SUPPLEMENTARY MATERIAL

Phytochemical and Biological Study of Callus Cultures of *Tulbaghia violacea* Harv. Cultivated in Egypt

Hanaa H. Eid[a] and Ghada F. Metwally[b*]

[a]*Faculty of Pharmacy, Department of Pharmacognosy, Cairo University, Cairo, Egypt*
[b]*Department of Plant Tissue Culture, National Organization for Drug Control & Research (NODCAR), Giza, Egypt*
*Corresponding author: ghadafarouk70@homail.com

Abstract

As *in vitro* plant cultures are used extensively to produce bioactive metabolites, our goal was to establish calli from *Tulbaghia violacea* Harv. flowers and assess the tissue phytochemically and biologically. Murashige & Skoog medium(MS)+22.6µM 2,4-dichlorophenoxyacetic acid+2.2µM benzylaminopurine induced callus from flowers. Gas chromatography/mass spectrometry(GC/MS) analyses of *n*-hexane extracts of calli(HC) and flowers(HF) revealed 33 and 32 components(92.6 and 98.5%, respectively). Hydrocarbons were predominant in HC(55.0%), whereas a higher percentage of oxygenated compounds was found in HF(74.6%). *Trans*(E)-anethole(39.1%) and 16-hentriacontanone(30.3%) dominated in HF and HC, respectively. However, sulfur compounds were only detected in HF. Quantitative estimation of thiosulfinates, phenolics, flavonoids and saponins in ethanolic extracts of calli(EC) and flowers(EF) showed much higher contents in EF. Antioxidant, antimicrobial and cytotoxic screening of extracts demonstrated that EF was the most potent, followed by HF and EC; conversely, HC was inactive. Although HC and EC were less biologically active, these calli could be an alternative source of bioactive metabolites.

Keywords: *Tulbaghia violacea* Harv.; callus cultures; GC/MS; secondary metabolites; biological activities.

Experimental

Plant material

Fresh leaves, stalks of inflorescences and flowers were obtained during the flowering stage from March to September (2013-2014) from plants cultivated at the Experimental Station of Medicinal Plants, Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Giza, Egypt. The plant was kindly authenticated by Mrs. Therese Labib, Herbarium Section, Orman Garden, Giza, Egypt. Identity was confirmed by Dr. Mohamed El Gebali (Ph.D., Botany specialist, Plant Taxonomy and Egyptian Flora Department, National Research Center, Dokki, Giza). Voucher samples are kept at the Museum of the Pharmacognosy Department, Faculty of Pharmacy, Cairo University (# 16.6.2014.1).

Material for tissue culture

Murashige and Skoog (MS) basal medium (1962) available as powder (Duchefa, The Netherlands); sucrose (Adwic, ARE), dilute hydrochloric acid and potassium hydroxide solutions for pH adjustment, which were prepared according to Egyptian Pharmacopoeia (2005); agar (Oxoid Bacteriological Agar No.1, Oxoid ltd, UK); 2,4-dichlorophenoxyacetic acid (2,4-D) and benzylaminopurine (BAP) were obtained from Sigma Chemicals, USA. Clorox at 1.5% sodium hypochlorite (Clorox company, Egypt), mercuric chloride and Tween 20 (Sigma Chemicals, USA) were used for sterilization of explants.

Induction of callus cultures

Fresh explants (leaves, stalks of the inflorescence and flowers) of *Tulbaghia violacea* Harv. were collected from field-grown plants and washed thoroughly with soap and water. Disinfection was then performed with 0.1% mercuric chloride solution for 1.5 minutes, followed by Clorox aqueous solution (30%) with the addition of one drop of Tween 20 for 10 min. The material was then washed 3 times with sterilized distilled water. The disinfected leaves and stalks were cut into small sections (1 cm^2) and applied to MS medium supplemented with different concentrations of 2,4-D and BAP; in contrast, small single flowers were separated from their umbels and applied as intact explants. For each concentration, 32 jars were prepared, with each containing 5 sectioned plants, and the cultures were incubated at 25˚C ±2 under a 16-h light/8-h dark photoperiod and a light intensity of 1500-2000 lux.

Preparation of *n*-hexane extracts

To prepare *n*-hexane (non-polar) extracts of calli (HC) and flowers (HF), approximately 250 g of each of the fresh callus cultures and flowers of the field-grown plants were, separately, extracted in *n*-hexane using sonication (Raypa ultrasonic bath, Spain) at 28ºC ±2 for ~30 min.

Preparation of ethanolic extracts

Ethanolic (polar) extracts of callus (EC) and flowers (EF) were prepared using defatted marc and ethanolic solution (70%). The extracts were then dried under vacuum using a rotatory evaporator at 40ºC, and the residues were stored in sealed amber bottles for phytochemical and biological investigation.

A 100-mg sample of the prepared ethanolic extracts (EC, EF) was dissolved by sonication in 10 ml distilled water then transferred to a 100-ml volumetric flask; the volume was adjusted using the same solvent to afford the stock solution (1 mg ml^{-1}). The prepared stock solution was used for determination of the total phenolic, flavonoid and saponin contents.

Material for phytochemical investigation

To determine thiosulfinates, Tris-hydrochloric acid buffer, L-cysteine and 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) (Thermo Scientific, USA) were used. For the assessment of the total phenolic content Folin-Ciocalteu reagent and sodium carbonate (Sigma Chemicals, USA) were utilized. For determination of flavonoids, sodium nitrite, sodium hydroxide and aluminium chloride (Sigma Chemicals, USA) were purchased, while vanillin, glacial acetic and perchloric acids were obtained for determination of saponins. Solvents of analytical grade were employed in this study: *n*-hexane, absolute ethanol, dimethylsulfoxide (DMSO) and methanol (Labscan analytical sciences, Poland). All procedures were performed using distilled water. In this investigation, reference standards *viz.* diosgenin, gallic acid and quercetin were purchased from Sigma Chemicals (USA).

Material for antioxidant activity

1,1-diphenyl-2-picrylhydrazyl radical (DPPH) was obtained from Sigma Chemicals (USA) for the determination of antioxidant activity (*in vitro*).

Material for antimicrobial screening

The tested microorganisms *viz.* gram-negative bacteria; *Escherichia coli* (NCTC-10416) and *Pseudomonas aeruginosa* (NCIB-9016), gram-positive bacteria; *Bacillus subtilis* (NCIB-3610) and *Staphylococcus aureus* (NCTC-7447), fungus; *Aspergillus*

niger (Ferm-BAMC-21) and *Candida albicans* (IMRU-3669), were kindly supplied by the Fermentation Biotechnology and Applied Microbiology Center, Al-Azhar University, Cairo, Egypt.

Material for evaluating cytotoxic activity

To examine *in vitro* cytotoxic activity, methylthiazolyl diphenyl-tetrazolium bromide (MTT) and Roswell Park Memorial Institute (RPMI-1640) medium (Sigma Chemicals, USA) were used. Experiments using human hepatocellular carcinoma (HepG2), prostate (PC-3) and breast (MCF-7) cell lines, which were obtained from American Type Culture Collection (ATCC, USA), were undertaken at the Regional Center for Mycology & Biotechnology, Al-Azhar University. The cells were grown on RPMI-1640 medium supplemented with 10% inactivated foetal calf serum and 50 µg ml^{-1} gentamycin. The cells were maintained at 37ºC in a humidified atmosphere with 5% CO_2.

Evaluation of biomass yield

The callus cultures established from flowers of field-grown plants on MS medium supplemented with 22.6 µM 2,4-D and 2.2 µM BAP were evaluated for their fresh and dry weights (g) every week during the growth period. In addition, the moisture content was calculated according to the following equation:

$$\text{moisture content}(\%) = [\text{fresh weight}(g) - \text{dry weight}(g)/\text{fresh weight}(g)]\text{x } 100$$

Phytochemical investigation

Both fresh calli and flowers of field-grown plants were subjected to phytochemical screening according to standard procedures (Trease and Evans 1989; Tiwari et al. 2011). The results are compiled in Table S1.

GC/MS analysis of *n*-hexane extracts of calli and flowers:

Analysis of *n*-hexane (non polar) extracts was performed using an Agilent 6890 gas chromatograph coupled with an Agilent 5973 mass spectrometric detector. The column used was an HP-5MS capillary column (30 m×320 µm×0.25 µm film thickness). Helium was used as the carrier gas, at a flow rate of 1 ml min^{-1}. The injected volume was 1 µl, and the injection mode was splitless. The oven temperature was programmed from 40°C (1 min), raised to 150°C at a rate of 7.5°C min^{-1} and finally increased to 250°C at 1.2°C min^{-1}. The injector and detector temperatures were 250°C and 280°C, respectively. The total run time was 40 minutes. EI/MS spectra were recorded at 70 eV, and the mass range was 50 to 500 m/z. Identification of the components was achieved by library database and Wiley mass spectral database

(Wiley 7 Nist 05 Lib. and W8N08 Lib.) searches and by comparison of their retention indices and mass fragmentation patterns with those of available references (Chen and Ho 1986; Rapior et al. 1997; Kouokam et al. 2001; Adams 2007). The percentage was determined by computerized measurement of the peak area. The results are presented in Table S2.

Evaluation of different secondary metabolites in ethanolic extracts of calli and flowers:

Ethanolic (polar) extracts were evaluated for different secondary metabolites by adopting the following methods.

Determination of the thiosulfinate content:

The thiosulfinate content in the ethanolic extracts was determined according to the methods described by Zhou et al. (2015), with slight modifications. This method is based on the fact that one molecule of thiosulfinate reacts with two molecules of cysteine to form two molecules of *S*-alkenyl or *S*-alkylmercaptocysteine. The residual concentration of cysteine was determined by measuring the amount of 2-nitro-5-thiobenzoate (NTB) formed as a result of its reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). Each extract (10 mg) was dissolved in 10 ml 50 mM Tris-HCl buffer (pH 7.5); 5 ml of 10 mM L-cysteine in 50 mM Tris-HCl buffer was mixed either with 1 ml of distilled water or 1 ml of the extract. One millilitre of each of the prepared mixtures was diluted to 100 ml with 50 mM Tris-HCl buffer, and 4.5 ml was mixed with 0.5 ml of 1.5 mM DTNB (prepared in 50 mM Tris-HCl buffer). All reactions were carried out at a temperature of 26-30°C. Absorbance was measured at 412 nm, using a UV spectrophotometer (Unicam, UK), after an incubation period of 15 min. The thiosulfinate content was determined, as allicin using 14,150 as its molar extinction coefficient in water, by the following equation (Abano et al. 2011): $\mathrm{C} = \frac{\beta(A°-A)\times 0.7\times 162}{2\times 14150}$

where C = the concentration of thiosulfinates (mg/ml); β = the dilution factor; $A°$ = the absorbance of water; A = the absorbance of the sample. The results are recorded in Table S3.

Determination of the total phenolic content:

Spectrophotometric determination of the total phenolic content was carried out according to Ndhlala et al. (2007), with slight modifications, using the Folin-Ciocalteu reagent. A volume of 0.5 ml of each stock solution (1mg ml^{-1}) was

separately added to a 5-ml volumetric flask; 0.5 ml Folin-Ciocalteu reagent and 1 ml sodium carbonate solution (4%) were added, and the volume was adjusted with distilled water. The absorbance of the solutions was measured at 760 nm after 30 min, using a UV spectrophotometer (Unicam, UK) against a blank solution (containing all reagents except the sample). The total phenolic content of the samples was deduced using a pre-established gallic acid (standard) calibration curve (2- 12 µg ml^{-1}) following the same procedure and by using the following equation:

$Y= 16.615x + 1.0767$, $R^2 = 0.9943$

where Y = the absorbance, x = the corresponding concentration (µg ml^{-1}), R^2 = the correlation coefficient.

Results are recorded in Table S3.

Determination of total flavonoids:

The total flavonoid content was determined according to the method reported by Atanassova et al. (2011), with some modifications. An aliquot (0.5 ml) of each stock solution (1 mg ml^{-1}) was used in the procedure; 2 ml of distilled water and 0.15 ml of $NaNO_2$ (5%) solution were added, followed by 0.15 ml of $AlCl_3$ (10%) solution after 5 min and 1 ml of 1 N NaOH after another 2 min. Finally, the volume was adjusted to 5 ml with distilled water. The absorbance was measured at 510 nm, utilizing a UV spectrophotometer (Unicam, UK), against a blank containing all of the above-mentioned reagents except for the sample. The calibration curve was established based on concentration (7-32 µg ml^{-1}) of quercetin, and absorbance was plotted versus concentration. The flavonoid contents of the samples are expressed as quercetin and calculated from the following equation: $Y = 152.9x + 3.3133$, $R^2 = 0.9687$. The results are recorded in Table S3.

Determination of saponins:

The content of saponin glycosides and aglycones in the studied samples was evaluated by adopting the method described by Chen et al. (1996) and by applying some modifications. A volume of 0.5 ml of the stock solution (1 mg ml^{-1}) prepared for each sample was hydrolysed using conc. HCl and then neutralized; the hydrolysate was evaporated to dryness and extracted with *n*-hexane. The volume of the extract was made up to 10 ml with the same solvent. One ml of the prepared extract was heated with 0.2 ml of 5% vanillin in glacial acetic acid and 0.8 ml of $HClO_4$ at 75°C for 15 min and cooled. The volume was adjusted to 5 ml by the addition of glacial acetic acid in an ice bath for 30 min. The absorbance of the samples was measured at 530

nm using a UV spectrophotometer (Unicam, UK),. Diosgenin (0.05-0.2 µg/ml) was used as a reference standard, and the total saponin content was calculated from its calibration curve (Y= 0.1438x + 0.0413, R^2 = 0.9741). The results are presented in Table S3.

Evaluation of biological activities

For comparison of biological properties, the *n*-hexane and ethanolic extracts of the callus cultures and flowers of the field-grown plants were examined for *in vitro* activities, including antioxidant, antimicrobial and cytotoxic activities.

***In vitro* antioxidant activity (DPPH assay)**

Antioxidant activity was evaluated by adopting the radical-scavenging DPPH method of Adib et al. (2010), with slight modifications. Briefly, 100 mg of each extract was separately dissolved in methanol by sonication (10 mg ml^{-1}). Aliquots (1, 2 and 3 ml) of each extract were separately mixed with 5 ml of a methanolic solution of DPPH (20 mg l^{-1}) and volume was completed to 10 ml with methanol. The absorbance of the samples was determined after 30 min using a spectrophotometer (Unicam, UK) at 517 nm. The results were compared to a control solution (containing all reagents except the tested sample). All experiments were carried out in triplicate. The radical-scavenging activity (Inhibition%) was calculated using the following equation:

$$\text{Radical} - \text{scavenging activity (Inhibition \%)} = (\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})/\text{Abs}_{\text{control}})\text{x } 100$$

Where $\text{Abs}_{\text{sample}}$ = absorbance of the tested extracts, $\text{Abs}_{\text{control}}$ = absorbance of the control solution.

The 50% inhibitory concentration (IC_{50}) was calculated in each case. Silymarin was used as a positive control, and the different concentrations (10-100 µg ml^{-1}) were treated similarly as the tested extracts. The results are given in Table S4.

Antimicrobial activity

The antimicrobial activity of the studied extracts (200 mg ml^{-1} in DMSO) was assessed using the agar well diffusion method described by Perez et al. (1990). DMSO was used as a negative control. After 24 h of incubation, the diameters of inhibition zones were measured. Paper discs impregnated with 200 µg of ciprofloxacin (for bacteria) or nystatin (for fungi) were used as standard antimicrobials (positive controls). The results are provided in Table S5.

Cytotoxic activity

Cytotoxicity of the extracts was assessed against three cell lines, hepatocellular (HepG2), prostate (PC-3) and breast (MCF-7), according to the method described by Mosmann (1983). The extracts were added to Corning® 96-well plates (three replicates). A control of untreated cells, lacking the tested extracts, was included. After incubating for 24 h, the number of viable cells was determined by the MTT assay. Briefly, the medium in each well was removed and replaced with 100 µl of RPMI-1640 medium without phenol red and 10 µl of 12 mM MTT stock solution (5 mg of MTT in 1 ml of phosphate- buffered saline: pH=7.4). The plates were incubated at 37°C and 5% CO_2 for 4 h. An aliquot of 85 µl of the medium was removed from the wells, and 50 µl of DMSO was added and mixed thoroughly. After incubation at 37°C for 10 min, optical density was measured at 590 nm using a microplate reader (SunRise, TECAN, Inc, USA) to determine the number of viable cells. The percentage of viability was calculated as follows:

$$\text{Viability } (\%) = [1 - (\text{ODt}/\text{ODc})]\text{x } 100$$

where ODt = the mean optical density of wells treated with the tested sample; ODc = the mean optical density of untreated control. The results are shown in Table S6.

References

Abano E E, Ma H, Qu W, Teye E. 2011. Modeling pre-treatments effect on drying kinetics of garlic (*Allium sativum L.*) slices in a convective hot air dryer. Afr J Food Sci. 5: 425 – 435.

Adams RP. 2007. Identification of Essential Oils Components by Gas Chromatography/ Quadrupole Mass Spectrometry. 4th Edition, Allured Publishing Corporation, 362 South Schmale Road, Carol Stream, Illinois, USA.

Adib MS, Rahman SM, Rahman ZM, Ahmed SK, Rashid AM. 2010. Free radical scavenging activities of some indigenous plants of Bangladesh. Bangladesh Pharm J. 13: 68-70.

Atanassova M, Georgieva S, Ivancheva K. 2011. Total phenolic and total flavonoid contents, antioxidant capacity and biological contaminants in medicinal herbs. J Chem Technol Metall. 46:81-88.

Chen ZG, Geng Z, Liu QG, Huang WH. 1996. Spectrophotometric determination of diosgenin in plants. Fenxi huaxue. 24: 227-229.

Chen C, Ho C. 1986. Identification of sulfurous compounds of Shiitake mushroom (*Lentinus edodes* Sing.). J Agric food chem. 34:830-833.

Egyptian Pharmacopoeia. 2005. General organization for governmental printing office. Cairo, Egypt.

Kouokam JC, Zapp J, Becker H. 2001. Isolation of New Alkylthiosulfides from the Essential Oil and Extracts from the Bark of *Scorodophloeus zenkeri* Harms. Z Naturforsch C. 56:1003-1007.

Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 65: 55-63**.**

Murashige T, Skoog F. 1962. A revised medium for rapid growth and biomass assays with tobacco tissue cultures. Plant Physiol. 15: 473-497.

Ndhlala AR, Kasiyamhuru A, Mupure C, Chitindingu K, Benhura MA, Muchuweti M. 2007. Phenolic composition of *Flacourtia indica, Opuntia megacantha* and *Sclerocarya birrea*. Food Chem. 103: 82–87.

Perez C, Paul M, Bazerque P. 1990. Antibiotic assay by agar-well diffusion method. Acta Biol Med Exp. 15: 113-115.

Rapior S, Breheret S, Talou T, Bessiere JM. 1997. Volatile flavor constituents of fresh *Marasmius alliaceus* (garlic Marasmius). J Agric Food Chem. 45:820-825.

Tiwari P, Kumar B, Kaur M, Kaur G, Kaur H. 2011. Phytochemical screening and Extraction: A Review. Int Pharm Sci. 1:98-106.

Trease GE, Evans WC. 1989. Pharmacognosy. 13th Edition, Bailliere Tindall, London, pp 176- 180.

Zhou C, Hu X, Chao C, Li H, Zhang S, Yan X, Yang F, Li Q. 2015. Quantitation of Allicin in Garlic-based Products: Comparisons among Spectrophotometry, GC and HPLC. Adv J Food Sci Technol. 9: 269-277.

Table S1. Results of preliminary phytochemical screening

Test	Callus cultures	Flowers of field-grown plants
Volatile constituents	+-	+
Alkaloids	-	-
Carbohydrates &/or glycosides	+	+
Anthraquinones	-	-
Cardiac glycosides	-	-
Sterols	+	+
Coumarins	+	+
Flavonoids	+-	+
Tannins	-	-
Saponins	+-	+

+:present, -: absent, +-: present in trace amounts

Table S2. GC/MS analysis of *n*-hexane extracts of callus (HC) and flowers of field-grown plants (HF).

No	RI	Name of compound	MF (MW)	Relative percentage(%)		ID
				HC	HF	
A) Non-oxygenated compounds						
Aliphatic hydrocarbons						
1	1114	Cyclohexane, 1,1-dimethyl-2-propyl-1	$C_{11}H_{22}$ (154)	0.3	-	MS, RI
2	1121	Decane, 2,4,6,-trimethyl	$C_{13}H_{28}$ (184)	0.1	-	MS, RI
3	1200	Dodecane	$C_{12}H_{26}$ (170)	0.4	-	MS, RI
4	1379	Dodecane, 2,6,10-trimethyl-	$C_{15}H_{32}$ (212)	0.5	-	MS, RI
5	1500	Pentadecane	$C_{15}H_{32}$ (212)	2.1	-	MS, RI
6	1600	Hexadecane	$C_{16}H_{34}$ (226)	1.0	-	MS, RI
7	1669	Cyclotetradecane	$C_{14}H_{28}$ (196)	0.1	-	MS, RI
8	1700	Heptadecane	$C_{17}H_{36}$ (240)	-	0.2	MS, RI
9	1887	Octadecane, 2-methyl-	$C_{19}H_{40}$ (268)	0.4	-	MS, RI
10	1900	Nonadecane	$C_{19}H_{40}$(268)	4.1	-	MS, RI
11	1985	1,19-Eicosadiene	$C_{20}H_{38}$ (278)	0.8	-	MS, RI
12	2000	Eicosane	$C_{20}H_{42}$ (282)	0.7	-	MS, RI
13	2063	Eicosane, 2-methyl	$C_{21}H_{44}$ (296)	0.6	-	MS, RI
14	2100	Heneicosane	$C_{21}H_{44}$ (296)	2.1	0.4	MS, RI
15	2188	1, 21-Docosadiene	$C_{22}H_{42}$ (306)	0.9	0.2	MS, RI
16	2200	Docosane	$C_{21}H_{44}$ (296)	2.7	-	MS, RI
17	2294	1-Tricosene	$C_{23}H_{46}$ (322)	0.2	-	MS, RI
18	2300	Tricosane	$C_{23}H_{48}$ (324)	5.6	0.9	MS, RI
19	2400	Tetracosane	$C_{24}H_{50}$ (338)	1.2	0.3	MS, RI
20	2471	9-Pentacosene	$C_{25}H_{50}$ (350)	-	0.4	MS, RI
21	2500	Pentacosane	$C_{25}H_{52}$ (352)	6.0	2.4	MS, RI
22	2600	Hexacosane	$C_{26}H_{54}$ (366)	0.7	0.6	MS, RI
23	2694	Heptacosene <1->	$C_{27}H_{54}$ (378)	-	0.6	MS, RI
24	2700	Heptacosane	$C_{27}H_{56}$ (380)	7.7	4.5	MS, RI
25	2753	Heptacosane, 5-methyl	$C_{28}H_{58}$ (394)	-	0.4	MS, RI
26	2800	Octacosane	$C_{28}H_{58}$ (394)	0.9	0.4	MS, RI
27	2900	Nonacosane	$C_{29}H_{60}$ (408)	12.6	2.9	MS, RI
28	3000	Triacontane	$C_{30}H_{62}$ (422)	3.3	-	MS, RI
Total				55.0	14.2	
Monoterpene hydrocarbons						
29	1029	dl-Limonene	$C_{10}H_{16}$ (136)	-	0.4	MS, RI
Sesquiterpene hydrocarbons						
30	1457	Humulene < α ->	$C_{15}H_{24}$ (204)	-	0.5	MS, RI
B) Oxygenated compounds						
Alcohols						
31	2282	1-Eicosanol	$C_{20}H_{42}O$ (298)	1.4	-	MS, RI
32	2577	Tricosanol <1->	$C_{23}H_{48}O$ (340)	-	0.3	MS, RI
Total				1.4	0.3	
Aldehyde						
33	2037	Octadecanal	$C_{18}H_{36}O$ (298)	1.0	-	MS, RI
Ketones						
34	888	2,5-Cyclohexadiene-1,4-dione	$C_6H_4O_2$ (108)	0.7	-	MS, RI
35	1490	β-Ionone<trans->	$C_{13}H_{20}O$ (192)	-	0.1	MS, RI
36	3304	16-Hentriacontanone	$C_{31}H_{62}O$ (450)	30.3	28.2	MS, RI
Total				31.0	28.3	
Phenolic compounds						
37	1361	Eugenol	$C_{10}H_{12}O_2$ (164)	-	0.8	MS, RI
38	1519	Phenol, 2,4-bis(1,1-dimethylethyl)	$C_{14}H_{22}O$ (206)	0.4	-	MS, RI
Total				0.4	0.8	
Fatty acids esters						
39	994	Butanoic acid, butyl ester	$C_8H_{16}O_2$ (144)	0.1	-	MS, RI
40	1823	Myristic acid, isopropyl ester	$C_{17}H_{34}O_2$ (270)	1.9	-	MS, RI

41	1925	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$ (270)	-	0.3	MS, RI
42	2177	9,12, 15-Octadecatrienoic acid, ethyl ester	$C_{20}H_{34}O_2$ (306)	-	0.2	MS, RI
Total				2.0	0.5	
Oxygenated aromatic compounds						
43	1196	Isoanethole	$C_{10}H_{12}O$ (148)	-	4.7	MS, RI
44	1251	Anethole <trans->	$C_{10}H_{12}O$ (148)	-	39.1	MS, RI
45	1869	1,2-Benzenedicarboxylic acid, bis (2-methylpropyl)	$C_{16}H_{22}O_4$ (278)	0.3	-	MS, RI
46	1940	1,2 Benzenedicarboxylic acid, dibutyl ester	$C_{16}H_{22}O_4$ (278)	-	0.5	MS, RI
47	2499	1,2 Benzendicarboxylic acid, bis (2-ethylhexyl)	$C_{24}H_{38}O_4$ (390)	1.5	0.4	MS, RI
Total				1.8	44.7	
C) Sulfur-containing compounds						
48	743	Dimethyl disulfide	$C_2H_6S_2$ (94)	-	0.8	MS, RI
49	1065	1,2,4-Trithiolane	$C_2H_4S_3$ (124)	-	1.2	MS, RI
50	1144	2,3,5-Trithiahexane	$C_3H_8S_3$ (140)	-	3.3	MS, RI
51	1315	Bis (3-chloropropyl)sulfide	$C_6H_{12}Cl_2S$ (187)	-	0.2	MS, RI
52	1355	2,3,4,6 Tetrathiaheptane	$C_3H_8S_4$ (172)	-	0.2	MS, RI
53	1480	2,4,5,7-tetrathiaoctane	$C_4H_{10}S_4$ (186)	-	2.3	MS, RI
54	1684	2,4,5,6,8-Pentathianonane	$C_4H_{10}S_5$ (218)	-	0.8	MS, RI
Total				-	8.8	
		Total identified compounds		92.6	98.5	
		Number of identified compounds		33	32	

RI: retention index values relative to C6-C24 *n*-alkanes calculated using a non-polar HP-5MS capillary column; MS: mass spectra; ID: identification method

Table S3. Results of evaluation of secondary metabolites in ethanolic extracts of callus (EC) and flowers of field-grown plants (EF)

Secondary metabolite content (µg mg^{-1}, F. wt)	EC	EF
Thiosulfinates	0.1±0.02	0.5±0.02
Total phenolic content	1.7±0.7	14.8±3.0
Flavonoids	2.3±1.7	132.6±1.5
Saponins	5.3±0.2	53.8±1.8

Results are the mean of 3 values ± standard deviation

Table S4 Antioxidant activity of *n*-hexane and ethanolic extracts of callus and flowers of field-grown plants

Concentration (µg ml^{-1})	Radical-scavenging activity (%)				Silymarin	
	HC	HF	EC	EF	Concentration (µg ml^{-1})	Radical scavenging activity (%)
1000	-	20.2	6.8	25.9	10	34
2000	-	40.1	13.6	51.7	50	65
3000	-	60.4	20.4	77.6	100	90
IC_{50} (µg ml^{-1})						
	-	2502.8	7349.5	1932.5		31.7

-: no activity was observed, HC: *n*-hexane extract of callus cultures, HF: *n*-hexane extract of flowers of field-grown plants, EC: ethanolic extract of callus cultures, EF: ethanolic extract of field-grown plants.

Table S5 Antimicrobial activity of *n*-hexane and ethanolic extracts of callus and flowers of field-grown plants

Microorganism	Zone of inhibition (mm) ± SD						
	DMSO	HC	HF	EC	EF	Ciprofloxacin	Nystatin
Escherichia coli	-	-	14.8±0.8	19.0±0.6	16.8±0.3	32.0±0.5	-
Pseudomonas aeruginosa	-	-	-	-	-	29.0±1.0	-
Bacillus subtilis	-	-	19.7±0.6	13.0±1.0	36.3±1.5	35.0±1.5	-
Staphylococcus aureus	-	-	30.3±1.5	11.5±0.5	33.2±0.8	27.0±1.0	-
Aspergillus niger	-	-	-	-	-	-	26.0±0.5
Candida albicans	-	-	31.0±1.0	10.8±0.3	30.3±0.6	-	30.0±1.0

Well diameter = 10 mm; 50 µl of extract (200 mg ml^{-1} in DMSO) was inoculated in each well. Results are the mean of 3 values ± standard deviation. -: no activity was observed, HC: *n*-hexane extract of callus cultures, HF: *n*-hexane extract of flowers of field-grown plants, EC: ethanolic extract of callus cultures, EF: ethanolic extract of field-grown plants.

Table S6 Cytotoxic activity of *n*-hexane and ethanolic extracts of callus and flowers of field-grown plants

Cancer cell line	Growth inhibition (%) ±SD				
	Extract (250 µg ml^{-1})				Doxorubicin
	HC	HF	EC	EF	(25 µg ml^{-1})
HepG-2	0	73.1±0.6	52.4±1.0	74.3±0.9	95.1±0.2
PC-3	0	63.3±2.0	19.6±0.6	61.0±1.4	92.6±0.1
MCF-7	1.2±0.1	60.4±1.8	21.7±3.1	73.3±0.9	93.5±0.1

Results are the mean of 3 values±standard deviation, HC: *n*-hexane extract of callus cultures, HF: *n*-hexane extract of flowers of field-grown plants, EC: ethanolic extract of callus cultures, EF: ethanolic extract of field-grown plants.

Figures captions

Figure S1. Development of callus cultures of *Tulbaghia violacea* Harv. flowers of field-grown plants (a) after 2 weeks of incubation, (b) after 4 weeks of incubation, (c) after 6 weeks of incubation, (d) after 8 weeks of incubation

Figure S2. Growth curve of flower-derived callus maintained on MS supplemented with 22.62 µM 2,4-D and 2.22 µM BAP (FW: fresh weight, DW: dry weight)

Figure S3. Moisture content of callus cultures during the incubation period

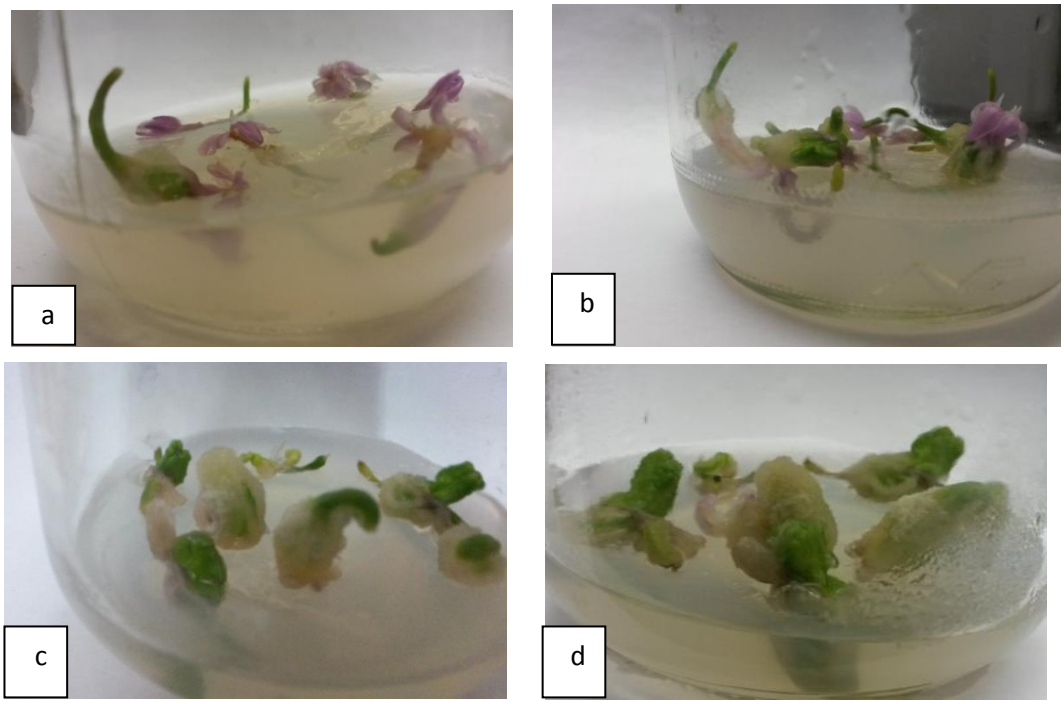

Figure S1.

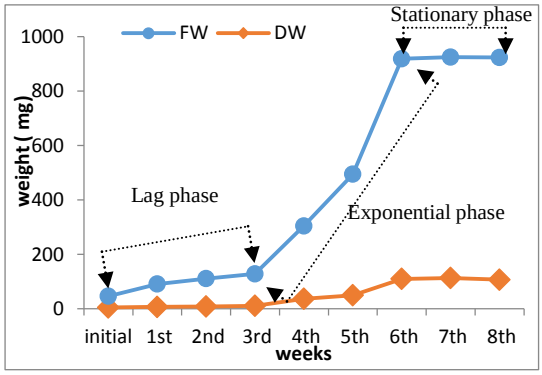

Figure S2

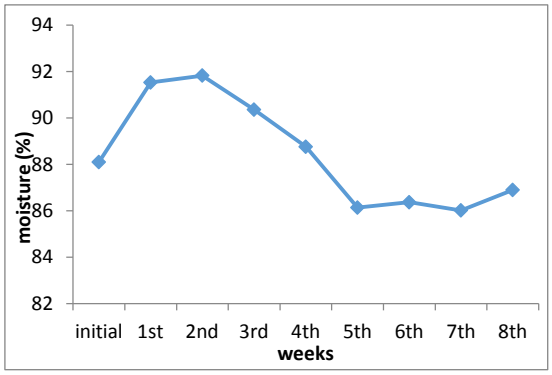

Figure S3