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Research Article

Polyphenols profiles and antioxidant activities of extracts from *Capsicum chinense* *in vitro* plants and callus cultures

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Abstract

In vitro plants of *Capsicum chinense* cv. Carolina Reaper have been obtained by planting seeds on sterile MS medium. The plants were used to initiate callus culture on half strength MS medium, supplied with 2 mg.L⁻¹ 1-Naphthaleneacetic acid and 0.5 mg.L⁻¹ 6-Benzylaminopurine. The polyphenol profiles (phenolic acids and flavonoids) of methanol extracts from obtained callus and *in vitro* plants have been analyzed by HPLC. The main constituents in plant extract were protocatechuic acid, sinapic acid, rutin, hesperetin and myricetin, whereas in callus extract the major compounds were found to be chlorogenic acid, ferulic acid, rutin, hyperoside, myricetin and hesperetin. The antioxidant capacity of both extracts have been evaluated by using four popular *in vitro* antioxidant methods. In our knowledge, this is the first report for obtaining of callus culture from *Capsicum chinense* cv. Carolina Reaper and evaluation of phytochemical profiles and antioxidant activities of its extract. Practical applications: The research outlines the potential of *Capsicum in vitro* systems as a renewable source of active ingredients for application in cosmetic and food products.

Keywords: chili pepper, plant *in vitro* systems, HPLC, phenolic acids, flavonoids

Abbreviations:

ABTS - 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
CUPRAC - Cupric reducing antioxidant capacity
DPPH - 1,1-diphenyl-2-picrylhydrazyl
FRAP - Ferric reducing antioxidant power
HPLC - High Performance Liquid Chromatography

MS medium - Murashige and Skoog medium
PCCT - Plant cell culture technology
TEAC - Trolox equivalents per gram dry biomass
SD - Standard deviations
SHU - Scoville Heat Units

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Introduction

Chili peppers (*Capsicum*) are genus of the *Solanaceae* family, which have been widely used in human diet, pharmacy, medicine and ethnomedicine worldwide. The genus *Capsicum* originated in South America, but after Columbus, it has been spread and adapted for growing all around the world. Currently, there are approximately 20 wild species, belonging to the genus *Capsicum*. However, only few of them - *Capsicum annuum*, *Capsicum frutescens*, *Capsicum chinense*, *Capsicum baccatum* and *Capsicum pubescens* have been commercialized. Moreover, as result of continuous breeding and selection procedures, thousands cultivars of these species with divers organoleptic properties have been developed (Haley and McDonald 2016). The interest in chili peppers is the presence of capsaicinoids in their fruit, which are chemesthetic compounds causing specific pungent sensation when eaten or contact with skin (Dalton and Byrnes 2016). Capsaicinoids are protoalkaloids which contain non-heterocyclic nitrogen and originate from the amino acids phenylalanine, valine or leucine. The most important capsaicinoids are capsaicin, dihydrocapsaicin, nordihydrocapsaicin and homocapsaicin, which have pungency of 16.1, 16.1, 9.3 and 6.9 million Scoville Heat Units (SHU) (Duelund and Mouritsen 2017). Beside as chemesthetics and food additives, the capsaicinoids possess various biological activities such as antimicrobial, stimulating lipid metabolism and weight reducing properties, which drive the interest to incorporate capsaicinoids as ingredients in formulations of wide range of pharmaceuticals (Omolo et al. 2014; Reyes-Escogido et al. 2011). As result of selective breeding, many *Capsicum* cultivars, having increased capsaicinoid contents have been developed. The world record, as is in the moment of preparation of this paper, belongs to *Capsicum chinense* cv. Carolina Reaper (average pungency of 1.5 million SHU), which is a hybrid cultivar of *Capsicum chinense* var. Pakistani Naga and *Capsicum chinense* var. Red Habanero (Haley and McDonald 2016). Phytochemical study of capsaicinoid contents in different chili peppers showed, that the cultivar Carolina Reaper

accumulates 131.76 $\mu\text{mol.g}^{-1}$ capsaicin, 68.47 $\mu\text{mol.g}^{-1}$ dihydrocapsaicin, 11.40 $\mu\text{mol.g}^{-1}$ nordihydrocapsaicin and 13.12 $\mu\text{mol.g}^{-1}$ homocapsaicin, which was the highest amount of capsaicinoids among the investigated cultivars (Duelund and Mouritsen 2017). However, at the time of preparation of this paper, there were no published data concerning development of *in vitro* cultures from *Capsicum chinense* cv. Carolina Reaper, nor for study on polyphenol profiles and antioxidant capacity of this species or of its *in vitro* systems. It is well known fact, that polyphenols are important phytochemicals with various bioactivities. The recent data from a large population-based cohort study showed, that inclusion of chili peppers in daily diet could reduce the instantaneous hazard of death and increase the lifespan with approximately 13% (Chopan and Littenberg 2017). It is highly possible this to be due to the combined biological effects of capsaicinoids and polyphenols, found in peppers. These potential health beneficial effects made Carolina Reaper an attractive model system for study on its polyphenol profile and for development of biotechnology for continuous production of pepper bioactive biomass by using plant cell culture techniques. Plant cell culture technology (PCCT) is an emerging multibillion industry, considered as reliable and environmental friendly alternative of field-grown plants used for production of valuable secondary metabolites (Paek et al. 2014). PCCT appears as one of the fastest developing area of plant biotechnology. Most of the commercial productions based on bioprocessing of plant cell cultures have been developed and manufactured in Japan, and only recently PCCT has been commercialized in Israel, US and Europe (Meyer and Schmidhalter 2014). The main limitations in commercialization of PCCT are the low concentrations of target metabolites, genetic instability and variability in yields, shear sensitivity and requirement of modified bioreactors. With the advancement of bioprocess engineering, many of those issues have been resolved in acceptable level (Paek et al. 2014). Moreover, a significant increase in growth, secondary metabolites accumulation and yields

could be achieved by optimization of nutrient medium composition, precursor feeding and development of fed-batch culture (Pavlov et al. 2000; Pavlov et al. 2007). Currently, many *in vitro* systems from various different *Capsicum* cultivars have been reported, including shoots (Kehie et al. 2012) and somatic embryos (Ochoa-Alejo 2016) used for micro propagation, callus (Santos et al. 2017) and cell suspension (Ferri et al. 2017; Kehie et al. 2016; Bhat and Bhat, 2016; Kehie et al. 2012) cultures, placental tissue culture (Aldana-Iuit et al. 2015) and immobilized cells (Kehie et al. 2014). However, no reports describing initiation of *in vitro* cultures from *Capsicum chinense* cv. Carolina Reaper have been reported yet. In this paper, the polyphenol profiles (phenolic acids and flavonoids) of *Capsicum chinense* cv. Carolina Reaper *in vitro* plants and callus cultures have been analyzed, and antioxidant capacity of methanol extracts, obtained from the above *in vitro* systems, have been evaluated.

Materials and Methods

Plant materials. Seeds of *Capsicum chinense* cv. Carolina Reaper (*Capsicum chinense* var. Pakistani Naga x *Capsicum chinense* var. Red Habanero) were bought from Chilli Wizards Ltd., UK. The seeds were surface sterilized in 70% ethanol for 30 sec., followed by sterilization in 4% calcium hypochlorite for 20 min. Sterilized seeds were rinsed in sterile water (three times) and dried on sterile filter paper before to be transferred on sterile semi-solid MS medium (supplemented with 3% sucrose and 3.5 g.L⁻¹ agar) for germination.

***In vitro* plants.** Germination of the seeds was carried out in darkness, at 26°C. When the seedlings were formed, the cultivation was performed under illumination. The used photoperiod was: light/dark – 16/8 hours. The growth of *in vitro* plants was realized under the same conditions by transferring them on fresh nutrient medium every month. For the needs of experiments, 21 days old *in vitro* plants were used. The entire plants were collected, freeze dried and powdered by laboratory grinder. The obtained powder was used for extraction of phenolic compounds by following the described procedure.

Callus culture. For initiation of callus cultures, explants from sterile *Capsicum chinense* cv. Carolina Reaper *in vitro* plants were used. The explants were cultured on solid MS medium, supplied with 3% sucrose, 5.5 g.L⁻¹ agar and various concentrations of auxins (1-Naphthaleneacetic acid, in a range of 0.5 to 2 mg.L⁻¹) and cytokinins (6-Benzylaminopurine, in a range of 0.5 to 2 mg.L⁻¹). The cultivation was performed both on darkness and under illumination (light/dark – 16/8 h), at 26°C. After separation of callus, the selected line was supported on the same conditions, with a sub-cultivation period of 24 days. For the needs of the experiments, 21 days old callus culture was used. The callus was carefully separated from the agar, freeze dried and powdered by laboratory grinder. The obtained powder was used for extraction of phenolic compounds by following the described procedure.

Preparation of extracts. Freeze-dried biomass from *in vitro* plants or callus culture (0.2 g) was extracted three times with 10 mL 70% methanol in ultrasonic bath for 15 min at 75°C. After filtration (Whatman #1 filter paper), the combined extracts were evaporated to dryness under vacuum and dissolved in 2 × 1 mL HPLC grade methanol. The obtained extracts were filtered through syringe filters (45µm) and used for analysis.

HPLC analysis. HPLC fingerprint and quantitative determinations of phenolic acids and flavonoids, were performed on Waters HPLC system (Waters, Milford, MA, USA), equipped with Waters 1525 Binary Pump, Waters 2484 dual-λ Absorbance Detector and Supelco Discovery HS C18 column (5 µm, 250 mm × 4.6 mm), and operated under the control of Breeze 3.30 software. For separation of phenolic acids, a mobile phase of 2% acetic acid (solvent A) and 0.5 % acetic acid: acetonitrile (1:1) (solvent B) was used. For separation of flavonoids the used mobile phase was 2.0 % acetic acid (solvent A) and methanol (solvent B). The used gradient programs were described elsewhere (Marchev et al. 2011). For compounds quantification, the standards of rosmarinic, gallic, protocatechuic, salicylic, chlorogenic, vanillic, caffeic, syringic, p-coumaric, sinapic, ferulic, and cinnamic acids, as well as for flavonoids myricetin,

hesperetin, quercetin, luteolin, kaempferol and apigenin, were used for creation of standard calibration curves. The quercetin glycosides rutin and hyperoside were analyzed on the same HPLC system, but a mobile phase of 2% acetic acid (solvent A) and acetonitrile (solvent B) was used and the elution was performed under gradient, described by Ivanov et al. (2014).

Antioxidant activity assays. DPPH assay. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity assay was performed according to the procedure described by Ivanov et al. (2014). The calibration curve was built by using Trolox at concentrations of 0.1; 0.2; 0.3; 0.4 and 0.5 mM. The antioxidant activity was expressed as mM Trolox equivalents per gram dry biomass.

TEAC assay. The procedure was previously described by Ivanov et al. (2014). The ABTS radical was generated by mixing in equal amounts of 7 mM 2,2'-azinobis (3)-ethylbenzthiazoline-6-sulfonic acid (ABTS) and 2.45 mM $K_2S_2O_8$ for 12 h at room temperature (21°C) in darkness. The calibration curve was built by using Trolox at concentrations of 0.1; 0.2; 0.3; 0.4 and 0.5 mM. The antioxidant activity was expressed as mM Trolox equivalents per gram dry biomass.

FRAP assay. Ferric reducing antioxidant power (FRAP) was performed according to the procedure described by Ivanov et al. (2014). The calibration curve was built by using Trolox at concentrations of 0.1; 0.2; 0.3; 0.4 and 0.5 mM. The antioxidant activity was expressed as mM Trolox equivalents per gram dry biomass.

CUPRAC assay. Cupric reducing antioxidant capacity (CUPRAC) assay was performed according to the procedure described by Ivanov et al. (2014). The calibration curve was built by using Trolox at concentrations of 0.1; 0.2; 0.3; 0.4 and 0.5 mM. The antioxidant activity was expressed as mM Trolox equivalents per gram dry biomass.

Statistical analyses. Results from antioxidant activity assays are presented as Mean values \pm standard deviations (SD) of three independent analyses, performed with three technical reads (n=3). The analysis of raw data was done by using MiniTab 17 software.

Results and Discussion

Initiation of *Capsicum chinense* cv. Carolina Reaper *in vitro* plants and callus cultures. The seeds started to germinate 7 days after planting on semi-solid MS medium (Figure 1A). To the end of the second week after planting, a good growing seedlings were obtained (Figure 1B). The seedlings were transferred for cultivation under illumination, and healthy *in vitro* plants were formed after 4 weeks of planting (Figure 1C). The resulting *in vitro* plants were supported by regular transfers on fresh semi-solid MS medium at every 30 days. Young leaves and stem segments from the *in vitro* plants were used as explants for initiation of callus cultures (Figure 1D). The best medium for formation of callus (95% of explants) was solid MS medium, supplemented with 3% sucrose, 5.5 g.L⁻¹ agar, 2 mg.L⁻¹ 1-Naphthaleneacetic acid and 0.5 mg.L⁻¹ 6-Benzylaminopurine. The first callus appeared 4 weeks after the transfer (Figure 1E). When the calli accumulated enough biomass, they were separated from explants for independent grow (Figure 1F). The selected callus lines were sub-cultured on fresh medium at every 24 days. The callus lines were supported for more than 6 months before the experiments.

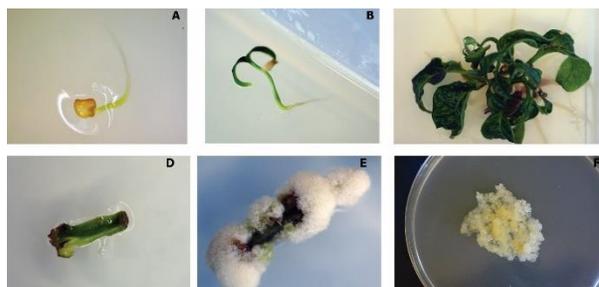


Figure 1. Initiation of *C. chinense* cv. Carolina Reaper *in vitro* systems: (A) – seeds germination; (B) – seedlings formation; (C) – *in vitro* plants; (D) – planting of explants; (E) – callus formation; (F) – selected callus line.

Polyphenols profiling of extracts from *Capsicum chinense* cv. Carolina Reaper *in vitro* plants and callus cultures. The polyphenol contents of *Capsicum chinense* cv. Carolina Reaper *in vitro*

plants and callus cultures were investigated by using High Performance Liquid Chromatography (HPLC) technique. The HPLC fingerprints of phenolic acids profiles of extracts from *in vitro* plants and callus culture showed significant differences in distribution of investigated compounds (Figure 2).

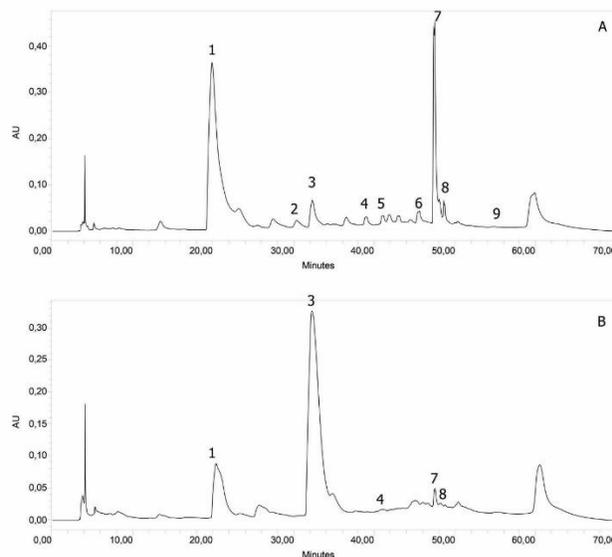


Figure 2. HPLC fingerprints of phenolic acids content in extracts from *C. chinense* cv. Carolina Reaper *in vitro* plants (A) and callus cultures (B): 1 - 3,4-Dihydroxybenzoic acid; 2 - 2-Hydroxybenzoic acid; 3 - Chlorogenic acid; 4 - Caffeic acid; 5 - Syringic acid; 6 - p-Coumaric acid; 7 - Sinapic acid; 8 - Ferulic acid; 9 - Cinnamic acid

Such differences in secondary metabolism of *in vitro* systems with different levels of differentiation, obtained from the same plant, was discussed elsewhere (Steingroewer et al. 2013). The results from quantification of phenolic acids and flavonoids, found in *Capsicum chinense* cv. Carolina Reaper *in vitro* plants and callus culture extracts are presented on Table 1. The main constituents in plant extract were protocatechuic acid, sinapic acid, rutin, hesperetin and myricetin, whereas in callus extract the major compounds were found to be protocatechuic acid, chlorogenic acid, ferulic acid, rutin, hyperoside, myricetin, hesperetin

and luteolin. It should be noted, that because of its high Scoville score, plants of *Capsicum chinense* cv. Carolina Reaper have been investigated only for their capsaicinoids profiles, and no research on phenolic or flavonoid contents have been published yet (Antonio et al. 2018). However, the content of luteolin in callus culture, reported here ($2.65 \mu\text{g}\cdot\text{g}^{-1}$ Dry Weight) is in agreement with the luteolin content in immature peppers of *Capsicum. chinense* L. cv. TAM Mild Habanero, reported by Bae and co-workers (Bae et al. 2014). Moreover, the callus culture showed a 4 times higher amount of chlorogenic acid than detected in *in vitro* plants ($1819.73 \mu\text{g}\cdot\text{g}^{-1}$ Dry Weight, vs. $457.19 \mu\text{g}\cdot\text{g}^{-1}$ Dry Weight; (Table 1). This is important finding, since chlorogenic acid possesses many health-promoting properties and is a perspective substance for development of dietary supplements and functional foods (Santana-Gálvez et al. 2017).

Antioxidant capacity of extracts from *Capsicum chinense* cv. Carolina Reaper *in vitro* plants and callus cultures. To evaluate the antioxidant capacity of *Capsicum chinense* cv. Carolina Reaper *in vitro* plants and callus cultures, four different methods were used. Two of them – DPPH and TEAC were based on mixed reaction mechanism, whereas the other two – FRAP and CUPRAC were based on single electron transfer reaction mechanism (Ivanov et al. 2014). The results are presented in Table 2. It is obviously that both *in vitro* plants and callus cultures showed moderate antioxidant activities and could not be classified as strong antioxidants. However, it should be noted that, the observed antioxidant activities concerns the polyphenol-enriched extracts and does not include the other cases of bioactive compounds, such as capsaicinoids, which were not a target of this study.

Table 1. HPLC quantification of polyphenolics in extracts from *C. chinense* cv. Carolina Reaper *in vitro* plants and callus cultures

Compound	Concentration, $\mu\text{g}\cdot\text{g}^{-1}$ Dry Weight	
	<i>in vitro</i> plants	callus culture
Rosmarinic acid	ND*	ND
Gallic acid	ND	ND
Protocatechuic acid	6327.84	930.93
Salicylic Acid	318.83	ND
Chlorogenic acid	457.19	1819.73
Vanillic acid	ND	ND
Caffeic acid	119.68	27.97
Syringic acid	17.24	ND
p-Coumaric acid	14.53	ND
Sinapic acid	1258.13	32.17
Ferulic acid	920.17	78.09
Cinnamic acid	5.83	ND
Rutin	295.37	28.08
Myricetin	55.24	30.57
Hesperetin	65.89	22.06
Quercetin	ND	ND
Luteolin	ND	2.65
Kaempferol	14.69	ND
Apigenin	ND	ND
Hyperosid	ND	12.72

* - not detected (ND).

Table 2. Antioxidant capacity of extracts from *C. chinense* cv. Carolina Reaper *in vitro* plants and callus cultures

Antioxidant activity assay	μM Trolox Equivalent / g Dry Weight	
	<i>in vitro</i> plants	callus culture
DPPH	7.57 \pm 0.16*	2.70 \pm 0.02
TEAC	15.17 \pm 0.20	13.04 \pm 0.09
CUPRAC	24.74 \pm 0.13	7.88 \pm 0.07
FRAP	2.86 \pm 0.27	1.79 \pm 0.15

* - Data were expressed as Mean \pm SD (n = 3).

Conclusions

In vitro plants and callus cultures of the world's hottest chilli pepper *C. chinense* cv. Carolina Reaper have been successfully initiated and subcultivated in our Lab. The data for polyphenol profiles of these - system showed, that callus culture of *C. chinense* cv. Carolina Reaper produces high amount of chlorogenic acid (1.8 mg.g⁻¹ DW) which define this *in vitro* system as perspective renewable source for production of active ingredients for the food and cosmetics industries. Moreover, the biomasses, obtained from Carolina Reaper *in vitro* plants and callus cultures, showed moderate antioxidant activities, which could increase their nutraceutical values. To our knowledge, this is the first report for initiation of callus culture from *Capsicum chinense* cv. Carolina Reaper and evaluation of phytochemical profiles and antioxidant activities of its extract. This data could be used as a base for future research on development of expression *in vitro* platform for continuous supply of bioactive biomass from *Capsicum* species.

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