and sorghum,[28] but there are no registered fungicides to control it.

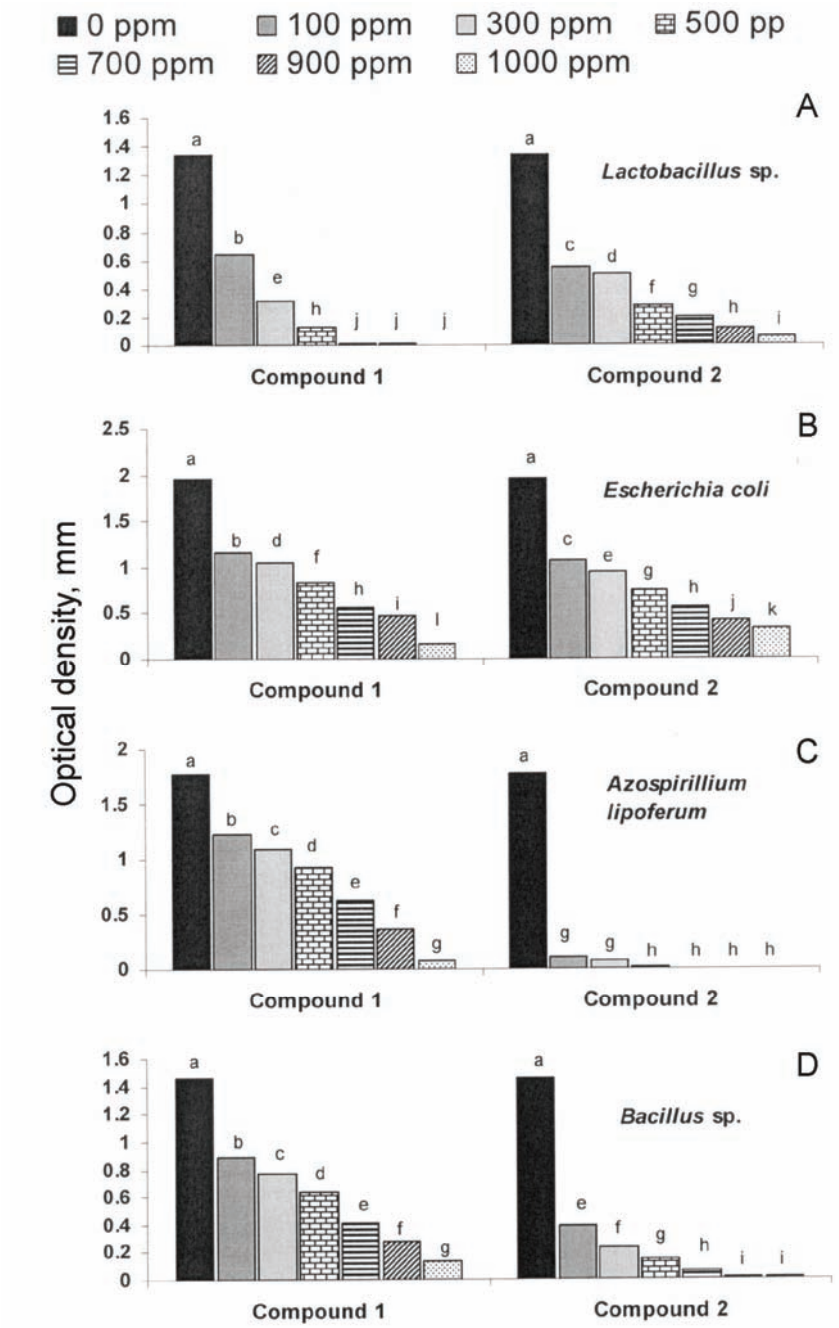

Fig. 2. Effect of different concentrations of two flavonoids on the *in vitro* growth of bacteria. Bars with different letters show significant difference ($P \leq 0.05$) as determined by the Duncan multiple range test. Compound **1**: genistein 7-*O*-glucoside; Compound **2**: (–)-epi-catechin.

ИЗВОД

АНТИМИКРОБНА АКТИВНОСТ ФЛАВОНОИДА ИЗОЛОВАНИХ ИЗ ЛИШЋА *Azadirachta indica*

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Антимикробна активност два флавоноида, генистеин-7-*O*-глукозида (**1**) и (–)-епи-катехина (**2**), изолованих из лишћа *Azadirachta indica* A. Juss тестирана је спрам пет врста гљива: *Alternaria alternata* (Fr.) Keissler, *Aspergillus fumigatus* Fresenius, *Aspergillus niger* van Tieghem, *Macrophomina phaseolina* (Tassi) Goid. и *Penicillium citrii*, и четири врсте бактерија: *Lactobacillus* sp., *Escherichia coli*, *Azospirillium lipoferum* и *Bacillus* sp. Коришћене су следеће концентрације сваког од флавоноида у подлози хранљивог агара: 100, 300, 500, 700, 900 и 1000 ppm. Оба флавоноида, у свим примењеним концентрацијама, су значајно инхибирала раст гљива и бактерија. Највећа концентрација (1000 ppm) једињења **1** и **2** смањила је раст различитих врста гљива за 83–99 %, односно 82–95 %. Једињење **1** је врло ефикасно смањило раст бактерија *Lactobacillus* sp. за 52–100 %, у зависности од конецентарције. Једињење **2** је било ефикасно спрам бактерија *A. lipoferum* и *Bacillus* sp., смањујући њихов раст за 94–100 %, односно 73–99 %.

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