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Bioactivity of *Myrtus communis* from the Montenegro coastline

[Bioactividad de *Myrtus communis* del litoral de Montenegro]

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Abstract: Although numerous studies have demonstrated the biomedical potential of *Myrtus communis* L., (Myrtaceae) data on myrtle from Montenegro are scarce. To evaluate antioxidant, antimutagenic and antibacterial activity of myrtle methanolic extracts. Antioxidant activity was evaluated by measuring free radicals scavenging activity, reducing power and enzyme inhibition. The strongest scavenging activity was towards DPPH radical (2,2-diphenyl-1-picrylhydrazyl) (IC_{50} 1.69-2.25 mg/mL) and superoxide anion (IC_{50} 0.56 to 0.88 mg/mL), followed by high reducing power (428-472 mgAA/g.DE) and inhibition of XOD (IC_{50} 0.308-0.6261mg/mL). Antimutagenic activity was evaluated in reverse mutation assays with *Escherichia coli* WP2 oxyR mutant IC202 and deficient in the induction of antioxidant enzymes. The myrtle extracts strongly inhibited mutagenesis induced by t-BOOH, reaching 70% at the highest concentration applied. Antimicrobial activity was examined on eight different bacterial strains. Gram-positive bacteria, *S. epidermis*, *S. aureus* and *M. flavus* demonstrated the highest sensitivity towards extracts (MICs 4.5-9 mg/mL), but significantly lower towards essential oil (MIC 0.42-3.32 mg/mL).

Keywords: Myrtle; Phenolics; Antioxidant; Mutagenesis; Bactericide.

Resumen: Aunque numerosos estudios han demostrado el potencial biomédico de *Myrtus communis* L., (Myrtaceae), los datos sobre el mirto de Montenegro son escasos. Evaluar la actividad antioxidante, antimutagénica y antibacteriana de extractos metanólicos de mirto. La actividad antioxidante se evaluó midiendo la actividad de eliminación de radicales libres, el poder reductor y la inhibición enzimática. La actividad secuestrante más fuerte fue hacia DPPH radical (IC_{50} 1.69-2.25 mg/mL) y radicales de anión superóxido (IC_{50} 0.56 a 0.88 mg/mL), seguido de alto poder reductor (428-472 mgAA/g.DE) e inhibición de XOD (IC_{50} 0,308-0,6261 mg/mL). La actividad antimutagénica se evaluó en ensayos de mutación inversa con *Escherichia coli* WP2 oxyR mutante IC202 y deficiente en la inducción de enzimas antioxidantes. Los extractos de mirto inhibieron fuertemente la mutagénesis inducida por t-BOOH, alcanzando el 70% a la mayor concentración aplicada. La actividad antimicrobiana se examinó en octo cepas bacterianas diferentes. Las bacterias grampositivas, *S. epidermis*, *S. aureus* y *M. flavus* demostraron la sensibilidad más alta hacia los extractos (MIC 4.5-9 mg/mL), pero significativamente más baja hacia el aceite esencial (MIC 0.42-3.32 mg/mL). Los resultados muestran la gran perspectiva nutrafarmacéutica de la especie montenegrina *Myrtus communis*.

Palabras clave: Myrto, Fenolicos, Antioxidante, Mutagénesis, Bactericida.

INTRODUCTION

The Myrtaceae family consists of 150 genera and 3500 species widely distributed in tropical and Mediterranean regions. Many of these plants are of economic importance especially for the pharmaceutical, cosmetic and food industries. In Europe, Myrtaceae plants are to be found mainly in the Mediterranean region. Myrtle (*Myrtus communis* L., Myrtaceae) is an important medicinal and aromatic plant growing spontaneously from the north-western to the eastern Mediterranean. They are traditionally used as antiseptic, hypoglycaemic, anti-inflammatory, anti-hemorrhoidal, and astringent agents for the treatment of candidiasis, healing wounds, diabetes, diarrhea and dysentery, eye diseases, liver injury etc. (Elfellah *et al*., 1984; Alipour *et al*., 2014; Sen *et al*., 2016; Ozcan *et al*., 2020; Torabi *et al*., 2022). The healing properties of myrtle are the results of the synergetic actions of diverse classes of secondary metabolites, among which the most important are essential oils, a complex mixture of volatile mono- and sesquiterpenes (Maxia *et al*., 2011). The main compounds in myrtle essential oil are monoterpenes: 1,8-cineole, myrtenyl acetate, α-pinene, myrtenol, limonene, linalool, α-terpinolene etc. (Bradesi *et al*., 1977; Mimica-Dukić *et al.,* 2010). Numerous studies show a wide range of biological activities for myrtle essential oils, making them commercially the most important myrtle products (Aleksić & Knežević, 2014; Mahboubi, 2016; Caputo *et al*., 2022; Seabai *et al*., 2022; Mansour *et al*., 2022). In addition, recent research shows non-volatile compounds, especially those with phenolic structures which are of great biological and pharmaceutical interest (Ozcan *et al*., 2020; Torabi *et al*., 2022; Mansour *et al*., 2022; Yangui *et al*., 2021). Polyphenolic in myrtle leaves extracts are grouped in three major classes: phenolic acid (ellagic, gallic, ferulic, caffeic, syringic, vanillic etc); flavonoids: flavonols (myricetin-3-O-rhamnoside, myricetin-3-O-galactoside, myricetin-3-O-arabinoside, quercetibe-3-O-glucoside, quercetine-3-Orhamnoside, quercetin and kaempferol), flavan-3-ols: [(+)-catechin, (-)-epicatechin-3-O-gallate, (-)-epigallocatechin-3-Ogallate etc.] and tannins comprise hydrolysable tannins (gallotannins) and proanthocyanidins. Besides, in myrtle leaves, coumarins and lignans compounds are also reported. (Taamalli *et al.,* 2014; Aleksić & Knežević, 2014; Babou *et al.*, 2016).

Although myrtle plants were already sufficiently investigated, interest in them is not waning, and the number of research articles increases yearly. According to the Web of Sciences Core collection, nearly 100 papers on this theme were published in 2021-2022. This could be the result of the commercial importance of myrtle and the great variation in chemical constituents among plants of different geographical origins.

In Montenegro, myrtle plants grow as natives around the seashores, river and river gorges. According to our information, data on their biological activities are very scarce. We therefore began research into the chemical composition and biological activities of plants collected from different localities along the Montenegrin coastline. Our focus was on essential oil and phenolic compounds. Previously we reported on the chemical profile and antimicrobial, antioxidant and antimutagenic activities of essential oils (EO). We found that α-pinene, linalool, 1.8-cineole and myrtenyl acetate were major compounds. EO of examined myrtle plants exhibited considerable antimutagenic activity which was correlated with DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity (Mimica-Dukić *et al*., 2010). In addition, we reported on the significant antimicrobial activity of myrtle *EO* and pointed out a possible mode of action (Aleksić *et al*., 2014). Intending to evaluate the individual contributions of these two classes of secondary metabolites to the overall healing properties of myrtle, this study examines the antimicrobial, antioxidant and antimutagenic effects of myrtle phenolic extracts.

MATERIAL AND METHODS

Plant material and chemicals

Aerial part of myrtle plants was collected from three localities on the Montenegro coastline around the towns of Herceg Novi (MHN), Bar (MB) and Kotor (MK). The specimen vouchers were prepared and identified by Prof. Goran Anačkov PhD and deposited at the Herbarium of the Department of Biology and Ecology (BUNS Herbarium), Faculty of Sciences, University of Novi Sad, Serbia, under numbers: *Myrtus communis* subsp. *tarantina* (L.) Nyman (1879): No 2-1819-Bar, No2-1821-Kotor; No2-1823-Herceg Novi; The leaves were separated and air-dried.

Chemicals: 1,1-Diphenyl-2-(2,4,6-trinitrophenyl) hydrazine (syn: 2,2-diphenyl-1-picrylhydrazyl, DPPH), 2-thiobarbituric acid (TBA), sulfanilamide, tert-butylated hydroxytoluene (BHT) were obtained from FlukaChemie GmbH (Buchs, Switzerland). Propyl galate, PG (Propyl 3,4,5-trihydroxybenzoate)

and tert-Butyl-4-hydroxyanisole (BHA) were obtained by ICN Biochemical (Clevelend, OH, USA). Trichloroacetic acid was purchased from Lach-Ners.r.o. (Neratovice, Czech Republic), n-hexane (Merck, Darmstadt, Germany); t-butyl hydroperoxide (t-BOOH, Aldrich, CAS No. 75-91-2).

Extract preparation and essential oil isolation

Air dried leaves weighing 100 g was extracted by 70% aqueous methanol (300 mL) for 48h at room temperature. Next, filtration solvent was evaporated in vacuo at 55ºC and crude residue was dissolved in hot, distilled water (1 g/mL). In order to remove non-polar compounds, the aqueous solution was re-extracted with petroleum ether (fraction 40-60ºC), concentrated to dryness under vacuum and dried to a constant weight. Dried extracts were dissolved in 70% aqueous methanol to obtain 300 mg/mL stock solutions for further analysis. The essential oil was isolated from air-dried leaves by hydrodistillation according to European Pharmacopoeia (2002) and analysed by means of the GC-MS method as described in our previous study (Mimica-Dukić *et al*., 2010).

Total phenolic and total flavonoid Content

The total phenolic content was determined by the Folin-Ciocalteu′s assay (Singleton *et al*., 1999), adapted for 96-well microplates. The results were expressed in milligrams of gallic acid equivalents per gram of dry extract (mg eq GA/g DE). The total flavonoid content was determined by the aluminium chloride colorimetric method (Chang, 2002). All samples were made in triplicate, and the mean values of flavonoid content were expressed in milligrams of quercetin equivalents per gram of dry extract (mg eq Quercetin/g DE).

Antioxidant activity (AOA)

Antioxidant activity was evaluated by several in vitro tests including: 2.2-diphenyl-1-picrylhydrazyl (DPPH), superoxide anion (O_2•-), nitric oxide (•NO) and hydroxyl (HO•) radicals scavenging capacity and FRAP assay for evaluation of reducing power of the leaf's methanol extracts. All tests, except superoxide anion (O_2•-), were performed according to previously described procedures (Pintać *et al.*, 2019).

Further, the effect on lipid peroxidation (LP) and xanthine oxidase (XOD) activity was conducted. The extent of lipid peroxidation (LP) was determined by measuring the colour adducts of 2-thiobarbituric acid (TBA) and malondialdehyde (MDA), on 512 nm. The commercial preparation of liposomes PRO-LIPO S (Lucas Meyer, Hamburg, Germany), pH 5-7, was used as a model system of cell membrane in which LP was induced by Fe^{2+}/ascorbate (Samojlik *et al*., 2010). The effect on the xanthine oxidase (XOD) and superoxide radical (O_2•-), scavenging activity was assessed by the same test, based on the oxidation of hypoxanthine and xanthine to uric acid (Cos, 1998). Molecular oxygen acts as an electron acceptor producing superoxide radicals and hydrogen peroxide. Inhibition of XOD results in a decrease of uric acid and can be measured spectrophotometrically, recording the increasing absorbance on 290 nm (Noro *et al*., 1983). In short, selected concentrations (5-100 μL) of plant extracts were mixed with 0.8 mL of xanthine (0.125 mM), 0.8 mL of hydroxylamine (0.26 mM), 0.8 mL of EDTA (0.25 mM) all dissolved in phosphate buffer pH 7.5. Reaction was started by adding 0.8 mL of xanthine oxidase (3.12 mU/mL) in a phosphate buffer solution pH 7.50. Reaction mixture (4 mL) was double diluted with phosphate buffer and incubated for 30 min at 37ºC. Prior to measuring uric acid production (recording the UV absorbance at 290 nm), reaction was stopped by adding 0.4 mL of HCl (8.97% v/v). Uric acid production was calculated from the differential absorbance with a blank solution in which the xanthine oxidase was replaced by buffer solution. Each measurement was done in 4 replications. 50% reduction of XOD was calculated by linear regression analysis. A test mixture containing no flavonoids was prepared to measure the total uric acid production. The effect of myrtle extracts on superoxide is evaluated by the nitrite method (Oyanagui, 1984) with slight modification. 0.4 mL of colouring reagent (3.3 mg/mL sulfanilic acid, 55 μg/mL of N-(1-naphthyl)- ethylenediamine dihydrochloride, and 16.7% (v/v) acetic acid in phosphate buffer solution pH 7.5) was added after the incubation. The mixture was allowed to stand for 30 min at room temperature, and absorbance at 550 nm was measured. The reduction of superoxide level was calculated from the differential absorbance with a blank solution in which XOD was replaced by buffer solution.

All results of antioxidant tests were compared to the synthetic antioxidants propylene glycol (PG) and butylated hydroxytoluene (BHT, 2,6-Di-tert-butyl-4-methylphenol). The percentage of inhibition achieved by different concentrations of extracts in the antioxidant assays was calculated by the following equation: I (%) = (A0 - A)/A0 × 100, where A0 is absorbance of the control reaction and A is

absorbance of the examined samples, corrected for the value of the control. The corresponding inhibition– concentration curves, as well as the calibration curves, were drawn using OriginLabs Origin Pro (ver. 8.0), and IC_{50} values, concentration of extracts that inhibited generation of DPPH•, •NO, HO•, O_2•-, MDA and uric acid production by 50% were determined.

Bacterial mutagenicity/antimutagenicity assay

Antimutagenic potential was evaluated in the reverse mutation assay with *Escherichia coli* WP2 IC202 *trpE65 oxyR/pKM101,* strain that is deficient in the induction of antioxidant enzymes (Urios & Blanco, 1996; Blanco *et al*., 1998; Bugarin *et al*., 2014). For this test, we selected the BHN sample, the same one whose essential oil was tested in our previous study, where detailed procedure is described (Mimica-Dukić *et al*., 2010). In brief, for the mutagenicity assay an overnight culture of *E. coli* IC202 strain (100 μL), and extract dilution in EtOH (0.05, 0.1, 0.25 and 0.5 μg/mL) were added to molten top agar (45ºC), mixed and poured onto minimal ET4. For the antimutagenicity assay, the overnight bacterial culture (100 μL), solution of *t*-BOOH (final concentration 25 μg/plate) and a extract dilution (0.05, 0.1, 0.25 and 0.5 mg/plate) were added to molten top agar, mixed and poured onto minimal ET4 plates. The number of Trp+revertants was scored after incubation for 48 h at 37ºC. Simultaneously, the influence of methanolic extract on spontaneous mutagenesis was examined. For the evaluation of pre-existing mutants plating on E4 medium was used, and only the cultures with a small number of revertants (<15 revertants per plate) were used for the experiments. EtOH was used as a negative control (Wall *et al*., 1988). Experiments were carried out at least 3 times. In experiments we calculated the percentage of inhibition of mutagenesis (%I).

Antibacterial activity

Bacteria and Media: The following bacterial strains were used: *Staphylococcus aureus* ATCC25923, *Staphylococcus epidermidis* ATCC12228, *Pseudomonas aeruginosa* ATCC27853, *Escherichia coli* ATCC25922, *Bacillus subtilis* ATCC10774, *Micrococcus flavus* ATCC10240, *Klebsiella pneumoniae* NCIB9111 and *Escherichia coli* SY252 and *Escherichia coli* IB112 strains from the laboratory collection of the Chair of Microbiology, University of Belgrade, Faculty of Biology. IB112 strain is characterized by increased permeability due to *lpc*A mutation. Bacteria were cultivated at 37ºC in Luria broth (LB) (yeast extract 5 g, bacto-tryptone 10 g, NaCl 5 g, distilled water 1 L), or Mueller Hinton Broth (MHB) from Oxoid (Basingstoke, UK). Luria Agar (LA, LB plus 15 g agar) and Mueller Hinton Agar (MHA) from Oxoid were used as solid media. Myrtle extracts dissolved in ethanol were applied in different concentrations.

Antibacterial activity was determined by disc-diffusion assays and MIC determination, described previously (Bugarin *et al*., 2014). Overnight bacterial cultures (100 μL) were spread onto MHA. Myrtle extracts were applied to 10 mm discs (Whatman paper No. 1). After 24 h of incubation at 37ºC, the diameter of growth inhibition zones was measured. Ethanol was used as a negative control and antibiotics: chloramphenicol (30 μg/disc), streptomycin (100 μg/disc), bacitracin (0.04 IU/disc) and gentamycin (40 μg/disc) as positive controls. For MIC determination the broth dilution test was performed in test tubes. In two-fold serial dilutions of extracts (32, 18, 9 and 4.5 mg/mL) a standardized suspension (McFarland turbidity standard) of test bacteria (100 μL) was added to obtain a final concentration of 5 × 105 CFU/mL. A growth control tube and sterility control tube were used in each test. After overnight incubation at 37ºC, the MIC was determined visually as the lowest concentration inhibiting growth, evidenced by the absence of turbidity.

Statistical analysis

The t-test was employed for statistical analysis. Significance was tested at $p<0.05$ level. Values in the tables are mean with standard errors.

RESULTS

In order to evaluate the biomedical value of myrtle from the Montenegrin area, we investigated the antioxidant, antimutagenic and antimicrobial effects of methanolic extracts of myrtle leaf samples collected from three locations in Montenegro. The content of phenolic compounds was determined as a chemical parameter.

Antioxidant activity (AOA)

AOA includes a variety of actions, such as scavenging harmful free radical species (ROS), chelation of transition metal ions, enzymatic detoxification of ROS, and inhibition of oxidising enzymes. Consequently, evaluation of the antioxidant capacity of a particular molecule or extract should be

done through different methods. In the present study, therefore, AOA of each myrtle extract was evaluated for scavenging capacity towards DPPH, hydroxyl, superoxide anion and nitric oxide radicals, lipid peroxidation, as well as the activity of xanthine oxidase and reducing power (FRAP). As phenolic compounds are crucial in antioxidant defence, the content of total phenolic (TPC) and total flavonoid (TFC) were also determined (Table No. 1). TPC significantly varied between samples, with MC-HN having the highest TPC (~260 mg eq. gallic acid/g DE), and MC-K the lowest (~ 184 mg eq. gallic acid/g DE). In contrast, we have not found a significant difference in TFC ranging from 37.1 to 42 mg eq. Quercetin/ g DE. HPLC/TLC preliminary analysis found flavonol-3-O and 7-O glucosides of quercetin and myricetin as the dominant flavonoids. Ellagic acid and its derivatives were also detected. The samples did not differ significantly in qualitative composition (Bugarin, 2010). Regarding TFC, one must bear in mind that the applied $AlCl_3$ colorimetric test does not measure flavonoids without characteristic chelating functional groups for Al^{3+} binding, e.g., flavanones and flavanonols (Vinson *et al*., 2001).

In all tests, AOA was concentration dependent, and IC_{50} values calculated are shown in Table 1. Synthetic antioxidants PG and BHT were used as positive controls. The strongest scavenging activity was towards DPPH IC_{50} ranging from 1.69 to 2.25 μg/mL, and superoxide anion radicals IC_{50} ranging from 0.56 to 0.88 μg/mL, which were similar or better than BHT or PG. However, myrtle extracts did not show increased activities towards nitrite oxide and hydroxyl radicals. In addition, they expressed a lower or moderate effect on LP. In contrast, all extracts expressed a high reducing power determined by the FRAP test (428-472 mgAA/g.DE). All extracts significantly reduced XOD activity.

Table No. 1
Antioxidant activities and the content of total phenolic and flavonoid in MeOH extracts of the leaves of *Myrtus communis*

AOA /samples	*DPPH$^{\bullet}$	*OH$^{\bullet}$	*NO$^{\bullet}$	*O_2^-	FRAP**	LP*	XOD*	TPC***	TFC***
MB	1.69±0.44^{ab}	339.9±38.6^{b}	979.3±61.0^{a}	0.88±0.18^{a}	428.4±15.8^{a}	96.3±8.0^{c}	0,626±0.12^{b}	217.9±9.8^{b}	37.1±3.4^{a}
MK	2.06±0.07^{b}	307.1±34.6^{b}	972.1±48.6^{a}	0.56±0.24^{a}	450.0±7.6^{ab}	105.0±6.1^{c}	0,608±0.14^{b}	183.9±8.3^{a}	42.0±2.8^{a}
MHN	2.25±0.13^{b}	282.7±62.1^{b}	994.0±40.1^{a}	0.66±0.19^{a}	472.2±9.8^{b}	112.9±11.3^{c}	0,308±0.08^{a}	259.9±5.1^{c}	37.8±3.0^{a}
PG	1.33±0,03^{a}	51.0±0.4^{a}	18.21±1.75	0.35±0.04^{a}	n.a	20.5±2.4^{b}	0.34±0.03^{a}		
BHT	8.41±0.40^{c}	233±19.28	724.82±50.58	n.a	25.32±2.5	7.65±0.12 ^{a}	n.t		

*** IC_{50} (μg/mL); **(mg Ascorbic acid eq/g.DE); ***Total phenolic content (TPC) in mg Gallic acid eq./g DE, total flavonoid content (TFC) in mg Quercetin eq. /g DE; n.t, not tested; n.a, 50% inhibition not achieved**
Values are means±SD of three measurements. Means within each column with different letters (a-c) differ significantly ($p<0.05$).
A comparison of the group means and the significance between the groups were verified by one-way ANOVA. Statistical significance was set at $p<0.05$.
MB, MeOH extract BAR;MK, MeOH extract KOTOR; MHN, MeoH extract HERCEG NOVI.

Antimutagenic activity

The antimutagenicity screening was performed in the co-treatment assay (mutagen–antimutagen), in *Escherichia coli* IC202 *oxyR*, with *trp*E65 mutation strain differing in the OxyR regulated response to oxidative stress. The effect of MHN methanolic extract on spontaneous and *t*-BOOH induced mutagenesis was evaluated by monitoring the percentage of Trp+ revertants (Table No. 2). The concentration of *t*-BOOH was chosen to give the highest mutagenic response with about 50% of lethality. For myrtle extract, the lowest non-toxic concentration was 0.5 μg/mL (data not shown), whilst in control, instead of extract, pure ethanol was used. The obtained results show that inhibition of spontaneous mutagenesis in the presence of myrtle extract was only moderate reaching 39% at the highest concentration applied. However, in the presence of oxidative mutagen, myrtle extract significantly reduced mutagenesis in tested *E. coli* strain. The inhibition was concentration dependent, ranging from 25 to 70%. Even in the lowest concentration myrtle extract reduced mutagenesis for 25%.

Table No. 2
Effect of myrtle MeOH extract (MHN) on spontaneous and *t*-BOOH-induced mutagenesis in *E. coli* IC202

Extract	-*t*-BOOH			+t-BOOH		
(μg/mL)	Revert/p[2]	M (%)	I (%)	Revert/p[2]	M (%)	I (%)
0[4]	99 ± 5	100	-	181 ± 6	100	-
0.05	98 ± 9	96	2	135 ± 2*	75	25
0.10	87 ± 7	85	15	119 ± 5*	66	44
0.25	77 ±11*	67	33	91 ± 7*	50	50
0.5	61 ± 4*	61	39	55 ± 4*	30	70

[1] *t*-BOOH induced mutagenesis, applied concentration 25 μg/p. These values are an average of duplicate samples from three independent experiments; [2] Trp/mL = Trp$^+$ revertants/p × 10; [3] *M*—mutagenesis (% M) = (Nt/Nc) × 100. [4]EtOH - solvent control Nt, sample with MHN; Nc control sample (EtOH); I (%) - inhibition mutagenesis; * (p<0.05).

Antibacterial activity

For preliminary screening of antimicrobial activity of myrtle extracts, we applied a disc-diffusion assay, and the results are presented in Table No. 3. Antimicrobial activity is expressed as the diameter of the inhibition zone (mm). In the experiment the following bacterial strains were tested: *Staphylococcus aureus* ATCC25923, *Staphylococcus epidermidis* ATCC12228, *Pseudomonas aeruginosa* ATCC27853, *Escherichia coli* ATCC25922, *Bacillus subtilis* ATCC10774, *Micrococcus flavus* ATCC10240, and *Escherichia coli* SY252 and IB112 (*lpcA*). In addition, we also tested *Klebsiella pneumoniae* NCIB9111, but no activity was obtained. The results demonstrated that myrtle extracts significantly reduce growth of Gram-positive bacteria, especially *S. aureus,* in the lowest concentration applied. In addition, the growth of *S. epidermis, B. subtuilis* and *M. flavus* was also reduced but in a higher concentration than 1.5 mg/disc. In contrast, all Gram-negative bacteria were mostly resistant and showed low sensitivity that was manifested by the presence of an enlightened rather than a clear zone around the discs. EtOH was used as negative control and did not show any antibacterial activity.

This test also demonstrated that analysed samples did not differ in antimicrobial activity. EtOH was used as negative control and did not show any antibacterial activity. Most of the tested bacteria were sensitive to streptomycin, which was chosen as positive control in the next MIC test.

In order to obtain more reliable results, minimal inhibition concentration (MIC) was determined. The results are presented in Table No. 4. MIC value was not established for *E. coli* ATCC25922 or *E. coli* SY252 for applied concentration. Obviously, lower MIC values were obtained towards Gram-positive bacteria. The highest sensitivity to all extracts was demonstrated by *S. epidermis* and *S. aureus* (except MHN, MIC 9 mg/mL), as well as *M. flavus* (except with MB, MIC 9 mg/mL) where MIC was 4.5 mg/mL. However, myrtle extract expressed considerable antimicrobial activity against two Gram-negative bacteria *E. coli* IB112 (*lpcA*), and *P. aeruginosa*, known as an antibiotics resistant strain. Only minor differences among samples were observed. Along with extracts, the antimicrobial effect of essential oils was also investigated, and results are presented in Table No. 4 and Table No. 5. Essential oils exhibited significantly higher antimicrobial effect, MICs ranged from 0.42 mg/mL (*S. epidermis, B. subtilus, K. pneumoniae* and *E. coli* ATCC 27853) to 3.32 mg/mL (*S. aureus* and *E. coli* IB112 (*lpc*A*)*. Unlike the extracts, the essential oil also had an effect on the *Klebsiella pneumoniae.*

Table No.3
Antimicrobial activity of myrtle leaves MeOH extracts tested by disc-diffusion method

Bacterial strain/EC (mg/disk)	0.30			0.60			0.75			1.50			3.0			Antibiotic[b]
Sample tested	MB	MK	MHN	MB	MK	MHN	MB	MK	MHN	MB	MK	MHN	MB	MK	MHN	
S. epidrmidis	0[1]	0	0	0	0	0	0	13	(17)	13	13	14	15	15	15	Gentamycin (35)
S. aureus	16	16	16	20	16	16	18	20	20	22	25	24	24	26	26	Streptomycin (22)
B. subtilis	0	0	0	0	(16)	0	13	(20)	(15)	14	12	14	16	16	16	Streptomycin (27)
M. flavus	0	0	0	0	0	0	(18)	(17)	0	13	14	(17)	16	16	13	Bacitracin (33)
E. coli ATCC 25922	0	0	0	0	0	0	0	0	0	0	0	0	(17)	(16)	(18)	Streptomycin (20)
P. aeruginosa	(17)	(17)	(15)	(20)	(18)	(17)	(20)	(20)	(20)	(22)	(20)	(23)	(26)	(25)	(27)	Streptomycin (20)
E. coli SY 252	(16)	(16)	(17)	(16)	(17)	(19)	(20)	(18)	(20)	(20)	(20)	(20)	(23)	(22)	(25)	Streptomycin(22)
E. coli lpcA	(16)	(17)	0	(16)	(17)	(16)	(18)	(18)	(17)	(18)	(22)	(18)	(13)	13	13	Streptomycin(23)

[1] Diameter of inhibition zone (mm); 0-no growth inhibition zone; bracket indicate incomplete inhibition of growth. [a] Extract concentration (mgL/disk);
[b] Sstreptomycin (100 µg/disc), bacitracin (0.04 IU/disc) and gentamycin (40 µg/disc); [2] Diameter of inhibition zone (mm); MB, MeOH extract BAR;MK, MeOH extract KOTOR; MHN, MeoH extract HERCEG NOVI

Table No. 4
Antimicrobial activity of myrtle leaves essential oils (*EO*s) tested by disc-diffusion method

Bacterial strain/EC (µL/disk)	3.2			1.66			0.83			0.41			Antibiotic[b]
Sample tested	MB	MK	MHN	MB	MK	MHN	MB	MK	MHN	MB	MK	MHN	
S. epidrmidis	26[1]	26	20	12	17	13	X	13	11	(12)	0	0	Gentamycin (35)
S. aureus	20	16	17	(15)	(10)	(15)	(12)	13)	(14)	0	0	0	Streptomycin (22)
B. subtilis	17	17	15	13	14	12	11	10	10	0	(12)	(10)	Streptomycin (27)
M. flavus	18	28	16	14	13	13	11	11	11	0	0	0	Bacitracin (33)
E. coli ATCC 25922	(28)	25	25	(20)	(20)	(27)	(18)	(20)	(16)	(18)	17	(18)	Streptomycin (20)
P. aeruginosa	0	0	0	0	0	0	0	0	0	0	0	0	Streptomycin (20)
Klebsiella pneumoniae NCIB9111	33	28	29	20	15	18	17	15	(19)	(10)	(13)	(17)	
E. coliSY 252	(27)	(35)	(26)	(18)	(24)	(18)	(17)	(19)	(27)	0	0	0	Streptomycin(22)
E. coli lpcA	32	30	20	23	25	20	21	20	20	17	(20)	8	Streptomycin(23)

[1] Diameter of inhibition zone (mm); 0-no growth inhibition zone; bracket indicate incomplete inhibition of growth. [a] Extract concentration (mgL/disk);
[b] Sstreptomycin (100 µg/disc), bacitracin (0.04 IU/disc) and gentamycin (40 µg/disc); [2] Diameter of inhibition zone (mm); MB, *EO* BAR; MK, *EO* KOTOR; MHN, *EO* HERCEG NOVI

Table No. 5
MIC values of myrtle leaves MeOH extracts (*MeOH-ex*), essential oil (EOs) and streptomycin* as reference antibiotic

Bacterial strain/	MB		MK		MHN		streptomycin
MIC (mg/mL)	*MeOH-ex*	*EO*	*MeOH-ex*	*EO*	*MeOH-ex*	*EO*	MIC (μg/mL)
S. epidermidis ATCC12228	4.50	0.83	4.5	0.42	4.5	0.83	25
S. aureus ATCC25923	4.50	3.32	4.5	3.32	9.0	3.32	50
M. flavus ATCC10240	9.00	0.83	4.5	0.42	4.5	0.83	25
B. subtilis ATCC10774	9.00	0.42	9.0	0.83	9.0	n.a	10
P. aeruginosa ATCC27853	18.0	n.t	18	n.t	18	n.t	12.5
E. coli IB112 (*lpcA*)	18	0.42	18	1.66	18	n.a	12.5
K. pneumoniae NCIB9111	n.t	3.32	n.t	0.42	n.t	0.42	19*
E. coli SY252	n.a	n.a	n.a	n.a	n.a	n.a	25
E. coli ATCC 27853	n.a	0.42	n.a	0.21	n.a	0.83	12.5

***chloramphenicol; n.a. not active in applied concentration range; n.t-not tested; MeOH, methanolic extracts, *EO*, essential oil; MB-Bar, MK-Kotor, MHN-Herceg Novi**

DISCUSSION

One of the most important features of phenolic compounds is their antioxidant potential. The protection of some plants against mutagenicity and cytotoxicity has been attributed to the various natural antioxidants they contain. Among them phenolic substances such as tocopherols, various classes of flavonoids, phenolic acid, tannins, lignans, etc., are of special significance (Cvejić Hogervorst *et al*., 2018). Numerous studies demonstrated significant variation of myrtle phenolics, depending on plant organ, geographical origin, maturation, extraction procedure, method of determination etc. (Vinson *et al*., 2001; Bakova *et al*., 2021; Snoussi *et al*., 2021; Yaghoobi *et al*., 2022). In generally, our results agree with those previously published, both in terms of amount and composition of myrtle phenolic compounds (Bugarin, 2010; Amensour *et al*., 2010; Sumbul *et al*., 2011; Babou *et al*., 2016). Considering the complexity and relevance of antioxidant assessments, especially in the case of complex mixtures, here we applied seven tests representing different mechanisms. Radical scavenging activity was evaluated by measuring the ability of the extract to neutralize DPPH,· OH, and ·NO radicals, mechanisms based on single electron transfer (ET) mechanism Commercial synthetic antioxidants PG and BHT were used as positive control. The results show that the myrtle extracts exhibited from significant (DPPH) to moderate (·NO) or poor (·OH) scavenging activity. In contrast, all extracts possess high reducing power assessed by FRAP test. Both DPPH and FRAP tests are based on a similar mechanism (ET-based test), measuring the capability of an antioxidant to reduce certain chromogenic redox reagents. Moreover, total phenol assay with Folin-Ciocalteu reagent (FCR), ·NO radical inhibition activity, and TBARS (thiobarbituric acid reactive substances) assay for OH, and O_2• are also based on ET, whereas lipid peroxidation assay is based on the hydrogen atom transfer (HAT) mechanism (Deepshikha, 2015; Dontha, 2016). The concentration of total phenolics and total flavonoid content affected AOA of myrtle leaf extracts. Total phenols were associated with antioxidant capacity towards OH radicals, reducing power (FRAP) and inhibition of XOD activity, while total flavonoids content influenced ·NO and. O_2^- radical scavenging capacity. The results were like those previously published (Medda *et al*., 2021). The XOD catalysed reaction of oxidation of xanthine and hypoxanthine to uric acid is used for the evaluation of the inhibitory activity of

myrtle extracts towards XOD and superoxide radicals (Cos *et al*., 1998). During reaction, superoxide radicals and hydrogen peroxide are generated. Investigating the structure-activity relationship of flavonoids, the authors demonstrated that the hydroxyl groups at C-5 and C-7 and the double bond between C-2 and C-3, were essential for a high inhibitory activity. On the other hand, high superoxide scavenging activity was shown by flavonoids having a hydroxyl group at C-3′ in ring B and at C-3. Simultaneously, determination of both parameters can provide more information about the activities of phenolic against the harmful effect of increased XOD activity in certain pathological conditions. Based on the results obtained, the authors classified flavonoids into four groups, depending on whether they inhibit only XOD or only superoxide radicals, or both, or neither. It is assumed that phenolics which act as both XOD inhibitors and superoxide scavengers, may be considered a promising drug in gout therapy instead of the widely used allopurinol, which inhibits XOD but does not remove superoxide radicals. Our results show that myrtle leaf extract inhibited XOD and scavenged superoxide radicals, which makes this plant interesting for further research.

The antimutagenic effect of the myrtle leaf extracts against the spontaneous and *t*-BOOH-induced mutagenesis was tested in *E. coli* IC202 *oxyR*, a bacterial strain deficient in removing ROS. Since the strain carries the *trpE65* mutation, we followed the antimutagenic effect by monitoring the percentage of Trp^+ revertants. Deficiency in removing ROS is a consequence of mutation in the *oxyR* gene, leading to deficiency in the *OxyR* function. The *OxyR* protein is a redox-sensitive transcriptional activator of genes encoding antioxidant enzymes: catalase, alkyl hydroperoxide reductase and glutathione reductase, which are produced by the cells in the response to oxidative stress (Blanco *et al*., 1998). Therefore, the IC202 strain is highly sensitive to oxidative DNA damage (Hayder *et al*., 2004).

Several reports revealing the antigenotoxic and antimutagenic activity of myrtle leaf extract are available. Most of them came from the same research group (Hayder *et al*., 2008a; Hayder *et al*., 2008b; Skandrani *et al*., 2011). Hayder *et al*. (2008a) demonstrated that methanol and ethyl acetate extracts from the leaves of *Myrtus communis* exhibited significant protective effect against the mutagenicity in the *Salmonella typhimurium* assay induced by aflatoxin B1 and sodium azide as indirect and direct mutagen, respectively. Furthermore, they show that myricetin-3-O-galactoside and myricetin-3-O-rhamnoside isolated from myrtle leaves, exhibited strong antioxidant activity, inhibiting XOD activity, lipid peroxidation and scavenging DPPH radicals. Both compounds inhibit mutagenicity induced by aflatoxin B1 and hydrogen peroxide. It was proposed that myricetin-3-O-galactoside and myricetin-3-O-rhamnoside modulated the expression of cellular genes involved in oxidative stress, DNA damage repair and apoptosis (Hayder *et al*., 2008b). Besides, it was found that 3.5-O-di-galloylquinic acid from myrtle leaves, exhibited a strong inhibitory effect against H_2O_2-induced genotoxicity, by increasing the activities of the antioxidant enzymes family and the activity of DNA repair proteins (Skandrani *et al*., 2011). The results of the present study support the presumption that the antimutagenic activity of myrtle extract is based on the antioxidant capacity of phenolic compounds. Antioxidant capacity of the myrtle extracts is proved by several AOA tests. Bearing in mind that the bacterial cells were treated with these extracts as antioxidant and mutagen (*t*-BOOH) simultaneously, we can presume that the reduction of mutagenesis is related either to scavenging of ROS or direct interaction with *t*-BOOH. In our previous study, we reported on the antioxidant and antimutagenic activities of essential oil (*EO*) obtained from the leaves of *Myrtus communis* from the Herceg Novi area (MHN). DPPH radical scavenging capacity of *EO* was nearly 1000 time lower (IC_{50} 5.99 x10^3 μg/mL) than that obtained by MeOH extract (2.25 μg/mL). Consequently, MeOH extract of the same plant exhibited significantly higher antimutagenic activity, and inhibited *t*-BOOH induced mutagenesis by 70%, whereas *EO* in the same system reduced the *t*-BOOH induced mutagenicity by 28%, respectively (Mimica-Dukić, 2010). We reported similar results with essential oil from the leaves of *Eucalyptus gunni* from the Montenegrin coastline, which (in the same system) reduced *t*-BOOH-induced mutagenesis by 23%. The capacity to scavenge DPPH radicals was also lower by IC50 7.19 x10^3 μg/mL (Bugarin *et al*., 2014).

The antibacterial properties of myrtle against pathogenic bacteria have been reported in many studies. Most of them confirmed high bactericidal activity of the volatile compounds and entire essential oil obtained from different parts of myrtle plants (Mansouri *et al*., 2001; Gortzi *et al*., 2008; Akin *et al*., 2010; Zanetti *et al*., 2010; Aleksić & Knežević, 2014; Aleksić *et al*., 2014). Furthermore, numerous studies

confirmed the antibacterial activity of the non-volatile compounds in myrtle leaves (De Laurentis *et al*., 2005; Taheri *et al*., 2013; Bokaeian *et al*., 2013; Ahmed *et al*., 2013; Casaburi *et al*., 2015). Crude extract of myrtle and 3 fractions (diethyl ether, ethyl acetate and ethanol) were tested against Gram positive (*Staphylococcus aureus, Micrococcus luteus, Streptococcus pneumoniae, Streptococcus pyogenes, Streptococcus agalactiae, Listeria monocytogenes*) and 4 Gram negative bacteria (*Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa* and *Campylobacter jejuni*). It was shown that crude extract inhibited the growth of all tested bacteria except *C. jejuni*. MICs range from 0.1 (*S. aureus*) and *M. luteus* to over 2 mg/mL for *E. coli*. Non-polar, diethyl ether fraction showed the highest activity with MIC 0.025 mg/mL for *S. aureus* and *M. luteus* and 0.1 mg/mL for *E. coli* and *P. aeruginosa* (Mansouri *et al*., 2001). It was also reported that hydroalcoholic extract of myrtle leaves inhibited *S. aureus* with very low MIC (0.2mg/mL) (Mansouri *et al*., 2001). Furthermore, excellent antibacterial activity of the methanolic myrtle leaf extract against foodborne pathogens was observed (Amensour *et al*., 2010). In addition, it was reported that after encapsulation in liposomes the antibacterial activity of myrtle extracts significantly increases. Evidently, liposomes improve bioavailability of myrtle extracts by increasing ingredient-solubility (easier incorporation of hydrophilic substances into the lipophilic membrane structure). This observation indicates great potential for encapsulated myrtle extract in preservation of food, cosmetics and drug products (Gortzi *et al*., 2008). This study is supported by the observation that water-ethanol extract of myrtle leaves inhibits the growth of meat spoilage strains *Brochothrix thermosphacta* and *Pseudomonas fragi*, with MICs values ranging from 12.5-50 mg/mL (*B. thermosphacta*) and 25-100 mg/mL (*P. fragi*) (Casaburi *et al*., 2015).

The present study shows that myrtle methanolic extracts were more active against Gram-positive bacteria. It is assumed that because lacking an outer membrane, Gram-positive bacteria are more susceptible to the myrtle extract. Furthermore, we demonstrated that essential oil of the same myrtle plant is active against both groups of bacteria, although the activity towards Gram-negative strains was lower (MICs range from 0.42 to 3.32 mg/mL.). Several studies demonstrated similar observations (Amensour *et al*., 2010; Taheri *et al*., 2013). Obviously, the mechanism of antibacterial activity between these two groups of secondary metabolites is different. One can conclude that the difference in their solubility significantly affects their antibacterial properties (Oydemi *et al*., 2009). Several mechanisms of antibacterial activity are proposed such as: disintegration of cytoplasmatic membrane, interaction with membrane proteins, disturbance of the outer membrane of Gram-negative bacteria, coagulation of cell content, inhibition of enzymes etc. (Aleksić & Knežević, 2014). It was proposed that phenolic compounds predominantly present in alcoholic extracts can interact with extracellular proteins and with the bacterial cell wall (Cuchine & Lamb, 2005). However, although numerous in vitro studies demonstrate the antibacterial properties of myrtle extracts, the mechanism of their action is poorly understood. It may be assumed that these compounds exert their activity primarily by inhibiting the functions of the cell membrane, inhibiting the activity of various enzyme systems and acting on the genetic material of bacteria (Shaheen *et al*., 2006). Evidently, more *in vivo* assays with singular phenolic compounds and entire extracts need to be explored.

CONCLUSION

Although many studies have demonstrated broad spectra of the biological activity of *Myrtus communis*, data on wild-growing myrtle plants in Montenegro are scarce. Therefore, we started with a detailed phytochemical and biological investigation of myrtle plants from the Montenegrin coastal area. Our previous study demonstrated a considerable antioxidant and antimutagenic potential of essential oil isolated from myrtle leaves. Here we report on the antioxidant, antimutagenic and antimicrobial properties of myrtle methanolic extracts. It was demonstrated that the extracts examined exhibit a strong antioxidant capacity, especially by scavenging DPPH and super oxide radicals. Antioxidant activity is also proved by high reducing power (FRAP) and inhibition of XOD activity. In the light of the results of antioxidant and antimutagenic activity, we can conclude that the antioxidant capacity of the examined extract is mostly responsible for significant inhibition of *t*-BOOH induced mutageneseis in *E. coli* IC202 *oxyR*, a bacterial strain deficient in removing ROS. Moreover, these extracts exhibited considerable antibacterial activity, mostly towards Gram-positive bacteria, but significantly lower than those achieved with essential oils. In summary, it may be concluded that both classes of secondary metabolites, essential oils and phenolics contribute to the overall biological

activity of myrtle plants.

To our best knowledge, this study was the first to assess the phytopharmaceutical potential of myrtle plants growing in Montenegro. However, the limited number of samples was insufficient for a more detailed statistical analysis that would provide a better insight into the mutual correlation of the bioactivity parameters. For a better assessment, we suggest further studies with greater numbers of samples and in vivo experiments on animal models. Nevertheless, the results obtained so far suggest that myrtle plants from Montenegro are a promising source of bioactive compounds for the pharmaceutical and foodstuff industries.

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ABBREVIATION

antioxidant activity (AOA); 1,1-Diphenyl-2-(2,4,6-trinitrophenyl) hydrazine (DPPH); 2-thiobarbituric acid (TBA); superoxide anion (O_2•-); nitric oxide (•NO); hydroxyl radical (HO•); lipid peroxidation (LP); xanthine oxidase (XOD); malondialdehyde (MDA); propylene glycol (PG); butylated hydroxytoluene, 2,6-Di-tert-butyl-4-methylphenol (BHT); reactive oxygen species (ROS), total phenolic content (TPC); total flavonoid content (TFC); minimum inhibitory concentration (MIC).

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