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ORIGINAL PAPER

Elevated antioxidant potential of chlorocholine chloride-treated in vitro grown *Stevia rebaudiana* Bertoni

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Abstract Medicinal plants contain a plethora of secondary metabolites, most of which are bioactive in nature. The role of a popular plant growth retardant CCC has been investigated to explore its impact on secondary metabolite production, particularly phenols and flavonoids from in vitro grown *Stevia rebaudiana*. CCC stimulated the production of total phenols and flavonoids in calli and leaves. Moreover, this elevated level of phenols and flavonoids was correlated with the antioxidant potential of the tissue extracts. Methanolic extracts from CCC-treated calli and leaves showed significant increment in antioxidant activity as determined by standard DPPH, ABTS, and hydroxyl radical scavenging assays. No significant antiproliferative effect of methanolic extracts from different tissue was noticed against THP-1 monocyte (ATCC-TIB202), Hela cell (ATCC-CCL2) lines endorses the issue of clinical safety of the extracts.

Keywords *Stevia rebaudiana* · Chlorocholine chloride (CCC) · Antioxidant activities · Cytotoxicity

Abbreviations

ABTS	2,2′-Azinobis-3-ethyl-benzothiazoline-6-sulfonic acid
BA	6-Benzyladenine
CCC	Chlorocholine chloride
DPPH	2,2-Diphenyl-1-picrylhydrazyl
lb/sq	Pound by square
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAA	1-Naphthalene acetic acid

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Introduction

Free radicals are created in normal biochemical processes and the body can normally keep them in check, but a problem is created when too many free radicals are present in the body for too long. Large generation of free radicals in the body, particularly reactive oxygen species and their high activity plays a vital role in the progression of a variety of human diseases like inflammation, atherosclerosis, stroke, heart disease, diabetes mellitus, multiple sclerosis, cancer, Parkinson's disease, and Alzheimer's disease (Mensor et al. 2001; Orhan et al. 2003; Tepe et al. 2005). Therefore, much attention has been focused on medicinal plants and phytochemicals due to their free radical scavenging activities (Hou et al. 2003; Galvez et al. 2005; Kukic et al. 2006).

Plants are the important source of natural compounds which differ widely in terms of structures, biological properties, and mechanisms of actions. Various phytochemical components, especially polyphenols (such as flavonoids, phenyl propanoids, phenolic acids, tannins, etc.) are known to be responsible for the free radical scavenging and antioxidant activities of plants. Flavonoids are a group of polyphenolic compounds, ubiquitously found in fruits, vegetables and medicinal plants. Flavonoids exhibit a wide range of biological activities, including anticarcinogenic,

anti-inflammatory, antiradical, and antioxidant actions, especially; they exert antioxidative effects as free radical scavengers.

Stevia (*Stevia rebaudiana* Bertoni), also known as "Sweet leaf (in USA)", "Sweet honey leaf (in Australia)" and "Sweet herb of Paraguay", is a semi bushy perennial herb belongs to the family Asteraceae. The plant is estimated to be 300 times sweeter than sucrose (Kinghorn 1987). Stevioside, a diterpene glycoside forms the largest part of sweetener molecules present in the leaves. Other compounds present but in lower concentration are steviolbioside 2, rebaudioside B, C, D, E, F, and dulcoside A (Starrat et al. 2002). Flavonols such as quercetin, myricetin, isorhamnetin, and kaempferol and the corresponding flavones, apigenin, and luteolin, have been well-established as potent antioxidants found in the extracts of *Stevia*, which prevent oxidation of low-density lipoprotein and inhibit lipid peroxidation (Srijani et al. 2007).

The present paper explores the role of a popular plant growth retardant CCC on production of secondary metabolites (especially phenols and flavonoids) from in vitro grown calli and microshoots *Stevia rebaudiana* Bertoni with special emphasis on their anti-oxidant potentialities. CCC, an onium compound, is a potent gibberellin biosynthesis inhibitor. Nowadays, several elicitor molecules like methyl jasmonate, abscisic acid, CCC, salicylic acid, phenylalanine and triazole compounds have been used to enhance the production of various medicinally important phytoconstituents (Ketchum et al. 1999; Kim et al. 1995). It had already been found that CCC can potentially enhance the alkaloid production of *Catharanthus roseus* in both tissue culture and field conditions (Choudhury and Gupta 1996). Taxol production in *Taxus globosa* shoot callus was also elevated by CCC treatment (Barrios et al. 2009).

The evaluation of the antioxidant activity of methanolic extracts from calli and microshoots of *S. rebaudiana* was undertaken using three standard parameters like DPPH, ABTS, and hydroxyl radical scavenging potentialities. There are reports that plant extracts containing different metabolites like alkaloids, glycosides, essential oils, proteins, amino acids, polyacetylenes, and furanocoumarins often cause potential toxicity to humans and other animals (Orech et al. 2005). In this context, it is quite justified to analyze the cytotoxicity of methanolic extracts using THP-1 monocyte (ATCC-TIB202), Hela cell (ATCC-CCL2) and preadipocytic cell line (3T3L1) through MTT assay method to evaluate the suitability of this plant's extract, particularly as a source of nutraceuticals. It is clear that CCC can stimulate secondary metabolite production, in general. This present investigation has been carried out to correlate the impact of CCC on elevation of total phenol and flavonoid content which in turn augmented the antioxidant potential in regenerated calli and leaves of *S. rebaudiana*.

Materials and methods

Plant material

Stevia rebaudiana Bertoni seeds were procured from medicinal plant nursery of Government Cinchona Factory located at Mungpoo, Darjeeling, West Bengal.

In vitro culture

Axenically grown cotyledonary leaves were used as explant in the tissue culture experiment. Seeds were first surface sterilized with 0.1 % (w/v) mercuric chloride solution followed by wash in distilled water for several times and then soaked in distilled water for 2–3 h. Soaked seeds were kept in filter paper inside the petri-plates moistened with water for germination. Regenerated cotyledonary leaves (6–7 days old) were then thoroughly washed in sterile distilled water followed by surface sterilization in 0.1 % (w/v) mercuric chloride for 1–1.5 min. After that, leaves were rinsed three to four times in sterile double-distilled water followed by inoculation in culture media. MS medium (Murashige and Skoog 1962) was used as the basal medium for in vitro culture and it was supplemented with different plant growth promoters like NAA, BA, and Kinetin singly or in combinations for callusing and shooting. After regeneration, calli and microshoots were transferred into fresh MS medium and primed with different concentrations of CCC (in test sets). The culture media were provided with 3 % (w/v) sucrose and gelled with 0.8–1 % (w/v) agar–agar. The pH of the media was adjusted to 5.7–5.9 by 0.1 (N) NaOH. The gelled media were poured into culture tubes, plugged with non-absorbent cotton and then autoclaved at 121 °C under 15 lb/sq. inch pressure for 15 min. Then the tubes were kept in culture room illuminated with white fluorescent light (2000 lux) under 16/8 h light/dark and 72 % RH condition. Subculturing was routinely carried out after every 4–5 weeks of inoculation.

Extraction of phytochemicals

Four-week-old calli (1 g fresh weight) and 7-week-old fully regenerated leaves (1 g fresh weight) from in vitro grown of *Stevia rebaudiana* were dried in room temperature, ground and extracted with 10 ml 85 % methanol separately. The extractions were evaporated under reduced pressure in rotary vacuum evaporator (Eyela, Japan). The remaining aqueous portion (approximately 1.5 ml) was lyophilized and was used for further analysis.

Estimation of total phenol

Total phenolic contents were determined according to the method of Slinkerd and Singleton (1977) with slight

modifications using caffeic acid as standard. Thus, 1 ml of 20 % Na_2CO_3 was added to 100 µl of different extracts (in sterile distilled water) and incubated at room temperature for 2 min. After that, 100 µl of Folin–Ciocalteu's phenol reagent [diluted to 9:1 (v/v) with distilled water] was added to the reaction mixture, mixed thoroughly and the absorbance was recorded at 650 nm by Systronics UV–Vis spectrophotometer.

Estimation of total flavonoid

Total flavonoid contents were determined following the method of Kim et al. (2003) using quercetin as authentic sample. Extracts from different control and test sets were diluted to 1 ml with distilled water (1:1 v/v). Then 100 µl of 5 % $NaNO_2$ was added to it and the reaction mixtures were kept at room temperatures for 5 min. After that, 1 ml of 10 % $AlCl_3$ was added to the mixtures and incubated for 5 min at room temperature followed by addition of 0.5 ml of 1 N NaOH. Absorbances were recorded at 510 nm using Systronics UV–Vis spectrophotometer.

DPPH radical scavenging activity

DPPH radical scavenging activity of methanolic extracts (500 and 1,000 µg/ml) from different calli and leaves (control and test sets) were determined through change in the absorbance of reaction mixtures (containing freshly prepared 100 mM DPPH solution + plant extracts) at 517 nm following the method of Kumar and Chattopadhyay (2007). Quercetin (1 mM) was used as authentic antioxidant. The blank or control reaction mixtures did not contain any plant extracts and radical scavenging activity (% inhibition) was determined using the following formula:

$$\% \text{ Inhibition} = [(\text{Abs. control} - \text{Abs. sample})/\text{Abs. control}] \times 100\ \%.$$

ABTS radical scavenging activity

ABTS radical scavenging activity was performed using methanolic extracts (500 and 1,000 µg/ml) from different calli and leaves (control and test sets) of *Stevia rebaudiana* following the method of Re et al. (1999). ABTS (7 mM) stock solution was made by reacting with potassium persulphate (2.45 mM) and allowing the reaction mixture to stand for 16 h in dark to produce $ABTS^+$ free radicals. Working solutions of ABTS were prepared by diluting the stock solutions with methanol to an absorbance value of nearly 0.7 ± 0.02 at 734 nm. These working solutions were used as control. Quercetin was used as standard antioxidant. The radical scavenging activity was determined by mixing 20 µl of plant extract and 980 µl ABTS working solution. The change of absorbance values was recorded at 734 nm just after 6 min and inhibition percentages (%) were calculated by the above-mentioned formula.

Hydroxyl radical scavenging activity

This was determined by preparation of reaction mixtures which contain methanolic extracts (500 and 1,000 µg/ml) from different calli and leaves, 3.6 mM deoxyribose, 0.1 mM EDTA, 0.1 mM L-ascorbic acid, 1 mM H_2O_2 and 0.1 mM $FeCl_3 \cdot 6H_2O$, and the volume was increased to 500 µl with 25 mM phosphate buffer (pH 7.4). The reaction mixtures were then incubated at 37 °C for 1 h. After incubation 500 µl of 1 % thiobarbituric acid and 500 µl of 1 % trichloroacetic acid were added to those mixtures, which was heated in a boiling water-bath for 15 min and then cooled. The absorbance was recorded at 532 nm. The main principle of this assay is degradation of deoxyribose in the presence of a low concentration of iron salt and thiobarbituric acid (Halliwell and Gutteridge 1981). Control reaction mixtures were not provided with any plant extracts. Quercetin was used as standard antioxidant for the assay and scavenging activity (% inhibition) was determined as described formula.

Measurement of cell viability (MTT assay)

Cell viability was measured following the MTT bioassay method (Bernas and Dobrucki 2002). Briefly, THP-1 monocyte (ATCC-TIB202), Hela cell (ATCC-CCL2), and preadipocytic cells (3T3L1) were seeded in the well (1×10^6 cells/well) of 96-well plate for 4 h. Then the cells were incubated with different plant extracts (500 and 1,000 µg/ml) for 24 h. After incubation, the MTT solution (5 mg/ml on PBS) was added (10 µl/well). This plate was incubated for additional 4 h at 37 °C, and the formazan crystals produced were dissolved in 100 µl of DMSO. Absorbance was measured in ELISA plate reader (Bio-Tek Instrument Co., WA, USA) at 540 nm and percentage (%) of cell viability was calculated as following:

$$\% \text{ Cell viability} = \text{Mean Abs. of the sample} \times 100/\text{mean Abs. of the control}$$

Statistical analysis

All the experiments were carried out in triplicate. Mean and standard deviations were calculated using Microsoft Office Excel 2007. One-way ANOVA with Duncan's post hoc tests were performed to determine the significant differences between the control sets and test sets of the respective experiment using SPSS version 17 for Windows. Significant differences were considered at $p = 0.05$.

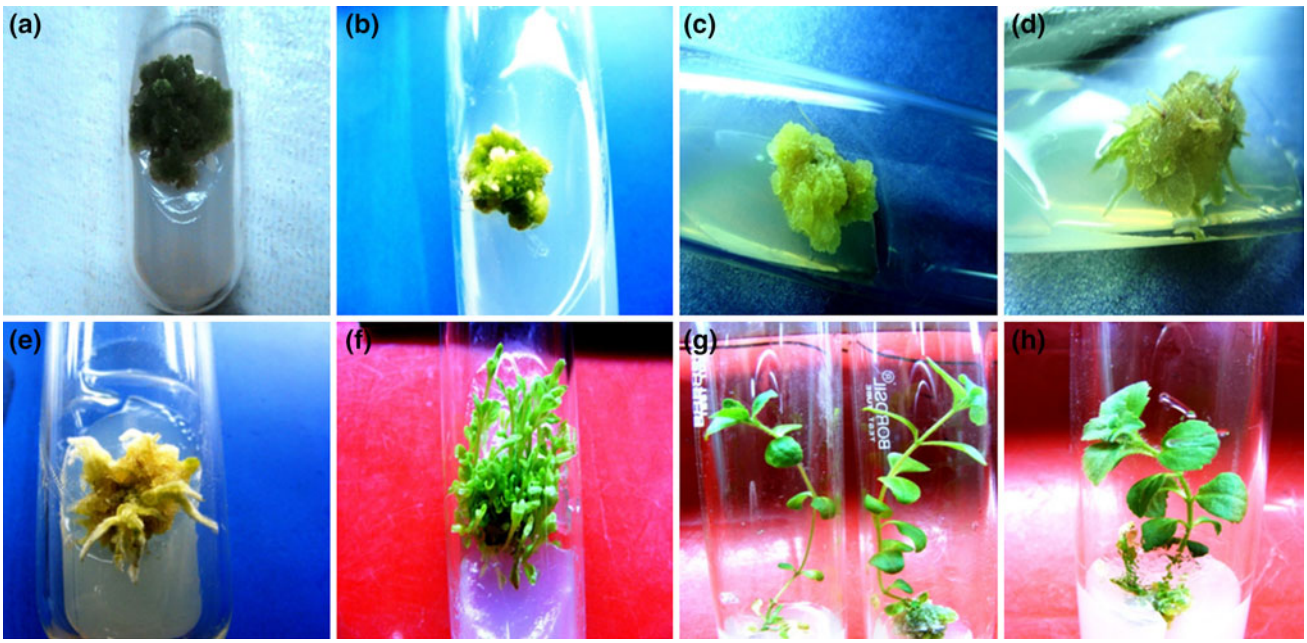
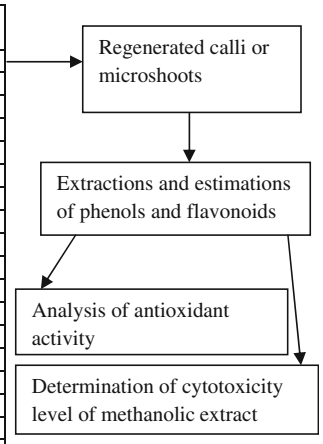


Fig. 1 Callusing and microshooting of *Stevia rebaudiana*; **a** callus regenerated in MS medium supplemented with NAA 3 mg/l + BA 1 mg/l, **b** callus grown in MS medium supplemented with NAA 3 mg/l + BA 1 mg/l, **c** callus regenerated in MS medium supplemented with NAA 5 mg/l + kinetin 3 mg/l, **d** callus grown in MS medium contained NAA 3 mg/l + BA 1 mg/l followed by subculture in MS with CCC 1 mg/l, **e** callus regenerated in MS medium supplemented with NAA 3 mg/l + kinetin 1 mg/l followed by subculture in MS with CCC 1 mg/l, **f** profuse multiple shooting in MS medium contained with BA 3 mg/l + kinetin 5 mg/l, **g** microshoots grown in MS medium supplemented with BA 3 mg/l + kinetin 3 mg/l followed by subculture in MS with same BA-kinetin combination (after 5 weeks), **h** microshoot regenerated in MS medium supplemented with BA 3 mg/l + kinetin 3 mg/l followed by subculture in MS with CCC 1 mg/l (after 5 weeks)

Table 1 Experimental design for tissue culture and subsequent investigations

Category	Combinations of growth regulators (mg/l)			CCC priming(mg/l)	Nature of response
Control sets (CS)	**NAA**	**BA**	**Kinetin**	**CCC**	
CS1	0.5	0.5	×	×*	Callus
CS2	0.5	1	×	×	Callus
CS3	1	1	×	×	Callus
CS4	3	1	×	×	Callus
CS5	0.5	×	1	×	Callus
CS6	1	×	1	×	Callus
CS7	3	×	1	×	Callus
CS8	5	×	3	×	Callus
CS9	×	1	1	×	Microshoot
CS10	×	1	3	×	Microshoot
CS11	×	3	3	×	Microshoot
CS12	×	3	5	×	Microshoot
Test sets (TS)	**NAA**	**BA**	**Kinetin**	**CCC**	
TS1	0.5	0.5	×	1	Callus
TS2	0.5	1	×	1	Callus
TS3	1	1	×	0.5	Callus with roots
TS4	3	1	×	1	Callus with roots
TS5	0.5	×	1	0.5	Callus
TS6	1	×	1	0.5	Callus with roots
TS7	3	×	1	1	Callus with roots
TS8	5	×	3	1	Callus with roots
TS9	×	1	1	0.5	Stunting of microshoots
TS10	×	1	3	1	Stunting of microshoots
TS11	×	3	3	1	Stunting of microshoots
TS12	×	3	5	1	Stunting of microshoots



* Medium without respective growth regulator or retardant

Table 2 Total phenol and flavonoid content in different control and test sets

Treatment sets	Amount of phenol (mg/g fresh wt.)	Amount of total flavonoids (mg/g fresh wt.)
CS 1	0.95 ± 0.04ab	0.88 ± 0.08a*
TS 1	1.05 ± 0.091abc	0.97 ± 0.09abc
CS 2	0.97 ± 0.06ab	0.94 ± 0.10abc
TS 2	1.08 ± 0.1007abcd	1.023 ± 0.06abc
CS 3	1.013 ± 0.07abc	0.93 ± 0.096abc
TS 3	1.12 ± 0.10bcd	1.09 ± 0.04bcd
CS 4	1.05 ± 0.07abc	0.96 ± 0.07abc
TS 4	1.23 ± 0.12de	1.116 ± 0.05cde
CS 5	0.91 ± 0.06a	0.88 ± 0.06a
TS 5	1.01 ± 0.04abc	0.98 ± 0.64abc
CS 6	0.93 ± 0.09ab	0.91 ± 0.091ab
TS 6	1.1 ± 0.07bcd	1.07 ± 0.04abcd
CS 7	1.05 ± 0.08abc	1.023 ± 0.1115abc
TS 7	1.16 ± 0.11cd	1.1167 ± 0.05cde
CS 8	1.15 ± 0.06cd	1.04 ± 0.08abc
TS 8	1.35 ± 0.13ef	1.23 ± 0.06def
CS 9	1.45 ± 0.08fg	1.29 ± 0.13efg
TS 9	1.63 ± 0.10hi	1.466 ± 0.146gh
CS 10	1.54 ± 0.11gh	1.31 ± 0.11fg
TS 10	1.75 ± 0.09ij	1.59 ± 0.14hi
CS 11	1.56 ± 0.09gh	1.4 ± 0.141fg
TS 11	1.8 ± 0.09j	1.72 ± 0.12i
CS 12	1.56 ± 0.1002gh	1.43 ± 0.13gh
TS 12	1.87 ± 0.15j	1.72 ± 0.122i

* Values are mean ± standard deviations of three replicates; means followed by same letter in a column are not significantly different ($p = 0.05$)

Results

In vitro callusing and microshooting

It was revealed in our previous work that MS medium supplemented with NAA and BA or NAA and Kinetin in different combinations produced profuse friable calli and BA and Kinetin together developed multiple shooting (Dey et al. 2010). We have selected few best combinations from the previous work for these present investigations. MS medium contained NAA (0.5–3 mg/l) and BA (0.5–1 mg/l) or NAA (0.5–5 mg/l) and Kinetin (1–3 mg/l) produced best friable calli (Fig. 1a–c). When these calli were further transferred into fresh MS medium supplemented with lower concentrations (0.5–1 mg/l) of CCC (in test sets), rooting of the calli was noticed (Fig. 1d, e). Profuse multiple shoots were developed in MS with BA (1–3 mg/l) and Kinetin (1–5 mg/l) combinations (Fig. 1f, g). Regenerated microshoots were then transferred into the fresh MS medium containing lower concentrations (0.5–mg/l) of CCC (in test sets), and it was noticed that CCC priming made the microshoots shorter and stouter in comparison with that of control sets (Fig. 1g, h). This paper mainly attempts to evaluate the antioxidant activity and cytotoxicity of the methanolic extracts from calli and microshoots of *S. rebaudiana*, so details of tissue culture were not included here (Table 1).

Total phenol and flavonoid content

Phenols and flavonoids are the most diverse and widespread secondary metabolites found in different medicinal plants and exhibited a broad spectrum of biological activities. Methanolic extracts from different in vitro grown calli and leaves (from both control and test sets) of *Stevia rebaudiana* showed significant variations in the accumulation of total phenol and flavonoid (Table 2). Proportionately, higher amount of phenol production (1.63 ± 0.10 mg/g fresh weight in TS9) was noticed in test sets (CCC treated) compared with control sets (1.45 ± 0.085 mg/g fresh weight in CS9). Leaves accumulated consistently greater amount of phenols compared with calli in both control and test sets. Highest amount of phenol (1.87 ± 0.15 mg/g fresh weight) was noticed in the leaves regenerated in MS medium supplemented with 3 mg/l BA, 5 mg/l Kinetin, and 1 mg/l CCC (Table 2).

Like phenols, flavonoid content was also found to increase in test sets (1.46 ± 0.14 mg/g fresh weight in TS9) compared with control sets (1.29 ± 0.13 mg/g fresh weight in CS9). Similarly, flavonoid accumulation remained steadily higher in leaves compared with calli in both control and test sets (Table 2). Maximum production (1.72 ± 0.12 mg/g fresh weight) was observed in the leaves regenerated in MS medium supplemented with 3 mg/l BA, 3 mg/l Kinetin and 1 mg/l CCC (Table 2).

DPPH radical scavenging activity

DPPH radical scavenging activities (% inhibition) of the methanolic extracts from different calli and microshoots of *S. rebaudiana* were performed and it was noticed that activities were varied according to CCC treatment (along with phenol and flavonoids content) and extract's concentration-dependent manner (Table 3). Significant increase was noticed in test sets (CCC treated) and highest activity (76.56 ± 1.4 %) was found in leaves (1,000 µg/ml) regenerated in MS medium containing 3 mg/l BA, 5 mg/l Kinetin, and 1 mg/l CCC, i.e. in TS 12 (Table 3).

ABTS radical scavenging activity

ABTS radical scavenging activity was also significantly increased in CCC-treated sets. Like DPPH, ABTS radical

Table 3 Estimation of DPPH, ABTS and hydroxyl radical scavenging activities of methanolic extract from different control and test sets

Treatment sets	DPPH scavenging activity (% inhibition)	ABTS scavenging activity (% inhibition)	Hydroxyl radical scavenging activity (% inhibition)
Quercetin (1 mM)	82.33333333	86.85	81.85
CS 1 (500 μg/ml)	35.16 ± 2.02a	46.34 ± 3.10b	34.6 ± 2.007a*
TS 1 (500 μg/ml)	42.93 ± 1.88c	55.7 ± 2.25de	54.4 ± 0.65de
CS 1 (1,000 μg/ml)	52.13 ± 1.20d	55.87 ± 3.65ef	51.77 ± 1.72de
TS 1 (1,000 μg/ml)	62.26 ± 2.38f	64.76 ± 1.07hi	60.97 ± 1.05gh
CS 3 (500 μg/ml)	34.46 ± 2.24a	47.5 ± 1.34b	37.27 ± 2.15ab
TS 3 (500 μg/ml)	43.33 ± 2.21c	55.93 ± 0.95ef	54.03 ± 4.02de
CS 3 (1,000 μg/ml)	57.03 ± 1.05e	57.33 ± 0.73efg	53.2 ± 2.02de
TS 3 (1,000 μg/ml)	65.9 ± 1.15gh	66.26 ± 1.02ij	61.03 ± 1.81gh
CS 5 (500 μg/ml)	37.67 ± 1.069b	40.6 ± 0.65a	39.43 ± 1.77bc
TS 5 (500 μg/ml)	49.9 ± 1.27d	51.23 ± 1.50c	51.3 ± 1.45d
CS 5 (1,000 μg/ml)	56.16 ± 0.75e	55.2 ± 0.95de	52.1 ± 1.57de
TS 5 (1,000 μg/ml)	63.26 ± 1.10f	62.96 ± 1.75h	61.73 ± 1.53h
CS 7 (500 μg/ml)	38.2 ± 0.81b	52.63 ± 1.006cd	41.93 ± 0.35c
TS 7 (500 μg/ml)	57.93 ± 0.6e	59.76 ± 1.22g	55.1 ± 2.16ef
CS 7 (1,000 μg/ml)	58.47 ± 0.97e	64.66 ± 0.56hi	55.26 ± 1.85ef
TS 7 (1,000 μg/ml)	66.4 ± 0.96h	68.33 ± 0.58jk	63.6 ± 3.24hi
CS 9 (500 μg/ml)	63.7 ± 1.9fg	58.63 ± 1.22fg	53.1 ± 1.4de
TS 9 (500 μg/ml)	68.36 ± 0.55hi	62.53 ± 0.56h	61.3 ± 1.4h
CS 9 (1,000 μg/ml)	69.76 ± 1.91ij	68.56 ± 1.06jk	65.56 ± 0.68ij
TS 9 (1,000 μg/ml)	71.9 ± 1.85jk	73.06 ± 0.95l	69.46 ± 2.25k
CS 12 (500 μg/ml)	66.63 ± 0.7h	59.76 ± 1.33g	57.93 ± 1.8fg
TS 12 (500 μg/ml)	71.3 ± 1.5jk	64.33 ± 0.77hi	63.8 ± 1.35hi
CS 12 (1,000 μg/ml)	73.23 ± 1.54k	69.43 ± 1.59k	68.03 ± 0.5jk
TS 12 (1,000 μg/ml)	76.56 ± 1.4l	75.3 ± 2.02l	74.4 ± 1.5l

* Values are mean ± standard deviations of three replicates; means followed by same letter in a column are not significantly different ($p = 0.05$)

scavenging activity was also comparatively higher [59.76 ± 1.33 % in CS12 (500 μg/ml), 64.33 ± 0.77 % in TS12 (500 μg/ml), 58.63 ± 1.22 % in CS9 (500 μg/ml), and 62.53 ± 0.56 % in TS9 (500 μg/ml)] in test sets (Table 3). Leaves consistently produced higher activities in both control and test sets. Maximum activity (75.3 ± 2.02 %) was produced in leaves (1,000 μg/ml) from CCC-treated TS 12 (Table 3).

Hydroxyl radical scavenging activity

Methanolic extracts of leaves and calli from test sets also produced higher hydroxyl radical scavenging activities compared with control sets (Table 3). It was clearly evidenced by reduced colour formation in the reaction mixtures of test sets. Maximum activity (74.4 ± 1.5 %) was consistently found in leaves (1,000 μg/ml) from CCC-treated TS 12 (Table 3).

Cell viability

Extracts from calli and leaves (from both control and test sets) did not produce any significant antiproliferative effect against THP-1 Monocyte cell line (ATCC-TIB202) and Hela cell line (ATCC-CCL2) (Table 4). It was noticed that viability percentages both THP-1 Monocyte [e.g. 95.83 ± 3.19 % in CS 10 (1,000 μg/ml) & 96.17 ± 3.17 % in TS 10 (1,000 μg/ml)] and Hela cell [e.g. 95.27 ± 0.971 % in CS 12 (500 μg/ml) & 95.07 ± 0.462 % in TS 12 (500 μg/ml)] were almost similar after the treatment with methanolic extract from control sets and test sets. However, viability percentages of preadipocytic cell line (3T3L1) were significantly decreased [39 ± 2 % in CS 6 (1,000 μg/ml), 39.66 ± 2.08 % in TS 6 (1,000 μg/ml)] upon treatment with different extracts (Table 4).

Discussion

Medicinal plants constitute an array of secondary metabolites, most of which are bioactive chemicals and used to fight against several human physiological disorders. Phenols and flavonoids are the most important phytoconstituents of medicinal plants. The novelty of this paper first lies in the applying of CCC to induce higher production of phenols and flavonoids in calli and leaves of *S. rebaudiana*

Table 4 Analysis of cellular toxicity level of methanolic extracts from different control and test sets

Treatment sets	Viability % of THP-1 monocyte cells (ATCC-TIB202)	Viability % of HeLa cells (ATCC-CCL2)	Viability % of preadipocytic cells (3T3L1)
CS 1 (500 μg/ml)	96.17 ± 0.907e	97.8 ± 1.03c	64 ± 2.64d*
TS 1 (500 μg/ml)	90.33 ± 2.082ab	97.37 ± 0.72c	62.66 ± 2.08d
CS 1 (1,000 μg/ml)	96.43 ± 1.45e	98.33 ± 0.75c	52.33 ± 2.51bcd
TS 1 (1,000 μg/ml)	89.8 ± 1.87a	98.31 ± 0.8c	51.66 ± 3.05bcd
CS 6 (500 μg/ml)	94.3 ± 1.99bcde	98.33 ± 0.55c	49.33 ± 2.51bc
TS 6 (500 μg/ml)	93.67 ± 1.87abcde	97.63 ± 0.61c	48 ± 2.64b
CS 6 (1,000 μg/ml)	94.83 ± 3.45cde	87.9 ± 0.98ab	39 ± 2a
TS 6 (1,000 μg/ml)	95.03 ± 3.66de	88.5 ± 2.32ab	39.66 ± 2.08a
CS 10 (500 μg/ml)	94.3 ± 1.17bcde	98.42 ± 0.911c	54.76 ± 2.04d
TS 10 (500 μg/ml)	93.83 ± 0.86abcde	97.77 ± 1.06c	53.66 ± 4.16cd
CS 10 (1,000 μg/ml)	95.83 ± 3.19e	90.53 ± 3.47b	39.66 ± 2.08a
TS 10 (1,000 μg/ml)	96.17 ± 3.17e	90.6 ± 4.223b	39.36 ± 1.70a
CS 12 (500 μg/ml)	91.51 ± 2.15abcd	95.27 ± 0.971c	60.66 ± 2.51d
TS 12 (500 μg/ml)	91.03 ± 1.36abcd	95.07 ± 0.462c	59.73 ± 1.72d
CS 12 (1,000 μg/ml)	90.77 ± 1.42abc	85.79 ± 2.707a	39.03 ± 2.45a
TS 12 (1,000 μg/ml)	90.43 ± 1.50ab	85.2 ± 2,08a	39.13 ± 3.83a

* Values are mean ± standard deviations of three replicates; means followed by same letter in a column are not significantly different ($p = 0.05$)

and second in exploring the associated changes in antioxidant potentialities of the methanolic extracts. Moreover, subsequent cellular toxicity levels of these methanolic extracts were also tested. Comparative analyses of test sets and control sets revealed that accumulations of phenols and flavonoids were increased after CCC treatment (Table 2). The above results find strong support in the effect of triazole, another growth retardant, that altered the phenylpropanoid pathway and caftaric acid, and cichoric acid content in of *Echinacea purpurea* L. grown in vitro (Jones et al. 2009).

Antioxidant activity of methanolic extracts from in vitro regenerated calli and leaves of *S. rebaudiana* were evaluated by DPPH, ABTS, and hydroxyl radical scavenging activity. It was observed that antioxidant activities were increased simultaneously with the increment of phenol and flavonoid content after CCC treatment. The use of DPPH radical provides an easy, rapid and convenient method to evaluate the antioxidant and radical scavenging potentialities (Argolo et al. 2004). Higher disappearance of DPPH (proportional to antioxidant activity) after adding methanolic extracts from CCC-treated calli and leaves into the reaction mixtures might be associated with better antioxidant potentialities [76.56 ± 1.4 % in TS 12 (1,000 μg/ml)] as compared with control sets [73.23 ± 1.54 % in CS12 (1,000 μg/ml)] (Table 3).

Increased ABTS scavenging activities of extracts from test sets reflected by decolourization of ABTS proved the higher donation of hydrogen atoms by antioxidants present in the extracts to inactivate these radicals. Like DPPH, extracts from test sets also produced higher ABTS radical scavenging activity in comparison with the control sets (Table 3).

Moreover, hydroxyl radical scavenging activities were also enhanced in calli and leaves grown in CCC-treated media (Table 3). Methanolic extracts from test sets produced higher scavenging activities consistently compared with control sets and highest (74.4 ± 1.5 %) activity was noticed in TS 12 (1,000 μg/ml). These greater scavenging activities might be helpful to provide better protection against hydroxyl radical-associated DNA damage (Srijani et al. 2007).

Observations regarding different radical scavenging potentialities might help us to predict a strong correlation between higher antioxidant activity and elevated total phenol or flavonoid content of calli and microshoots that was achieved through priming with CCC. It was further noticed that phenol content and different radical scavenging activity followed a linear regression curve. The coefficient of determination values ($R^2 = 0.839$ in DPPH scavenging activity) were found to be positive and almost close to one (Fig. 2a–c). These results indicate the positive correlation between antioxidant activity and total phenol content. Such correlation between the phenol content and antioxidant activity was widely studied in Bulgarian *Veronica officinalis* (Valyova et al., 2009), in fruit extracts of citron and blood orange (Jayaprakasha and Patil 2007), and *Lygodium flexuosum* (Jeetendra and Manish 2011).

Furthermore, methanolic extracts from calli and leaves grown in different control and test sets were used to study the cytotoxicity of plant extracts which was expressed in terms of percentage of cell viability. This experiment was designed with a view to investigate the cellular toxicity of extracts from calli and leaves and it was noticed that extracts from both control sets and test sets did not produce

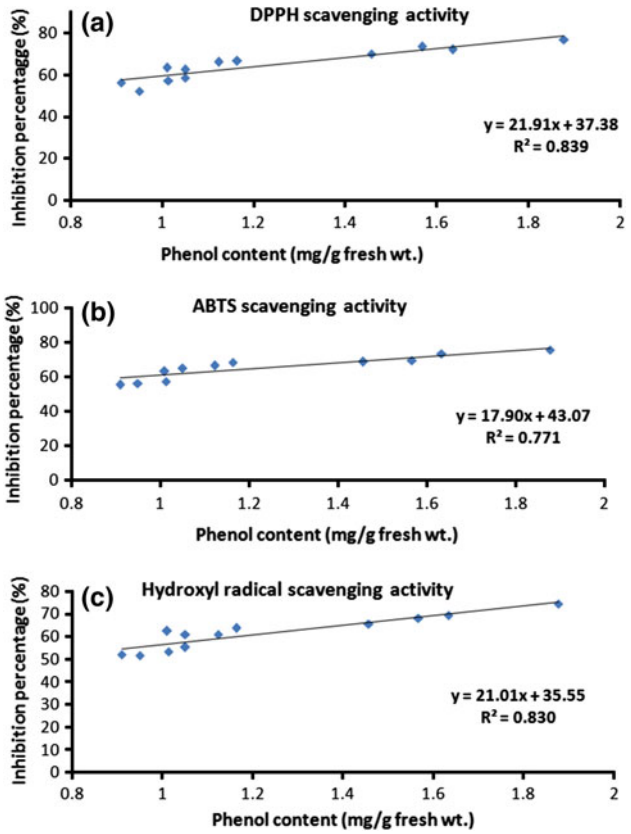


Fig. 2 Determination of correlation between phenol content and different radical scavenging assay; **a** phenol content and DPPH scavenging activity showing linear regression curve with positive coefficient value. **b** Phenol content and ABTS scavenging activity also showing linear regression curve with positive coefficient value. **c** Linear regression between phenol content and hydroxyl radical scavenging activity

any significant cellular toxicity in THP-1 monocyte (ATCC-TIB202) and Hela (ATCC-CCL2) cells (Table 4). However, cell viability percentages of preadipocytic cells (3T3L1) were decreased after treatment with extracts from both control and test sets (Table 4). This reduction of viability percentage of preadipocytic cell line (3T3L1) may be an important anti-obesity factor, though further research is necessary in this regard.

This paper emphasizes that priming of calli and microshoots of *S. rebaudiana* with plant growth retardant like CCC in the tissue culture media stimulates the accumulations of biologically active secondary metabolites like phenols and flavonoids. This elevated level of phenol and flavonoids in turn provides higher antioxidant potentialities without generating any cytotoxic phytochemicals. Significantly, such elevation of antioxidant potentialities is strongly correlated with the increasing metabolite concentrations in the extracts (Fig. 2a–c). Thus, this study provides scope for further research regarding the application of plant growth retardants in tissue culture system for enhancement of bioactive and medicinally important phytochemicals.

Author contribution A. Dey and A. Bandyopadhyay designed the research. A. Dey and S. Kundu established in vitro culture, estimated secondary metabolites and analyzed hydroxyl radical scavenging activity. S. Paul performed MTT assay as well as DPPH and ABTS scavenging assay. A. Dey wrote the paper (including figures and tables) and contributed statistical analysis. A. Bandyopadhyay and A. Bhattacharjee performed critical corrections in the paper.

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