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Phytochemical Profile, Antioxidant Capacity and Anticancer Potential of Water Extracts from In Vitro Cultivated *Salvia aethiopis*

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Abstract: *Salvia aethiopis* L. (Mediterranean sage) is a medicinal plant known for its rich phenolic content and different therapeutic properties. This study evaluated the phytochemical composition, antioxidant capacity and anticancer potential of water extracts from in vitro cultivated *S. aethiopis*. The extract exhibited a high total polyphenol (110.03 ± 0.7 mg GAE/g) and flavonoid (7.88 ± 0.25 mg QE/g) content, along with a strong oxygen radical absorbance capacity (an ORAC value of 3677.9 ± 24.8 µmol TE/g). LC-HRMS analysis identified 21 bioactive compounds, including salvianic acid C, rosmarinic acid, salvianolic acid K and various organic acids. A cytotoxicity evaluation using the Neutral Red Uptake assay showed that the extract had a low toxicity to non-cancerous BALB/3T3 cells. An antiproliferative activity assessment via the MTT assay revealed selective cytotoxicity against Hep G2 hepatocellular carcinoma cells (IC_{50} = 353.8 ± 21.8 µg/mL) and lung (A549) and prostate (PC-3) carcinoma cell lines. Migration assays and cytopathological evaluations confirmed the significant inhibition of cancer cell proliferation, the suppression of migration and G2/M cell cycle arrest. Flow cytometry revealed considerable increases in apoptotic and necrotic cell populations following treatment with *S. aethiopis* extract. These findings showed the potential of *S. aethiopis* as a promising source of bioactive compounds with antioxidant and anticancer properties, supporting its further exploration for therapeutic applications.

Keywords: *Salvia aethiopis*; phytochemicals; antioxidant activity; hepatocellular carcinoma; cytotoxicity; apoptosis; LC-HRMS

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Antitumor Activity of Whole-Plant Extracts from In Vitro Cultured and Wild-Growing *Clinopodium vulgare* Plants on a Panel of Human Tumor Cell Lines

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Abstract: *Clinopodium vulgare* L. is a valuable medicinal plant with various beneficial effects on health. In this study, water extracts from the aerial part of the wild and in vitro cultured *C. vulgare* plants were obtained. The polyphenol, flavonoid content and antioxidant activity of the extracts as well as their antitumor efficiency against a panel of cell lines were analyzed. The ability of *C. vulgare* to inhibit cancer cell migration and induce apoptosis in the tumor cells was examined by wound healing assay and fluorescence microscopic methods. The effect of the extracts on the cell cycle progression of the tumor cells was analyzed by flow cytometry. The presented results show that the antitumor activity of the extracts from in vitro cultured plants was similar to and even exceeded that of the wild plants. The cell viability and migration assays demonstrate the selective anticancer effect of the extract and significant inhibition of cancer cell proliferation and motility. The fluorescence microscopy and cell cycle analyses indicate that the antitumor activity of the in vitro plant extract was related to both antiproliferative and proapoptotic effects. These results show that *C. vulgare* plants obtained by in vitro micropropagation and cultivated ex vitro are promising candidates for anticancer drug therapy.

Keywords: *Clinopodium vulgare*; in vitro cultured plants; antitumor activity; cell viability; apoptosis

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1. Introduction

Globally, there has been a significant increase in the number of malignant neoplasms, defined as diseases of modern lifestyle and one of the main causes of premature death. These are serious, socially significant diseases that have a physical, psychological, social and economic impact on patients, their families and caregivers and society as a whole. According to the International Agency for Research on Cancer, both new cancer cases (over 26 million) and cancer deaths (17 million cancer deaths per year) are expected to double by 2030 [1]. In recent years, many new synthetic chemotherapeutic agents have

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Extracts of *Sideritis scardica* and *Clinopodium vulgare* Alleviate Cognitive Impairments in Scopolamine-Induced Rat Dementia

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Abstract: *Sideritis scardica* Griseb. and *Clinopodium vulgare* L., belonging to the Lamiaceae family, are rich in terpenoids and phenolics and exhibit various pharmacological effects, including antioxidant, anti-inflammatory and anti-cancer activities. While the memory-enhancing impacts of *S. scardica* are well documented, the cognitive benefits of *C. vulgare* remain unexplored. This study assessed the potential effect of *C. vulgare* on learning and memory in healthy and scopolamine (Sco)-induced memory-impaired male Wistar rats, comparing it with the effects of *S. scardica*. Over a 21-day period, rats orally received extracts of cultivated *S. scardica* (200 mg/kg) and *C. vulgare* (100 mg/kg), either individually or in combination, with administration starting 10 days before and continuing 11 days simultaneously with Sco injection at a dose of 2 mg/kg intraperitoneally. The results showed that both extracts effectively mitigated Sco-induced memory impairment. Their combination significantly improved recognition memory and maintained monoaminergic function. *S. scardica* excelled in preserving spatial working memory, while *C. vulgare* exhibited comparable retention of recognition memory, robust antioxidant activity and acetylcholinesterase inhibitory activity. The extracts alleviated Sco-induced downregulation of p-CREB/BDNF signaling, suggesting neuroprotective mechanisms. The extract combination positively affected most of the Sco-induced impairments, underscoring the potential for further investigation of these extracts for therapeutic development.

Keywords: recognition memory; antioxidant system; acetylcholinesterase; biogenic amines; BDNF; pCREB

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1. Introduction

Alzheimer disease (AD) is one of the most widespread age-related progressive neurodegenerative diseases [1]. Over the years, this disease gradually impairs memory, affects language and leads to abnormalities in the personality and behavior of patients [2]. The neurodegenerative process damages cognitive function in several ways, with a primary cause being the compromised functionality of the brain cholinergic system. This deficiency is characterized by a substantial loss of cholinergic neurons in the basal forebrain along with their projections to the cortex and hippocampus—integral brain structures essential for the maintenance and regulation of memory [3–8]. Additionally, decreased levels of acetylcholine (ACh) and choline acetyltransferase (CAT), coupled with elevated acetylcholinesterase (AChE) in the brains of dementia patients, have been well established [9,10]. AChE terminates Ach before it reaches the ACh receptor, hydrolyzing it to choline and acetic acid [11]. Therefore, the use of acetylcholinesterase inhibitors (AChEIs) is the most popular pharmacological strategy for the symptomatic treatment of AD [12].

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Neuroprotective Effect of *Marrubium vulgare* Extract in Scopolamine-Induced Cognitive Impairment in Rats: Behavioral and Biochemical Approaches

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Simple Summary: Cognitive deficits, including spatial working and recognition memory impairment, are a common feature of Alzheimer's disease with current therapies offering limited efficacy. *Marrubium vulgare*, a member of the Lamiaceae family, has shown potential to alleviate spatial memory impairment in a model of experimental dementia in rats through its antioxidant and acetylcholinesterase inhibitory activities. The aim of this study was to examine the effect of *M. vulgare* on recognition memory in healthy and dementia-affected rats after 21 days of oral administration. Memory performance was evaluated by the novel object recognition test. Levels of neurotransmitters acetylcholine, noradrenaline (NA), and serotonin, as well as the protein expression of the brain-derived neurotrophic factor (BDNF) and the phosphorylation of the cAMP response element-binding protein (p-CREB), were measured. The expression levels of *BDNF* and *CREB* were evaluated via RT-PCR in the cortex and hippocampus. Our result revealed that *M. vulgare* ameliorated recognition memory impairment in dementia rats by preserving cholinergic function in the hippocampus, increasing NA levels in the brain, and restoring pCREB expression in the cortex following their reduction in the experimental model used. In healthy rats, the extract upregulated the expression of *BDNF* and *pCREB* in the cortex. These findings suggest that *M. vulgare* has potential as a therapeutic agent for cognitive impairments in various neurodegenerative diseases.

Abstract: The potential of *Marrubium vulgare* to alleviate scopolamine (Sco)-induced deficits in spatial working memory has drawn considerable scientific interest. This effect is partly attributed to its potent antioxidant and acetylcholinesterase inhibitory (AChEI) activities. This study examined the effects of *M. vulgare* extract, standardized to marrubiin content, on recognition memory in healthy and Sco-treated rats. Male Wistar rats (200–250 g) were divided into four groups. The extract was orally administered for 21 days and Sco (2 mg/kg) was intraperitoneally injected for 11 consecutive days. Memory performance was assessed using the novel object recognition test. Levels of acetylcholine (ACh), noradrenaline (NA), serotonin (Sero), and brain-derived neurotrophic factor (BDNF) and the phosphorylation of cAMP response element-binding protein (p-CREB) were evaluated in the cortex and hippocampus via ELISA. *BDNF* and *CREB* expression levels were assessed using RT-PCR. The results showed that *M. vulgare* significantly alleviated Sco-induced memory impairment, preserved cholinergic function in the hippocampus, increased NA levels in the brain, and restored pCREB expression in the cortex following Sco-induced reduction. In healthy rats, the extract upregulated *BDNF*, *pCREB*, and *Bcl2* expression. Our findings indicate that the neuroprotective effects of *M. vulgare* may be linked to the modulation of cholinergic function, regulation of NA neurotransmission, and influence on key memory-related molecules.

Keywords: *Marrubium vulgare*; phytotherapy; dementia; cognitive enhancement; cholinergic pathways; biogenic amines; neurotrophic factors

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Marrubium vulgare Extract Improves Spatial Working Memory and Oxidative Stress Damage in Scopolamine-Treated Rats

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Abstract.
Background: The cholinergic neuronal loss in the basal forebrain and increasing brain oxidative stress are one of the main features of the brain suffering from Alzheimer's disease. *Marrubium vulgare* (*M. vulgare*), commonly known as 'white horehound,' possesses a variety of valuable properties, such as antioxidative, anti-inflammatory, and antidiabetic activities. Moreover, it possesses neuromodulatory properties that could potentially impact short-term memory functions.
Objective: The present study was undertaken to investigate the preventive effects of water *M. vulgare* extract on working memory, cholinergic neurotransmission, and oxidative stress in rats with scopolamine (Sco)-induced dementia.
Methods: Male Wistar rats (200–250 g) were divided into four experimental groups. The plant extract was administered orally for 21 days, and Sco (2 mg/kg) was administered intraperitoneally for 11 consecutive days. The behavioral performance of the animals was evaluated by the T-maze test. The effect of the extract on acetylcholinesterase (AChE) activity and antioxidant status in cortex and hippocampus were also monitored.
Results: Our experimental data revealed that treatment with *M. vulgare* significantly increased the percentage of correct choices of rats with Sco-induced dementia in the T maze test (by 38%, $p < 0.05$). Additionally, it reduced AChE activity in the hippocampus (by 20%, $p < 0.05$) and alleviated oxidative stress induced by Sco, particularly in the cortex.
Conclusions: *M. vulgare* water extract demonstrated working memory preserving effect in rats with Sco-induced dementia, AChE inhibitory activity and *in vivo* antioxidant potential, and deserve further attention.

Keywords: Acetylcholinesterase, Alzheimer's disease, *Marrubium vulgare*, oxidative stress, scopolamine-induced dementia, T-maze test

INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia worldwide. Amyloid-β peptide (Aβ) accumulation, oxidative stress, neuroinflammation, affected synaptic function, and neuronal loss are the main neuropathological features of the disease and are closely related with learning and memory impairment [1–7]. AD patients typically experience loss of working, spatial, and anterograde memory [8].

Increasing oxidative stress in the brain is a key pathological hallmark in the multicomponent etiology of AD. It is widely accepted that inflammation caused by the Aβ accumulation is a major source of free radical formation [9, 10]. The overproduction of reactive oxygen species such as hydrogen perox-

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Effects of *Sideritis scardica* Extract on Scopolamine-Induced Learning and Memory Impairment in Mice

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Abstract.

Background: The neurodegenerative process in Alzheimer's disease, one of the most common types of dementia worldwide, mostly affects the cholinergic neurotransmitter system and, to a lesser extent, the monoaminergic one. The antioxidant acetylcholinesterase (AChE) and triple monoamine reuptake inhibitory activity of *Sideritis scardica (S. scardica)* and other *Sideritis* species has already been reported.

Objective: To investigate the effects of *S. scardica* water extracts on the learning and memory processes, anxiety-like behavior, and locomotor activities in scopolamine (Sco)-induced dementia in mice.

Methods: Male Albino IRC mice were used. The plant extract was administered for 11 consecutive days in the presence or absence of Sco (1 mg/kg, i.p). The behavioural performance of the animals was evaluated by passive avoidance, T-maze, and hole-board tests. The effects of extract on AChE activity, brain noradrenalin (NA), and serotonin (Sero) content, and antioxidant status were also monitored.

Results: Our experimental data revealed that the *S. scardica* water extract caused a reduction in degree of memory impairment and anxiety-like behaviour in mice with scopolamine-induced dementia. The extract did not affect changed by the Sco AChE activity but impact reduced brain NA and Sero levels and demonstrated moderate antioxidant activity. In healthy mice we did not confirm the presence of anxiolytic-like and AChE inhibitory effects of the *S. scardica* water extract. The extract did not change the control Sero brain levels and reduce those of NA.

Conclusion: *S. scardica* water extract demonstrated memory preserving effect in mice with scopolamine-induced dementia and deserve further attention.

Keywords: Anxiety, behaviour, cholinergic function, dementia, mice, monoaminergic function, scopolamine

INTRODUCTION

Alzheimer's disease (AD) is one of the most common neurodegenerative disorders worldwide. It is a *chronic, debilitating condition of great social impor-*

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Antioxidant and Antitumor Potential of Micropropagated Balkan Endemic *Sideritis scardica* Griseb

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Abstract: *Sideritis scardica* Griseb. is a critically endangered Balkan endemic species, known for its antioxidant, neuroprotective and anti-inflammatory properties. The aim of the present study was to detail an efficient protocol for the micropropagation of *S. scardica*. In vitro cultures were initiated from the shoot tips of 40 days-old in vivo seedlings and the effects of different plant growth regulator treatments were examined. A Murashige and Skoog nutrient medium (MS) containing 1 mg/L zeatin and 0.1 mg/L indole-3-acetic acid (IAA) proved to be the most efficient for shoot multiplication as it produced quality, vigorous shoots with a mean number of six shoots per explant. For the first time, the antioxidant and antitumor activities of extracts from in vitro-obtained plants were evaluated. In vitro cultivated plants grown in the field revealed a higher total polyphenol content (3929.1 ± 112.2 mg GAE/100 g vs. 3563.5 ± 52.8 mg GAE/100 g) and higher ORAC antioxidant activity (1211.6 ± 27.3 µmol TE/g vs. 939.9 ± 52.4 µmol TE/g) than in situ cultivated plants. A comparison of the antitumor activities of extracts from in vitro propagated shoots, field-grown in vitro-obtained plants and in situ plants on HeLa (cervical adenocarcinoma), HT-29 (colorectal adenocarcinoma) and MCF-7 (breast cancer) human cancer cell lines showed that in vitro propagated shoots had a significant concentration-dependent cytotoxic effect on the cervical adenocarcinoma cell line HeLa, while the field-grown in vitro-obtained and in situ-collected samples induced the highest reduction in the viability of the mammary carcinoma cell line MCF-7. In both cases, the cells of the control non-tumor cell line, BALB/3T3, were significantly less affected. The results showed that the in vitro multiplication protocol ensured the obtainment of numerous plants with antioxidant and antitumor potential.

Keywords: Mursala tea (*Sideritis scardica*); multiplication; antioxidant activity; tumor cell line

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1. Introduction

In recent years, attention has been largely focused on the problem of preserving plant biodiversity endangered by habitat destruction and ecological fragmentation, the uncontrolled collection of wild plants, inappropriate agricultural and forestry practices, urbanization and pollution [1]. Special consideration is given to the conservation of the gene pool of plant species with a high rate of extinction, not only to maintain botanical heritage, but also to preserve rare plants with valuable, and sometimes unique, medicinal properties [2,3]. Biotechnology is especially useful for plant genetic preservation, as well as

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Antitumor and Antioxidant Activities of In Vitro Cultivated and Wild-Growing *Clinopodium vulgare* L. Plants

Maria Petrova [1], Lyudmila Dimitrova [1], Margarita Dimitrova [1], Petko Denev [2], Desislava Teneva [2], Ani Georgieva [3], Polina Petkova-Kirova [4], Maria Lazarova [4] and Krasimira Tasheva [1,*]

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Abstract: *Clinopodium vulgare* L. is a valuable medicinal plant used for its anti-inflammatory, antibacterial and wound-healing properties. The present study describes an efficient protocol for the micropropagation of *C. vulgare* and compares, for the first time, the chemical content and composition and antitumor and antioxidant activities of extracts from in vitro cultivated and wild-growing plants. The best nutrient medium was found to be Murashige and Skoog (MS) supplemented with 1 mg/L BAP and 0.1 IBA mg/L, yielding on average 6.9 shoots per nodal segment. Flower aqueous extracts from in vitro plants had higher total polyphenol content (29,927.6 ± 592.1 mg/100 g vs. 27,292.8 ± 85.3 mg/100 g) and ORAC antioxidant activity (7281.3 ± 82.9 µmol TE/g vs. 7246.3 ± 62.4 µmol TE/g) compared to the flowers of wild plants. HPLC detected qualitative and quantitative differences in phenolic constituents between the in vitro cultivated and wild-growing plants' extracts. Rosmarinic acid was the major phenolic constituent, being accumulated mainly in leaves, while neochlorogenic acid was a major compound in the flowers of cultivated plants. Catechin was found only in cultivated plants, but not in wild plants or cultivated plants' stems. Aqueous extracts of both cultivated and wild plants showed significant in vitro antitumor activity against human HeLa (cervical adenocarcinoma), HT-29 (colorectal adenocarcinoma) and MCF-7 (breast cancer) cell lines. The best cytotoxic activity against most of the cancer cell lines, combined with the least detrimental effects on a non-tumor human keratinocyte cell line (HaCaT), was shown by the leaf (250 µg/mL) and flower (500 µg/mL) extracts of cultivated plants, making cultivated plants a valuable source of bioactive compounds and a suitable candidate for anticancer therapy.

Keywords: wild basil; micropropagation; polyphenols; HPLC; antitumor activity

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1. Introduction

Medicinal plants have been used for centuries to treat a number of diseases and represent an important source for identifying and obtaining novel pharmaceutical drugs [1]. Scientific evidence for the benefits of using medicinal plants and plant-derived active compounds as safe and non-toxic remedies is steadily growing. Due to their wide range of biological activities and favorable effects, plants of the Lamiaceae family are particularly suitable as a source of new phytochemicals. *Clinopodium vulgare* L. (wild basil), spread throughout most of Europe, Western and Central Asia, North America and Northern Africa, is an aromatic herb belonging to the Lamiaceae family. In Bulgaria, the species is used in

antioxidants

MDPI

Article

Memory Recovery Effect of a New Bioactive Innovative Combination in Rats with Experimental Dementia

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Abstract: Alzheimer's disease manifests as a complex pathological condition, with neuroinflammation, oxidative stress and cholinergic dysfunction being a few of the many pathological changes. Due to the complexity of the disease, current therapeutic strategies aim at a multitargeted approach, often relying on a combination of substances with versatile and complementary effects. In the present study, a unique combination of α-lipoic acid, citicoline, extracts of leaves from olive tree and green tea, vitamin D3, selenium and an immune-supporting complex was tested in scopolamine-induced dementia in rats. Using behavioral and biochemical methods, we assessed the effects of the combination on learning and memory, and elucidated the mechanisms of these effects. Our results showed that, compared to its components, the experimental combination was most efficient in improving short- and long-term memory as assessed by the step-through method as well as spatial memory as assessed by T-maze and Barnes maze underlined by decreases in AChE activity ($p < 0.05$) and LPO ($p < 0.001$), increases in SOD activity in the cortex ($p < 0.05$) and increases in catalase ($p < 0.05$) and GPx ($p < 0.01$) activities and BDNF ($p < 0.001$) and pCREB ($p < 0.05$) levels in the hippocampus. No significant histopathological changes or blood parameter changes were detected, making the experimental combination an effective and safe candidate in a multitargeted treatment of AD.

Keywords: Alzheimer's disease; α-lipoic acid; citicoline; extract of leaves green tea; extract of leaves olive tree; vitamin D_3; selenium; scopolamine

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1. Introduction

Alzheimer's disease (AD), the most common form of dementia (contributing to 60–70% of cases), is often referred to as the "epidemic of the 21st century". According to the World Health Organization, at present, more than 55 million people suffer from dementia, the number increasing with 10 million new cases each year. AD and other forms of dementia are revealed as leading causes for death and disability, being classified among the top 10 causes of death worldwide and ranking 3rd in both the Americas and Europe in 2019 and described

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PRODUCTION OF THE PHENOLS SALIDROSIDE AND ROSAVINS IN *RHODIOLA ROSEA* REGENERANTS EX VITRO ADAPTED TO NATURAL CONDITIONS

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Abstract

Rhodiola rosea L. is a high-altitude medicinal plant with various health-beneficial effects, associated mainly with the phenolic substances salidroside and rosavins (rosavin, rosin and rosarin). The intensive collection of *R. rosea* rhizomes and roots to be used in folk medicine and pharmacy has led to a decrease of the species natural resources. New opportunities for species conservation and accelerated synthesis of biologically active substances have been provided by various systems for in vitro and ex vitro cultivation. In this study, the salidroside and rosavins content was determined using HPLC analysis in in vitro cultures of *R. rosea* and further monitored in adapted regenerants grown ex vitro. The targeted secondary metabolites were not detected in the calli and in the roots of 1-month-old regenerants obtained on different nutrient media. The roots/rhizomes of adapted regenerants grown ex vitro in a greenhouse for 1 year and in the mountain for 1, 2 and 3 years produced increasing amounts of salidroside (from 0.4 to 1.2%) and rosavins (from 4.3 to 4.5%). The salidroside to rosavins ratio (1:3.75) in 3-year-old regenerants grown in the mountain was close to the pharmacopoeial standard ratio of 1:3. These results show that *R. rosea* plants, obtainted by in vitro micropropagation and grown ex vitro are promising for drug production.

Key words: ex vitro, HPLC, in vitro, medicinal plant, *Rhodiola rosea*

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Natural substances with therapeutic potential in wound healing

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Untreated wounds are a significant health problem that affects the whole world. The healing wounds capacity of different plants and animals extracts is due to their wide variety of bioactive compounds with epithelial cell regenerative effects on wounds. The present study aims to investigate the efficacy of 3 combined preparations (extract 1, extract 2 and extract 3) as potential regenerative products used for healing of skin wounds. The gel compositions were prepared by mixing the extracts of garden snail mucus *H. aspersa* and plants (leaf extract of *Plantago major* and/or flower extract *of Calendula officinalis)* in 5:1:1 ratio. Their wound healing activity was evaluated using the excision wound model on female Wistar rats. The percentage of wound contraction area was calculated precisely on the 3rd, 6th, 10th, 14th and 19th day. Our results demonstrated that extract 3- treated group (containing extract from *P. major* and snail mucus extract) exerted the best rate of wound closer in the period between 6th and 10th day post injury. The reduction of wound area was by 55% on the 6th day and by 21% on the 10th day after injury versus the control untreated group, respectively. extract 1 was the best for wound closing in the period between 3rd and 6th day post injury. The wound area was reduced by extract 1 on the 3th post injury day by 133% and by 61% on the 6th day versus the control. In conclusion the best wound healing effect of Gel 1 suggests a synergic mechanism of action among the three ingredients: mucus from *H. aspersa*, *P. major* leaf extract and extract of *Calendula* flowers.

Key words: snail *Helix aspersa* mucus extract; *Calendula* flower extract; *Plantago major* leaf extract; healing effects

INTRODUCTION

Untreated wounds are one of the most significant health problems in the world and most often lead to complications and limb amputation [1]. Wound healing is a complex process that goes through four partially overlapping stages: hemostasis, inflammation, proliferation and maturation or tissue modelling [1, 2]. The efficiency of healing depends on the synchrony of the four phases, and it can be influenced by many internal (endogenous) and external (exogenous) factors.

Although there are good clinical practices in the world that prevent delayed of chronic wounds/ulcers healing, their effectiveness is still unsatisfactory. In this regard, numerous studies have been conducted on folk methods of treatment as an alternative to modern clinical practices [3, 4]. A number of herbal preparations including extracts and/or purified biologically active compounds with plant origin have been used to treat skin lesions and are applied in the form of emulsions, creams and ointments [5-7].

Extracts of *Plantago major* (*Plantagiaceae)* and *Calendula officinalis* (Asteraceae) are ones of the most widely used natural products for the treatment of skin wounds. *Plantago major* leaves have been used as a wound healing herbal agent for many years in folk and traditional medicine [5]. Flowers of *Calendula officinalis* (calendula, marigold) is another medicinal plant which is also used in the modern world due to its pharmacological actions including wound healing and antioxidant. Some authors provide evidence that they increase the activity of white blood cells and accelerate tissue repair [8]. Stimulating angiogenesis, granulation, epithelialization and wound contraction effects were also proven [9, 10].

The combination of plant extracts with some animal products like a honey, propolis or snail extract significantly increases the effectiveness of treatment [7, 11, 12]. The positive wounds healing effects of different plants extracts is due to their

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A SOFT COMPUTING QSAR ADAPTED MODEL FOR IMPROVEMENT OF GOLDEN ROOT *IN VITRO* CULTURE GROWTH

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ABSTRACT

Golden Root is a rare medicine plant, difficult for cultivation, with a wide potential in treating cardiovascular and other diseases because of its biologically active compounds. Therefore there are researches about in vitro cultivation of Rhodiola rosea. It is well known that biotechnological experiments provide slowly and in a non-efficient way the needed protocols about multiplication, growing and rooting in in vitro nutrient media. The previously collected data of such experiments were analyzed and computationally trained in order to identify the nutrient media that give the best results for growing and rooting, taking into account the limitations such as insufficiency of the plant material and in vitro nutrient media cost.

The proposed analysis contributes to the optimization of biotechnology experiments giving new directions for the theoretically fitting quantity of nutrient medium ingredients necessary for in vitro growth and rooting of Golden root, taking into account criteria such as biology and cost effectiveness concerning the experiment and results.

"Similar biological activity is provided by common structural properties" – this is the main concept of modeling by Quantitative structure–activity relationship model (QSAR), which is a common method used for property prediction of biochemistry molecules and drugs. Based on this we adapted the concept, making the following assumptions: "biological activity" can be every result from an in vitro experiment such as the type of developed tissue and percentage of necrotic tissues; "structural properties" can be the properties of the nutrient medium, i.e. the concentrations of chemical compounds in the medium. QSAR could be defined as a method based either on regression models, or on artificial intelligence models. Here Artificial Neural Networks is proposed as a soft computing method for creating a model of dependences between nutrient medium, type of explant, type of nutrient medium, cultivation days, medium price and the initial response during the in vitro cultivation. Forecast results were subjected to applied basic statistical analysis, clustering and graphical interpretations.

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Keywords: QSAR, AINN, multiplication coefficient, *Rhodiola rosea*, *in vitro* development, soft computing

Abbreviations: ABA: adapted biological activities; AC ratio: cytokinin /auxin ratio; AI: artificial intelligence; ANN: artificial neural network; AQSAR: adaptation of QSAR; ASP: adapted structural properties; BAP: N6-benzylaminopurine; CM: culture meduim/a; 2,4-D: 2,4-dichlorophenoxyacetic acid; GA_3: gibberellic acid; IAA: indolyl-3-acetic acid; 2-iP: 6-(γ,γ-dimethylallyl amino) purine; IBA: indole 3-butyric acid; IPM: initial plant medium/a; MC: media combination; NAA: a-naphthyl acetic acid; TDZ: thidiazuron; QSAR: Quantitative structure–activity relationship

Introduction

Golden root is an endangered plant which is protected by law in Bulgaria and some other countries (UK, Finland, Russia, Mongolia). The extracts from the roots and rhizomes are used in prevention and treatment of socially important diseases of the cardiovascular and central nervous system, cancer and others. Difficult seed propagation (6) as well as intensive and unregulated harvesting/collection of the plants from their natural habitat in many countries leads to disappearance of the species from its natural areas of existence (1).

Protocols for *in vitro* plant organogenesis, regeneration and propagation using plants growing in nature were established previously (8). *In vitro* cultures with different characteristics were obtained using a large number of nutrient media and explants, allowing flexibility in research. These schemes varied in length, time execution, laboratory chemicals and consumables as well as in their cost. The suitable concentration and combination of the plant growth regulators depends on the genotype, the ecotype, the explant type and its stage of development. The obtained results did not need to be further improved experimentally in order to establish efficient schemes for mass propagation and callusogenesis from the wild growing Golden root plants.

By definition the Quantity Structure–Activity Relationship (QSAR) analysis is a set of soft computing and statistical tools and approaches, applied to data from pharmacology, molecular biology, organic and quantum chemistry (2). QSAR is based on the hypothesis that similar biological activity is provided by common structural properties. It aims quantitative characterization of the relationship between the chemical

Chapter 11

Role of Biotechnology for Protection of Endangered Medicinal Plants

Krasimira Tasheva and Georgina Kosturkova

Additional information is available at the end of the chapter

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1. Introduction

The last two centuries of industrialization, urbanization and changes in land use converting agricultural and natural areas to artificial surface have led to European plants being considered amongst the most threatened in the world. In some countries, more than two-thirds of the existing habitat types are considered endangered. Human activity is the primary cause of risk for 83% of endangered plant species. Habitat destruction and loss are also a problem because they lead to the fragmentation of the remaining habitat resulting in futher isolation of plant population [1]. From another side during the last 10 years an intense interest has emerged in "nutraceuticals" (or "functional foods") in which phytochemical constituents can have long-term health promoting or medicinal qualities. Although the distinction between medicinal plants and nutraceuticals can sometimes be vague, a primary characteristic of the latter is that nutraceuticals have a nutritional role in the diet and the benefits to health may arise from long-term use as foods (i.e. chemoprevention) [2]. In contrast, many medicinal plants possess specific medicinal benefits without serving a nutritional role in the human diet and may be used in response to specific health problems over short- or long-term intervals [3].

There is indisputable interest towards traditional and alternative medicine world-wide [4] and at the same time an increasing application of herbs in medical practices, reported by World Health Organization (WHO) [5]. Nowadays the centuries-old tradition of medicinal plants application has turned into a highly profitable business on the world market. Numerous herbal products have been released like patented medical goods, food additives, herbal teas, extracts, essential oils, etc [6 - 9].

There is an expansion of the market of herbs and herbs based medical preparations all over the world. The income a decade ago in the North American market for sales of medicinal plants has climbed to about $3 billion/year [10]. In South America, Brazil is outstanding with 160

Establishment of Callus Cultures of *Rhodiola rosea* Bulgarian Ecotype

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Keywords: Golden Root, callus induction, long-term cultures, auxin, cytokinin

Abstract

***Rhodiola rosea* (Roseroot, Golden Root or Arctic Root) is a herbaceous perennial plant of the *Crassulaceae* family. *Rhodiola* radix and rhizome is a multipurpose medicinal herb with antidepressant, anticancer, cardio protective and central nervous system stimulating effects. For callus induction, leaves were excised from in vitro micropropagated *Rhodiola rosea* and placed on 18 variants of nutrient media containing MS basic medium supplement with BAP, 2-ip, kinetin, 2,4-D, IAA, NAA, casein hydrolysate and glutamine in different combinations and concentrations. The highest responses for callus formation (62.85 and 73.17%) were observed on two media - containing 1 mg/L BAP and 1 mg/L or 0.5 mg/L 2,4-D. These two combinations of plant growth regulators proved less efficient when 1 g/L casein hydrolysate was added. Callogenesis was not observed in any of the variants containing 2-ip and most of the variants containing NAA. Callus growth rate and tissue characteristics varied depending on the culture media composition with a tendency of positive correlation between callus induction efficiency and callus growth in most of the variants. BAP in concentration of 1 mg/L favoured fast growing compact tissue with grain structure easy to be maintained. Callus growth continued for more than 6 months being subcultured each 20 days on the same initial medium. In the present pioneer study, optimal combinations and concentrations of phyto-regulators were determined for efficient induction and long-term callus cultivation allowing accumulation of biomass and biochemical analysis of valuable secondary metabolites.**

INTRODUCTION

Rhodiola rosea L. (Roseroot, Golden Root or Arctic Root) is an herbaceous perennial plant of the family *Crassulaceae*. The yellow-flowered Roseroot is a circumpolar species of cool temperate and sub-arctic areas of the northern hemisphere, including Europe, North America, Greenland, Iceland and the Altai, Tien-Shan, Himalaya mountains in Asia. The European distribution includes Scandinavia and most of the mountains of Central Europe (2000-2600 m altitude). Roots (rhizomes) and calli of *Rhodiola rosea* contain 6 different groups of chemical components with pharmaceutical activities. Rosavin (from phenylpropanoid group) and salidroside (from phenylethanol derivatives group) are of major importance for pharmaceutical preparation (Satsiperova et al., 1993; Ganzera et al., 2000; Linh et al., 2002). Roseroot has traditionally been used in Russia and Mongolia for the treatment of long-term illness and weakness caused by infection. *Rhodiola* radix and rhizome is a multipurpose medicinal herb with adaptogenic properties: it increases the body's nonspecific resistance and regularizes body functions. It has antidepressant, anticancer, cardio protective and central nervous system stimulating effect (Kelly, 2001; Brown et al., 2002). The great importance of *Rhodiola* is also determined by the intensive phytochemistry research of wild plants and, to a lesser extent, of in vitro cultures. The latter could offer possibilities for bioreactor production of secondary substances and metabolic engineering of their pathways. Despite the great potential of biotechnology methods, the investigations are limited. Kirichenko et al. (1994) and Yin et al. (2004) studied callogenesis and regeneration ability of *Rhodiola*

Antioxidant Activities of Bulgarian Golden Root – Endangered Medicinal Species

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Keywords: *Rhodiola rosea*, Roseroot, phytoregulators, antioxidants

Abstract

***Rhodiola rosea* (Golden Root, Roseroot or Arctic Root) is a yellow-flowered herbaceous cool climate species distributed in the sub-arctic areas of the northern hemisphere (North America, Greenland, Iceland, Scandinavia), in high mountains of Asia (the Altai, the Tien-Shan, and the Himalayas) and in Europe (Central Europe and the Balkan peninsula) above 1800 m. In Bulgaria, this species is endangered and is included in the Red Book. It is protected by law in several other countries (e.g. UK, Finland, Russia, Mongolia). *Rhodiola* is a multipurpose medicinal herb with adaptogenic properties. Its radix and rhizome extracts increase the body's nonspecific resistance and normalize body functions, having antidepressant, anticancer, cardio protective and central nervous system stimulating effects. The present study aims at establishing the optimal in vitro conditions for in vitro cultivation to stimulate bioactive substances production. Calli were cultivated on various phytoregulators and concentrations media. Total phenolic/flavonoid content and radical scavenging activity was determined in these calli. Antioxidant properties were influenced by the composition of the cultivation media. A good correlation was obtained between the scavenging activity of 1,1-diphenyl-2-picrylhydrazyl radical with both the total phenolic content and the total flavonoid content. No strict relation between the callus colour/structure/texture and the secondary metabolite amounts was found. Despite the low accumulation of phenolic and flavonoid compounds in the calli of Bulgarian *R. rosea*, the presence of these metabolites indicates the possibility for the production of biologically active substances by non differentiated cells. This could be a basis for in vitro metabolic engineering and biotransformation for alternative production of valuable substances in Roseroot.**

INTRODUCTION

Rhodiola rosea L. (Roseroot, Golden root or Arctic root) is an endangered medicinal species that is included in the Red Book of Bulgaria and is protected by law in other countries. *Rhodiola* radix and rhizome is a multipurpose medicinal herb with adaptogenic properties: it increases the body's nonspecific resistance and normalizes body functions. It has antidepressant, anticancer, cardio protective and central nervous system stimulating effects (Kelly, 2001; Brown et al., 2002).

Rhizomes and roots of *Rhodiola rosea* contain 6 different groups of chemical components with pharmaceutical value (Satsiperova et al., 1993; Ganzera et al., 2000; Linh et al., 2002). Beneficial health effects are mainly due to the antioxidant content in Golden Root tissues. Phenolic compounds like flavonoids have a very important antioxidant activity by inactivating free radicals and preventing decomposition of hydroperoxides into free radicals. Phenolic compounds have received considerable attention as potentially protective factors against cancer and heart diseases because of their antioxidant potential and their ubiquity in a wide range of commonly consumed foods of plant origin (Burda and Oleszek, 2001; Edreva et al., 2008). The antioxidant activity of *Rhodiola imbricata* rhizome extracts was proved by examining total phenolic

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Review Article

The Role of Biotechnology for Conservation and Biologically Active Substances Production of *Rhodiola rosea*: Endangered Medicinal Species

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At present, more than 50 000 plant species are used in phytotherapy and medicine. About 2/3 of them are harvested from nature leading to local extinction of many species or degradation of their habitats. Biotechnological methods offer possibilities not only for faster cloning and conservation of the genotype of the plants but for modification of their gene information, regulation, and expression for production of valuable substances in higher amounts or with better properties. *Rhodiola rosea* is an endangered medicinal species with limited distribution. It has outstanding importance for pharmaceutical industry for prevention and cure of cancer, heart and nervous system diseases, and so forth. Despite the great interest in golden root and the wide investigations in the area of phytochemistry, plant biotechnology remained less endeavoured and exploited. The paper presents research on initiation of *in vitro* cultures in *Rhodiola rosea* and some other *Rhodiola species*. Achievements in induction of organogenic and callus cultures, regeneration, and micropropagation varied but were a good basis for alternative *in vitro* synthesis of the desired metabolites and for the development of efficient systems for micropropagation for conservation of the species.

1. Introduction

Resent investigation of the World Health Organization (WHO) recorded increasing utilization of herbs in the medical practice [1]. The European market of medicinal plant preparations and remedies has been stimulated by the national policies for better exploitation of the advantages of the traditional and the alternative medicine [2, 3]. The short period of time from 1999 to 2001 marked a considerable increase in the medicinal plants trade in many countries: in the Czech Republic by 22%, in Turkmenistan by 100%, and in Bulgaria by 170%. Similarly increasing herbs use in the developed countries was recorded [4]. More than 25% of the British population is taking them regularly [5]. The business becomes more stable with sales income reaching 5 billion USD for the period of 2003-2004 in Western Europe; 14 billion USD in China for the year of 2005, and 160 million USD in Brazil for the year of 2007.

At present more than 50 000 plant species are used in the two major fields: the contemporary phytotherapy and the modern allopathic medicine [4]. World-wide, about 2/3 of those 50 000 medicinal plants are harvested from nature [6]. The share of the cultivated plants used in the pharmaceutical industry is quite small, yet, being only 10% in Europe [5]. The number of natural populations is decreasing progressively leading to local extinction of many species or degradation of their habitats [7]. According to the data of the Food and Agricultural Organization (FAO) at the United Nations annually the flora bares irretrievable losses which destroy the natural resources and the ecological equilibrium [8]. Four thousand to 10 000 medicinal species were endangered of disappearing during the last years [6].

Bulgaria is in the leading world positions in export of wild medicinal plants. Annual harvest varies between 15 000 t and 17 000 t. Half of them are collected in the mountains while 80% of them are exported [9–12]. Bulgarian medicinal plants are famous for their high content of biologically active substances. They are ranked in the first world positions considering quality which is due to the specific climatic and soil conditions of the country [13].

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ORIGINAL PAPER

Rhodiola rosea L. *in vitro* plants morphophysiological and cytological characteristics

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Abstract

Rhodiola rosea L. (Golden Root, Roseroot) is an endangered species and is protected by law in Bulgaria and other countries. Rhizome and roots extracts are used as a stimulant of immune system, adaptogen, and for prophylactics and cure of socially important diseases – cancer, cardiovascular, etc.

Schemes for in vitro propagation and plant regeneration were established previously. The aim of the present work was to morphologically and cytologically characterize the regenerants. Concerning flowers, leaves, stem and rhizomes no differences were recorded between the wild plants and the regenerated plants grown ex vitro in the adaptation room, in the green house and in the mountain. To detect chromosome number, commonly used techniques for squash preparations were modified to be used for root tip meristem cells of Rhodiola rosea regenerants. In vitro plants obtained on different culture media were subjected to cytological analysis. The chromosome number in all samples was 2n = 22, which confirms the diploid level of plant regenerants and cytogenetic identity with the wild type.

The study indicates that the established in vitro schemes are suitable for mass propagation and production of identical plants of this endangered medicinal species, which could be a basis for restoration of the natural habitats and for establishment of fields for growing plants for production of pharmacologically valuable substances.

Key words: *medicinal plant, golden root, in vitro, cytological and morphological characteristics*

Introduction

Bulgarian medicinal plants are famous for their high content of valuable substances and outstanding quality, due to the country specific soil and climate conditions [1]. More than half of the annual production of 15 000 – 17 000 t is coming from the mountains [2, 3, 4]. Eighty per cent of the harvests are exported, which situates the country in one of the leading world positions in export of medicinal plants collected from nature.

According to the Food and Agricultural Organization (FAO) [5] the world flora becomes poorer from year to year, thus destroying the ecological balance in nature. During the last years, 4000 to 10 000 medicinal species are in danger of disappearing [6].

Rhodiola rosea L. (*Sedum roseum* (L.) Scop., *S. rhodiola* DC.), with popular names Golden root, Roseroot or Arctic root, is a rare plant belonging to family *Crassulaceae*. It is a valuable species of the Bulgarian medicinal plants gene fund. The family *Crassulaceae* includes six genera. Genus *Rhodiola* enlists 200 species. The Golden root spread in Bulgaria has distinguished pharmacological value and is widely used in practice.

Rhodiola rosea L. is a succulent herbaceous plant with a thick quite branched scaly rhizome (rootstock) with average weight of 70 – 400 g, but reaching 3.5 kg as well. Root and rhizomes have rose scent. The stem is straight, 10 – 30 cm in height with rare leaves, cylindrical in shape [7, 8]. The natural habitats of *Rh. rosea* decrease in their number progressively. At the same time restoration ability of wild plants is limited due to the low seed germination and the low vegetative coefficient of reproduction [9]. Roots and rhizomes