UNIVERSITY OF BELGRADE FACULTY OF BIOLOGY Microtitre plate-based antibacterial assay with resazurin for detection of antibacterial agents Master thesis Student Salahaldin Abdulkarim Omar Alfurjany Belgrade, Serbia, 2016 Acknowledgement This work was done at Chair of Microbiology, University of Belgrade – Faculty of Biology, Serbia.Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances.Nutrient media In this study the following nutrient media were used: o MHB – Mueller-Hinton Broth: MHB powder (21 g), distilled water (1000 ml); o MHA – Mueller-Hinton Agar: MHB powder (21g), Agar "Difco" (15g), distilled water (1000ml); o BHI – Brain-Heart Infusion Broth: BHI powder (37g) , distilled water (1000 ml); o BHA – Brain-Heart Infusion Agar: BHI powder (37g) , Agar "Difco" (15g), distilled water (1000 ml); Nutrient media were sterilized in an autoclave for 20 minutes at a temperature of 121?C and a pressure of 101.3 kPa. 5.1.2. Antibiotics and other reagents used in the MIC assay o Streptomicin (Str): antibiotic solution in MHB; concentration of working stock 500 ug/ml o Rifampicin (Rif): antibiotic solution in BHI, concentration of working stock 200 ug/ml o Dimethyl sulfoxide (DMSO): solvent, used for EO o Resazurin Sodium Salt: distilled water solution, concentration of working stock 0,675 mg/mL 5.1.3. Bacterial strains The following bacterial strains were used to determine antibacterial activity of the EO and PDW: Gram – positive bacteria: o Staphylococcus aureus ATCC25923 o Listeria innocua ATCC33090 Gram – negative bacteria: o Salmonella enteritidis ATCC13076 o Pseudomonas aeruginosa ATCC15442 5.1.4. Plant material and preparation of EO and PDW Plant material was collected in July 2011 at Stara Planina Mountain, Serbia. The voucher specimen (Juniperus communis var. saxatilis, No. 16693) was prepared, identified and deposited at the Herbarium of University of Belgrade, Faculty of Biology, Institute of Botany and Botanical Garden "Jevremovac" (BEOU Herbarium).Figure 3: Apperance of multi-drug resistant pathogens (from Madigan and Martinko, 2006) BACTERIAL RESISTANCE TO ANTIBIOTICS Resistence mechanism Antibiotic example Genetic basis of resistance Mechanism presented in Reduced permeability Penicillin Chromosomal Salmonella enteritidis Pseudomonas aeruginosa Chloramphenicol Erytrommycin Lincomycin Plasmid Listerya innocua Inactivation of antibiotic Penicillin Plasmid and chromosomal Staphylococcus aureus Salmonella enteritidis Chloramphenicol Plasmid and chromosomal Staphylococcus aureus Salmonella enteritidis Alteration of target Eritromycin Rifamycin Streptomycin Norfloxacin Chromosomal Staphylococcus aureus Salmonella enteritidis Development of resistant biochemical pathaway Sulfonamides Chromosomal Staphylococcus aureus Salmonella enteritidis Efflux(pumping out of cell) Tetracycline Chloramphenicol Plasmid Chromosomal Salmonella enteritidis Staphylococcus aureus Table 1.As advised, these guidelines provide a uniform procedure for testing that is practical to perform in most clinical microbiology laboratories and bioassay to be performed in a standardized approach in order to evaluate the clinical relevance of results Nevertheless if dilution is prepared in agar or in broth, the range of antibiotic concentrations used for determining MICs is universally accepted to be in doubling dilution steps up and down from 1 mg/mL, as required (Balouiri, 2016).Studying of biological properties indicates that Juniperus species are endowed with numerous activities including antioxidant, antiseptic, diuretic, anticancer, antirheumatic, antihelmintic, anti-inflammatory, immunomodulatory, analgesic, antituberculotic and abortifacient activities (Glisic et al. 2007; Orphan et al., 2011; Swanston-Flatt et al. 1990).MIC and MBC values* Bacterial strains EO PDW

Str Rif MIC MBC MIC MBC MIC MBC MIC MBC Staphylococcus aureus 6.25 12.5 3.125 6.25 12.5 25 nt nt Listeria innocua 6.25 6.25 1.56 1.56 nt nt / 0.19 Salmonella enteritidis 50 50 6.25 6.25 3.125 6.25 nt nt Pseudomonas aeruginosa / / 12.5 25 25 50 nt nt / – not determined in used concentration range nt – not tested *MIC and MBC in mg/mL for EO and PDW, and in ug/mL for Str and Rif.In such environments the multi-drug resistant pathogens appear, making huge difficulties in curing of infections caused by them (Figure 3.) The mechanisms of bacterial resistance to antibiotics include reduced permeability, inactivation of antibiotics, alteration of target, development of specific biochemical pathways, as well as the efflux of antibiotics (Table 1).Interestingly, although the antibacterial effect is not high, the importance is the fact that Juniperus communis extracts possess antibacterial effect against important patogenic bacteria Mycobacterium tuberculosis, a causative agent of tuberculosis disesase (Carpenter et al., 2012; Jimenez-Arellanes et al., 2003).In vitro methods for antimicrobial evaluation Two parameters are important to determine by antimicrobial susceptibility assays – minimal inhibitory concentration (MIC) and minimal bactericidal/fungicidal concentration (MBC/MFC).The interpretative standards for this method are published by different national organizations such as the Clinical and Laboratory Standards Institute (CLSI) in the USA and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (Wiegand et al. 2008).Evaluate and quantitatively determine the antibacterial potential of J. communis essential oil and post-distillation waste against Staphylococcus aureus, Listeria innocua, Salmonella enteritidis and Pseudomonas aeruginosa.RESULTS AND DISCUSSION 21 CONCLUSIONS 25 REFERENCES 26 INTRODUCTION High mortality and morbidity worldwide, especially in developing countries, are frequently caused by microbial infections that occur as a result of poor sanitation, unhygienic and overcrowded living conditions.One variant of diffusion method is followed by placing of filter paper discs (about 6 mm in diameter), containing the test compound at a desired concentration (ug/disk), on the agar surface (Balouiri, 2016).The principal disadvantages of the macrodilution method are the tedious, manual task of preparing the antibiotic solutions for each test, the possibility of errors in preparation of the antibiotic solutions, and the relatively large amount of reagents and space required for each test (Jorgensen, 2009).Although agar dilution method is significantly more expensive and laborious to perform, it is especially suitable for testing the compounds (or extracts) which masks the detection of microbial growth in the liquid medium by their coloring (Balouiri, 2016).Essential oils (EOs) of many aromatic plants, such as Origanum vulgare, Thumus capitatus and Ocimum basilicum, are proved as potent antibacterial agents (Dzamic et al., 2015; Sokovic et al., 2010).If colonization of P. aeruginosa occurs in critical body organs, such as lungs, urinary tract and kidneys, the results could be fatal (https://en.wikipedia.org/wiki/Pseudomonas aeruginosa).Low antibacterial effect of J. communis derivatives is in line with previously published data, indicating moderate and low antibacterial effect of Juniperus species (Andogan et al., 2002; Angioni et al., 2003; Glisic et al., 2007; Karaman et al., 2003; Nikolic et al, 2016; Lesjak et al., 2014).Nevertheless, an approximate MIC can be calculated for some microorganisms and antibiotics by comparing the inhibition zones with stored algorithms (Jorgensen and Ferraro, 2009).Microtitre plate-based antibacterial assay incorporating resazurin (Figure 2.) is a method that is generally accepted for application in the in vitro antibacterial screening of different antibiotics and phytochemicals (Sarker et al., 2007).Taking into account the widespread occurrence of this genus, as

well as the richness in the active substances, it is not surprising to find extensive use of EO and differently prepared extracts of many Juniperus species (Adams, 2014).Furthermore, study of Khan et al. (2012) provides a pharmacological basis for traditional use of Juniperus excelsa against respiratory disorders and gut hyperactivity, such as asthma, diarrhoea and colic.For that purpose, additional plating of suspension from all the wells without visible growth onto the corresponding agar medium (BHA for L. innocua, and MHA for all the rest bacteria) was performed.Judged by the dominant constituents of EO, obtained antibacterial effect could be at least partially attributed to ?-pinene and sabinene (Arunkumar et al., 2014; Da Silva et al. 2012).Comparing to antibiotic controls (Str or Rif), the antibacterial potential of EO and PDW was several times lower, indicating that J. communis derivatives could be defined as substances with low antibacterial potential.Moreover, the agar diffusion method is not appropriate to determine the minimum inhibitory concentration (MIC), as it is impossible to quantify the amount of the antimicrobial agent diffused into the agar medium.1.2 Dilution methods Dilution methods are the most appropriate ones for the determination of MIC values, since they offer the possibility to estimate the concentration of the tested antimicrobial agent in the agar (agar dilution) or broth medium (macrodilution or microdilution).Broth macrodilution method The antibiotic-containing tubes are inoculated with a standardized bacterial suspension of 1-5x105CFU/mL.Broth microdilution method Microtitre plate based antibacterial assay is the dilution method, consisted of series of small test tubes arranged in a regular matrix pattern on a plastic plate, usually made from transparent polystyrene.In resazurin-incorporated microtitre plate-based antibacterial assay, MIC value is determines as the lowest concentration that induces no color change, i.e. well remains blue (Sarker et al., 2007).In order to facilitate the curing of hospital-acquired infections in the future, the international, national, and local antibiotic stewardship campaigns have been developed to encourage prudent use and limit unnecessary exposure to antibiotics.Advanced antibiotic resistance mechanisms could be associated with serious illnesses - especially nosocomial infections such as pneumonia and various sepsis syndromes.The high abundance of ?-pinene and sabinene was consistent with previous studies regarding J. communis (Adams, 2014; Seca and Silva, 2006; Shahmir et al., 2003).Libyan Thymus capitatus essential oil: antioxidant, antimicrobial, cytotoxic and colon pathogen adhesion-inhibition properties.The test is performed by applying a bacterial inoculum of approximately (1-2) x 108 CFU/mL to the surface of a large (100 mm diameter) Mueller-Hinton agar plate.The standard agar disc-diffusion test is also known as Kirby-Bauer method, Kirby-Bauer antibiotic testing, KB testing, disc-diffusion antibiotic sensitivity testing, or antibiogram method (Qi et al., 2006).Agar disc diffusion test (from Madigan and Martinko, 2006) Although disk-diffusion method is standardized variant of testing, another variant of diffusion method is also frequently applied.Diffusion methods provides qualitative results, by categorizing bacteria as susceptible, intermediate or resistant (Qi et al 2006).1.1.2 Antimicrobial gradient method (E-test) The E-test is a commercial version of antimicrobial gradient method that combines the principle of dilution methods with diffusion methods, in order to determine the MIC value by this technique.In the procedure, a strip impregnated with an increasing concentration gradient of the antimicrobial agent from one end to the other is deposited on the agar surface, previously inoculated with the microorganism tested (Balouiri, 2016).Microdilution assay reduced the sample volumes from milliliters, used in test tubes in

macrodilution assay, to microliters in 96-wells microtiter plates, and even to nanoliters in plates with thousands wells.He was the first to have the notion to apply calibrated spiral wire loops for multiple simultaneous serial dilutions in plastic multiwell strips (Manns, 1999 ommited in the list).Juniperus derivatives have been used for fragrance and flavoring in alcoholic beverage industry, in food preparation, as well as for insecticidal and cosmetic purposes (Lawless, 2013).The chosen bacterial indicator strains were Staphylococcus aureus, Listeria innocua, Salmonella enteritidis and Pseudomonas aeruginosa.Although it is not always pathogenic, it could be causative agent of some skin and respiratory infections, and also could be food poisoning (https://en.wikipedia.org/wiki/Staphylococcus_aureus).The two main clinical manifestations of listeriosis are sepsis and meningitis (https://en.wikipedia.org/wiki/Listeria).Indicator bacterial strains: A. Staphylococcus aureus; B. Listeria innocua; C. Salmonella enteritidis; D. Pseudomonas aeruginosa 4.Chemical compositions of EO and PDW were determined by GC-MS and LC-MS/MS analysis, respectively, as previously described (Lesjak et al., 2013; Orcic et al., 2014).Bacterial suspension preparation Bacterial cultures were freshly prepared for every experiment in corresponding medium (BHI for L. innocua, MHB for all the rest bacteria).GC-MS analysis determined 93.95 % of total EO composition and revealed exclusively monoterpene (40.7 %) and sesquiterpene (59.3 %) hydrocarbon as dominant constituents.This could be attributed to the high proportion of the hydrocarbon monoterpenes, which possess the lowest effect compared to other terpenoid compounds, including oxigenated ones (Griffin et al., 1999).Quantitative determination of plant phenolics in Urtica dioica extracts by high-performance liquid chromatography coupled with tandem mass spectrometric detection.I owe a great debt of gratitude to Prof. Dr. Jelena Knezevic-Vukcevic, Prof. Dr. Slavisa Stankovic, Prof. Dr Tanja Beric , Dr Ivica Dimkic, Dr Stoimir Kolarevic, Dr Karolina Sunjog, Ivan Nikolic and Jovana Kostic.Most often, the antimicrobial susceptibility tests complements the Gram staining and assessment of cultural properties, the results of which are obtained previously.Agar dilution method The agar dilution method is based on the incorporation of variable concentrations of the antimicrobial agent into molten agar medium, using serial two-fold dilutions.Plant derivatives as potent antimicrobial agents Due to the growing occurrence of microbial resistance to currently available antimicrobials, there is a continuous need for new agents, which can serve as a potential alternative (Davies and Davies, 2010).Consider if microtitre plate-based antibacterial assay with resazurin is a reliable and accurate method to detect antibacterial properties of agents from plant origin.Briefly, air-dried and finely ground needles and seed cones of the J. communis plant sample were submitted to hydro-distillation using an apparatus of Clevenger type.Bacterial suspensions were centrifuged at 4000 rpm for 10 min and resuspended in 0.01M $MgSO_4$ to achieve 106 CFU/mL.Regardless to J. communis derivative that was used, the analysis of bacterial susceptibility revealed the same pattern: Gram-positive bacteria were more sensitive than Gram-negative ones, against both test substances.The microtitre plate-based antibacterial assay with resazurin is a sensitive, reliable and accurate method, which could be used to detect antibacterial properties of agents from plant origin.Antibacterial effects of theaflavin and synergy with epicatechin against clinical isolates of Acinetobacter baumannii and Stenotrophomonas maltophilia.Levy, S. B., O'Brien, T. F., Davey, P. G., McEwen, S. A., Barrett, J. F., Avorn, J., World Health Organisation., Alliance for the Prudent Use of

Antibiotics., 2001.Screening of the antibacterial effect of Juniperus sibirica and Juniperus sabina essential oils in a microtitre plate-based MIC assay.On the other hand, the MBC/MFC concentration is defined as a minimal concentration of antibacterial/antifungal agent that completely (>=99.9%) killed particular microorganism (Levinson, 2010).This variant is so-called 'disk-diffusion method', since test compounds are applied by filter paper discs as carriers (Figure 1).Generally, antimicrobial agent diffuses into the agar and inhibits the growth of the test microorganism, leading to the formation of growth zones inhibition.Based on obtained results, i.e. inhibition zones sizes, bacteria used as test organisms are then interpreted into susceptibility categories.There are many approved guidelines for dilution antimicrobial susceptibility testing of bacteria, yeast and filamentous fungi.In addition, MBC value is determined by taking a small sample (0.01 or 0.1 mL) from the tubes used for the MIC assay and spreading it over the surface of appropriate agar plate (the plate does not contain the antimicrobial agent).Following overnight incubation at appropriate temperature, optimized for every particular bacterial species, the tubes are examined for visible bacterial growth, as evidenced by turbidity.In the environments with high presence of antibiotics, the prevalence of resistance increases rapidly as a result of mutation, genetic exchange and natural selection (Aminov, 2007 omitted in the list).saxatilis derivatives against chosen bacterial species in resazurin-incorporated microtitre plate-based antibacterial assay.After distillation of EO, residual aqueous solution was evaporated in vacuum at 45?C in order to prepare dried PDW extract.o Columns with EO and PDW: In the first well, 160 uL of MHB (or BHI for L. innocua) was added, while 100 uL of medium was added in all the remaining wells.The final volume of samples in the wells was 200 uL. Prepared concentration ranges were 50-0.39 mg/mL and 25-0.19 mg/mL for EO and PDW, respectively.o Positive controls (antibiotics Str and Rif): The columns with gradient concentrations of antibiotics were prepared as previously explained, but instead of test-substance solution, antibiotic stock solution was used.Among the quantified constituents, rutin and quinic acid were the most abundant, determined at 12.2 mg/g and 11.1 mg/g, respectively, and followed by catechin (5.53 mg/g) and epicatechin (1.74 mg/g).saxatilis EO and PDW were tested against two Gram-positive (S. aureus and L. innocua) and two Gram-negative (S. enteritidis and P. aeruginosa) bacteria.The stronger activity against Gram-positive bacteria could be attributed to the cell wall structure and is in accordance with numerous literature data (Burt, 2004; Dzamic et al., 2015; Lesjak et al, 2014).The most sensitive was Gram-positive L. innocua, with equal MIC and MBC values determined at 6.25 mg/mL and 1.56, for EO and PDW, respectively.Furthermore, in the case of the most resistant P. aeruginosa, it was not possible to determine MIC and MBC values of EO in applied concentration range.In the case of PDW, literature data indicate that catechin and epicatechin could contribute to its antibacterial effect (Betts et al., 2011; Taylor et al., 2005).Phytochemical composition and antioxidant, anti-inflammatory and antimicrobial activities of Juniperus macrocarpa Sibth.Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the in vitro antibacterial screening of phytochemicals.saxatilis sensitizes lung cancer cells to the anticancer effects of doxorubicin in vitro, in press Wiegand, I., Hilpert, K., Hancock, R.E., 2008.https://en.wikipedia.org/wiki/ Staphylococcus_aureus https://en.wikipedia.org/wiki/Listeria https://en.wikipedia.org/wiki/Salmonella https://en.wikipedia.org/wiki/Pseudomonas_aeruginosaPreviously to the antibiotic therapy practice, it is

necessary to determine correctly the antimicrobial potential of antibiotics, since it is the only way to define doses of antibiotics that should be use in therapy.The MIC value is defined as the lowest concentration of a drug that will inhibit the visible growth of a microorganism after overnight incubation.In general, assessment of antimicrobial activity is performed by three basic types of tests: diffusion, dilution and bioautography (Fennell et al. 2004).Agar diffusion method In general, in agar diffusion methods agar plates are inoculated with a standardized inoculum of the test microorganism.Since bacterial growth inhibition does not obligatory mean the bacterial death, this method cannot distinguish bactericidal and bacteriostatic effects (Balouiri, 2016).When applied to the surface of an inoculated agar plate, the gradient is transferred from the strip to the agar plate and remains stable for a period that covers the wide variation of critical times associated with the growth characteristics of different microorganisms.The MIC value in ug/ml can be read as the point where the ellipse edge intersects the precalibrated E-test strip, providing a precise MIC value (Balouiri, 2016).1, 2, 4, 8, and 16 ug/mL) in a liquid growth medium dispensed in test tubes (macrodilution) or in the microtitre-plate wells (microdilution).Bactericidal antibiotics usually have an MBC equal or very similar to the MIC, whereas bacteriostatic antibiotics usually have an MBC significantly higher than the MIC (Levinson, 2010).In the broth microdilution assay, the microorganisms are grown in the plate wells, to which various concentrations of the tested compound are added.Resazurin is an oxidation-reduction indicator used for the evaluation of cell growth, particularly in various cytotoxicity assays.It is a blue non-fuorescent and non-toxic dye that becomes pink and fluorescent when reduced to resorufin by oxidoreductases within viable cells.Listeria species are gram-positive, rod-shaped, and facultative anaerobic bacteria, and do not produce endospores (Figure 4B).1.1.1.2.1.1.2.1.1.1.2.1.2.2.2.5.5.2.5.2.1.5.2.2.5.2.3.6.2.3.4.1806.40.2, 71.