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Antioxidant phenolic constituents, antimicrobial and cytotoxic properties of *Stachys annua* L. from both natural resources and micropropagated plantlets

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An efficient micropropagation protocol was developed to produce *Stachys annua* plantlets and the extracts obtained from both micropropagated and naturally growing individuals were evaluated for their possible antimicrobial, antioxidant and cytotoxic activities. Mean number of shoot (4.5 ± 0.54 per explant) and node number (4.56 ± 0.5 per explant) as well as biomass yield based on fresh (0.29 ± 0.02 gm per explant) and dry weight (0.029 ± 0.003 gm per explant) were found to be the highest on MS medium with 6-BA, whilst the highest mean shoot length (36.65 ± 1.58 mm) was obtained from MS medium containing 2iP. Hexane extracts from both sources showed activity against *Staphylococcus aureus* whilst methanol extracts of micropropagated plantlets exerted activity on *Pseudomonas aeruginosa*. In antioxidant activity assays, the best antioxidant activity was up to IC_{50} 9.41 mg/mL in DPPH and 1409.5 (mg/100 gm trolox equivalent) was found in FRAP. Extracts from natural plantlets showed higher cytotoxic activity micropropagated ones, with IC_{50} of 0.099 µg/mL and $IC_{50}$of 0.211 µg/mL on HeLa cells, respectively. Total phenolics ranged from 87.47 to 605.12 in micropropagated samples while 771.46 (mg/100 gm gallic acid equivalent) in natural resources.

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Stachys annua subsp. *annua* L., commonly known as *Hacıosmanotu* by the local people in Giresun-Turkey, is an aromatic herbaceous perennial plant belonging to the *Lamiaceae* family which is represented by 45 genera, 565 species and 735 taxa in the flora of Turkey, among which *Stachys* is considered to be one of the largest genera comprising 89 species and 126 taxa, 45 of them being endemic[1]. *Stachys* species have high secondary metabolite content as iridoids[2], flavonoids, phenolic acids, diterpenoids[3] and essential oils[4], but *S. annua* doesn't contain iridioids which are chemotaxonomic markers of this taxon[5]. On the other hand, phenylethanoid glycosides (i.e., verbascoside and related compounds) which are a class of natural compounds with chemosystematic relevance in this taxon[6-8]. These species have been used in traditional medication as wound-healing, anti-diarrheal, and astringent agents[9]. Moreover, antibacterial[10], anti-inflammatory, anti-toxic, anti-nephritic[11] and anti-hepatitis[12] effects of different species of *Stachys* have also been reported. The different secondary metabolites isolated from *Stachys* species have been well-documented elsewhere[13]. However, no attempt has yet been made on its phenolics production capacity from the plantlets propagated *in vitro*.

Plant biotechnology methods are powerful tools for the conservation, and rapid multiplication of many valuable aromatic and medicinal plant species[14]. Especially, the micropropagation technique is useful for the production of active compounds during formation of secondary metabolites (i.e., biosynthesis during *in vitro* culture) in aromatic plants and in particular increasing the levels of some significant metabolites[15,16]. Also, natural collection methods are likely to impact negatively on the natural habitat of these plants and lead to a dramatic decrease in plant

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populations; however, with these techniques, it is possible to prevent the use of natural plants for that purpose and keep nature's beauty intact[17,18].

The aims of this study were to develop an efficient and rapid micropropagation protocol for producing *S. annua* plantlets and thereafter analyze their phenolics contents, together with antioxidant, antimicrobial and cytotoxic effects. We have therefore, developed a micropropagation protocol for *S. annua* that can be used to avoid using natural plant for the production of chemicals in question.

Materials and methods

Plant material

S. annua seeds were collected from the wild populations spread between Şebinkarahisar-Alucra (40° 19′ 114″ N, 38° 40′ 110″ E; 1474 m), Giresun, Turkey, during the August-September 2013, and followed by storage in darkness at 4 °C until use. Before surface sterilization, seeds were incubated with 5 % sucrose solution containing a few drops of commercial bleach for 12 hrs. The solution was removed, and seeds were sterilized with 36.5 % (v/v) hydrogen peroxide solution (H_2O_2) for 30 min. The seeds were cultured on approximately 30 ml nutrient media in 98.5 × 59 mm glass containers for germination. Cultured seeds were kept in the dark growth chamber at 24 ± 2 ˚C, under a 16/8 hrs photoperiod with a photosynthetic photon flux density of 50 µmol m^{-2} s^{-1}.

Experimental

Seed germination

Initially, two basal media MS[19] and Gamborg's B-5 (B5)[20] as cytokinin free (control), half strength (HS) and full strength (FS) each supplemented with 1.0 mg/L6-benzyladenine (6-BA) were employed for the seed germination. After 30 days of incubation, the percentage of germination and shoot length were calculated for each application.

Shoot proliferation

Nodal segments of shoots which were originated from seed germination were cut and placed on MS basal media, each containing 2 % (w/v)sucrose (Duchefa), 0.8 % (w/v) andphyto agar (Duchefa) supplemented with different plant growth regulators (PGRs), such as 6-benzyladenine (6-BA), 6-furfurylaminopurine (kinetin), N 6-[2-isopentyl] adenine (2iP) and 1-phenyl-3-(1,2,3,thidiazol-5-yl) urea (thidiazuron) (TDZ) (2.0 mg/L, each)in combination with indole-3-butyric acid (IBA) (0.1 mg/L) and cytokinin free (control) medium. All plant growth regulators used in this study were filter-sterilized with 0.22 µm filters and added to the cooled media after autoclaving. The *p*H of the media was adjusted to 5.8 with 1 N HCl or 1 N NaOH before autoclaving. All cultures were maintained at 24 ± 2 °C under a 16 hrs photoperiod at a photosynthetic flux of 50 µmol m^{-2} s^{-1}, provided by cool daylight fluorescent lamps. After culturing for a month, the proliferation ability was defined by evaluating number of micro shoots, length of shoots, and number of nodes, biomass yield based on fresh and dry weight. Micropropagation applications were coded as below:

Sample 1; 2.0 mg/L Kinetin + 0.1 mg/L IBA, Sample 2; 2.0 mg/L 2 iP + 0.1 mg/L IBA, Sample 3; Control (No PGR), Sample 4; 2.0 mg/L 6-BA + 0.1 mg/L IBA, Sample 5; 2.0 mg/L TDZ + 0.1 mg/L IBA.

Extraction

In vitro grown biomass was extracted as reported elsewhere[21,22]. Briefly, 200 mg of each sample was macerated in *n*-hexane (HE) and dichloromethane (DCM) (10 mLeach) for 10 min, solvent was filtered out and residue was removed via methanol (MeOH) extraction for 30 min, and methanol was evaporated *in vacuo*. The obtained methanol extract was then dipped in *p*H 2.0 ± 0.1 water and shaken vigorously followed by extraction three times with 5 mL diethyl ether and ethyl acetate. Organic phases were combined, evaporated and made up in methanol (2.0 mL) for HPLC analysis after passing through 0.45 µm filter.

Determination of total phenolic contents

Total phenolic contents were determined with previously reported method[23]. Briefly, 750 µL of Folin-Ciocalteu's/water mixture (1:14) were added to 50 µL sample and after 3 min, 200 µL of 20 % Na_2CO_3 was added. Then the reaction mixture was incubated in dark for 30 min. Absorbance was measured on an ultraviolet-visible (UV–Vis) spectrophotometer (Unicam UV2-100) at 760 nm and methanol was used as blank. Gallic acid was used as standard and total phenol contents in extracts were calculated as mg gallic acid equivalent total phenolic in mg Gallic Acid Equivalent/100 (GAEq mg/100 gm) gm dry weight of plant.

Antioxidant activity

DPPH

The DPPH free radical scavenging activity of the extract was determined by previously reported

method[24]. Briefly, 0.5 mL of methanolic extracts of *S. annua* methanol extracts (or BHT standard solutions) was added to 5 mL of a freshly prepared 0.004 % (w/v) DPPH methanol solution. The mixture was shaken vigorously and left in dark for 30 min. The absorbance was measured at 517 nm against methanol as reagent blank.

The per cent inhibitions (I %) of DPPH radical was calculated as follows:

$$I\ \% = \frac{(A\ blank - A\ sample)}{A\ blank} \times 100$$

Where, A blank is the absorbance of the blank containing all reagents except the test compound and A sample is the absorbance value of the extract. Extract concentration providing 50 % inhibition (IC_{50}) was calculated from the graph plotted inhibition percentage against extract concentration. Tests were carried out in triplicate and butylated hydroxyl toluene (BHT) was used as positive control.

FRAP (Reducing ability) assay

The measure of reducing ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex is also considered as antioxidant activity of any substance. The assay protocol was carried out according to the methods described by Benzie and Strain[25] with some modifications. The test involves the reduction of ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex to a blue colored Fe (II) TPTZ by antioxidant constituents of extracts. The fresh FRAP reagent was prepared by mixing (10:1:1) of 300 mM acetate buffer (*p*H 3.6) with of 10 mM TPTZ solution in 40 mM HCl and 20 mM $FeCl_3$ $6H_2O$ solution. Three mL freshly prepared FRAP reagent and 100 µL of the samples were mixed to incubate for 4 min at 37 °C which gave blue color. The absorbance was noted at 595 nm against blank containing distilled water instead of sample or positive control (Trolox). Constructions of a reference calibration curves (62.5-1000 mg/L) by using absorbance of control were done and FRAP values were expressed as mg Trolox Equivalent/100 gm (mgTEq/100 gm).

HPLC analysis of phenolic compounds

A previously developed and validated HPLC method[26] was used in the quantification of phenolics. The analysis was carried out on an ELITE LaChrome (Hitachi HPLC system), quaternary pumps (L-2130 model), auto injector (model L-2200) and variable wavelength PDA detector (photo diode array (L-2455 Model). A C-18 reverse phase column (250 mm × 4.6 mm id, 5 µm particle size, Agilent (USA)) was used in the analysis which was fixed in column oven (Model-2300). Mobile phase was a mixture of solvent A (2 % acetic acid in water) and solvent B (70:30, acetonitrile/water). The injection volume was 20 µL and column was kept at 30 °C. The flow rate was kept constant at 1 mL min^{-1} using gradient programming; starting the flow of mobile phase as B (5 %) to three min, gradual increase (up-to 15, 20, 25, 40 and 80 % at 8, 10, 18, 25 and 35 min, respectively) and drop back to 5 % at 40 min and left for 10 min to equilibrate in column. Eluent was continuously monitored through PDA by measuring at three different wavelengths, i.e., 280, 315 and 350 nm.

Activity tests

Antimicrobial activity

Microbial strains

The antimicrobial activities of the HE, DCM and MeOH extracts were tested against *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Acinetobacter haemolyticus* ATCC 19002, *Enterobacter aerogenes* ATCC 13048, *Klebsiella pneumoniae* ATCC 13883, *Salmonella typhimurium* ATCC 10708, *Candida albicans* ATCC 10231 and *Candida parapsilosis* ATCC 22019 strains.

Disc diffusion assay

The assay was performed by following the protocols of Clinical and Laboratory Standards Institute[27] and The European Committee on Antimicrobial Susceptibility Testing[28]. Briefly, the dried plant extract was dissolved in methanol to a final concentration of 30 mg/mL and sterilized by filtration through a 0.45 µm Millipore filter. Antimicrobial tests were then carried out by the disc diffusion method using 100 µL of suspension containing 1 x 10^8 CFU/ mL bacteria, 1 x 10^6 CFU/mL yeast spread on nutrient agar (NA), sabouraud dextrose agar (SDA) or potato dextrose agar (PDA) medium respectively. The discs (6 mm in diameter) were impregnated with 30 mg/mL extract (300 µg per disc) placed on the inoculated agar. Negative controls were prepared using methanol. Ofloxacin (10 µg per disc), sulbactam (30 µg)+ cefoperazone (75 µg); (105 µg per disc) and/or netilmicin (30 µg per disc) were used as positive reference standards to determine the sensitivity of each strain tested. The inoculated plates were

incubated at 37 °C for 24 hrs for bacterial strains, and 48-72 hrs for yeasts. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms. In this experiment, each assay was repeated twice.

Microdilution assay

Minimum inhibitory concentration (MIC) values were determined for the bacterial strains which were sensitive to the extracts in the disc diffusion assay. Inocula of the bacterial strains were prepared from 12 hrs broth cultures, and suspensions were adjusted to 0.5 McFarland standard turbidity. The extracts dissolved in 10 % dimethylsulphoxide (DMSO), were first diluted to the highest concentration (500 µg/mL)to be tested, then serial two fold dilutions were made and MIC values of the extracts were determined by the broth microdilution method. A dilution of 1/10 bacterial suspension was prepared from $1x10^8$cfu/mL bacteria and then 5 µL was added to each well from this dilution to make final concentration $5x10^5$cfu/mL per well. Microbial growth was determined by reading the respective absorbance (Abs) at 600 nm using an ELx 800 universal microplate reader (Biotek Instrument Inc, Highland Park, VT, USA) and confirmed by plating 5 µL samples from clear wells on nutrient agar medium. The extracts tested in this study were screened twice against each organism. The MIC value was defined as the lowest concentration required to inhibit the growth of microorganisms.

Cytotoxic activity assay

Cell culture

Human HeLa cervical carcinoma cells were cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 10 % (v/v) heat-inactivated fetal bovine serum, and antibiotic–antimycotic mixture [penicillin (100 U/mL), streptomycin (100 µg/mL), amphotericin B (0.25 µg/mL)]. Cells were seeded at a concentration of $1x10^5$ cells/mL and maintained at 37 ^oC in an atmosphere with 5 % CO_2. The samples were added to the growth medium, after dissolving in EMEM.

Cytotoxicity Test (MTT Viability Assay)

Cytotoxic activities of the MeOH extracts of sample 4 were tested by using the MTT assay with minor modifications[29]. The assay based on the reduction of MTT (the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to a colored formazan product by mitochondrial dehydrogenase, which is active only in living cells[30]. The stock solutions of the samples were diluted with EMEM. The cells were maintained in 96 well-plates (each well contained 200 µL cell suspension at a density of 1×10^5 cells/mL). After reaching confluence (1 day later), the cells were treated with increasing concentrations (1 µg/mL-1000 µg/mL) of the samples diluted with EMEM. After growth of the cells for 48 hrs at 37 °C in a humidified 5 % CO_2 atmosphere, the adherent cells were washed with phosphate buffered saline (PBS), then 10 µL of MTT stock solution (5 mg/mL) and 90 µL PBS buffer was added to each well and the plates were further incubated at 37 °C for 4hrs. At the end of this period, supernatants were discarded, DMSO (200 µL) was added to each well to solubilize the water-insoluble purple formazan crystals. The absorbance was measured at 570 and 690 nm in a microplate reader (µQuant, Bio-Tek Instruments, Inc. Highland Park, USA). The cell viability was calculated using the following equation:

Cell viability (%) = (A sample / A control) x 100

A sample was the absorbance of the experimental group and A control was the absorbance of the control group. The half maximal inhibitory concentration (IC_{50}) of the extracts on HeLa cells were calculated from a graph of cell viability versus the sample concentrations.

Statistical analysis

For all germination experiments, 10 seeds were placed per magenta and 3 magenta were prepared per treatment and each treatment was repeated triplicates. All data were statistically evaluated to analysis of variance (ANOVA) using SPSS (version 21.0) software. Analysis of variance (ANOVA) was used to calculate statistical significance, and the mean ± SE (standard error) by using Duncan's multiple range test (DMRT). For cytotoxicity experiments, data are given as mean values ± SD with 'n' denoting the number of experiments. Statistical comparisons were made using one-way analysis of variance (ANOVA) module of GraphPad Prism 5. The statistical significance of differences between treatments was considered significant at $P < 0.05$. For shoot proliferation, each experiment was repeated as triplicates and for each treatment of a replicate experiment, sixteen explants were cultured.

Results

Micropropagation

S. annua seeds generally germinated a few days within 30 days. The highest germination percentage

was obtained from the control group (no PGR is available) as well as FS of MS with 48.89 ± 1.92 % and 34.45 ± 3.85 %, respectively, followed by FS of B5 (25.53 ± 3.68 %) and HS of B5 (24.45 ± 3.85 %) media. Among all basal media tested, HS of MS and control of B5 gave the lowest germination percentage with 23.32 ± 3.33 %.

Statistical analysis also shows great significant differences between MS and B5 media in terms of the germinating seed number ($P < 0.05$), although the highest of shoot length was obtained from FS of B5 (Fig. 1). However, due to weak shoot production, lower fresh and dry weight efficiency and higher germination percentage FS of MS medium was chosen again for subsequent shoot proliferation studies.

Many different PGRs were tested for plant regeneration from lateral explants; namely 6-BA, kinetin, TDZ and 2iP were individually employed with the presence of IBA (0.1 mg/L) and compared with basal medium without PGR (control). Among the all PGRs tested, sample 4 was found to be more effective in terms of the shoot number, node number and biomass, whereas sample 1 and 2 was suitable for the best shoot length. Significant stimulation of the shoot number (4.5 ± 0.54 shoots per explant) was obtained from sample 4, followed by sample 1 (3.18 ± 0.55 shoots), sample 5 (3.02 ± 0.46 shoots), sample 2 (2.83 ± 0.36 shoots) in comparison with sample 3 (1.5 ± 0.36 shoots) per explant (Table 1). Of all PGRs tested, 2iP produced maximum shoot length 36.65 ± 1.58, followed by kinetin 35.97 ± 1.74 mm (shoot length per explant) whilst TDZ led to the lowest average of shoot length with 19.11 ± 1.19 mm (length/shoot) (Table 1).These results revealed that TDZ shows negative effect in terms of the shoot length possessing significant statistical difference from other media ($P < 0.05$).As far as, node numbers are concerned, 2iP, kinetin and 6-BA gave the higher node number with (4.31 ± 0.46, 4.5 ± 0.43 and 4.56 ± 0.49 node per explant) respectively and no statistical difference was observed between these three PGRs ($P < 0.05$) (Table 1).

In such studies biomass yield is one of the most important parameters. In this study, 6-BA gave the highest fresh weight (0.29 ± 0.024 gm) (Fig. 2) and dry weight (0.029 ± 0.0027 gm) (Table 1). A significant statistical difference was observed between 6-BA and other tested PGRs ($P < 0.05$). As a result, the type of cytokinin employed greatly influenced the shoot proliferation for *S. annua* (Table 1).

Total phenolics and Antioxidant activity

As presented in Table 2, the highest total phenolic value was highest in natural sample, followed by micropropagated samples, e.g. sample 1, samples 2, 5,

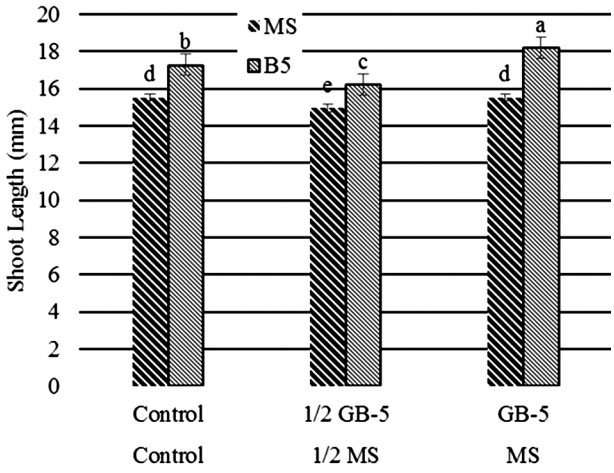

Fig. 1—Effect of differentmedia on germination of *S. annua* seeds. Data obtainedfrom 4 week-oldcultures and represent a total of threereplicates of 30 seeds per treatment. Bars represent S.E. according to Duncan'smultiplerange test ($P < 0.05$)

Table 1—Effects of different PGRs in the presence of IBA (0.1 mg/L) on shoot numbers, shoot length and node numbers, fresh and dry weight of *S. annua*

PGRs(2.0 mg/L)	Shootnumber/Explant	Shootlength(mm)	Nodenumber/Explant	Freshweight(gm)	Dryweight(gm)
CONTROL	1.5±0.37d	28.57±1.99c	3.17±0.37b	0.098± 0.017d	0.005±0.0004d
6-BA	4.5±0.54a	30.59±1.42b	4.56±0.5a	0.290±0.024a	0.029±0.003a
KINETIN	3.18±0.56b	35.97±1.74a	4.5±0.44a	0.153± 0.017c	0.012±0.0023c
2iP	2.83±0.37c	36.65±1.58a	4.31±0.46a	0.140± 0.023c	0.014±0.0004c
TDZ	3.02±0.46bc	19.11±1.19d	2.93±0.44b	0.235± 0.038b	0.023±0.0021b

Data recorded on the 4 weeks after the culture and a total of three replicates of 16 plants per treatment for shoot regeneration.
Values with the same letter(s) in the same line are not significantly different according to Duncan's multiple range test at $P < 0.05$.
Sample 1; 2.0 mg/L Kinetin + 0.1 mg/L IBA, Sample 2; 2.0 mg/L 2 iP + 0.1 mg/L IBA, Sample 3; Control (No PGR), Sample 4; 2.0 mg/L 6-BA + 0.1 mg/L IBA, Sample 5; 2.0 mg/L TDZ + 0.1 mg/L IBA.

control whilst sample 4 contained the lowest. Similarly, antioxidant activity was the most prominent in natural sample in the assays.

Antioxidant activity

Antioxidant potential of extracts was evaluated by two assays (i.e. DPPH and FRAP). The most active samples as antioxidant were natural and sample 1 showing IC_{50} value 3.85 and 9.41 in DPPH while 1409.5 and 823.8 mg/100 gm trolox equivalent, respectively. Following these, sample exerted slightly weaker ability against DPPH with IC_{50} value 10.86 and FRAP 884.5 mg/100 gm trolox equivalent. None of the remaining samples showed remarkable activity. The antioxidant potential was found weaker in control sample, which shows supplement of PGRs during *in vitro* growth of *S. annua* has positive effect on its antioxidant potential. The lowest activity was shown by sample 4 with IC_{50} 23.70 mg/mL and 334.5 mg/100 trolox equivalent in FRAP.

Determination of phenolic contents

Free phenolics were analyzed by HPLC and the data were summarized in Table 3. Gallic acid was highest in control followed by sample 2. Catechin was highest in sample 2 and lowest in sample 4 and sample 5. Catechin amount of natural sample and sample 2, 3 are comparable. Chlorogenic was not found in sample 5, low in sample 1, 3 and 4, whereas sample 2 was comparable with that of natural sample, which contained highest amount (18.08 mg/100 gm). The highest Caffeic acid was found with 34.51 ± 2 in sample 3. Vanillic acid was not found in sample 1, 4 and low amounts in sample 2 and 5 whilst control and natural sample possess high amount. Epicatechin was found to be highest in sample 3. Ferulic acid was found to be highest in sample 1, 2 and 5 while low in natural sample as well as sample 3 and sample 5. *o*-Coumaric acid was found only in two samples (i.e. control and sample 4). Quercetin was highest in

Fig. 2—*In vitro* propagation of *Stachys annua*. Shoot proliferation after 4 weeks; (A) on culture medium from nodal explant on free MS medium (control). (B) on culture medium from nodal explant on MS medium supplemented with 2.0/0.1 mg/L 2iP/IBA. (C) with 2.0/0.1 mg/L KIN/IBA. (D) with 2.0/0.1 mg/L 6-BA/IBA. (E) with 2.0/0.1 mg/L TDZ/IBA. Bar = 1.28 cm

Table 2—Total phenolics and antioxidant activity of *S. annua*

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Natural
TPC	605.12±4.16	413.94±8.32	140.41±4.16	87.47±4.16	366.88±8.32	771.46±4.99
FRAP	1409.5±17.68	884.5±10.61	554.5±10.61	334.5±17.68	659.5±17.68	823.8±7.07
DPPH	9.41±0.09	10.86±0.08	17.58±0.07	23.70±0.02	9.41±0.02	3.85±0.02

Sample 1; 2.0 mg/L Kinetin + 0.1 mg/L IBA, Sample 2; 2.0 mg/L 2 iP + 0.1 mg/L IBA, Sample 3; Control (No PGR), Sample 4; 2.0 mg/L 6-BA + 0.1 mg/L IBA, Sample 5; 2.0 mg/L TDZ + 0.1 mg/L IBA.
TPC; Total Phenolic Content, FRAP; Ferric reducing/antioxidant power, DPPH; 2,2-diphenyl-1-picryl-hydrazyl-hydrat

Table 3—Phenolics identified though HPLC (mg/100 gm)

Phenolics	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Natural
Gallic acid	3.60±0.32	4.29±0.36	17.78±0.11	0.26±0.019	2.79±0.25	6.05±0.51
Catechin	3.22±0.29	12.22±0.87	4.17±0.39	1.89±0.12	0.79±0.06	4.02±0.38
Chlorogenic acid	1.26±0.09	4.88±0.37	0.72±0.066	0.54±0.044	-	18.08±1.19
Caffeic acid	-	-	34.51±2.5	-	1.62±0.11	-
Vanillic acid	-	0.34±0.027	4.69±0.42	-	0.61±0.055	8.72±0.83
Epicatechin	-	1.08±0.091	2.11±0.19	3.59±0.31	1.42±0.13	1.08±0.92
Ferulic acid	3.71±0.27	3.38±0.32	0.31±0.027	1.26±0.11	2.39±0.21	0.38±0.036
o-coumaric acid	-	-	1.17±0.10	2.43±0.23	-	-
Quercetin	-	2.43±0.21	14.15±1.22	24.12±2.44	-	-

Sample 1; 2.0 mg/L Kinetin + 0.1 mg/L IBA, Sample 2; 2.0 mg/L 2 iP + 0.1 mg/L IBA, Sample 3; Control (No PGR), Sample 4; 2.0 mg/L 6-BA + 0.1 mg/L IBA, Sample 5; 2.0 mg/L TDZ + 0.1 mg/L IBA

sample 4, followed by control, whereas sample 2 contained lowest amount.

Antimicrobial activity

Amongst all microorganisms selected, only *S. aureus* ATCC 25923 was found to be sensitive against the *n*-hexane extracts of natural and micropropagated seedlings with MIC values of 0.78 and 1.45 mg/mL, respectively, and *P. aeruginosa* ATCC 27853 was found to be sensitive against the methanol extracts of micropropagated seedlings with MIC values at 6.25 mg/mL. It could be concluded that extracts obtained from both natural and micropropagated seedlings had efficient antimicrobial activities.

Cytotoxic activity

In order to determine the cytotoxic activity of the samples, we evaluated its effect on HeLa cell line by MTT assay. Cytotoxicity data are presented as mean percentages of control ± SD and linear regression analysis was used to calculate the IC_{50} values. All samples exhibited cytotoxic activity against the HeLa cells. Results are shown as IC_{50} of the HeLa cervical cancer cell line and illustrated in Fig. 3.

Cytotoxic activities of natural sample was higher than micropropagated sample (sample 4) by presenting IC_{50} of 0.099 µg/mL the former and IC_{50} of 0.211 µg/mL in the latter case on HeLa cells.

Discussion

Micropropagation

The aim of the study was to establish an *in vitro* propagation method for *S.annua* and subsequently compare the accumulation of phenolic compounds, antioxidant, antibacterial and cytotoxic effects of the extracts obtained from naturally growing and micropropagated plantlets. The use of secondary metabolites has gained an increasing important in the medical field in recent years. For this reason the new plant breeding technologies are used for increasing the amount of these valuable products and their biological activities at the present time. Although secondary metabolite profiles of many Lamiaceae[31,32,33,34] and other plant family members were previously studied[35,36], those of *in vitro* produced plantlets are scarce in the literature[15,37]. Many researchers reported that the MS medium supplemented with 6-BA is very effective in terms of the shoot multiplication for some species[38,39] as emphasized in our study.

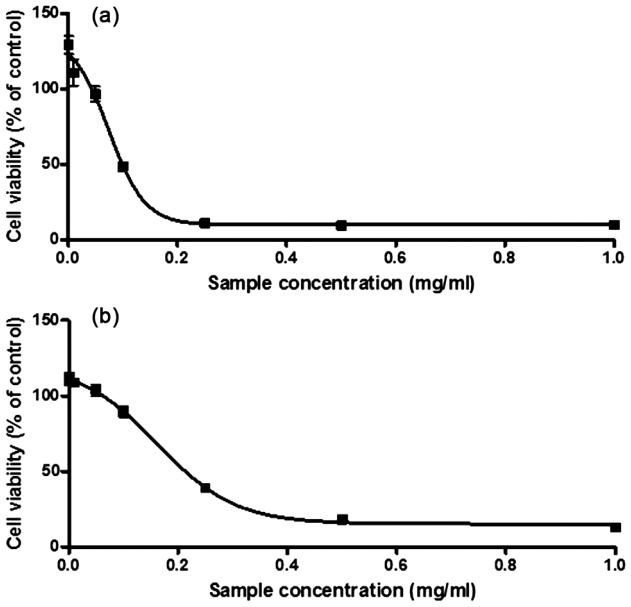

Fig. 3—Cytotoxic effect of the samples on HeLa cells. a) *S.annua* (natural sample) ($P < 0.0001$, $R^2=0.911$), b) *S. annua* (cultured sample) ($P < 0.0001$, $R^2=0.950$)

Although, Pistelli *et al.*[39] obtained the highest rate of shoot length in MS medium supplemented with 6-BA, whereas 2iP was proven to be the best for this approach in our experiments, there was no statistical differences between these two cytokinins. The highest shoot and node number, fresh and dry weight values were obtained MS medium supplemented with 6-BA similarly as in the study of Pistelli *et al.*[39]. As far as the highest multiple shoot formation is concerned, our findings are in accordance with the previous report published elsewhere[40].

Total phenolics and antioxidant activity

Total phenolic content (TPC) and antioxidant activity show positive correlation with a few exceptions; i.e., although the extract from naturally growing plant possessed the highest TPC amount and, as expected, showed the strongest activity in DPPH, it exerted moderate activity that slightly lower than sample 1 and 2 in FRAP. Similarly, sample 2 had considerably high TPC and extended better activity in FRAP, but in DPPH, slightly weaker than sample 1 and 5. This difference is not significant but still it can be attributed to possible synthesis of some unidentifiable phenolic which is quite obvious as in present study. None of PGR could lead to produce caffeic acid, except for sample 5. If we consider the TPC values of present study, these are comparable or even better than previous reports[41,42]. Likewise,

antioxidant activity of the natural *S. annua* as well micropropagated ones is comparable with previous reports[26].

Phenolic contents

Although sample 1 contains low number of phenolics, it shows highest total phenolic content compared to control sample as well as other biomass samples grown in media with different PGRs. This indicates that some unidentified phenolics are present which must be biosynthesized via these PGRs (i.e. 2.0 ppm Kinetin + 0.1 ppm IBA). At first glance the comparison of control sample and natural is interesting. It can be noticed that identified phenolics of control sample are comparable or almost double than those of natural samples in number as well as quantity except vanillic acid and chlorogenic acid. It shows that there is great potential if this plant is grown via *in vitro* tissue culture and to be used as source of these valuable compounds. The higher amounts of phenolics comparing with their parent natural plant have already been reported[43] where no PGRs were used during *in vitro* propagation methods, to produce biomass to have significant potential for the production of high-value phytochemicals with no seasonal constraints and independent of geographical and soil conditions. Whereas, the effects of PGRs are contrary in almost all cases. In a previous report[44] caffeic acid has been reported as 2.05 % (extract basis) in *S. annua*. However, in present study no caffeic acid was found which might be due to climatic or regional variation effect, while control sample shows considerable amount of caffeic acid (i.e. 34.51 mg/100 gm), and also sample 3 contains 1.62 mg/100 gm. Hence, it can be assumed that this plant has capacity to produce caffeic acid in particular conditions as it is dependent of climate, region and PGRs *in vitro* tissue culture production of *S. annua*. As already found in the report, gallic acid is also found here in natural as well control sample (thrice in quantity) while in the media supplemented with PGRs, concentration of gallic acid is reduced. In general, the lower amounts of phenolics with PGR 2ip were found which is in agreement with a previous report[45].

Antimicrobial activity

Although some researchers reported a few study about antimicrobial effect of *Stachys* species[46,47], there was not any comprehensive antimicrobial activity study about to compare natural and micropropagated plantlets of *S. annua.* The use of *n*-hexane extract of natural *S. annua* is more effective than *n*-hexane extract of micropropagated plantlets. While we found 15 mm zone diameter for natural *n*-hexane extract, we obtained 17 mm zone diameter for *n*-hexane of micropropagated plantlets.

Cytotoxic activity

The researchers reported a few study about cytotoxic activities of different *Stachys* species[48-50], but the comparison of cytotoxic effects of natural and micropropagated *S. annua* plantlets was firstly evaluated in this study. Previous studies have shown that *S. pilifera* dichloromethane extract had the highest cytotoxic activity as they contain non-polar compounds such as terpenoids[49]. It was revealed that iridoid glycosides (aucubin and harpagide) in *Stachys* species have probably exhibited cytotoxic effects on the cells[51]. But *S. annua* does not contain these compounds[5]. Phenolic compounds, in particular glycosidic flavonoids related to isoscutellarein and hypolatetin which are known to possess interesting biological activities and the possibility to be the active components can be responsible for cytotoxic effects on the cells in *S. annua*[2,5,52]. Our results showed that all samples have significant cytotoxic effect. The toxic effect of natural *S. annua* was higher than micropropagated sample (sample 4) on the HeLa cells. According to the results, total phenolic constituents of natural plant were different from micropropagated *S. annua* plantlets (Tables 2&3). Natural sample has the highest amounts of phenolic compounds. Therefore cytotoxic effect of natural *S. annua* plant could be higher than micropropagated plantlets. An improved tissue culture protocol has been developed in this report from the nodal segments of *S. annua*.

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