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Comparative determination of antimicrobial activity of the Balkan endemic species *Stachys thracica* Davidov during the process of ex situ conservation

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Abstract

Stachys thracica Davidov – Thracian woundwort is a Balkan endemic plant included in The Red Data Book of Bulgaria with conservational status "rare". The plants from genus *Stachys* have a long history of use to treat various diseases, inflammatory conditions, coughs, ulcers, genital tumors, and infected wounds. Due to its limited distribution the information on the biological activity and chemical composition of *S. thracica* is rather scarce. The aim of the present research is the comparative determination of the antimicrobial activity of methanolic extracts obtained from in situ wild, in vitro cultivated and ex vitro adapted *S. thracica* plants. The in vitro shoot culture of the Thracian woundwort was maintained in hormone-free MS medium under controlled environmental conditions. The methanolic extracts from in situ, in vitro cultivated and ex vitro adapted *S. thracica* plants were active mainly against Gram-negative bacteria. All three extracts showed equal activity against *Acinetobacter calcoaceticus.* The establishment of in vitro shoot culture and its subsequent adaptation in ex vitro conditions was an appropriate alternative approach for the ex situ conservation of *S. thracica* as well as for the study of its biological activity.

Keywords

Antimicrobial activity, in vitro cultivation, Thracian woundwort

Introduction

Genus *Stachys*. L, or woundworts, comprises more than 300 herbs and shrubs and is considered one of the largest genera from *Lamiaceae* family (Tomou et al. 2020). Most of the species are distributed in temperate and tropical regions of the world especially in the Mediterranean. There are 22 species from *Stachys* genus in Bulgaria, 5 of which are under the protection of the Bulgarian Biodiversity Law. The natural habitats of some of these species are located in some of the Bulgarian national parks and others are within localities included in NATURA 2000. The plants from genus *Stachys* have a long history of use in ethnomedicine for various diseases, coughs, ulcers, genital tumors, inflammatory conditions, and infected wounds (Tundis et al. 2007; Conforti et al. 2009; Goren et al. 2014).

It is reported that woundworts exhibit various biological effects such as antioxidant, antibacterial, anti-inflammatory, wound healing, cytotoxic, hepatoprotective properties (Khanavi et al. 2005; Vundać et al. 2007; Háznagy-Radnai et al. 2012; Tundis et al. 2014; Tomou et al. 2020). According to different phytochemical studies, the plants from the *Stachys* genus are sources mainly of phenylethanoid glycosides (Karioti et al. 2010; Delazar et al. 2011), iridoids (Murata et al. 2008; Tundis et al. 2014) and phenolic acids (Venditti et al. 2014).

Taking into account the research done so far, *Stachys* species may be considered a favourable subject for exploration and discovery of secondary metabolites with antimicrobial potential.

The inconsistent application of antibiotics poses a great risk of antibiotic resistance in most of the microbial species that cause human infections (Ventola et al. 2015). This creates an urgency for the research and discovery of alternative sources of antimicrobial agents.

Plants have been used by humanity since ancient times for the treatment of various bacterial infections even without scientific proof of their effectiveness. As a potential source of numerous biologically active substances, plant species have always been potential candidates for alternative agents with antimicrobial activity.

In recent years the antimicrobial potential of some *Stachys* species was a great point of interest among different research groups. Published data indicate that different polar extracts, as well as essential oils, show antimicrobial activity against human pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Dulger et al. 2005; Aleebrahim-Dehkordy et al. 2016; Cuce et al. 2016).

Stachys thracica Davidov (The Plant List) or Thracian woundwort is a Balkan endemic plant distributed in Bulgaria, Greece and Turkey. In Bulgaria, it is classified as "rare" and some of its localities are within Natura 2000 ecological network. The populations of the Thracian woundwort are comprised of a small number of individuals and are located in the Strandja Mountain, Black Sea coast and Sofia region. There is no available data on ex-situ conservation of the species and its chemical composition and biological activity are not well studied.

The aim of the present research is a comparative determination of the antimicrobial activity of methanolic extracts obtained from in situ wild, in vitro cultivated and ex vitro adapted *Stachys thracica* plants.

Materials and methods

Plant material

S. thracica Davidov plants grew in situ in their natural habitat near the village of Sinemorets, Tsarevo municipality, Bulgaria. A small set of samples from aerial parts of the plants in the period of active blooming (in June) and seeds (in September) were collected with the permission of the Ministry of Environment and Water of Bulgaria. A voucher specimen SO107847 was deposited in the Herbarium of Sofia University "St. Kliment Ohridski".

In vitro shoot culture from *S. thracica* was induced by sterilisation of seeds with 70% ethanol for 5 min. The sterilised seeds were placed on a germination medium containing water and agar (WA) and further, the sprouting seedlings were transferred on MS medium (Murashige and Skoog 1962) supplemented with 3% sucrose and 0.7% agar,without growth regulators. The in vitro collection was maintained under controlled environmental conditions (16 h light/8 h dark, 60 mmol/(m^2s) photosynthetic photon flux density, Philips TLD-33, temperature 25 °C and 60–70% relative air humidity).

Ex vitro adaptation was performed in three stages with plants having well-developed root systems. At the first step, the regenerated plants were planted in pots and subjected to acclimation in a phytotron chamber for a period of one month. After that, they were transferred to a greenhouse for another month and at a final stage were planted on the experimental field of Sofia University "St. Kliment Ohridski".

Methanolic extracts preparation

Three grams (3 g) of finely powdered dry plant material from aboveground parts of in situ grown, in vitro cultivated and ex vitro adapted *S. thracica* were subjected to triple sonication extraction with 30 ml chloroform (Sigma-Aldrich, Spain) in ultrasonic bath for 10 minutes. In the next step, the dried biomass was extracted three times with methanol for 30 minutes. The final plant extract from each variant was concentrated through a vacuum evaporator (IKA, Germany) and dried to constant dry weight. The yields of extracts from in situ, in vitro cultivated and ex vitro adapted plants were 13.8%, 28.46% and 13.6% respectively. For the current study, each methanolic extract was dissolved in 5% DMSO.

Antimicrobial activity

Microbial strains

The methanolic extracts from in situ, in vitro cultivated and ex vitro adapted *S. thracica* plants were individually tested against seven Gram-negative microbial strains – *Pseudomonas aeruginosa* NBIMCC 3700, *Proteus mirabilis* NBIMCC 8690, *Proteus hauseri* NBIMCC 1393, *Enterobacter cloacea* NBIMCC 8570, *Acinetobacter calcoaceticus* NBIMCC 3730, *Escherichia coli* NBIMCC 8954, *Klebsiella pneumoniae* NBIMCC 3670

and three Gram-positive bacteria – *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermitis* NBIMCC 3360, *Enterococcus faecalis* NBIMCC 1093 microbial species and the yeast *Candida albicans* NBIMCC 74. The microbial specimens were purchased from The Bulgarian Collection for Industrial Microorganisms and Cell cultures (NBCIMCC). The bacterial strains were cultured overnight at 37 °C on Muller-Hinton agar (MHA) and the yeast was cultured on Sabouraud Dextrose Agar (SDA).

Disk diffusion assay

An initial screening of the antimicrobial activity of the dried methanolic extracts from *S. thracica* was performed by agar disk diffusion method according to the guidelines of CLSI (Clinical and Laboratory Standards Institute). The dried extracts from in situ, in vitro and ex vitro adapted plants were dissolved in 5% DMSO to a final concentration of 200 mg/ml and filtered by 0.45 μm Millipore filters for sterilization. Briefly, 100 μl of each suspension containing 10^7 cell/ml was inoculated in 25 ml MHA for bacterial strains and SDA for the yest respectively. Sterile paper disks (6 mm diameter) were impregnated with the extracts (8 mg/disk) and allowed to dry under aseptic conditions before placing them on the inoculated agar. DMSO at concentration 5% was used as a negative control. The antibiotics tetracycline and amikacin were used as a positive control for the bacterial strains and nystatin for *C. albicans*. The samples were incubated at 37 °C for 24 hours and 48 hours for bacterial strains and *C. albicans* respectively. The antimicrobial activity of the extracts was related to the inhibition zones.

Micro-well dilution assay

Bacterial strains which were sensitive to the methanolic extracts in the disk diffusion assay were studied for their minimal inhibitory concentration (MIC) using the micro-well dilution assay (Wiegand 2008, EUCAST). For the experiment, the bacterial suspension was prepared in a liquid MH-Muller Hinton medium with a density of 0.5 on the McFarland scale, corresponding to 10^7 cells/ml. The 96-well plates were prepared by dispersing 50 μl MH broth in each well. The serial dilutions of each extract were prepared directly in the wells as the starting concentration was 64 mg/ml. Then, 50 μl of each well was transferred to the next and the final dilution of each extract was 2 mg/ml. Finally, 50 μl of the bacterial suspension was added to each well and the final volume of each well was 150 μl.

Prior to incubation, the absorbance of each microplate was measured using ELISA reader (Uscn Kit Inc., China) at λ=630 nm and this was considered the absorption at 0 h.

Results

In vitro multiplication and ex situ conservation of *S. thracica*

In vitro shoot culture from *S. thracica* was successfully induced by the sterilisation and subsequent germination of ripe dried seeds. The in vitro regenerated plants

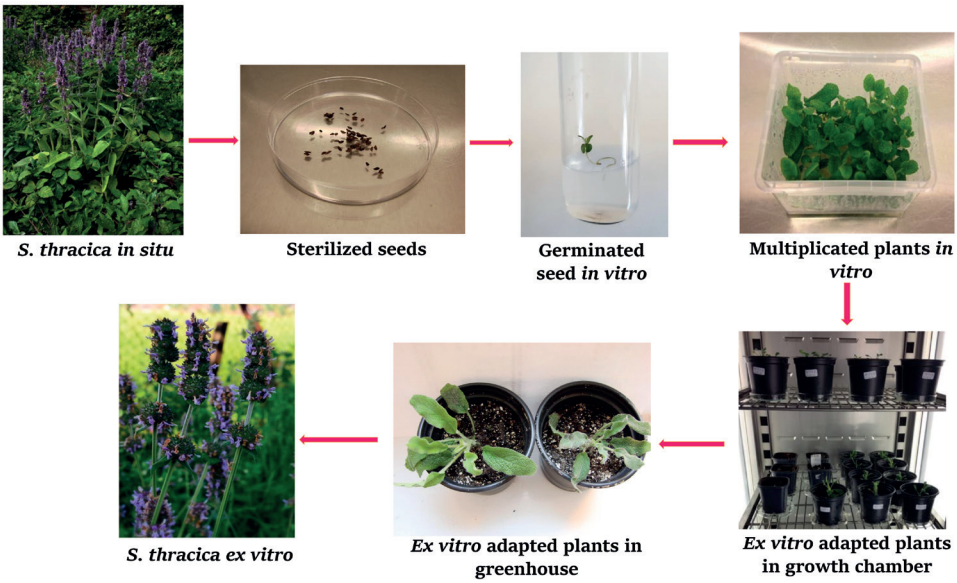

Figure 1. Ex situ conservation of *S. thracica.*

were further propagated and maintained on hormone-free MS medium and characterised with plentiful leaf biomass and well-developed roots. This allowed their further 3-stage acclimatisation – in a phytotron chamber, in a greenhouse and an experimental field. For the current research, the collection of ex vitro adapted *S. thracica* plants were successfully maintained on the experimental field with 83% survival rate (Fig. 1).

Antimicrobial activity

The antimicrobial activity of the methanolic extracts from in situ, in vitro cultivated and ex vitro adapted *S. thracica* plants was evaluated against 11 microorganisms that are frequently related to human infections and are typically present in infected wounds.

The results from the preliminary screening by the disk diffusion assay as well as the microdilution assay are presented in Table 1. All tested extracts show tendency to be active against Gram-negative bacterial strains rather than Gram-positive strains. Overall, the extracts of *S. thracica* showed activity against only 4 of the tested microbial strains and no dependency between the type of extract and its activity was observed (Fig. 2). The most sensitive microbial species appeared to be *A. calcoaceticus* as all three extracts showed bactericidal zones and MIC values of 8 mg/ml. The other bacterial strains that were sensitive to either of the extracts were *K. pneumoniae*, *P. mirabilis* and *E. faecalis.* The highest MIC value – 16 mg/ml and the smallest inhibitory zone – 7 mm were established against *K. pneumoniae*. Although the zones in *P. aeruginosa* were seen as bacteriostatic, no activity was detected in the microdilution assay.

Discussion

In vitro multiplication and ex vitro adaptation of *S. thracica*

The conservational status of the Thracian woundwort and its limited distribution enforced us to apply an alternative method which would allow simultaneously the conservation of the species and the determination of its biological activity. The in vitro micropropagation is a reliable method for ex-situ conservation of endemic and threatened plant species and it is successfully applied for the investigation of such, without disturbing their natural population and habitats.

For the current study we successfully initiated in vitro shoot cultures of *S. thracica* from sterilised ripe dried seeds. The in vitro culture is successfully grown on MS medium without the addition of plant growth regulators and the micropropagated plants are characterised with vigorous growth, plentiful leaf biomass and very well-developed root system. This in turn led to the successful ex vitro acclimation of the Thracian woundwort with 83% survival rate.

Table 1. Antimicrobial activity of methanolic extracts from in situ wild, in vitro cultivated and ex vitro adapted *Stachys thracica* plants.

Test microorganisms	*Stachys thracica* methanolic extracts						Antibiotics		
	In situ		In vitro		Ex vitro		Amicacine	Tetracycline	Nystatine
	DD[a]	MIC[b]	DD[a]	MIC[b]	DD[a]	MIC[b]	DD[a]	DD[a]	NA
Acinetobacter calcoaceticus	9	8	8	8	7.5	8	11	12	NA
Enterobacter cloacea	-	-	-	-	-	-	8	21	NA
Proteus mirabilis	6*	-	8*	-	8*	8	13	8	NA
Proteus hauseri	-	NA	-	NA	-	NA	20	19	NA
Staphylococcus aureus	-	NA	-	NA	-	NA	20	28	NA
Staphylococcus epidermitis	-	NA	-	NA	-	NA	20	12	NA
Klebsiela pneumoniae	7	16	9	4	-	NA	15	29	NA
Pseudomonas aeruginosa	15*	-	12*	-	10*	-	25	12	NA
Escherichia coli	-	NA	-	NA	-	NA	12	22	NA
Enterococcus feacalis	-	NA	9*	4	-	NA	8	25	NA
Candida albicans	-	NA	-	NA	-	NA	NA	NA	18

(-) – no antimicrobial activity; (*) – bacteriostatic zone; aDD – disc diffusion method; Inhibition zones (mm); bMIC – minimal inhibitory concentration (mg/ml); NA – not tested.

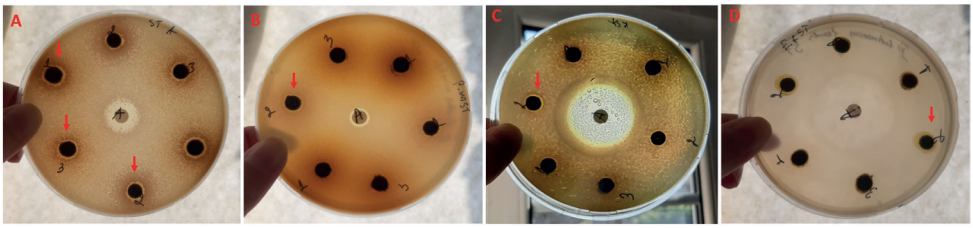

Figure 2. Antimicrobial activity of methanolic extracts from in situ [1], in vitro [2] and ex vitro [3] *S. thracica* plants measured by the disk diffusion assay **A** *A. calcoaceticus* **B** *P. mirabilis* **C** *K. pneumoniae* **D** *E. faecalis.*

Similar to our results, the successfully initiated in vitro culture from the Balkan endemic *S. maritima* was maintained on a hormone-free MS medium and the plants showed an excellent regeneration rate (Panayotova et al. 2008).

Antimicrobial activity

There is a high possibility that different growth conditions would affect the biological activity of plants extracts. *S. thracica* is a source of pharmacologically active secondary metabolites such as phenylethanoid glycosides (Bankova et al. 1999). To evaluate the changes in the antimicrobial activity of methanolic extracts from *S. thracica*, a comparison between the in situ grown, in vitro cultivated and ex vitro adapted plants was made.

The antimicrobial activity was evaluated against 10 bacterial strains and 1 yeast strain – *C. albicans*. The disk diffusion method was used for preliminary study of the antibacterial activity and the microdilution assay was applied afterwards for determination of MIC and verification of the results obtained by the initial screening. All the extracts were more active against Gram-negative bacteria which may be due to the different structure of the cell wall of gram-negative and gram-positive bacteria. We observed no visible trend in the antimicrobial activity of the methanolic extracts obtained from in situ, in vitro cultivated and ex vitro adapted plants with the exception that all the extracts were equally active against *A. calcoaceticus* showing inhibitory zones of 8 mm and MIC values – 8 mg/ml.

Typically, *A. calcoaceticus* is a soil bacterium but it is very frequently associated with infections within hospitals due to its ability to form a complex with another species – *Acinetobacter baumanii* and it is usually used in laboratory testing instead of *Acinetobacter baumanii* (Mancilla-Rojano et al. 2020).

Ebrahimabadi et al. (2010) reported that the polar fraction of *Stachys inflata* Benth. was active against only two microbial species which in parts overlaps with our results. In another study, Dulger et al. (2004) demonstrated that methanolic extracts from *Stachys* species were active against *E. coli*, *P. aeruginosa*, *S. aureus* and *B. cereus* but no activity was established against the tested yeast cultures – *C. albicans*, *K. fragilis* and *R. rubra*. Contrary to our results, Cüce et. al. (2017) reported that methanolic and hexane extracts from in situ and in vitro cultivated *S. annua* plants were active against *S. aureus* and the methanolic extract showed activity against *P. aeruginosa.*

Conclusions

The initiated in vitro culture from *S. thracica* was successfully maintained on hormone-free MS medium under controlled environmental conditions and the micropropagated plants continued to form plenty of biomass and well-developed roots. The methanolic extracts from the Thracian woundwort showed activity mostly against Gram-negative bacteria and the most sensitive bacterial strain was *A. calcoaceticus* against which all three different extracts exhibit equal antimicrobial activity. Further research on the

chemical profile would be necessary in order to reveal which compounds are responsible for the antimicrobial activity of *S. thracica.*

The established in vitro and ex vitro plant cultures serve as an effective alternative approach for the preservation of the rare *S. thracica* and at the same time represents a model system for the study of its biological activity and pharmacological potential.

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