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Original article

In vitro callus propagation and antibacterial activities of callus an edible endemic and medicinal plant *Scutellaria orientalis* L. subsp. *bicolor*

Fethi Ahmet Ozdemir[1], Omer Kilic[2], Ekrem Atalan[3]

[1]Department of Molecular Biology and Genetics, Faculty of Science and Art, Bingol University, 12000, Bingol, Turkey - E-mail: ozdemirfethiahmet23@yahoo.com; [2] Technical Science Vocational College, Bingol University, 12000, Bingol, Turkey; [3]Department of Biology, Faculty of Science, Inonu University, 44000, Malatya, Turkey

Summary. Plants are a stupendous source for exploration of new medicinal products for drug development. Today several drugs used to treat many diseases are simply synthetic modifications or copies of naturally obtained plant substances or extracts. Medicinal plants offer best source to obtain a variety of new drugs; and new plants need to be investigated for their potential use against various microorganisms including bacteria. *Scutellaria orientalis* subsp. bicolor in the Lamiaceae (mint) family, is an endemic plant species that grows widely on abondoned lands at altitude of 1000-2800 meters in Eastern Anatolia. The plant or extracts are popularly used in preparation of various traditional medicines to treat many diseases including stress, breast and ovarian cancers; however there is need to establish validity of these methodologies scientifically as plant tissue culture techniques could serve as an alternative way to produce them. This study aimed to documents crude callus extracts from hypocotyl explants obtained from 17 days old plantlets of *Scutellaria orientalis* subsp. *bicolor* for antibacterial activities. Research findings clearly showed inhibition of growth of *Staphylococcus aureus* COWAN 1 and *Bacillus subtilis* var. *niger* ATCC 10 in the extracts determined by disc diffusion methodology. Antibacterial activity was evaluated by measuring zone diameters in mm. No antibacterial activities were detected in control group. The hexane, acetone and methanol extracts of *S. orientalis* subsp. *bicolor* calli exhibited inhibitory action against both bacterial strains. Moreover, callus extracts showed less anti bacterial activities on calli developed on MS medium containing NAA compared to calli obtained on MS medium containing BAP. Furthermore, the results confirmed inhibitory effects of increased BAP concentrations on callus weight, size and quantity of extracts. Moreover, methanol extracts showed more antibacterial activity compared to acetone and hexane extracts. Better knowledge about antibacterial activities of this endemic plant sub species could be highly useful for understanding ways for cheap, commercial production of these extracts at extensive level under controlled conditions.

Key words: *Scutellaria orientalis* subsp. *bicolor*, callus, antibacterial activity

Introduction

Turkey has suitable climate and soil charachteristics to grow many medicinal plants that features a whole continent (1). About 12000 vascular plant taxa are grown in Turkey. Approximately 3000 of them are endemic (2). Among these, the plants belonging to Lamiaceae family are a stupendous source for exploration of new medicinal products for drug development. Medicinal plants offer best source to obtain a variety of new drugs; and new plants need to be investigated for better understanding of their safe use, efficienty and charachteristics for use against various types of bacteria (3). Most of the drugs used to treat many diseases are simply synthetic modifications or copies of naturally occuring plant substances or extracts (4) *Scutellaria orientalis* subsp. *bicolor* in the Lamiaceae (mint) family, is an endemic plant species of Eastern Turkey that grows

widely on moorlands, limestones, volcanic and misty slopes in Eastern Anatolia at 1000-2800 meters above sea level. Plant parts like herbs, leaves, fruits and seeds are popularly used in preparation of various traditional medicines to treat constipation, wound healing, cardio vascular diseases stress (5), and to induce apoptosis of breast and ovarian cancers (6). *Scutellaria* taxa contain sesquiterpenoids, monoterpenoids and phenylpropane derivatives iridoid glycosides (7) and glucuronide or aglucuronide type phenolic compounds (8). All taxa of *Scutellaria* are rich in scutellarin, that is known to have anti cancer properties and induce apoptosis of breast and ovarian tumor cells *in vitro* and is long time used in treatment of cardiovascular diseases. Plants grown through tissue culture are excellent source of obtaining secondary metabolites in alternative way (9). Many of these species contain compounds with ecological properties, which modulate the feeding behaviour of insects or are anti-fungal (10-17). Plant tissue culture techniques have become an excellent source to identify and obtain medicinally important extracts on commercial scale during last 50 years (18-24). There is need to continue such studies for identification of such principle compounds identifying new plant species and developing new methodologies for production of natural or new synthetic antibiotic drugs.

This study aimed to optimize and identify best conditions necessarry for hypocotyl induced callus production and obtain raw callus extracts of *Scutellaria orientalis* subsp. *bicolor* that could inhibit growth of *Staphylococcus aureus* COWAN 1 and *Bacillus subtilis* var. *niger* ATCC 10 that may have a greater application and potential in treatment of infectious diseases.

Materials and Methods

The seeds of *S. orientalis* subsp. *bicolor* were obtained from the Park and Garden Plants of Bingol University, Bingol, Turkey. Voucher specimens of these plants are deposited at the Herbarium of the Faculty of Science, Hacettepe University, Ankara, Turkey.

Surface sterilizatrion of seeds

The seeds were treated with 100% commercial bleach (5% NaOCl Ace, Turkey) for 20 min. followed by 3 × 3 min. rinsing with sterilized distilled water. The plants were cultured in Petri dishes (100 ×10 mm) containing agar solidified Murashige and Skoog (MS) medium (25) contained supplemented with 3% sucrose to sprout them under 16 h light photoperiod (35µmol $m^{-2}s^{-1}$) in Aralab versatile growth chamber at 24 ± 1 ˚C.

Selection of explant and autoclaving

Hypocotyl explants were obtained from 17 days old young seedlings cultured on MS medium containing 1, 2, 4 mg/l NAA and 0, 0.1, 0.2 mg/l BAP (9 combinations) suplemented with 3% (w/v) sucrose and 0.65% (w/v) plant agar (Duchefa). All media were autoclaved for 20 min. at 121˚C and 1.4 kg cm^{-2} pressure. The pH of all media was adjusted to 5.7± 0.1 with 1N NaOH or 1 N HCl.

Extraction of crude extracts from Calli

The calli obtained from each treatment were powdered under steril conditions. Each of the powdered calli (5 g) were extracted in 25 ml with hot water (control), acetone, hexane and methanol (98.1) solvents by keeping on a rotary shaker (100 rpm) for 24 h. The solvents used in the study were removed with the help of a rotary vacuum evaporator at 35˚C. After carefully determining the percentage yield of each extract. The extracts were filtered using Whatman filter paper. Then, 500 µg extracts were injected into empty antibiotic paper discs having a diameter of 6 mm (Bioanalyse). The experimental studies were repeated triplicate.

Bacterial strains

Antibacterial activities for *Staphylococcus aureus* COWAN 1 and *Bacillus subtilis* var. *niger* ATCC 10 strains of bacteria were studied.

Study of antibacterial activity

100 µl of suspension containing 10^6 cells/ml of bacteria were inoculated into petri plates containing Mueller Hinton Agar (Difco) using disc diffusion method for antibacterial activity.

Disc diffusion method was prepared using the discs (6 mm in diameter) containing 25 µl (500 µg) of the extracts (20 mg/ml) and placed on the inoculated Mueller Hinton Agar (Difco). Petri dishes were placed at 4°C for 2 h. Then, the inoculated plates were incubated at 37°C, at 24 h for bacterial strains. Antibacterial activity was evaluated by measuring zone diameters in mm.

Statistical analysis

Each treatment used 30 explants divided into 3 replications. Callus induction observations were recorded after 18 days. Experimental values for each parameter were compared using One Way Anova of IBM SPSS 22 for Windows. Standard error was computed using descriptive statistics function. Means were compared using Tukey's b test. All values expressed as 0.00 were not subjected to statistical analysis.

Results

Callus induction on MS medium containing NAA + BAP

The most effective way to induce calli *in vitro* cultures for *Scutellaria orientalis* L. subsp. *bicolor* is not reported yet therefore, initially hypocotyl explants were treated with 9 different concentrations of NAA with and without BAP for selection of optimum medium for *in vitro* culture and measure quantitative and qualitative differences in the yield of hypoctyl explants. The findings of the results showed that 100% calli were induced on hypocotyl explants irrespective of NAA concentrations with or without BAP in the culture medium (Tab. 1). However the callus weight varied significantly depending on concentrations of plant growth regulators and their ratio (NAA: BAP). MS medium (control) without NAA or BAP plant growth regulators failed to induce any callus (Tab. 1). The callus weight ranged 496.73 ± 0.12 mg to 304.63 ± 1.16 mg (Tab. 1) on all concentations of NAA with or without BAP. Maximum callus weight (496.73 ± 0.12) was noted on MS medium containing 4 mg/l NAA. Each increase in the concentration of NAA (with or without 0.10 or 0.20 mg/l BAP) was accompanied with significant increase in callus weight. However, any concentration of NAA with 0.20 mg/l BAP was more ihibitory conpared to any concentration of NAA with 0.10 mg/l BAP. Minimum callus weight (304.63 ± 1.16) was noted on MS medium containing 1 mg/l NAA + 0.20 mg/l BAP.

Table 1. The effect of NAA and BAP concentrations on callus induction on hypocotyl explants.

Plant growth regulator concentrations and combinations		Rate of callus formation (%)	Callus weight (mg)*
NAA (mg/l)	**BAP (mg/l)**		
1.00	0.00	100.00 ± 0.00	461.76 ± 0.87c
2.00	0.00	100.00 ± 0.00	473.34 ± 0.64b
4.00	0.00	100.00 ± 0.00	496.73 ± 0.12a
1.00	0.10	100.00 ± 0.00	412.53 ± 1.16f
2.00	0.10	100.00 ± 0.00	439.56 ± 0.98e
4.00	0.10	100.00 ± 0.00	451.03 ± 0.73d
1.00	0.20	100.00 ± 0.00	304.63 ± 1.16i
2.00	0.20	100.00 ± 0.00	323.54 ± 0.23h
4.00	0.20	100.00 ± 0.00	346.09 ± 1.02g
Control (MS medium)		0.00 ± 0.00	0 ± 0.00

**All values in a single column showed by different small letters are statistically different at 0.05 level of significance; ± = Standard error*

Measurement of antibacterial activity

Recounted proof and the customary utilization of plants as medications give the premise to showing which key oils and plant concentrates might be helpful for particular therapeutic conditions. Verifiably, numerous plant oils have been utilized as a part of conventional prescriptions as potential sources of antimicrobial activities (26). The antibacterial activities of hypocotyls induced calli extracts from endemic *S. orientalis* subsp. *bicolor* are given in Table 2. No antibacterial activities were detected using extracts obtained from control group. The hexane, acetone and methanol extracts of *S. orientalis* subsp. *bicolor* calli exhibited inhibitory action against both bacterial strains variably.

When we compared the antibacterial activity of the acetone, methanol and hexane extracts of the calli developed on MS medium containing 4 mg/l NAA with those on NAA + 0.10 or 0.20 mg/l BAP induced calli extracts it was noted that the obtained on MS medium containing NAA had less antibacterial activity. Comparatively, extracts of the calli obtained on culture medium with 0.10 or 0.20 mg/l BAP had high antibacterial activity. The increase of BAP concentration in the callus production environment resulted in an increase of antibacterial activity. Similarly, methanol extracts showed more antibacterial activity compared to acetone and hexane extracts.

Discussion

S. orientalis subsp. *bicolor* is a perennial endemic shrub bearing pale grey-white flowers an annual herbaceous plant that contain a large number of volatile and aromatic compounds was reported to be used externally and internally used for treatment of constipation, haemostatic, tonic, and wound healing purposes in Anatolian folk medicine (6). We studied callus induction in juvenile hypocotyl explants raised from seeds and tested their crude callus extracts for antibacterial activity.

It is important to know about plant growth regulator concentrations suitable for callus induction to achieve valuable secondary metabolites of high pharmaceutical importance in *S. orientalis* subsp. *bicolor*. The results of this study clearly indicate the role of different plant growth regulators for callus induction. Similar studies were performed with various species in order to achieve the best hormone combinations for callus induction.

Zhang et al. (27) and Zouzou et al. (28) pointed out that hypocotyls more callogenic explants. This results agreement with our study. Kumari et al.(29) found that different combinations of BA and NAA on MS medium resulted in effective callus induction for *Cotyledon orbiculata*. Calli regeneration was affected markedly by combinations of auxins (30). The combined favourable influence of cytokinins and auxins were

Table 2. Effects of extracts from NAA + BAP induced calli on antibacterial activity against *S. aureus* and *B. subtilis*

Plant growth regulator combinations			**Antimicrobial activity**		
	Extract type	**Yield (%)**	**Control**	***S. aureus***	***B. subtilis***
4 mg/l NAA	Acetone	1.13	Nil	7	12
	Hexane	0.62	Nil	7	13
	Methanol	15.18	Nil	16	23
4 mg/l NAA + 0.10 mg/l BAP	Acetone	1.51	Nil	7	11
	Hexane	0.76	Nil	10	15
	Methanol	12.63	Nil	19	25
4 mg/l NAA + 0.20 mg/l BAP	Acetone	1.93	Nil	9	13
	Hexane	1.06	Nil	11	17
	Methanol	10.13	Nil	21	27

Inhibition zone diameters were measured in mm.

in accordance with the culture response of other medicinal plant species (31, 32). Lemraski et al. (33) used BA-NAA for callus induction on *Salvia* species. Bolta et al. (34) reported best callus induction on MS medium with 10.47 m.mol NAA and 4.5 m.mol BA-6. Dronne et al. (35) also point out that lavender (*Lavandula officinalis*) could best regenerate on MS medium containing NAA + BA as the callusing medium. Researches have shown that the ability of tissue culture and plant regeneration from callus is related to the genetics and many genes in the nucleus and cytoplasm could control it (36).

Analysis of bioactivity of tissue culture developed plant tissues is required to support its use in traditional medicine and to multiply to control their extinction (37). In general, tissue cultured and cultivated plants are not as therapeutically potent as naturally grown plants, however this is not always the case (38, 39). In the current study, antibacterial activity was evaluated in *in vitro* endemic *S. orientalis* subsp. *bicolor* hypocotyl induced calli for the first time.

The results indicate that bioactivity is dependent on plant tissue types and plant growth regulators with which the plants were treated in accordance with reports in *Drimia robusta*, *Catha edulis* and *Cotyledon orbiculata* (39, 40, 29).

Antimicrobial properties were afected by secondary compounds as put forth by Banasiuk et al. (41). In addition, the accumulation of secondary metabolites was regulated by plant growth regulators (39, 29). The bioactivity in *ex vitro* plants may have been affected with secondary compounds which regulated by plant growth regulators during *in vitro* cultures and acclimatization process. However, the mechanism of biochemical changes needs to be investigated in this species. Our study indicates that NAA with or without 0.10 or 0.20 mg/l BAP combinations used for induction of cali on hypocotyl explants of endemic *S. orientalis* subsp. *bicolor* are good for callus induction. Calli from hypocotyl explants with 0.10 or 0.20 mg/l BAP are evaluated more positive for their antibacterial activities against *S. aureus* and *B. subtilis* as examples of gram positive and gram negative bacteria, respectively. The present results suggest that *in vitro* grown plant tissues with specific plant growth regulators combinations can more effectively ihibit grpwth of bacteria compared to others. As a conclusion, this is the first report on *in vitro* callus propagation and antibacterial activity in endemic *S. orientalis* subsp. *bicolor* using hypocoyl explants. *In vitro* callus grown *S. orientalis* subsp. *bicolor* plant tissues treated with one combination of plant growth regulators could yielded a higher antibacterial activity compared to the other plant growth regulator combination. Furthermore, this study also indicate that *in vitro* propagated calli from hypocotyls of *S. orientalis* subsp. *bicolor* have therapeutic potential, which can be used in modern and traditional medicine systems very effectively.

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Correspondence:
Fethi Ahmet Ozdemir
Department of Molecular Biology and Genetics,
Faculty of Science and Art, Bingol University, 12000 Bingol, Turkey
Tel: +90 4262160012 Fax: +90 4262160022
E-mail: ozdemirfethiahmet23@yahoo.com