Original research article

Soil Protein as a Potential Antimicrobial Agent Against Methicillin –Resistant *Staphylococcus aureus*

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Abstract

Recently, the interest is increasing to find alternatives to replace the usage of antibiotics since their massive and improper usage enhance the antibiotic resistance in human pathogens. In this study, for the first time we showed that the soil proteins have very high antibacterial activity (98% of growth inhibition) against methicillin resistant *Staphylococcus aureus* (MRSA), one of the most threatening human pathogens. We found that the protein extract (C3) from the forest with past intensive management showed higher antibacterial activity than that of unmanaged forest. The MIC and IC_{50} were found to be 30 and 15.0 μg protein g^{-1} dry soil respectively. C3 was found to kill the bacteria by cell wall disruption and genotoxicity which was confirmed by optical and fluorescent microscopy and comet assay. According to qPCR study, the mecA (the antibiotic resistant gene) expression in MRSA was found to be down-regulated after C3 treatment. In contrast, C3 showed no hemolytic toxicity on human red blood cells which was confirmed by hemolytic assay. According to ultra-high performance liquid chromatography- mass spectrometry (UHPLC-MS), 144 proteins were identified in C3 among which the majority belonged to Gram negative bacteria (45.8%). Altogether, our results will help to develop novel, cost-effective, non-toxic and highly efficient antibacterial medicines from natural sources against antibiotic resistant infections.

Keywords: soil protein, forest soil, MRSA, natural products, antibacterial

1. Introduction

Over the last ten years, the antimicrobial resistance (AMR) has received serious attention since several bacterial species start to develop resistance against the available antibiotics, where the resulted infections might become more complicated, and thence modern medical interventions may become more dangerous for patients (Wells and Piddock, 2017). The healthcare associated infections and AMR are growing challenges to public health and healthcare providers worldwide (Watkins and Bonomo, 2016) . The bacterial infections mostly caused by commensal bacteria which acts as a reservoir of antibiotics resistant genes which then can be transferred to pathogenic species (von Wintersdorff et al., 2016). The antibiotics treatment enhances the development of microbial resistance and change the competitive balance between organisms (Crémieux et al., 2003; van Bijnen et al., 2015). Generally, it is well known that the extensive use of antibiotics, the long duration of treatment, and sometimes the early stopping of antibiotics leads to AMR strains emergence (Chastre *et al.*, 2003; Fontela and Papenburg, 2018). In addition, many other factors might speed up the emergence and the prevalence of AMR such as the improper use of antimicrobial medication, absence of a comprehensive and coordinated response, lack the surveillance and controlling systems of antimicrobial resistance, insufficient systems to ensure quality and continuous supply of medicines, substandard infection control practices, and the vast use of these agents as a growth stimulator in animal feed (Jindal et al., 2015).

Staphylococcus aureus raked among the highest extensively drug resistant gram-positive bacteria, it was included with high priority tier according to the WHO priority list of the antibiotics resistant bacteria that urgently require enhanced and focused research and development investments of new antibiotics (Tacconelli et al., 2018). Since 1960, around 80% of *S.aureus* isolates have been resistant to penicillin, thereafter, *S. aureus* methicillin

and vancomycin resistant strains emergence had been reported (Deurenberg et al., 2007). Methicillin *S. aureus* is the pathogen of greatest interest for the healthcare frameworks, it considers the major cause of the community and nosocomial acquired infections, and the most invasive pathogen among the healthcare facilities worldwide. The resistance of *S. aureus* to methicillin is acquires by the *mec A* gene which is located on a mobile genetic element Staphylococcal Cassette Chromosome *mec* (SCC*mec*) (Ito et al., 2003) and encodes the penicillin binding protein (PBP 2A) with decreased affinity for β-lactam antibiotics (Deurenberg et al., 2007). Apparently, the antimicrobial resistance genes and their genetic vectors once developed in certain bacterial strains in some places might be spread indirectly among the world's interconnecting commensal, environmental, and pathogenic bacterial populations to other bacterial strains or species anywhere else (O'brien, 2002).

During the past few decades, the pharmaceutical research and development was ineffective to meet the clinical requirements for new antibiotics even though the prevalence of antibiotic resistant bacteria cause a considerable challenges to morbidity and mortality worldwide (Tacconelli et al., 2018). Thence, inadequate pipeline of new antibiotics, the unsustainable production and supply of old antibiotics is becoming a serious global problem that restricts the treatment options for common bacterial infections (Tängdén et al., 2018). In 2016, due to risen of global awareness for the necessity for new antibiotics, WHO created a priority list of antibiotic resistant bacteria to direct research and development of new and effective medication (Tacconelli et al., 2018). Recently, the interest is risen in use the natural products as antibiotics or antimicrobial compounds against multidrug resistant pathogens including Methicillin-resistant *Staphylococcus aureus* (MRSA). Finding new natural sources for the antibiotic scaffolds is one of the suggested strategy for screening a new antibiotic compounds for refilling the dried

pipeline (Spellberg, 2014). Antimicrobial proteins or peptides (AMPs) are one of these natural compounds that are widely distributed in nature and produced by organisms among all kingdoms (prokaryotes and eukaryotes) (Hegedüs and Marx, 2013). Presently, there are more than 3000 experimentally reported antimicrobial peptides including both the synthesized and naturally produced compounds (Ageitos et al., 2017).

Soil is very rich habitat with wide variety of natural antimicrobial compounds including proteins and peptides that originated from different sources such as plants, animals, and microbes. In addition, anthropogenic activities have a significant role in modification and alteration of soil microbial communities and their metabolic activities (Ananbeh et al., 2019). Thus, the soils that are subjected to sever management (coppicing) or hard environmental conditions (i.e. desert soil) could be the best choice to study their pharmacological properties since a numerous primary and secondary metabolites will be produced by the microbial community to adapt the stressful conditions. Furthermore, the antimicrobial properties of soil microbial enzymes are overlooked although they have crucial roles in environmental pollutants and toxins degradation. From this point of view and due to arisen interest in the biologically produced compounds, these enzymes might be a hotspot for the pharmacological studies. In addition to the microbial secondary metabolites, that includes antibiotics, antimicrobial agents, and others, which are not essential for the microbial growth but important for human health (Ruiz et al., 2010). About 75% of commercially and clinically valuable antibiotics were produced by different species of soil *Streptomycetes* (Ceylan et al., 2008; Saadoun et al., 2017). Since the antibiotics resistant and producing genes are found in environment mainly soils (de Castro et al., 2014) and many of soil organisms showed resistance against synthetic antibiotics including *Staphylococcus* species (Tomasz, 2006), thence the natural products that found in soil can be the ideal solution against such organisms. From this point of

view, soil proteins might possess a unique structures and properties that enable them to act as safe antimicrobial agents on human health and the environment. Especially that they are 100% natural and easy degraded if they release to the ecosystem.

To our knowledge, the use of soil proteins that directly extracted from soil as antimicrobial agent against AMR microbes has never been reported previously. Due to this reason, the main aim of this study was to assess the antimicrobial properties of soil proteins as a natural antimicrobial compounds against MRSA. For this purpose, soil proteins were extracted from different soil samples and analyzed for their antimicrobial activity using different methods.

2. Materials and methods

2.1. Soils

A total of 24 soil samples were collected randomly from two sessile oak (*Quercus petraea*) forests high (H) and abandoned coppice (C) forests, (12 soil samples from each). To avoid confounding of the results, the high forest developed spontaneously without any human intervention while coppice forest was under intensive management until the beginning of 20th century (Kadavý et al., 2011)). Both forest stands are located in South Moravian region of the Czech Republic, the average annual precipitation ranges from 500 to 550 mm and the mean annual temperature is 8.5 ºC. All soils were acidic cambisol developed from granodiorite (Němeček, 2001). All soil samples were taken from the upper 6 cm of the soil, sieved at 4 mm and stored at -20 °C until analysis. For detailed description of forest stands and soils see Stojanović et al. (2017) and Ananbeh et al. (2019), respectively.

2.2. General soil properties

Soil pH were measured in a soil: water suspension (1 part soil to 2 parts pure water) (Hanlon and Bartos, 1993). The total carbon (TC) and total nitrogen (TN) contents were determined in air-dried, ground samples by respectively potassium dichromate oxidation in acid medium and Kjeldahl digestion (Guitián Ojea and Sacro, 1976).

2.3. Soil protein extraction

Soil proteins were extracted according to Ogunseitan (1993) with the modification of Singleton et al. (2003). Briefly, 1 g of soil (at 50% WHC) were weighed in an Eppendorf centrifuge tube (1.5 ml) and mixed with 100 µl protease inhibitors cocktail (Sigma-Aldrich, St. Louis, MO, USA) and 1 ml of extraction buffer. Thence, the mixture was subjected to four cycles of snap freezing in liquid nitrogen and thawing to 25 °C. After centrifugation at 20000xg for 15 min at 4 °C, 600 µl of the supernatant was pipetting to a clean centrifuge tube and kept at -80 °C for further analysis.

2.4. The estimation of protein concentration

Prior to the total protein concentration estimation, the proteins existing within the extract were concentrated using acetone precipitation method. The supernatant of extracted proteins were mixed gently with 1500 µl cold acetone and kept overnight at -20 °C and then centrifuged at 20000xg for 15 min at 4 °C. The supernatant was then discarded and the protein pellet were retained and resuspended in ACS water (HPLC grade) and stored at -80 °C. The total protein concentration was estimated using the modification of the Lowry assay (Redmile-Gordon et al., 2013).

2.3.1. The application of the extracted soil protein on the methicillin resistant Staphylococcus aureus

2.3.1.1. Cultivation of methicillin resistant Staphylococcus aureus

The methicillin resistant *Staphylococcus aureus* (ST239:SCCmec IIIA) strain using in this study obtained from the Czech collections of Microorganisms, Faculty of Science, Masaryk University in Brno, Czech Republic. The bacterial strain was stored in 80% (v/v) glycerol at -80 °C. The bacterial strain was inoculated into 25 ml Erlenmeyer flasks with sterilized culture media (Nutrient Broth, pH 7.4) and incubated in a shaker incubator at 600 rpm, for 24 h at 37 °C. Prior to experiment, the bacterial culture was diluted to OD_{600} nm= 0.5 McF (0.1 absorbance) using the same cultivation media (Jelinkova et al., 2018).

2.3.1.2. Bacterial growth curve determination

The estimation of the antimicrobial effect of the extracted soil proteins was performed by the apparatus Multiskan EX (Thermo Fisher Scientific, Germany) according to the protocol of our previous article (Jelinkova et al., 2018). MRSA culture was diluted with Muller- Hinton (MH) medium to OD_{600}nm= 0.5 McF after cultivation in MH broth for 24 h at 37 °C. Different soil protein extracted from different soil samples with 30 µg protein g^{-1} dry soil concentration were applied to the diluted bacterial culture into the microtiter plate with a total volume of 300 µl in each well. After 24 hours, the absorbance were measured for 24 h with 30 min interval at 600nm (Jelinkova et al., 2018). Among all the applied proteinaceous extracts, C3 extract that caused the highest percentage of inhibition in MRSA culture was selected to perform the rest of the experimental part.

2.3.1.3. Minimum inhibitory concentration (MIC) estimation

The MIC defined as the lowest concentration of a given compound that inhibits the growth of the tested microbe. The MIC of the most active soil protein extract (C3) was determined by the standard broth microdilution technique (EUCAST) using the 96 well microtiter plate and detected by naked eyes. The soil protein extract (30 µg protein g^{-1} dry soil) was pipetted into the microplate wells and serially diluted with the two fold

dilutions to produce different concentrations (30, 15, 7.5, 3.75, 1.875, and 0.935 μg protein g^{-1} dry soil) which then mixed with bacterial culture (0.5 McF) and incubated at 37 °C for 24 hours. The well with the lowest protein extract concentration at which there is no visible bacterial growth was considered the MIC. The bacterial culture without any soil protein was used as a positive control.

2.5. Haemolytic assay

Human red blood cells (RBCs) were washed with 150 mM NaCl and centrifuged repeatedly at 5000xg for 5 min until a clear supernatant was obtained. The Triton X-100 (0.1%) was used as a positive control due to its high haemolytic activity and PBS was used as a negative control. Different concentrations of soil protein extracts (30, 15, and 7.5 μg protein g^{-1} dry soil) were added to the suspension of washed RBCs in PBS (pH 7.4). The samples were mixed and incubated for 1 h at 37 °C and centrifuged at 5000xg for 5 min. After centrifugation, the absorbance was measured for the supernatant at 540nm and used to calculate the percentage of haemolysis using the following equation: % haemolysis = $[(A_t - A_c) / (A_{100\%} - A_c)] \times 100$, where A_t is the absorbance of the samples incubated with the protein extract; A_c is the absorbance of the negative control; and $A_{100\%}$ is the absorbance of the positive control.

2.6. Microscopy of MRSA after the application by soil protein extract in Ambient Light and Live/ dead cell assay

The evaluation of live/dead bacterial cells was performed using an inverted Olympus IX 71S8F-3 fluorescence microscope (Olympus, Tokyo, Japan) equipped with Olympus UIS2 series objective LUCPlanFLN 40× (N.A. 0.6, WD 2.7 – 4 mm, F.N. 22) and a mercury arc lamp X-cite 12 (120 W, Lumen Dynamics, Mississauga, Canada). Two fluorescent dyes were used for the live/dead cell assay: propidium iodide (PI) for staining of cells with damaged membranes and SYTO9 (Invitrogen AG, Basel, Switzerland) for

permeating both intact and damaged membranes of the cells (Berney et al., 2007b). Images were obtained by Camera Olympus DP73 (Olympus, Tokyo, Japan) and managed by Stream Basic 1.7 software (Olympus Soft Imaging Solutions GmbH, Münster, Germany) with the software resolution of 1.600 × 1.200 pixels. After 24 h of incubation at 37 °C with the soil protein extract (30 µg protein g^{-1} dry soil), 5 µl of bacterial culture was stained with 0.5 µl of fluorescent dye.

2.7. Comet assay (single cell gel electrophoresis)

After 24 h incubation at 37 °C, the bacterial culture was diluted at a density of 0.5 McF and treated with the soil protein extract for two hours. The applied concentration of the soil protein extract was 30 µg protein g^{-1} dry soil. As a positive control, 250 µM H_2O_2 was applied. To break down the bacterial cell wall, the lysozyme with 20 µg ml^{-1} concentration was added to the diluted bacterial culture and shaken together for 1 hour. Thereafter, about 10 µl of the cell suspension was mixed with 75 µl of 0.8% low melting point agarose in an Eppendorf tube and added on one end of a frosted plain glass slide precoated with 200 µl of normal agarose (1%) and then cover slip was placed over it (~ 5 – 10 min). The cover slip was removed and the third layer of low melting agarose (100 µl) was added. After gel solidification, the slides were immersed in a lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10 containing 1% Triton X-100 and 10% DMSO) for 2 hours at 4 °C. A cold alkaline electrophoresis buffer was poured into the chamber and incubated for 30 min at 4 °C. The electrophoresis was carried at 4 °C for 30 min, at 1.25 V/cm and 300 mA. The slides were neutralized (0.4 M Tris, pH 7.5) and then stained with ethidium bromide (EtBr, 2 µg/ml). The cells were analyzed using a fluorescence microscope Olympus IX 71S8F-3 (Olympus, Tokyo, Japan) and classified according to the shape of the fluorescence of the comet tail [0 (no visible tail) to 4 (significant DNA in the tail)].

253 *2.8. RNA isolation*

254 MRSA was cultured in nutrient broth with shaking at 37 °C for overnight. After that, 3
255 ml of overnight bacterial culture was centrifuged at 2000xg for 5 minutes at room
256 temperature, and then the bacterial pellet was resuspended with the soil protein extract
257 (30 µg protein g^{-1} dry soil) and incubated at 37 °C for 24 hours. Then, the bacterial culture
258 was centrifuged at 2000xg for 5 minutes for 10 minutes at room temperature. The
259 harvested bacterial pellet was resuspended in 100 µl of Lysozyme buffer (Lysozyme: 1
260 mg/ml; 10 mM Tris; pH 8) and incubated at 37 °C for 30 minutes. Then after, 300 µl of
261 the lysis buffer from High Pure RNA Isolation Kit (Roche, Basel, Switzerland) was
262 added, sample was mixed by pipette and incubated at 37 °C for 30 minutes. The isolations
263 were performed using High Pure RNA Isolation Kit according to manufacturer's protocol.
264 Furthermore, one extra DNase I (NEB, Ipswich, MA, USA) treatment was added to avoid
265 the DNA contamination of isolated samples. The RNA concentration and purity was
266 assessed with Infinite 200 PRO NanoQuant instrument (Tecan, Zürich, Switzerland). The
267 RNA integrity was inspected by Bleach gel (Aranda et al., 2012).

268 *2.9. Quantitative real-time PCR (qRT-PCR)*

269 The expression of *mecA* gene (MRSA antibiotic resistant gene) and *16S rRNA* (reference
270 gene) was studied by one step quantitative PCR using SYBR® Green Quantitative RT-
271 PCR Kit (Sigma-Aldrich, St.Louis, MO, USA) and Mastercycler® ep ealplex4 instrument
272 (Eppendorf, Hamburg, Germany). The RT-PCR reaction mix (20 µl) RT-PCR was
273 prepared according to manufacturer's instructions and was composed of 100 ng total
274 RNA, 10 µl of 2xSYBR Green Ready mix, M-MLV reverse transcriptase (1 unit/µl) and
275 set of primers with 0.5 µM final concentration. Primers sets used to amplify *mecA* gene
276 were (Forward: 5′-CCTCTGCTCAACAAGTTCCA-3′, Reverse: 5′-
277 ACGTTGTAACCACCCCAAGA -3′) and *16S rRNA* (Forward: 5'-

CTCGTGTCGTGAGATGTTGG -3', Reverse: 5'-TTCGCTGCCCTTTGTATTGT -3'). The gene expression and high resolution melting curves evaluation were performed using Realplex software (Eppendorf, Hamburg, Switzerland). Threshold was determined by noise band with automatic baseline drift correction. The expression level of *mecA* gene was normalized against the *16S rRNA* expression level. Fold change differences were determined using the $2^{-\Delta\Delta CT}$ method compared with untreated bacterial cells as a control (Livak and Schmittgen, 2001). The differences between the treated and untreated groups were analyzed by unpaired two-tailed Student's t-test and $p< 0.05$ was considered significant. All samples were performed in triplicate.

2.10. Identification of the total proteins in the active extract (C3)

2.10.1. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis SDS-PAGE

The SDS-PAGE was performed using polyacrylamide gels (6% stacking, 12% separating) run at 200 V for 40 min at 23 °C. Prior to analysis, the concentrated protein extract was thawed and mixed with reducing buffer (5% b-mercaptoethanol) in 2:1 ratio and heated at 95 °C for 5 min. The mixture then loaded on to the gel along with SDS-PAGE standard (Precision plus protein standards, BIORAD). The electrophoresis was run at 200 V for 40 min at 23 °C (Power Basic, Biorad, Hercules, CA, USA) in tris–glycine buffer (0.025 M Trizma-base, 0.19 M glycine and 3.5 mM SDS, pH = 8.3). After separation, the gels were stained using Coomassie brilliant blue R-250, followed by silver staining using a commercial kit and following manufacturer's instructions (Sigma silver stain kit Ag-2).

2.10.2. Ultra-high performance liquid chromatography-Mass spectrometric analysis (UHPLC-MS), data processing and protein identification

The Peptide lysates were separated on a UHPLC system (Ultimate 3000 RSLCnano, Dionex/Thermo Fisher Scientific, Idstein, Germany). The samples (5 µl) were first loaded

for 5 min on the pre-column with a maximum loading capacity of 2 μg (μ- precolumn, Acclaim PepMap, 75 μm inner diameter, 2 cm, C18, Thermo Scientific), at 4% mobile phase B (80% acetonitrile in nanopure water with 0.08% formic acid) and 96% mobile phase A (nanopure water with 0.1% formic acid), and then were eluted from the analytical column (PepMap Acclaim C18 LC Column, 25 cm, 3 μm particle size, Thermo Scientific) over a 120-min linear gradient of mobile phase B (4–55% B). Mass spectrometry was performed on a Q Exactive HF mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) with a TriVersa NanoMate (Advion, Ltd., Harlow, UK) source in LC-chip coupling mode. Mass spectrometry scans were measured at a resolution of 120000 in the scan range of 400- 1600 m/z (Starke et al., 2017).

Proteome Discover (Thermo Fisher Scientific, v1.4, San Jose, CA, USA) was used for protein identification and the acquired MS/MS spectra were searched with Sequest HT against the Uniport bacteria and fungi database, and RefSoil database. Only peptides that passed the FDR thresholds set in the Percolator node of <1% FDR q value and that were rank 1 peptide were considered for protein identification (Ros et al., 2018).

3. Results and discussion

Over the last two centuries, the infectious diseases caused by MRSA showed a progressive increase in the health care facilities. Recently, MRSA infections are the recurrent causative agent of both the community and the nosocomial infection. MRSA is known as a multidrug resistant pathogen and they acquire resistant rapidly after the introduction of new antibiotics (Ventola, 2015). The developing or discovery of new antibiotics or antimicrobial compounds is in demand due the continuous increase of newly multidrug resistant pathogens emergence.

3.1. The total protein concentration

All soils were acidic and they vary in their TC and TN contents (Supplementary information, Table S1). The total protein was extracted from 24 different soil samples collected from two differently managed forest ecosystem high (H) and abandoned coppice (C). The soil samples under the two forests vary in their proteins extract concentration from 65 to 320 µg protein g^{-1} dry soil, with highest concentration in soils collected from the abandoned coppice (C) (Fig.1). The variations in the protein concentration between the different soils might be related to the management practice, and soil chemical and biological properties such as pH, organic matter content, and the microbial community diversity (Shen et al., 2015). In addition, the extraction and analysis of soil proteins can be a good indicator for the microbial biomass and the soil ecosystem stress (Singleton et al., 2003).

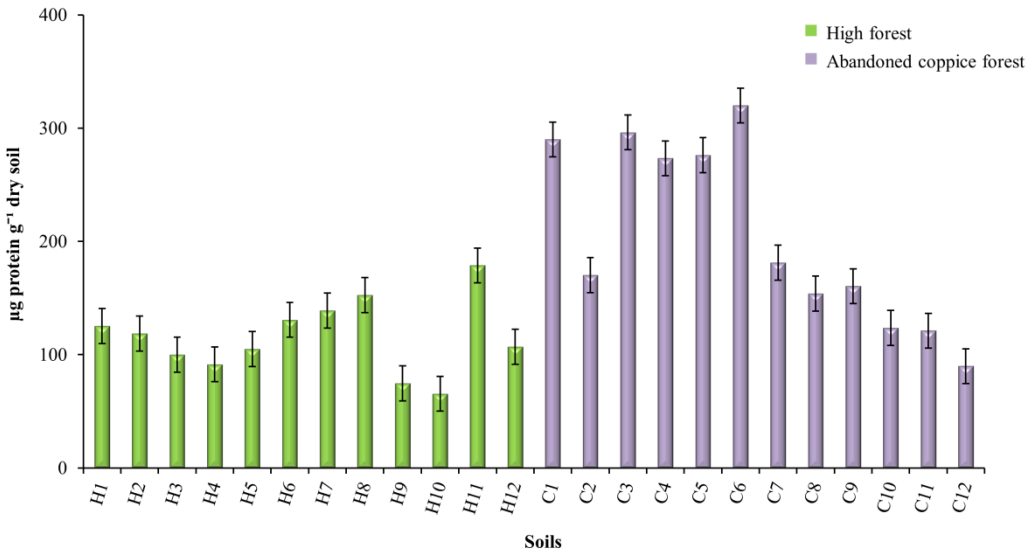


Fig.1. Protein concentration in soil samples collected from high (H) and abandoned coppice sessile oak (C) forest. Data represent the mean ± SD, n=5.

3.2. Antimicrobial properties estimation of the soil protein extracts

The growth curve analysis was used to determine the inhibitory effects of the soil protein extracts. Minimum inhibitory concentration (MIC) and IC_{50} were also estimated for better

345 understanding of the antimicrobial effect of the soil protein. Totally 24 protein extracts
346 were obtained from different forests' soils and applied against MRSA, and then the active
347 extract with highest inhibitory activity were used for the rest of the analysis. All the
348 applied proteinaceous extracts showed inhibitory effects against MRSA compared to the
349 control (Fig. 2). The percentage of inhibition varies from 79% to 98%. The highest
350 percentage of inhibition (98%) was found in total protein extracted from C3, followed by
351 the samples C4, C2 with 95% and H4 with 94% of inhibition from the coppice and high
352 forests, respectively (Fig. 3). The variations in the inhibitory effects of the different
353 extracts might be related to the variation in the chemical properties (i.e. pH and organic
354 matter) of the soil samples that may affect the microbial population and their related
355 properties (Shen et al., 2015). This result could be a consequence of the previously
356 intensive used management practice in the abandoned coppiced site. The forest
357 management practice especially the biomass harvesting (i.e. coppicing) alters different
358 soil properties which affect their microbial community composition and related functions
359 (Nave et al., 2010; Ananbeh et al., 2019). During the management rotation period, the
360 microclimate changed in the site (i.e. light, temperature, moisture content, organic matter
361 content, substrate availability, etc.) (Mitchell, 1992), which might cause a competition
362 between the microbial community to survive on the limited resources through secreting
363 some compounds that enhance the growth of one group of microbes and suppress the
364 other. This type of competition changes the structure and functions of the microbial
365 community and may be the composition of their products. These products might possess
366 a special characteristic in their composition, structure and function, which make them
367 acting as antimicrobial agents when they applied to human pathogens and it can be a good
368 impact of the coppicing practice.

The minimum inhibitory concentration of the extract (C3) that exhibit the highest percentage of exhibition and the IC_{50} was calculated after the application of different concentration of the active extract against MRSA. The MIC and IC_{50} were found to be 30 µg protein g^{-1} dry soil and 15.0 µg protein g^{-1} dry soil respectively. The MIC of our natural proteinaceous extract was found to be much lower than that of some synthetic and innate peptides like β-defensins (>1000 µgml^{-1}) (hBD1 to hBD3) (Midorikawa et al., 2003). In addition, many researchers showed the efficiency of some natural products against different MRSA strains such as the inhibitory and bactericidal effects of bee venom (Han et al., 2016), and the antimicrobial activity of two peptides produced by a halotolerant *Bacillus subtilis* strains isolated from a rhizosphere soil sample against different Gram positive bacteria including *S. aureus* (Baindara et al., 2013). Similarly, two peptides (subpeptin JM4-A and subpeptin JM4-P) produced by *Bacillus subtilis* with the antibacterial effect against different species of Gram positive and Gram negative bacteria (i.e. *Staphylococcus aureus* CFMCC 1.2645, *Escherichia coli* CMCC44104) were revealed by Wu et al. (2005).

3.3. The estimation of haemolytic activity of the protein extract

Haemolysis identified as the breakdown of RBC membranes that causes the release of haemoglobin, which is also known as erythrocyte necrosis (Wilson, 2012). The haemolytic activity of the soil proteins is a critical parameter to evaluate their therapeutic index and cytotoxicity for their usefulness in pharmacological preparations (Zohra and Fawzia, 2014). Different concentrations of the C3 extract were applied to the human red blood cells. The complete haemolysis of RBCs was obtained using the 0.1% Triton X-100 (positive control) and the non- haemolysis was observed in case of PBS buffer (negative control). The haemolytic activity of the extract was 0% at all applied concentrations, which means that our extract showed non-toxic effects that make it

suitable for future use as antimicrobial medicine against MRSA and maybe other pathogenic bacterial strains (Fig. 4). Similar results had been reported for different natural products extracted from different medicinal plants (Karou et al., 2011; Khalili et al., 2014; Zohra and Fawzia 2014; Dima et al., 2017; Chansiw et al., 2018), this might gave us a clear idea that most of the natural products from soil or plant are safe on human health. In contrast, many synthetic and commercial compounds that used as antimicrobial agents have toxic effects on human cells at certain concentrations (Harder et al., 2000; Shin et al., 2001; Aranda et al., 2005).

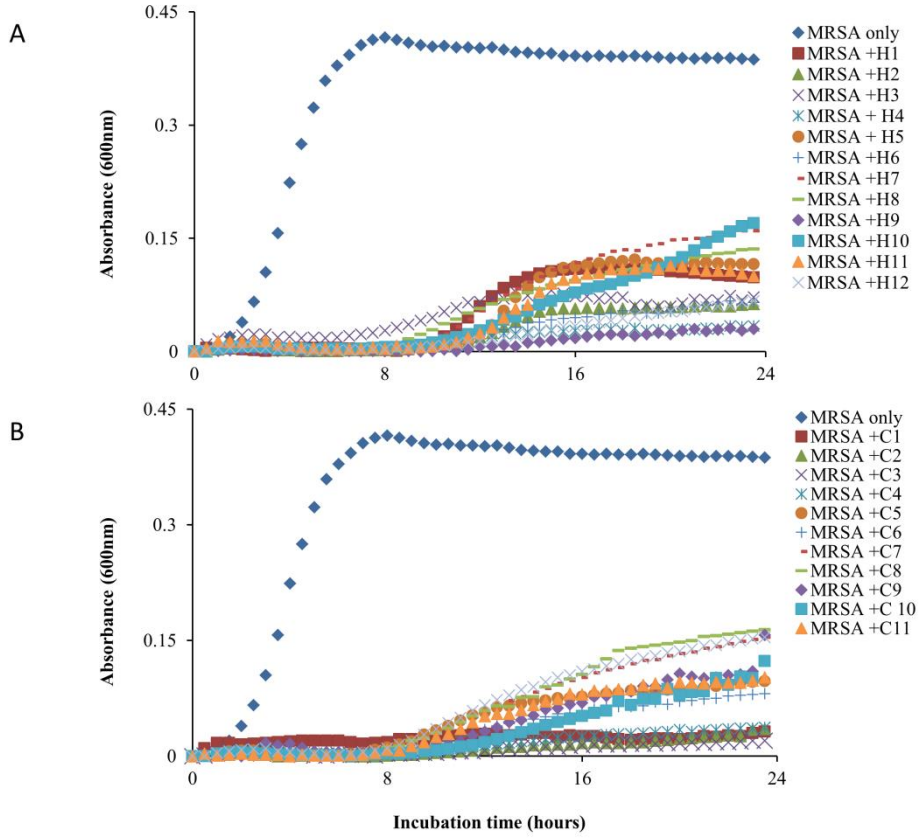
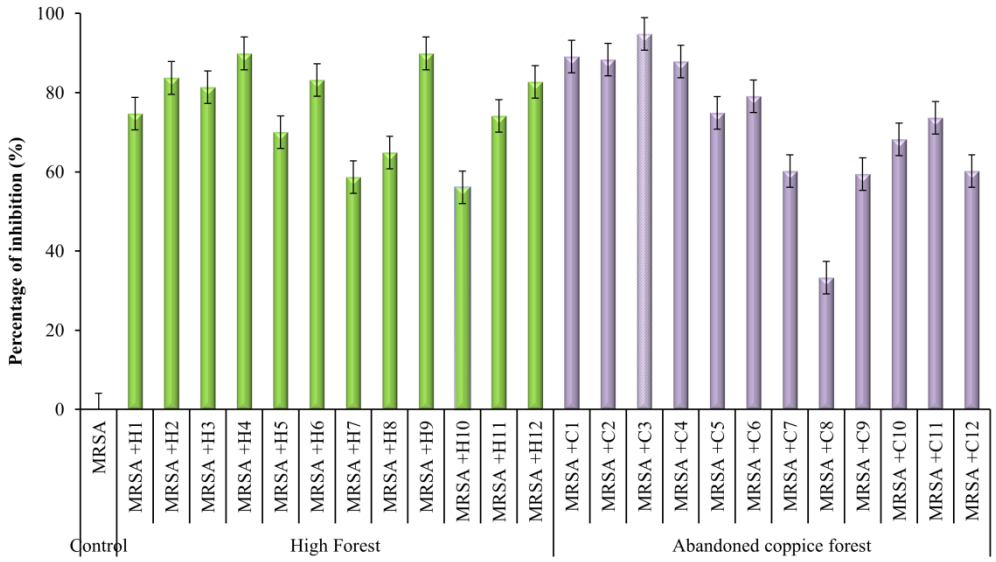


Fig. 2. Growth changes after application of the extracted protein from soil samples collected from high (H) and abandoned coppice (C) sessile oak forest on MRSA. The bacterial growths were represented by the absorbance at 600nm. A) Represents the growth changes in MRSA after application of protein extracted form soil samples that collected from high sessile oak forest (H) compared to the control. B) Represents the growth changes in MRSA after application of protein extracted form soil samples that collected from abandoned coppice sessile oak forest (C) compared to the control.

411 *3.4. Microscopic assessment of live/dead and the genotoxicity of the bacterial cells*

412 The antibacterial effect of the soil protein extract was confirmed under ambient light
413 illumination by optical microscope after its application against MRSA. The number of
414 bacterial cells decreased significantly after the treatment with the soil extract (C3)
415 compared to the control group (Fig. 5). The bacterial cells appear in clots with broken cell
416 wall (Berney et al., 2007a), which indicates the antibacterial effects of the applied extract
417 against the tested pathogen.



418

419 **Fig. 3.** Percentage of inhibition in MRSA growth after application the proteins extracted
420 from different soil samples collected from high (H) and abandoned coppice (C) sessile
421 oak forest. Data represent the mean ± SD, n=5.

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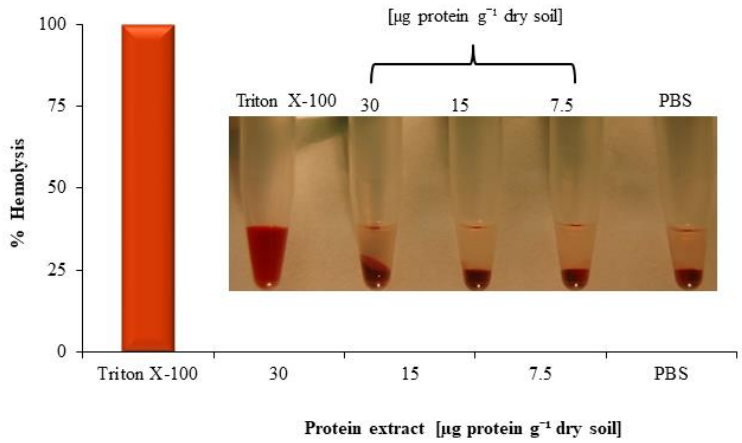


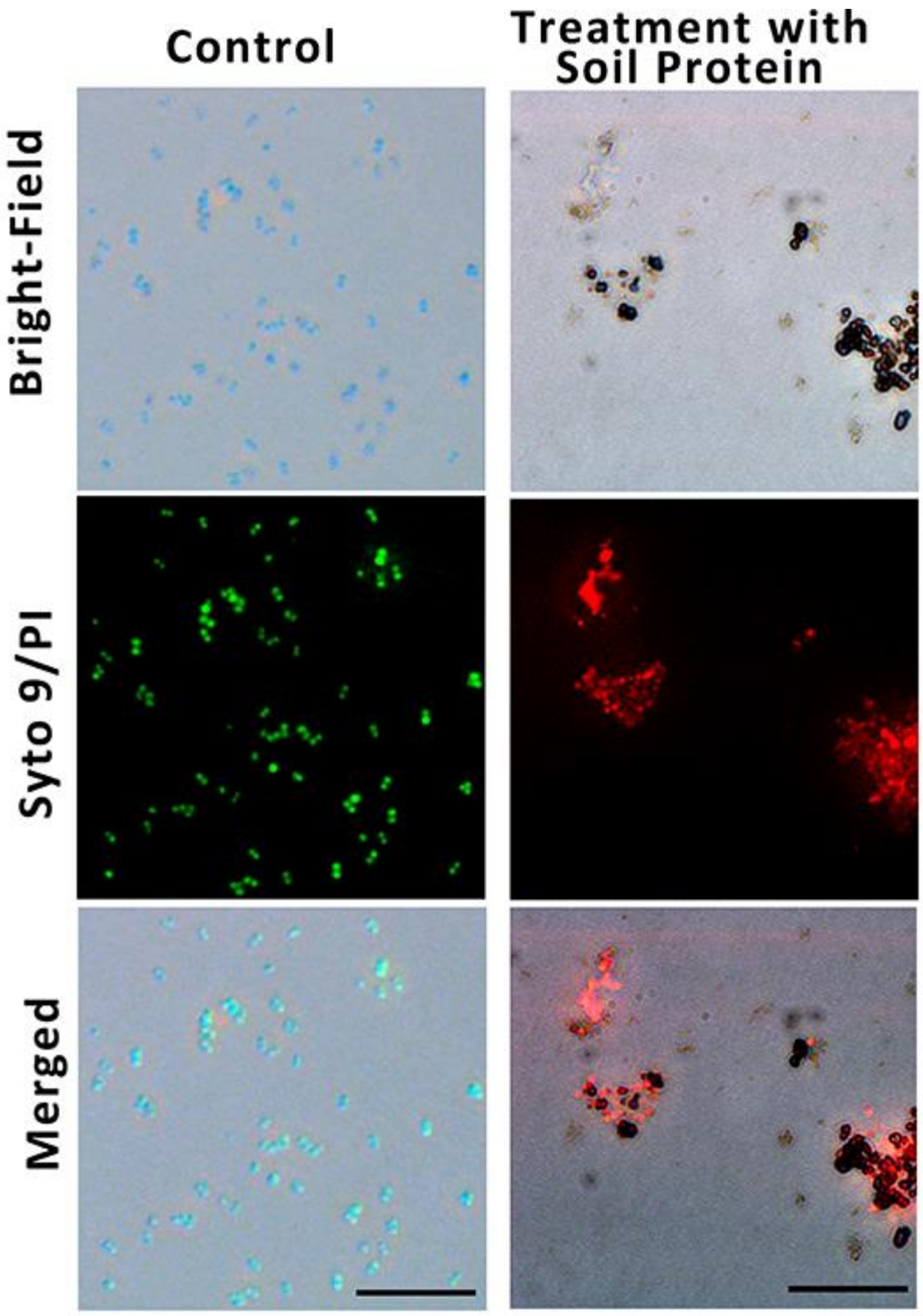
425

426 **Fig. 4.** Haemolysis assay of the soil protein extract (C3). Triton X-100 which causes
427 100% haemolysis was used as a positive control, and PBS (pH 7.4) was used as a negative
428 control.

429

430 The viability of bacterial cells was estimated after the application of the soil extract using
431 the live/ dead assay by the fluorescent microscope. Two fluorescent stains were used and
432 both stained the nucleic acids. Propidium iodide (PI) red fluorescing enters only cells with
433 damaged cytoplasmic membranes (Moulick et al., 2018), while SYTO9 green fluorescing
434 and able to enters all the cells and is used for assessing total cell counts (Berney et al.,
435 2007b). The bacterial cells without the protein extract were used as control and they
436 stained with the green fluorescent dye (Fig. 5). On the other hand, the decrease in the
437 viability of the bacterial cells after the application of the extracts indicated by the red
438 fluorescent of PI dye. These results are in good agreement with the results obtained by
439 ambient microscope and both the results prove the efficiency of the soil protein extract
440 against the tested pathogen through damaging its membrane.

441



442

443 **Fig. 5.** Microscopic assessment of MRSA after the application of 30 µg protein g^{-1}dry
444 soil of protein extracts using optical microscope in ambient light and Live/ dead assay
445 using two fluorescent dyes (PI/SYTO9). Scale bar is 10µm.

446

447 The genotoxicity effect of soil extract on the tested pathogen was estimated by DNA

448 damage through the single gel electrophoresis (comet assay). The bacterial DNA treated

with 250 µM H_2O_2 (positive control) caused the DNA damage and lost its supercoil structure, whereas the bacterial DNA without treatment used as a negative control. The DNA damage measured by tail length and intensity compared to the control group (Garaj-Vrhovac and Zeljezic, 2000). Comet tails were indicated after two hours treatment of the bacterial cells with the tested extract. Comet grade and index of damage of MRSA was found large (grade 4) and visible after the treatment with the extract C3 (Fig. 6). Our results demonstrated that our soil extract C3 was very effective against the tested MRSA strain and it was strongly affecting its DNA structure which may affect its resistant gene. Therefore, the soil protein extract might be considered as a promising antimicrobial agent against MRSA and other pathogenic strains.

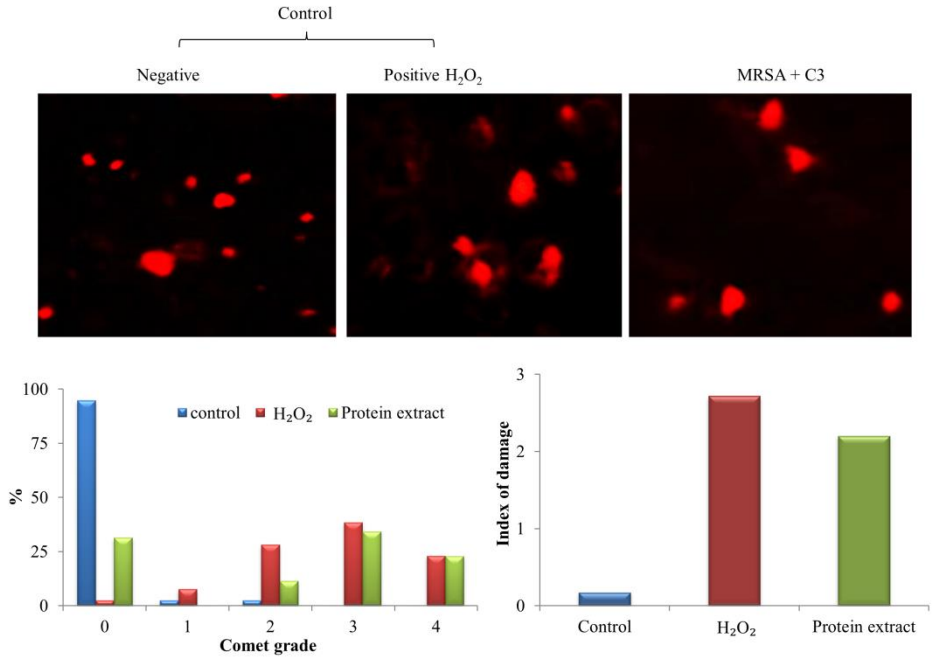


Fig. 6. Single cell gel electrophoresis assay (Comet assay) showing DNA damage of MRSA after application of 30 µg protein g^{-1} dry soil of protein extract (C3).

3.5. Evaluation of mecA expression after application of soil protein extract

High-level resistance to methicillin is caused by the *mecA* gene, which encodes an alternative penicillin-binding protein, PBP 2A (Wielders et al., 2002). The statistically significant decrease in *mecA* expression was observed in MRSA after 24 h soil protein

extract (C3) treatment in comparison with untreated control (0.125-fold *vs* control) (Fig. 7). This result from qPCR was in accordance with observation from MRSA growth curve, comet and live/dead assays that also described anti MRSA effects of tested soil protein extract. The down-regulation of *mecA* gene reduces one of major defense mechanisms used by MRSA against antibiotics (Foster, 2017). Our data revealed the antibacterial potential of soil extract for their use as a candidate for the therapy against MRSA infections.

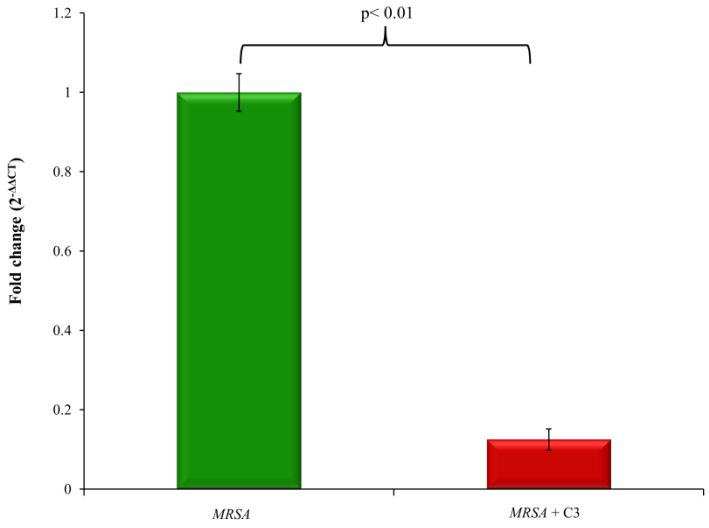


Fig. 7. Comparison of mecA gene expression between untreated *MRSA* (control) and treated *MRSA* by soil protein extract (C3). Data are presented as the fold change ($2^{-\Delta\Delta CT}$ method) in mecA gene expression normalized to a *16S rRNA* (reference gene) and relative to the untreated control (value 1). Data are presented as median±SD of three biological replicates.

3.6. Protein identification by UHPLC-MS

A total of 144 proteins originated from different bacterial (Table 1) and fungal (Table 2) species were identified in C3 protein extract using UHPLC-MS. The majority of the identified proteins were produced by gram negative bacteria (45.8%) (Fig. 8A). Out of 144 total proteins, 23 possess antimicrobial properties, and the majority of them were

produced by different fungal species (52.2%), while the rest were produced by gram positive and gram negative bacteria with 30.4% and 17.4%, respectively (Fig. 8B).

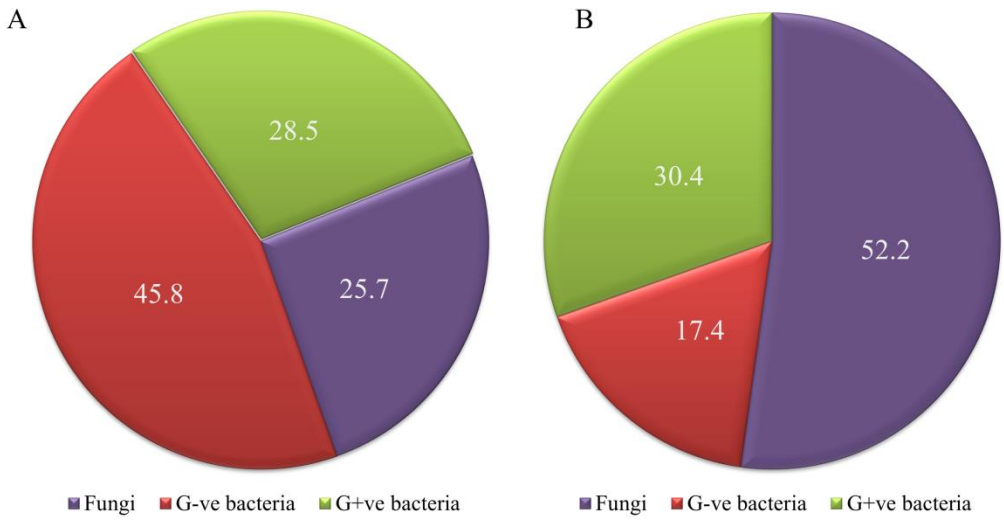


Fig. 8. The identified microbial proteins by UHPLC-MS. A) Percentage of proteins that produced by different fungal, Gram positive (G+ve) and Gram negative (G-ve) bacterial species. B) Percentage of protein components with antimicrobial properties that produced by different fungal, Gram positive (G+ve) and Gram negative (G-ve) bacterial species.

Around eleven metallopeptidase proteins (i.e. M1 family peptidase, M23 peptidase, M24 methionine peptidase, etc.) have been identified in different bacterial and fungal species (Table 1, Table 2). Among these metallopeptidase, the M23 family peptidases that contains endopeptidases which used by particular bacterial species to lyse cell walls of other bacteria, either as a defense or feeding mechanism (Kang et al., 2017). The antimicrobial activity of M23 peptidase proteins (i.e. M23B) have been reported against several pathogenic bacteria such as *Neisseria gonorrhoeae* (Stohl et al., 2012), *Helicobacter pylori* (Sycuro *et al.*, 2010) and *Staphylococcus aureus* (Kang et al., 2017). Therefore, these proteins might perform potential novel anti-MRSA agents by lysing the bacterial cell wall peptidoglycans by cleave the pentaglycine cross bridges that found in staphylococcal peptidoglycan (Odintsov et al., 2004).

Microorganisms including bacteria and fungi are well known to produce a wide range of natural products that also known as secondary metabolites (Niehaus et al., 2014). Normally, they are produced by plants and microorganisms in response to environmental stress or as a defense against any predictable threats (Lyu et al., 2019) and they play a significant role in human health and drug discovery. In this study, a group of enzymes that have a crucial role in the secondary metabolites biosynthesis were identified such as M24 peptidase, 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase, clavaminate synthase, and equisetin and lovastatin synthase that involved in trepenoid, clavulanic acid, equisetin and lovastatin acid biosynthesis processes, respectively (Table 1, Table 2). Trepenoid compounds, clavulanic acid, equisetin and lovastatin acid are secondary metabolites known by their antimicrobial and antifungal activities (Qiao et al., 2007; Mousa and Raizada, 2013; Hennessy et al., 2016). The presence of clavaminate synthase in our soils have a special interest since its play a crucial role in biosynthesis pathway of clavulanic acid, the clinically used inhibitor of serine β-lactamases (Zhang et al., 2000). Polyketides are secondary metabolites produced by numerous microorganisms with various biological functions including pharmacological properties like anticancer, antifungal and antibiotics (Tae et al., 2007; Gomes et al., 2013). They are synthesized by serial reaction of a group of enzymes known as polyketides synthase (PKs) (Tae et al., 2007).

523 **Table 1.** Bacterial soil proteins identified in (C3) soil by UHPLC-MS

Gene name	Description	Organism	Phenotypic classification	Identity (%)	Mass
SAMN04488505_102705	Beta-glucosidase	*Chitinophaga rupis*	G-ve	100	1307.6
leuD	3-isopropylmalate dehydratase small subunit	*Gammaproteobacteria bacterium*	G-ve	100	2723.6
ispE	4-diphosphocytidyl-2-C-methyl-D-erythritol kinase	*Aliivibrio salmonicida*	G-ve	100	1277.6
rplF	50S ribosomal protein L6	*Chlorobium phaeovibrioides*	G-ve	100	1029.5
iolC	5-dehydro-2-deoxygluconokinase	*Chloroflexi bacterium*	G-ve	100	1301.6
C5O28_00810	Phosphoesterase	*Lactobacillus fermentum*	G+ve	100	1192.6
ilvB	Acetolactate synthase	*Oceanicaulis sp.*	G-ve	100	1044.6
apc4	Acetophenone carboxylase delta subunit	*Aromatoleum aromaticum*	G-ve	100	4590.1
B7Y43_04665	Aldehyde oxidase	*Sphingomonas sp.*	G-ve	100	869.5
EHM40_06970	AraC family transcriptional regulator	*Chloroflexi bacterium*	G-ve	100	1548.7
argS	Arginine-tRNA ligase	*Leptothrix cholodnii, Leptothrix discophora*	G-ve	100	1253.6
bioB	Biotin synthase	*Cyanothece sp.*	G+ve	100	1133.6
SAMN04488101_10169	Branched-chain amino acid aminotransferase	*Pedobacter nyackensis*	G-ve	100	1001.6
DC74_748	Carboxylic ester hydrolase	*Streptomyces albulus, Pusillimonas noertemannii*	G+ve, G-ve	100	1030.6
Pedsa_2825	Carboxyl-terminal protease	*Pseudopedobacter saltans*	G-ve	100	1192.6
CMC99_01860	Carnitine dehydratase	*Flavobacteriales bacterium*	G-ve	100	1438.8

CK553_03225	**Cobalamin biosynthesis protein CbiX**	*Opitutae bacterium*	G-ve	100	3263.5
EEJ42_03270	**Cobalamin biosynthesis protein CobW**	*Streptomyces sp.*	G+ve	100	1380.6
Mflv_4343	Cytochrome P450	*Mycobacterium gilvum*	G+ve	100	1656.8
ddl	D-alanine--D-alanine ligase	*Peptostreptococcaceae bacterium*	G+ve	100	1392.7
gyrA	DNA gyrase subunit A	*Mycobacterium xenopi*	G+ve	100	1748.7
A0A2S9F9N0_9MYCO	DNA gyrase subunit A	*Mycobacterium sp.*	G+ve	100	1081.6
C4E04_05600	DNA helicase	*Microvirga sp.*	G-ve	100	2064.0
AOZ06_37110	Exopolyphosphatase	*Kibdelosporangium phytohabitans*	G+ve	100	1189.6
CAP37_00120	Glutamine synthetase	*Hydrogenophaga sp., Comamonadaceae bacteriu, Neptuniibacter caesariensi, Marinomonas primoryensis*	G-ve	100	1356.7
BSY239_78	Glutamine synthetase	*Hydrogenophaga sp.*	G-ve	100	2006.0
BKE56_025590	Glycerol-3-phosphate dehydrogenase	*Rhodococcus sp.*	G+ve	100	1553.6
glcE	Glycolate oxidase, subunit GlcE	*Agrobacterium fabrum*	G-ve	100	873.5
BK123_17575	Histidine kinase	*Paenibacillus lautus (Bacillus lautus)*	G+ve	100	3166.1
CMM48_05295	Histidine kinase	*Rhodospirillaceae bacterium*	G-ve	100	1105.6
ruvB	Holliday junction ATP-dependent DNA helicase RuvB	*Gloeobacter violaceus*	G-ve	100	1728.9
BTT61001_00491	**Hybrid polyketide synthase/nonribosomal peptide synthetase**	*Bacillus thuringiensis*	G+ve	100	977.5

menF	Isochorismate synthase MenF	*Chlorobium limicola*	G-ve	100	2145.9
araA1	L-arabinose isomerase 1	*Bacillus licheniformis*	G+ve	100	1171.5
BH718_00236	**Lipoprotein**	*Brachyspira hyodysenteriae (Treponema hyodysenteriae)*	G-ve	100	1390.7
BH718_00236	**Lipoprotein**	*Brachyspira hyodysenteriae (Treponema hyodysenteriae)*	G-ve	100	1765.7
CWE27_05520	**Lipoprotein**	*Streptomyces sp.*	G+ve	100	1575.8
EGC82_15695	M1 family peptidase	*Shewanella livingstonensis*	G-ve	100	1121.6
SSOG_00479	**Modular polyketide synthase***	*Streptomyces himastatinicus*	G+ve	100	1032.5
B446_04650	**Modular polyketide synthase***	*Streptomyces collinus*	G+ve	100	1462.6
B0I18_11039	Murein tripeptide amidase MpaA	*Taibaiella chishuiensis*	G-ve	100	2415.5
CU635_16875	Oligopeptide ABC transporter substrate-binding protein	*Bacillus canaveralius*	G+ve	100	1040.6
AUG47_05405	Ornithine decarboxylase	*Alphaproteobacteria bacterium*	G-ve	100	1108.5
CMP24_02880	Oxidoreductase	*Rickettsiales bacterium*	G-ve	100	898.5
V512_013135	Patatin	*Mesotoga sp.*	G-ve	100	832.4
DBX38_07545	Peptidase M24	*Clostridiales Family XIII bacterium*	G+ve	100	1306.7
DS901_14945	Peptidase M3	*Loktanella sp.*	G-ve	100	1319.6
EDC38_0807	Peptidylprolyl isomerase	*Marinimicrobium koreense*	G-ve	100	983.4
SAMN05216202_1463	**Polyketide synthase PksN**	*Pseudomonas mucidolens*	G-ve	100	1207.6
HMPREF0240_01497	Porphyrin biosynthesis protein HemD	*Clostridium sp.*	G+ve	100	1335.7
A6302_00081	Putative multidrug export ATP-binding/permease protein	*Methylobrevis pamukkalensis*	G-ve	100	1393.7
tgt	Queuine tRNA-ribosyltransferase	*Lachnospiraceae bacterium*	G-ve	100	1389.7
ER33_06675	Ribonuclease E	*Cyanobium sp.*	G+ve	100	1059.6

ELQ87_34570	SDR family NAD(P)-dependent oxidoreductase	*Streptomyces griseoviridis*	G+ve	100	1339.7
HK11_01835	Secretion protein	*Acetobacter sp.*	G-ve	100	1141.7
A7A08_00960	Small heat shock protein IbpA	*Methyloligella halotolerans*	G-ve	100	806.4
SAMN02787100_2510	Tetracycline resistance monooxygenase	*Chryseobacterium sp.*	G-ve	100	1033.5
C789_1799	Tetratricopeptide repeat family protein	*Microcystis aeruginosa*	G-ve	100	1015.5
BSTER_0936	TraG-like protein	*Bifidobacterium stercoris*	G+ve	100	1127.5
nusG	Transcription termination/antitermination protein NusG	*Parcubacteria group bacterium*	G-+ve	100	1110.5
MA47_05740	Transposase	*Corynebacterium auriscanis*	G+ve	100	1169.5
dusC	tRNA-dihydrouridine(16) synthase	*Moraxella nonliquefaciens*	G-ve	100	1138.5
gspD	Type II secretion system protein GspD	*Burkholderia cenocepacia*	G-ve	100	1063.6
DBX38_07545	Peptidase M24	*Clostridiales Family XIII bacterium*	G+ve	100	1330.6
ureG	Urease accessory protein UreG	*Frankia sp.strain, Actinoplanes regularis*	G+ve	100	931.5
ASE55_14235	Zinc permease	*Chryseobacterium sp.*	G-ve	100	1221.6
AS202_03115	Methanol dehydrogenase	*Myroides odoratimimus*	G-ve	93.3	1021.6
BEN48_11985	Galactokinase	*Hymenobacter glacialis*	G-ve	90.9	1553.6
SAMN04488528_102417	Glycine reductase complex selenoprotein A	*Clostridium frigidicarnis*	G+ve	90.9	1478.8
SAMN05421797_1089	Multidrug efflux pump subunit AcrA	*Maribacter ulvicola*	G-ve	90.9	830.5

VL15_20815	Pentapeptide repeats family protein	*Burkholderia cepacia, Pseudomonas cepacia*	G-ve	90.9	1978.0
ASD37_24655	Peptidase M48, Ste24p	*Mycobacterium sp.*	G+ve	90.9	2019.0
SAMN06272789_1538	Pseudouridine synthase	*Streptomyces sp.*	G+ve	90.9	1234.5
crcB	Putative fluoride ion transporter CrcB	*Corynebacterium xerosis*	G+ve	90.9	1146.5
radA	DNA repair protein RadA	*Corynebacterium durum*	G+ve	90	1064.5
hemH	Ferrochelatase	*Synechococcus sp.*	G-ve	90	1595.7
DCY59_10565	**M23 family peptidase**	*Micrococcaceae bacterium*	G+ve	90	841.5
DI544_02580	Protein dehydratase	*Sphingomonas taxi*	G-ve	90	2308.1
ERS852554_02209	Putative lipoprotein	*Bacteroides uniformis*	G-ve	90	1492.7
DW039_00730	Tetratricopeptide repeat protein	*Bacteroides sp.*	G-ve	90	1164.6
A176_002281	Beta-1,3-glucosyltransferase	*Myxococcus hansupus*	G-ve	88.9	1286.6
EKK46_16395	Chemotaxis protein CheW	*Rhodocyclaceae bacterium*	G-ve	88.9	1124.5
pyrF	Orotidine 5'-phosphate decarboxylase	*Devosia sp.*	G-ve	88.9	1236.6
OLMES_2929	Strictosidine synthase family protein	*Oleiphilus messinensis*	G-ve	88.9	1598.8
DW352_06045	LysM peptidoglycan-binding domain-containing protein	*Pseudolabrys taiwanensis*	G-ve	88.2	1349.7
BAU28_19195	Acyltransferase	*Bacillus paramycoides, Bacillus cereus*	G+ve	87.5	1746.7
hutH_1	Histidine ammonia-lyase	*Vibrio mangrovi*	G-ve	87.5	1682.8
nadB	L-aspartate oxidase	*Bacteroidales bacterium*	G+ve	87.5	1005.4
A9Q85_05135	Phenol hydroxylase	*Cycloclasticus sp.*	G+ve	87.5	1091.5
priA	Primosomal protein N'	*Ignavibacteriae bacterium HGW-Ignavibacteriae-3*	G-ve	87.5	1160.6
AZ34_12410	**Lipoprotein**	*Hylemonella gracilis str.*	G-ve	84.6	1525.7

grpE	Protein GrpE	*Roseateles aquatilis*	G-ve	84.6	1764.7
DCR23_03620	Alpha-mannosidase	*Ruminococcaceae bacterium*	G+ve	83.3	1091.5
I568_01086	Penicillin-binding protein 1A	*Enterococcus columbae*	G+ve	83.3	1926.8
NGAL_HAMBI2605_29050	Peptidase C14 caspase catalytic subunit p20	*Neorhizobium galegae*	G-ve	83.3	1301.7
DMG39_11560	Peptidase S10	*Acidobacteria bacterium*	G-ve	81.8	1746.7
SAMN04488126_106113	Peroxiredoxin	*Bhargavaea beijingensis*	G+ve	81.8	1474.7
B9N62_05110	**Riboflavin biosynthesis protein RibD**	*Campylobacter concisus*	G-ve	81.8	2246.9
ASE19_16010	Peptidase	*Nocardioides sp.*	G+ve	81.3	1349.7
CWM47_16065	Methanol dehydrogenase	*Spirosoma pollinicola*	G-ve	80.8	1638.9
A6035_11085	DNA helicase	*Dietzia lutea*	G+ve	80.6	1900.9
pepF	Oligoendopeptidase F	*Rhodobacteraceae bacterium*	G+ve	80	1110.6
A9Q87_01310	Methanol dehydrogenase	*Flavobacteriales bacterium*	G-ve	77.3	1404.7
Chro_5729	**Peptidase M23**	*Chroococcidiopsis thermalis*	G-ve	75	1418.7
GA0115251_121312	Pseudouridine synthase	*Streptomyces sp.*	G+ve	65.1	830.5
A3G29_02480	Universal stress protein	*Burkholderiales bacterium*	G-ve	84.6	1248.6
SAMN02745121_08438	Dihydroxyacetone kinase	*Nannocystis exedens*	G-ve	81.3	1433.8

Bold color indicates proteins and enzymes which have a role in secondary metabolites biosynthesis process

Table 2. Fungal soil proteins identified in (C3) soil by UHPLC-MS

Gene Accession Uniprot	Description	Species	Identity (%)	Mass
A9K55_005725	Alpha-ketoglutarate-dependent sulfonate dioxygenase	*Cordyceps militaris, Clavaria militaris*	100	1548.7
BN946_scf184611.g7	Aryl-alcohol oxidase	*Pycnoporus cinnabarinus*	100	1001.6
DL98DRAFT_515201	Beta-glucosidase	*Cadophora sp.*	100	1030.6
BP5796_05907	Beta-glucosidase	*Coleophoma crateriformis*	100	1192.6
Ptr86124_12611	Cell surface protein (Mas1)	*Pyrenophora tritici-repentis*	100	1081.6
OH76DRAFT_1404752	**Clavaminate synthase-like protein***	*Polyporus brumalis*	100	2064.0
NEUTE2DRAFT_108181	Coatomer subunit beta	*Neurospora tetrasperma*	100	1189.6
DAD1	DASH complex subunit DAD1	*Debaryomyces hansenii*	100	3166.1
BBA_00775	Glutamate dehydrogenase	*Beauveria bassiana ,Tritirachium shiotae*	100	1390.7
DDE83_008222	Glycoside hydrolase family 13 protein	*Stemphylium lycopersici*	100	1462.6
AYI68_g4887	GPI-anchored wall transfer protein	*Smittium mucronatum*	100	2415.5
BNA5	Kynureninase	*Fusarium oxysporum, Fusarium vascular wilt*	100	1319.6
F503_02830	**Lovastatin nonaketide synthase**	*Ophiostoma piceae*	100	1389.7
MEX67	Mex67p	*Saccharomyces cerevisiae*	100	1339.7
PGRI_015640	NAD(P)H-hydrate epimerase	*Penicillium patulum, Penicillium roqueforti, Penicillium solitum*	100	1015.5
A0A0A2ILR0_PENEN	Peptidase C19, ubiquitin carboxyl-terminal hydrolase 2	Penicillium *expansum*	100	1063.6
CCHL11_04557	Pisatin demethylase 15	*Colletotrichum chlorophyti*	100	1021.6
TCAP_03404	**Polyketide synthase***	*Tolypocladium capitatum*	100	1553.6
AUD_8391	Probable beta-glucosidase M	*Aspergillus udagawae*	100	1978.0
ALT_2439	**Proline dehydrogenase**	*Aspergillus lentulus*	100	2019.0
M747DRAFT_296186	**Proteinase aspergillopepsin II**	*Aspergillus niger*	100	1234.5

ABOM_001287	RBR-type E3 ubiquitin transferase	*Aspergillus bombycis*	100	1595.7
kojR	Transcription factor kojR	*Aspergillus oryzae*	100	1598.8
B9Z65_2538	Lysophospholipase 2	*Elsinoe australis*	92.3	1091.5
STUB1	STIP1 like proteiny and U box-containing protein 1	*Cladophialophora carrionii*	90	1248.6
BJ508DRAFT_344280	Ankyrin	*Ascobolus immersus*	88.9	1004.5
BS50DRAFT_616188	**Clavaminate synthase-like protein***	*Corynespora cassiicola*	87.5	1474.7
TEQG_04484	**Equisetin synthetase***	*Trichophyton equinum*	87.5	1036.5
BS50DRAFT_588398	Leptomycin B resistance protein pmd1	*Corynespora cassiicola Philippines*	87.5	1186.7
TD95_002415	**Peroxidase**	*Thielaviopsis punctulata*	87.5	1089.5
F503_02093	Zinc knuckle transcription factor	*phiostoma piceae*	86.7	1460.7
FSPOR_4523	Alkylglycerol monooxygenase	*Fusarium sporotrichioides*	86.4	1740.7
TSTA_079690	**Polyketide synthase, putative***	*Talaromyces stipitatus, Penicillium stipitatum*	85.7	896.4
EMCG_00433	Isopenicillin-N N-acyltransferase	*Emmonsia crescens*	60	1746.7
leu1	3-isopropylmalate dehydrogenase	*Schizosaccharomyces pombe*	100	2723.6
HEM2	Delta-aminolevulinic acid dehydratase	*Saccharomyces cerevisiae*	100	1105.6

Bold color indicates proteins and enzymes which have a role in secondary metabolites biosynthesis process

The presence of several polyketide synthase enzymes (PKs) in our sample might be an indicator for the availability of several polyketides either in our protein mixture or in the soil samples where this protein was extracted.

Screening for microbial proteins with novel properties from naturally occurring habitats like soil might be useful in providing more information about their antimicrobial properties. Alpha mannosidase is one of these proteins which belong to glycoside hydrolase families that involved in N-linked glycoproteins turnover and produced by several bacterial species (i.e. *Ruminococcaceae bacterium*, *Bacillus sp.*, *Mycobacterium tuberculosis*, etc.) (Angelov et al., 2006). Bacterial alpha mannosidase are rarely studied but it is similar to those of eukaryotic families (Nankai et al., 2002). The antimicrobial properties of α-mannosidase was previously reported for plants mannosidase but never for bacterial mannosidase (Banar et al., 2016). Proline dehydrogenase (ProDH) is another interesting enzyme that was detected in our soil especially that the human homolog ProDH plays critical roles in cancer prevention and schizophrenia (Lee et al., 2003), therefore it might also possess some antimicrobial properties. In addition, fungal proteases are well documented for their biological properties and some of them showed excellent anti-cancer and anti-microbial activities while others exhibited good potential in biotechnological field (Cavello et al., 2013). However, the studies about antimicrobial properties of bacterial proteases are uncommon. Moreover, peroxidases are a group of enzymes of great interest because of their important role in detoxification of environmental pollutants (Bansal and Kanwar, 2013). Microbial peroxidase antimicrobial properties never been reported and its presence among our extracted protein that exhibit anti MRSA activity might be an indicator about the role of this enzyme as antibacterial agent.

Vitamins are organic and essential molecules that needed by organism in a small quantities (Halver, 2003). Proteins that involves in riboflavin (vitamin B2) and cobalamin (vitamin B12) biosynthesis process were found in our soil pretentious mixture. The presence of these two proteins is an indicator that soil might be a natural habitat to isolate these vitamins or their producing organisms. The antimicrobial properties of riboflavin and cobalamin against several human pathogens (i.e. *Staphylococcus aureus*, *Enterococcus faecalis*, *Salmonella typhi*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*) was previously reported by many authors (Selvakumar et al., 2012; Ahgilan et al., 2016).

Finally, the natural habitat might be an excellent and sustainable source for a novel proteins or natural compounds that could have a role in drug discovery. The presence of all the above mentioned proteins in our extracted protein plays a direct or indirect role in its anti MRSA inhibitory effects.

4. Conclusion

In the present study, for the first time we reported that that the soil proteins have very high antibacterial activity against MRSA. The protein extract from the forest with past intensive management showed higher antibacterial activity than that of unmanaged forest. Optical and fluorescent microscopic data confirmed that the soil protein killed the bacteria with disruption of their cell wall. Comet assay showed its genotoxic effect against MRSA but not toxic to human red blood cells. The qPCR study showed that the mecA (the antibiotic resistant gene) expression was down-regulated by the soil protein. The UHPLC-MS identified 144 proteins in C3 among which the majority belonged to gram negative bacteria. The rest of the protein was found to be originated from gram positive bacteria and fungi. Taken together, these results will help to develop efficient, non-toxic and natural antibacterial medicine against antibiotic resistant pathogens.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

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