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PHYTOCHEMICAL AND ANTIMICROBIAL SCREENING OF LEAVES AND STEMS OF BALKAN ENDEMIC SPECIES *DAPHNE MALYANA* BLEČIĆ

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ABSTRACT

Daphne malyana Blečić (Thymelaeaceae) is an endemic species of the western part of the Balkan Peninsula. No previous detailed studies exist on D. malyana Blečić. The aim of this study was to provide information on the anatomy of the leaf and stem as well as to perform phytochemical screening and to assay the antimicrobial activity of methanol extracts of the leaves and stems of D. malyana Blečić. The phytochemical analysis showed that coumarins and flavonoids are the major classes of secondary metabolites in both leaves and stems. The antimicrobial activity of the methanol extracts from leaves and stems of D. malyana was also evaluated against six Gram-positive and six Gram-negative bacteria by employing both microdilution and disc diffusion methods. The results from the well-diffusion assay showed significant antimicrobial activity at all tested concentrations of the extracts. The results showed that methanol extracts of leaves and twigs have similar chemical compositions and similar antimicrobial activity. To our knowledge this is the first report on the phytochemistry and antimicrobial activity of this plant species.

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Keywords: *Daphne malyana* Blečić, anatomy, coumarins, flavonoids, antimicrobial activity

Introduction

The Balkan endemic relict species *Daphne malyana* Blečić belongs to the family Thymelaeaceae (3), section Daphnanthes C.A. Mayer, subsection Oleoides (13) and was first recorded and described from limestone rocks in the canyon of the Piva River in Montenegro (2). The presence of the species *D. malyana* has recently been recorded also in Serbia (27). The plant grows in rock crevices of inaccessible limestone cliffs as a typical chasmophyte (11).

Representatives of the *Daphne* genus have been of interest on account of their notable medicinal value. Extracts of some of *Daphne* species are widely used for treating cancers, and various compounds isolated from these plants have shown cytotoxic, antimicrobial and antileukemic activities (14, 15, 18). Different classes of secondary metabolites have been isolated from species belonging to this genus including flavonoids, coumarins, and diterpenoids (1, 23, 25, 26, 29). Previous studies have shown that coumarins daphnetin, daphnin, daphnoretin, umbelliferon, and 4-hydroxy-coumarin exhibited good antimicrobial activity (4, 5, 7, 8).

The aim of this study was to provide information on the anatomy of the leaf and stem as well as to perform phytochemical screening and to assay the antimicrobial activity of methanol extracts of the leaves and stems of *D. malyana*. To the best of our knowledge, we present here for the first time information about the chemical and antimicrobial properties of this endemic species.

Materials and Methods

Collection and identification of plant sample

The plant *Daphne malyana* Blečić was collected from Mt Tara (Vranjak – Zaovine), in southwest Serbia (UTM CP 75) during the flowering season in May 2008. The collected plant material was either placed in the herbarium or fixed in 50% ethanol. A voucher specimen was deposited in the Herbarium of the Institute of Botany and Botanical Garden "Jevremovac", Faculty of Biology in Belgrade, Serbia (BEOU).

Morpho-anatomical analysis

Anatomical analyses of leaves and stems were done on permanent slides prepared by the standard method for light microscopy. Cross-sections of the leaves (50 samples) and stems (30 samples) were cut on a Reichert sliding microtome (up to 10 mm thick). All morpho-anatomical measurements were done with the microscope Leica DM 2500-Leica DFC490-Leica Qwin Standard (Leica Microsystem, Germany).

Preparation of plant extracts

The plant material (leaves and stems) was air dried at room temperature (26 °C) for two weeks, and after that the dried material was separately grinded to a uniform powder. The methanol extract of leaves was prepared in a Soxhlet apparatus using 20 g of the material in 200 ml of solvent for 2 h. The extract from stems was prepared by the same procedure. Both extracts were filtered through a Whatman filter paper No 42

(125 mm) and concentrated *in vacuo* using a rotary evaporator. The extracts were used for chemical and antimicrobial analysis.

Phytochemical screening

Phytochemical screening was performed using standard procedures (24).

Anthraquinones were assayed by the following procedure: 0.5 g of the extract was boiled with 10 ml of sulphuric acid (H_2SO_4) and filtered while hot. The filtrate was shaken with 5 ml of chloroform. The chloroform layer was pipetted into another test tube and 1 ml of dilute ammonia was added. The resulting solution was observed for color changes.

Coumarins were assayed as follows: 0.2 g of plant material was shacked with 5 ml of 70% methanol and filtered. A few drops of 1 M NaOH were added in the filtrate. The solution was then exposed to UV light and the presence of coumarins was indicated by the fluorescence.

Terpenoids were assayed by adding 2 ml of chloroform to 0.5 g of the extract; then concentrated H_2SO_4 (3 ml) was carefully added to form a layer. The presence of terpenoids was indicated by a reddish brown coloration of the interface.

Flavonoids were assayed using three methods. First, dilute ammonia (5 ml) was added to a portion of an aqueous filtrate of the extract. After that, concentrated sulphuric acid (1 ml) was added. The presence of flavonoids was indicated by a yellow coloration that disappears on standing. Second, a few drops of 1% aluminum solution were added to a portion of the filtrate. The presence of flavonoids was indicated by a yellow coloration. Third, a portion of the extract was heated with 10 ml of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution. The presence of flavonoids was indicated by a yellow coloration.

Alkaloids were assayed by diluting 0.5 g of the extract to 10 ml with acid alcohol, boiled and filtered. To 5 ml of the filtrate were added 2 ml of dilute ammonia. 5 ml of chloroform were added and shaken gently to extract the alkaloidal base. The chloroform layer was extracted with 10 ml of acetic acid. This was divided into two portions. Mayer's reagent was added to one portion and Draggendorff's reagent to the other. The formation of a cream (with Mayer's reagent) or reddish brown precipitate (with Draggendorff's reagent) was regarded as positive for the presence of alkaloids.

High-performance liquid chromatography (HPLC)

HPLC was used for determination of secondary metabolites. HPLC analysis was carried out on Agilent 1200 Series HPLC instrument with: C18 column (C18; 25 cm × 4.6 mm, 10 m) and UV spectophotometric detector as described previously by Manojlovic et al. (19). Acetonitrile-water-phosphoric acid (90:10:0.1, v/v/v) was used as a solvent system because it provides excellent separation of peaks in chromatograms. Acetonitrile was of HPLC grade, and was purchased from Merck (Darmstadt, Germany). Phosphoric acid was analytical grade reagent. Deionized water used throughout the experiments was generated by a Milli-Q academic water purification system (Milford, MA, USA). The sample injection volume was 10 µl. The flow rate was 1.0 ml/min. The constituents of the extracts were analyzed on the basis of their retention times and absorption spectra (200 – 400 nm).

Antimicrobial activity

The antimicrobial activity of the tested extracts was evaluated using laboratory control strains: *Clostridium perfringens* ATCC 19404, *Bacillus subtilis* ATCC 6633, *Listeria innocua* ATCC 33090, *Staphylococcus aureus* ATCC 6538, *Sarcina lutea* ATCC 9341 and *Micrococcus flavus* ATCC 40240 (Gram-positive bacteria), *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella enteritidis* ATCC 13076, *Shigella sonnei* ATCC 25931, *Klebsiella pneumoniae* ATCC 10031 and *Proteus vulgaris* ATCC 8427 (Gram-negative bacteria) obtained from the American Type Culture Collection. The inocula of the bacterial strains were prepared from overnight broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity (corresponding to 10^7-10^8 CFU/ml, depending on genera – consensus standard by the National Committee for Clinical Laboratory Standards, NCCLS).

Well-diffusion assay

Preliminary antimicrobial tests were carried out by a modified disc diffusion method using 100 µl of bacterial suspension spread on Mueller-Hinton agar (20 ml) in sterilized Petri dishes (90 mm in diameter). The tested extracts were diluted in methanol to the test concentration (10.0, 5.0 and 2.5 µg/well). With a metal borer, wells (5 mm in diameter) were made in the inoculated agar and 50 µl of the extract dilution was added to each well. The Petri dishes were incubated for 24 h at 37 °C. Reference antibiotic, tetracycline (30 µg/disc) served as a positive control, while the solvent (methanol – 50 µl/well) was used as a negative control. It was found that the solvent showed no inhibitory activity. All the tests were performed in triplicate. Antibacterial activity was evaluated by measuring the zone of inhibition (in mm) against the test bacterial strains.

Broth microdilution method

A broth microdilution method was used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) according to the NCCLS (21). The inocula of the bacterial strains were prepared from overnight broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. Serial doubling dilutions of the tested extracts were prepared in a 96-well microtiter plate over the range of 22.00 - 0.02 µg/ml in inoculated nutrient broth (the final volume was 100 µl and the final bacterial concentration, 10^6 CFU/ml in each well). The plate was incubated for 24 h at 37 °C. All experiments were performed in triplicate. Two growth controls consisting of medium with solvent (negative control) and medium with tetracycline (positive control) were also included. The microbial growth was determined by absorbance at 620 nm using the universal microplate reader

(ThermoLabsystems, Multiskan EX, Software for Multiscan ver.2.6.). MIC was defined as the lowest concentration of tested extracts at which microorganisms showed no visible growth. In order to determine MBC, broth was taken from each well without visible growth and inoculated on Mueller Hinton agar (MHA) for 24 h at 37 °C. The MBC is defined as the lowest concentration of the extract dilution at which 99.9% of the inoculated microorganisms were killed.

Statistical analysis

Analysis of variance (ANOVA) was used to determine the significance ($P \leq 0.05$) of the data obtained in all experiments. All results were determined to be within the 95.0% confidence level for reproducibility.

Results and Discussion

Leaf morphology and anatomy

Leaves of *Daphne malyana* are simple, small, slightly curved toward the adaxial side at the edges, leathery, with a short stem or sessile. On the surface of the leaves there are some mechanical, unicellular hairs, with denser distribution around the main nerve (**Fig. 1**). Hairs are wider at the base and tapering toward the top. The hairs are always at the abaxial side of the leaves.

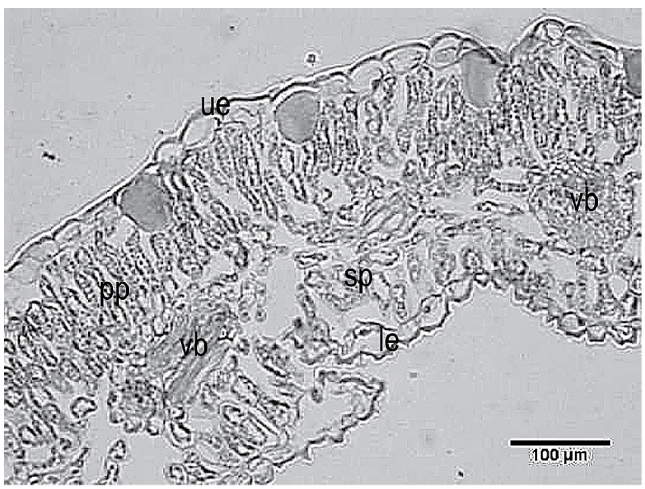


Fig. 1. Cross section of the leaves of *Daphne malyana*: ue: upper epidermis; pp: palisade parenchyma; sp: spongy parenchyma; le: lower; vb: vascular bundle.

As seen in Fig. 1, the leaves have a dorsiventral structure. The cross-sections of leaves show a single layer of epidermis, with a well-developed, thickened and strongly wrinkled cuticle both on the abaxial and adaxial sides of the leaf. These leaves have a hypostomatic character. The stomata are anomocytic, much indented and therefore at the level of the spongy parenchyma cells. The mesophyll has differentiated palisade and spongy tissue and a number of vascular bundles. The palisade tissue is composed of short, densely packed cells distributed in two or three, rarely even four layers. The mesophyll also includes rare sclerenchymatous idioblasts. The spongy tissue is composed of more or less globular cells, separated by large intercellular space. The strongly developed xeromorphic characteristics include reduced leaf size, thick wrinkled cuticle, mucous epidermis, thickened outer wall of epidermal cells, straight anticlinal walls of epidermal cells and strongly indented stomata (6).

Stem morphology and anatomy

The dwarf bush of *D. malyana* reaches over 30 cm in height, with numerous, short and wrinkled branches and twigs, bearing lump-like leaf scars. The stems are round in cross section, with a single layer of epidermis with thickened walls and well-developed cuticle, as well as the partial formation of peridermis (**Fig. 2**). The thick primary bark is formed by two or three layers of collenchyma and about a dozen layers of parenchyma cells, globular to elongated-oval in shape. The vascular tissue commonly forms (more or less complete) cylinder between the stem cortex and the pith. Pith parenchyma was compact, composed of parenchyma cells.

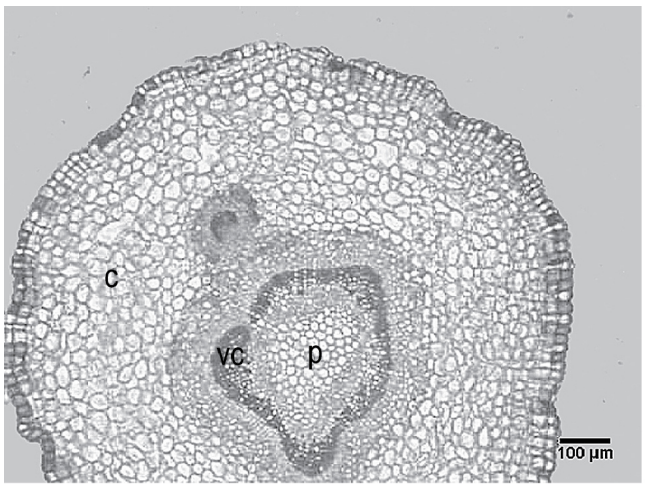


Fig. 2. Cross section of the stem of *Daphne malyana*: c: stem cortex; vc: vascular cylinder; p: pith.

The anatomical and micromorphological characteristics of leaves and stems in the studied population of *Daphne malyana* Blečić indicating the stable xeromorphic features that are also characteristic for other endemic plants of the Balkan Peninsula, such as the species *Satureja horvatii*, *Teucrium*, *Fibigia*, *Degenia* (11, 17, 20, 28).

Phytochemical studies

The preliminary phytochemical screening by color reactions of the leaves and twigs of the studied plant showed the presence of flavonoids and coumarins. *D. malyana* methanol extracts of the leaves and stems showed the absence of anthraquinones and the extracts were negative for the presence of alkaloids.

Fig. 3 shows the HPLC chromatograms of the methanol extracts of the leaves and stems of *D. malayana* and selected UV spectra typical for coumarins and flavonoids. Thus, coumarins and flavonoids were the major secondary metabolites presented in the methanol extracts. Peaks 1-7 in both chromatograms (A and B) are the same compounds, since the retention times of these peaks in chromatogram A are very similar with the retention times for the corresponding peaks in chromatogram B, and the UV spectra of these peaks are the same.

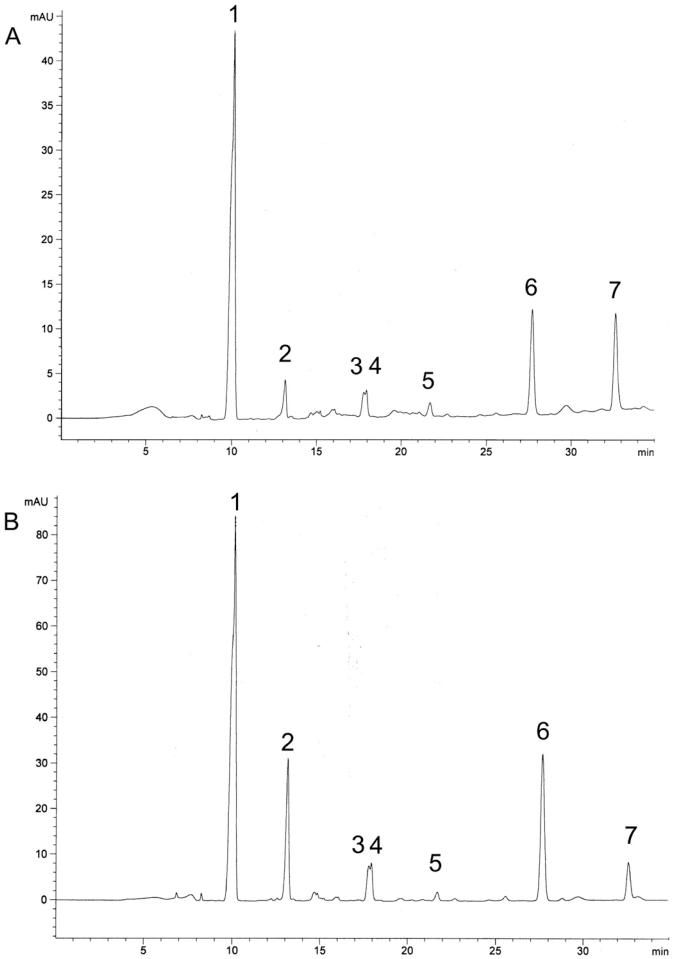


Fig. 3. HPLC chromatograms of the leaves (**A**) and twigs (**B**) of *Daphne malyana* methanol extract. Wavelength detection at 320 nm. Peaks 2, 3, 4, 6 and 7 were characterized as coumarins, and 1 and 5, as flavonoids.

Chromatograms show the presence of five coumarin compounds. The t_R values for these coumarins 2, 3, 4, 6 and 7 amounts to 13.15, 17.80, 17.95, 27.67 and 32.63 min, respectively. The compounds 3 and 4 have very similar t_R values and UV spectra, indicating that their structures are very slightly different.

In addition to these coumarin compounds, two flavonoids 1 and 5 (t_R = 10.14 and 21.71 min) were also detected in the extracts. From the chromatogram, flavonoid 1 was the most abundant secondary metabolite in both extracts with a wavelength of maximum absorption of the peak being 320 nm (**Fig. 4**).

The results showed that methanol extracts of leaves and twigs have a similar chemical composition. The amount of flavonoid 5 was higher in the stem extract than in the extract from leaves. On the other hand, the amounts of coumarins 6 and 7 were higher in the extract from leaves, than in the stem extract. Both classes of compounds were identified by their specific UV absorption spectra recorded in the range of 200 to 400 nm.

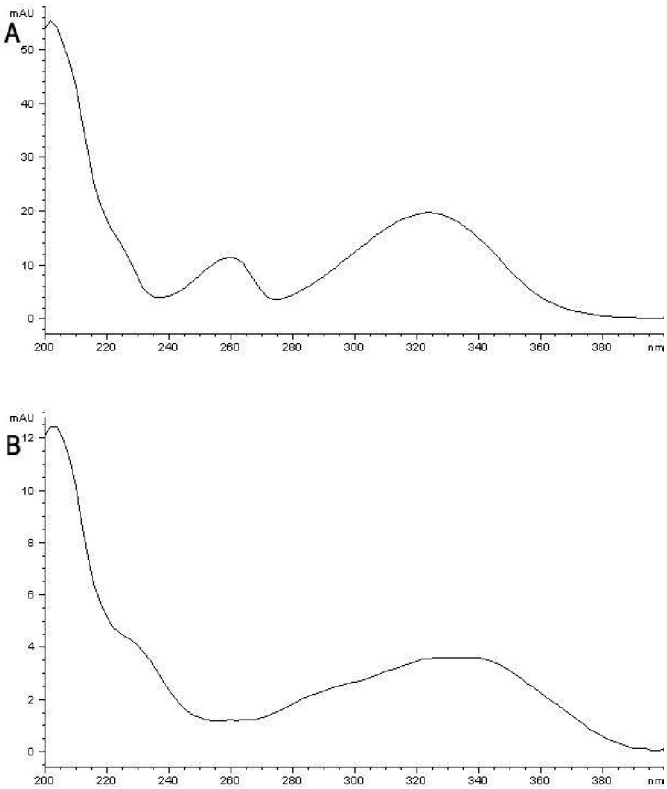


Fig. 4. Typical UV spectra of coumarins (**A**) (t_R = 10.14 min) and flavonoids (**B**) (t_R = 17.95 min).

Antimicrobial activity

The results obtained by the well-diffusion assay showed significant antimicrobial activity at all tested concentrations of the extracts (**Fig. 5**). Also, the inhibition zones were concentration dependent and were proportionally larger. Significant difference between the activities of the leaf methanol and stem methanol extracts was not detected. The solvent (methanol) showed no inhibitory activity, while the antibiotic exhibited much higher antimicrobial activity when compared with the extracts. The most sensitive strains were *K. pneumoniae* ATCC 10031 and *P. vulgaris* ATCC 8427.

The minimal inhibitory/bactericidal concentrations were in the range from 2.75 – 22.0 µg/ml, which can be considered as high activity in comparison with the reference antibiotic which was active in the range from 1.37 – 11 µg/ml (**Fig. 6**). The leaf methanol extract exhibited slightly higher activity than the stem methanol one. The most susceptible strains were *S. enteritidis* ATCC 13076, *K. pneumoniae* ATCC 10031, *P. vulgaris* ATCC 8427, *S. lutea* ATCC 9341 and *M. flavus* ATCC 40240, while *Sh. sonnei* and *L. innocua* showed the lowest sensitivity to the presence of the tested extracts.

The results of the microbiological screening indicated good antimicrobial potential of the extracts of *D. malyana*. It could be supposed that flavonoids might be responsible for this activity. This is supported by the overall poor activity shown by pure coumarins (22), although in earlier studies some antibacterial inhibitory effects have been found for xanthotoxin, herniarin,

umbelliferone and scopoletin, and antifungal activity for umbelliferone, scopoletin and coumarin (9, 10, 12, 16).

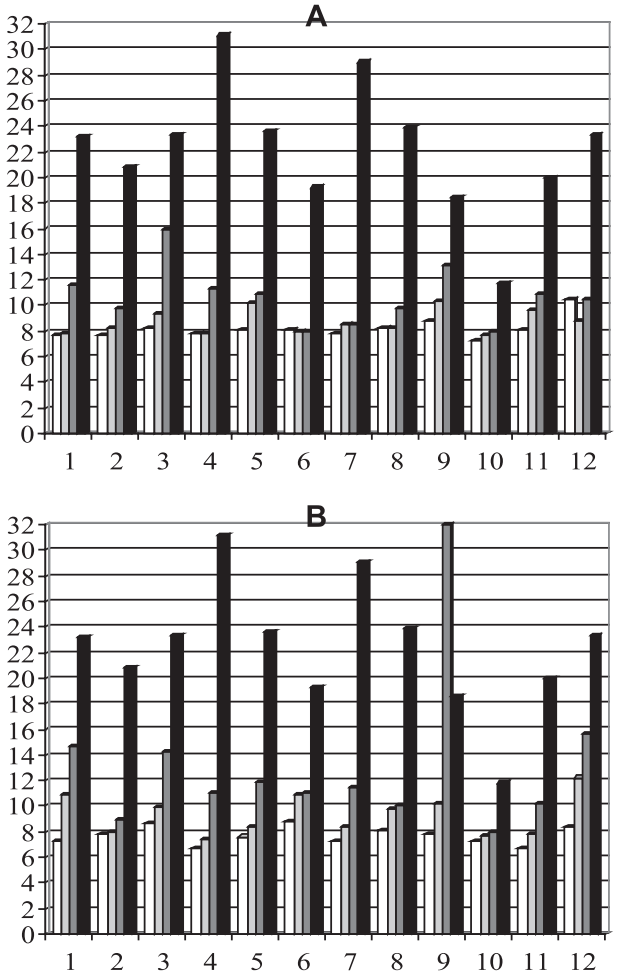
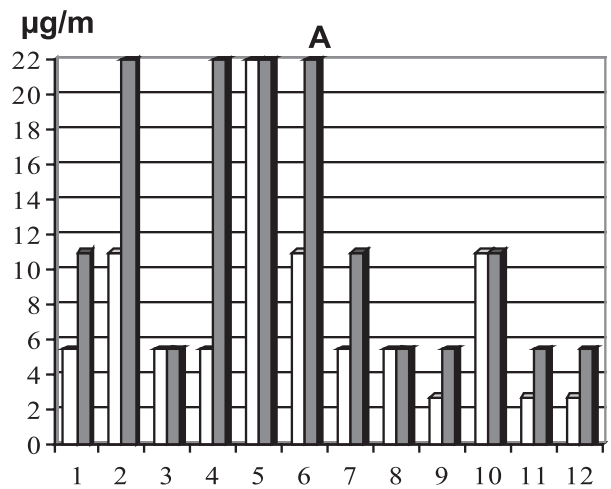


Fig. 5. Antimicrobial activity (zone of inhibition in mm) of *D. malyana* leaf (**A**) and stem methanol extract **(B)**, against six Gram-negative bacteria: *Escherichia coli* (**1**), *Pseudomonas aeruginosa* (**2**), *Salmonella enteritidis* (**3**), *Shigella sonnei* (**4**), *Klebsiella pneumoniae* (**5**) and *Proteus vulgaris* (**6**), and six Gram-positive bacteria: *Clostridium perfringens* (**7**), *Bacillus subtilis* (**8**), *Staphylococcus aureus* (**9**), *Listeria inoccua* (**10**), *Sarcina lutea* (**11**) and *Micrococcus flavus* (**12**); 10.0 μg/well (□), 5.0 μg/well (▪), 2.5 μg/well (■); antibiotic tetracycline (30 μg/well) (■).



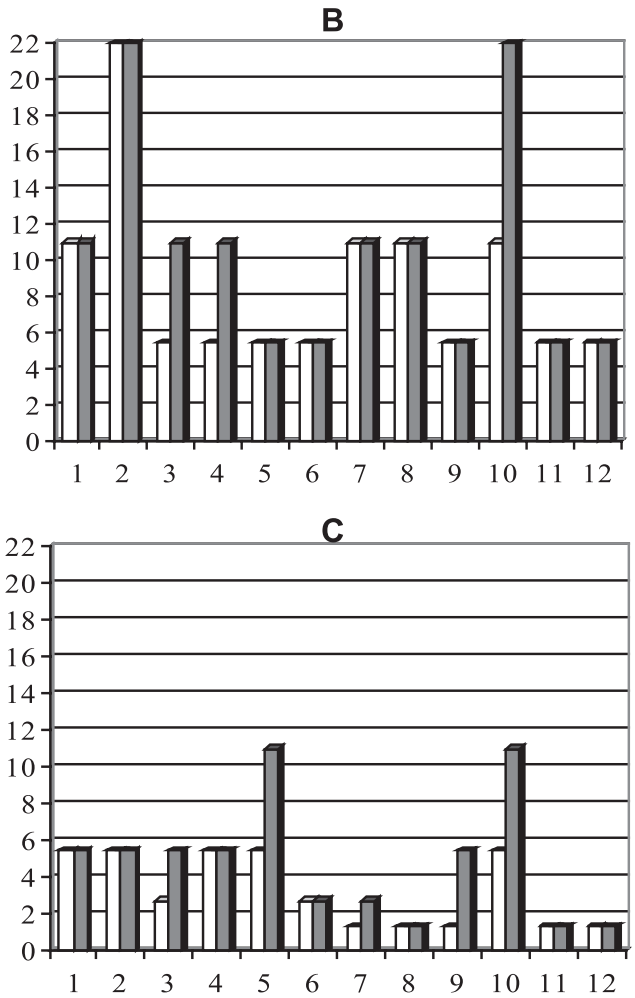


Fig. 6. Antimicrobial activity (MIC/MBC in mg/ml) of *D. malyana* leaf methanol extract (**A**), stem methanol extract (**B**) and reference antibiotic tetracycline (**C**), against six Gram-negative bacteria: *Escherichia coli* (**1**), *Pseudomonas aeruginosa* (**2**), *Salmonella enteritidis* (**3**), *Shigella sonnei* (**4**), *Klebsiella pneumoniae* (**5**) and *Proteus vulgaris* (**6**), and six Gram-positive bacteria: *Clostridium perfringens* (**7**), *Bacillus subtilis* (**8**), *Staphylococcus aureus* (**9**), *Listeria inoccua* (**10**), *Sarcina lutea* (**11**) and *Micrococcus flavus* (**12**); MIC (□), MBC (■).

Further research will be focused on the identification and antimicrobial activity of the isolated metabolites of this plant.

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