



Thidiazuron-induced efficient biosynthesis of phenolic compounds in callus culture of *Ipomoea turbinata* Lagasca and Segura

Waqar Ahmad¹ · Adnan Zahir¹ · Muhammad Nadeem¹ · Muhammad Zia¹ · Christophe Hano² · Bilal Haider Abbasi^{1,2,3}

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Abstract

Ipomoea turbinata Lagasca and Segura (Purple Moonflower) belongs to the largest flowering genus *Ipomoea* in the *Convolvulaceae* family. *Ipomoea turbinata* has not been previously explored for its *in vitro* potential. This is the first study focused on thidiazuron-induced callus culture for efficient biosynthesis of commercially important phenolic compounds in this plant species. Among the two plant growth regulators tested on leaf, stem, and root explants, 5 mg L⁻¹ thidiazuron (TDZ) induced the highest biomass accumulation (61.4 g L⁻¹ fresh weight, 6.3 g L⁻¹ dry weight) in leaf-derived callus cultures after 5 wk of culture. The highest total phenolic and flavonoid contents recorded were 9.04 mg g⁻¹ and 1.16 mg g⁻¹, respectively, in optimized callus cultures. High-performance liquid chromatography analysis indicated high levels of pharmacologically important anticancer compounds such as chlorogenic acid (13.48 mg g⁻¹), arctigenin (11.67 mg g⁻¹), quercetin (6.19 mg g⁻¹), and kaempferol (5.48 mg g⁻¹), along with other phenolic acids. Furthermore, the antioxidant activity was also evaluated, and leaf-derived callus culture displayed a maximum of 62.6% antioxidant potential. The induction of improved biomass accumulation in callus culture and the production of multipotent bioactive metabolites shows the potential of the multifunctional thidiazuron hormone as an efficient elicitation tool in callus culture of *I. turbinata*.

Keywords *Ipomoea turbinata* · Thidiazuron · Phenolics · Flavonoids · Anticancer

Introduction

Decades of clinical understanding and indigenous facts of traditional medicines are still pivotal sources for current drug discovery. In the ancient culture of medicine, the extracts of medicinal plants established the foundation for the treatment of a wide variety of disorders. The unique nature and phytochemical diversity in extracts of medicinal plants plays an important role in the search for novel drug discovery and development (Awale et al. 2006; Osbourn and Lanzotti 2009; Wink 2012).

Plants produce a variety of secondary metabolites to adapt to environmental fluctuations, to survive in stressful conditions, and to defend themselves against pathogenic invasions. Of these metabolites, phenolics have gained considerable attention because of the multidimensional applications discovered over the past few years including anti-mutagenic, anti-inflammatory, antioxidant and anti-clotting ability, which have potential roles to treat cardiovascular disorders and cancer (Han et al. 2007; Fresco et al. 2010; Loke et al. 2010; Ostertag et al. 2010).

Plant *in vitro* culture techniques provide valuable insights for the production of phenolics (Hiroyuki et al. 2002). *In vitro* cultures are more profitable than whole plant extracts in several ways, such as continuous production of valuable metabolites (Blando et al. 2004), large-scale production depending on market needs (Smith and Pépin 1999) and low expenditure and extrapolation of biosynthetic pathways (Curtin et al. 2003; Plata et al. 2003).

Phenolics are naturally synthesized during a plant's metabolic reactions. These are bioactive compounds that act as antioxidants by scavenging reactive oxygen species (ROS)

✉ Bilal Haider Abbasi
bhabbasi@qau.edu.pk

¹ Department of Biotechnology, Quaid-I-Azam University, Islamabad 45320, Pakistan

² Laboratoire de Biologie des Ligneux et des Grandes Cultures (LBLGC), Plant Lignans Team, INRA USC1328, Université d'Orléans, Chartres, France

³ EA2106 Biomolécules et Biotechnologies Vegetales, Université Francois-Rabelais de Tours, Tours, France



and free radicals that are produced during metabolic reactions in the body (Gill and Tuteja 2010; Nadeem et al. 2018a). Phenolic acids such as chlorogenic acid, caffeic acid, and ferulic acid have been shown to inhibit the promotion of skin tumors in female CD-1 mice (Huang et al. 1988). Similarly, the antiviral properties of these phenolic compounds are also reported to act against hepatitis B virus (Wang et al. 2009). Quercetin and kaempferol have anti-inflammatory potential (Hämäläinen et al. 2007). Protocatechuic acid has been shown to inhibit hepatotoxicity, due to its antioxidant and anti-inflammatory properties (Liu et al. 2002). In addition to these properties, phenolic compounds also exhibit antibacterial, anti-allergenic and antithrombotic properties (Ahmad et al. 2012).

Ipomoea turbinata Lagasca and Segura, also known as Purple Moonflower, belongs to the family *Convolvulaceae*. *Ipomoea* genus is known for its entheogenic potential and presence of psychoactive compounds (Meira et al. 2012). This genus grows across the tropical and subtropical countries. The *Ipomoea* genus is composed of annual and perennial climbing shrubs and herbaceous plants. According to Gunn (1969), *I. turbinata* was introduced as an ornamental plant in the USA, but now, it is listed as introduced weed species in 46 of the 50 United States. In China and Sri Lanka, the pedicels, fruits and juvenile seeds of *I. turbinata* are consumed. Its leaves are consumed for treating stomach aches and trauma in China (Gunn 1969; Chandler et al. 1977). There is no previous report available on callus culture and phenolic profiles of *I. turbinata*. Therefore, the objective of the current study was to explore and establish an *in vitro* callus culture system to produce phenolic compounds. This study will possibly open new areas to produce multifunctional health-promoting bioactive ingredients in callus cultures of *I. turbinata*.

Materials and Methods

Seed germination Seeds of *I. turbinata* were collected from their natural habitat in the rural region of Peshawar, Khyber Pakhtunkhwa, Pakistan. Seed inoculation was performed as described by Zahir et al. (2018a) with minor modifications. Briefly, the seeds were surface sterilized with 0.1% (*w/v*) mercuric chloride and 70% (*v/v*) ethanol for 60 s each treatment, followed by washing with autoclaved distilled water three times. Sterilized seeds were dried on autoclaved filter paper and inoculated onto MS basal medium (Murashige and Skoog 1962) modified with 30 g L⁻¹ sucrose, 8 g L⁻¹ plant tissue culture micropropagation grade agar (Phytotech Laboratories® Lenexa, KS), and the pH was adjusted to 5.6 using 2 M NaOH. For seed inoculation, locally manufactured jam glass jars tightly closed with plastic caps were used.

Each jar contained 40 mL of medium and was autoclaved at 121 °C under 103 kPa for 20 min prior to inoculation. The seed-containing jars were maintained at 25 ± 2 °C with a 16-h photoperiod and light intensity of 40 μmol m⁻² s⁻¹ supplied by Philips TLD 35 fluorescent lamps (Signify Philips, Eindhoven, Netherlands) in a growth room. All the chemicals used in the current study were purchased from Sigma-Aldrich® (St. Louis, MO).

Establishment of callus cultures To establish callus cultures, stem, leaf and root explants (approximately 1.0 cm each) were excised from 4-wk-old *in vitro* germinated seedlings. All of the leaf, stem and root explants were separately inoculated onto MS basal medium containing 30 g L⁻¹ sucrose and augmented with 0.1 to 20 mg L⁻¹ thidiazuron (TDZ) alone or in combination with 1.0 mg L⁻¹ α-naphthaleneacetic acid (NAA), and the pH of all media was adjusted to 5.6 using 2 M NaOH followed by the addition of 8 g L⁻¹ plant tissue culture micropropagation grade agar (Phytotech Laboratories®) in 100-mL Erlenmeyer flasks (Pyrex®, Corning, NY) containing 30-mL medium, tightly closed with sterile cotton plugs and aluminum foil. The medium-containing flasks were then autoclaved at 121°C under 103 kPa for 20 min. Plant growth regulator (PGR)-free medium (MS0) was used as the control. The explanted tissues were cultured in the same culture conditions previously described. The experiment was executed in triplicate and repeated two times. Optimum growing conditions were determined after initial culture incubation for 35 d. Afterwards, growth curves were obtained by recording growth-associated parameters periodically at an interval of 7 d for a total of 49 d.

Biomass determination For biomass determination, the respective calluses were harvested and washed with distilled water to remove any attached medium. Callus tissue was placed and rubbed gently on filter paper to remove any extra water and weighed to determine the fresh weight (FW) and subsequently oven-dried at 45°C for 48 h for dry weight (DW) determination.

Extract preparation Extracts of callus cultures were prepared according to the protocol of Zahir et al. (2018b) with minor modifications. Briefly, 100 mg of powdered callus was mixed with 500 μL of methanol in a 1.5-mL Eppendorf tube. These mixtures were vortexed for 1 min each, and then sonicated at 60 kHz and 25°C for 30 min (Elmasonic P, Elma Schmidbauer GmbH, Singen, Germany). This process was repeated twice, and the resultant mixtures were maintained at constant agitation at 80 rpm, on a gyratory shaker at 25 ± 2 °C for 24 h. The vortexing and sonication step was repeated. Finally, the mixtures were centrifuged (Spectrafuge™ 24D, Labnet International, Edison, NJ) at 9200×g for 10 min. The

resultant supernatants were syringe filtered (0.45 μm , Merck Millipore, Saint Quentin Falavier, France) and stored at 4 °C for further analysis.

Total phenolic content and production To determine the total phenolic content (TPC), the protocol described by Zahir et al. (2014) was followed using Folin-Ciocalteu (FC) reagent. Absorbance of the mixture was recorded at 630 nm, with the aid of an ELx808 microplate reader (BioTek, Winooski, VT). Total phenolic content was expressed as equivalent of gallic acid (GAE g^{-1} of DW). Equation 1 was then used to calculate the total phenolic production (TPP). Total phenolic content was expressed in mg gallic acid g^{-1} DW and TPP in mg gallic acid L^{-1} .

$$\begin{aligned} \text{Total phenolic production (mg L}^{-1}\text{)} \\ = \text{DW (g L}^{-1}\text{)} \times \text{TPC (mg g}^{-1}\text{)} \end{aligned} \quad (1)$$

Total flavonoid content and production To determine the total flavonoid content (TFC), the protocol described by Nadeem et al. (2018b) was followed. Absorbance Microplate Reader (ELx808, BioTek) was used to evaluate the absorbance of the samples at 415 nm. Total flavonoid content (TFC) was expressed as equivalent of quercetin (QE g^{-1} DW). Equation 2 was then used to calculate the total flavonoid production (TFP). Total flavonoid content was expressed in mg quercetin

g^{-1} of DW and TFP in mg quercetin L^{-1} .

$$\begin{aligned} \text{Total flavonoid production (mg L}^{-1}\text{)} \\ = \text{DW (g L}^{-1}\text{)} \times \text{TFC (mg g}^{-1}\text{)} \end{aligned} \quad (2)$$

Free radical scavenging assay To determine the antioxidant potential, the free radical scavenging assay (FRSA) was performed using 1,1-diphenyl-2-picrylhydrazyl (DPPH) as described by Amarowicz et al. (2004). Methanolic extracts of DPPH solution were used as the control. Equation 3 was then used to calculate FRSA. The antioxidant potential was expressed as a percentage. Equation ID=t=t b 15pt

$$\begin{aligned} \text{Free radical scavenging activity (\%)} \\ = 100 \times (1 - (A_C \div A_S)) \end{aligned} \quad (3)$$

where A_C is the absorbance of the solution when the sample extract was mixed at a specific concentration and A_S is the absorbance of the standard (DPPH solution).

Reverse phase high-performance liquid chromatography (RP-HPLC) analysis To identify the flavonoids and phenolic acids in *I. turbinata* callus extracts, HPLC analysis was conducted. Following ultrasound-assisted extraction as described previously in the ‘‘Extract Preparation’’, extracts were evaporated and subjected to enzymatic hydrolysis using 5 U mL^{-1} of β -

Table 1. Effect of different plant growth regulators (PGR) on callogenesis in callus culture of *Ipomoea turbinata* Lagasca and Segura

PGR	Concentration (mg L^{-1})	Callus initiation (d)			Callus induction frequency (%)			Callus color			Morphology		
		Stem	Leaf	Root	Stem	Leaf	Root	Stem	Leaf	Root	Stem	Leaf	Root
MS0	0	–	–	–	0	0	0	–	–	–	–	–	–
TDZ	0.1	8	8	9	60 \pm 3.2 ^d	60 \pm 3.3 ^d	58 \pm 2.6 ^d	YG	G	LB	C	C	C
	0.5	7	7	7	64 \pm 3.3 ^{cd}	65 \pm 3.5 ^{cd}	60 \pm 3.2 ^{cd}	DG	DG	LB	C	C	C
	1.0	6	6	7	73 \pm 3.8 ^{cb}	75 \pm 3.9 ^{cb}	72 \pm 3.6 ^{cb}	LG	DG	LB	C	C	C
	5.0	6	6	6	90 \pm 4.7 ^a	92 \pm 4.2 ^a	85 \pm 4.1 ^a	LG	DG	B	C	C	C
	10.0	7	7	7	82 \pm 4.0 ^b	80 \pm 3.8 ^b	84 \pm 4.0 ^a	LG	DG	YG	C	C	C
TDZ + NAA	0.1 + 1.0	8	8	8	74 \pm 3.8 ^{cb}	74 \pm 3.2 ^{cb}	75 \pm 3.3 ^b	YG	LB	YG	C	C	C
	0.5 + 1.0	8	8	9	70 \pm 3.7 ^c	72 \pm 3.8 ^c	72 \pm 3.6 ^{cb}	LB	YG	LB	C	G	G
	1.0 + 1.0	9	9	10	62 \pm 3.2 ^{cd}	64 \pm 3.5 ^{cd}	64 \pm 3.4 ^c	LB	OW	LB	G	G	G
	5.0 + 1.0	11	11	11	50 \pm 2.5 ^e	52 \pm 2.7 ^e	48 \pm 2.3 ^e	LB	LB	LB	F	F	F
	10.0 + 1.0	11	11	11	30 \pm 2.0 ^f	30 \pm 1.9 ^f	28 \pm 1.8 ^f	B	B	B	F	F	F
NAA	1.0	9	8	9	30 \pm 2.1 ^f	32 \pm 2.2 ^f	28 \pm 1.7 ^f	G	G	G	C	C	C
	2.0	10	9	10	26 \pm 1.3 ^{fg}	27 \pm 1.6 ^{fg}	26 \pm 1.6 ^f	YG	YG	YG	C	C	C
	3.0	10	10	10	21 \pm 1.1 ^g	22 \pm 1.2 ^g	20 \pm 1.2 ^{gh}	YG	YG	LB	G	G	G
	4.0	11	10	12	20 \pm 1.0 ^g	18 \pm 0.9 ^h	18 \pm 1.0 ^h	LB	LB	B	G	G	F
	5.0	12	12	14	19 \pm 1.0 ^g	18 \pm 0.9 ^h	18 \pm 0.9 ^h	B	B	B	F	F	F

MS0 Murashige and Skoog (1962) medium without PGR, TDZ thidiazuron, NAA α -naphthaleneacetic acid, YG yellowish green, LG light green, G green, DG dark green, LB light brown, B brown, C compact, G granular, F friable

Values are means of three replicates \pm SD. Means with different letter/s are statistically different ($P < 0.05$)

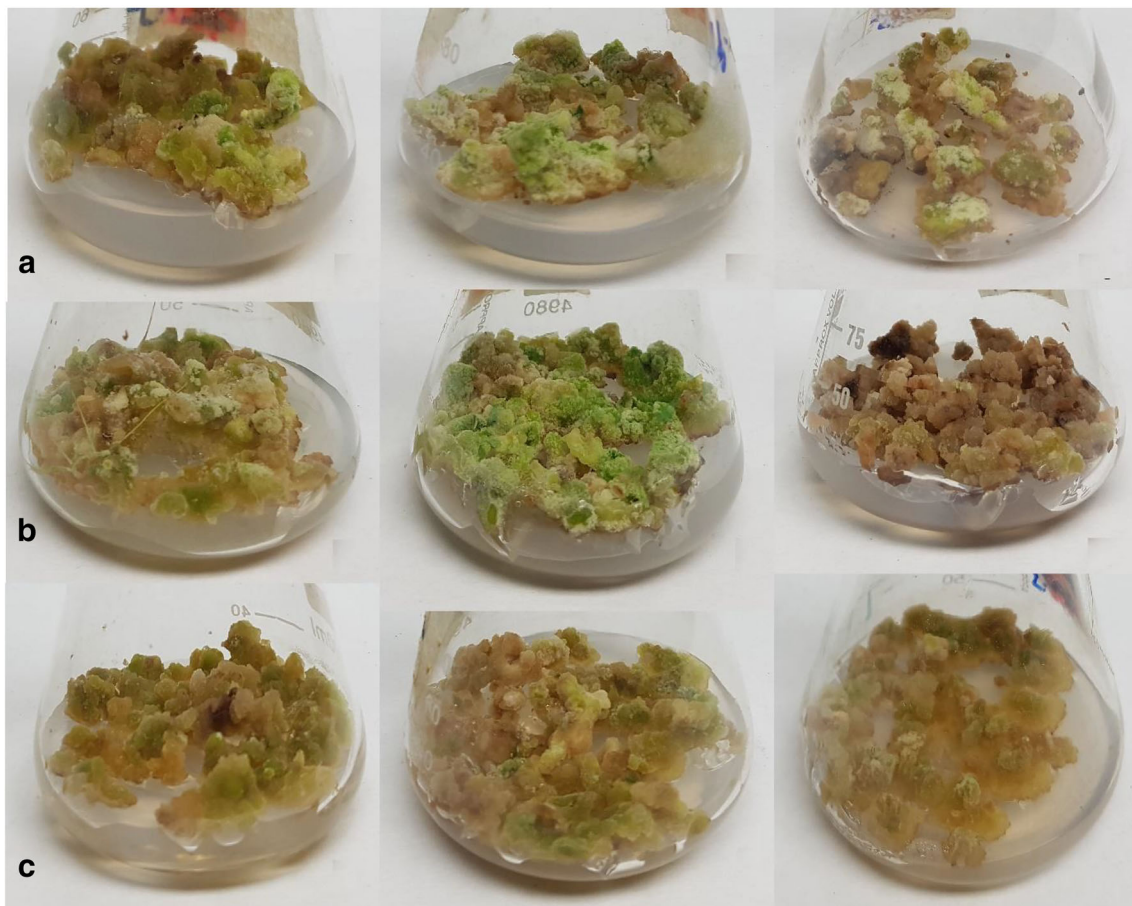


Figure 1. Effect of thidiazuron (TDZ) on biomass accumulation and callus morphology from stem, leaf, and root explant, respectively, of *Ipomoea turbinata* Lagasca and Segura after 5 wk of culture. (a) 1 mg L^{-1} TDZ; (b) 5 mg L^{-1} TDZ; (c) 10 mg L^{-1} TDZ.

glucosidase from almond (Sigma-Aldrich®, Saint Quentin Falavier, France), in 1 mL of pH 4.8 0.1 M citrate–phosphate buffer, for 6 h at 37°C , to release aglycones and simplify the analysis. The samples were then centrifuged (Spectrafuge™ 24D) at $12,000\times g$ for 5 min, and the supernatant was filtered using a $0.45\text{-}\mu\text{m}$ syringe filter (Merck Millipore) prior to HPLC analysis. The separation was performed on a Varian ProStar system (Agilent Technology, Les Ulis, France) comprising a pump Varian Prostar 230, a Degasser MetaChem Degasit, an autosampler Varian Prostar 410 and a Photodiode Array Detector PAD, Varian Prostar 335. The system was driven by Galaxie v1.9.3.2 software (Agilent Technology). A Purospher® RP-18 column ($250 \times 4 \text{ mm i.d.}; 5 \mu\text{m}$) (Merck Millipore) was used for the separation at 40°C . The mobile phase was composed of 0.2% (v/v) acetic acid-acidified water as solvent A and HPLC-grade methanol as solvent B. The mobile phase composition varied according to a nonlinear gradient at a flow rate of 0.8 mL min^{-1} as follows: from 0 to 40 min of A–B: 90:10 (v/v) to 30:70 (v/v), from 41 to 50 min of A–B: 30:70 (v/v) to 0:100 (v/v), and A–B: 0:100 (v/v) from 51 to 60 min. A range of authentic standards were used, and compounds were

identified by comparing their retention times, and UV spectra to those of authentic standards, and by standard additions.

Identification of metabolites Quantification was performed at 280 nm using calibration curves of each identified standard, ranging from 0.0125 to 0.5 mg mL^{-1} with a correlation coefficient of at least 0.999. Quantification was done on the basis of retention times compared to commercial reference standards of chlorogenic acid, caffeic acid, ferulic acid, vanillic acid, protocatechuic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, quercetin, kaempferol and arctigenin (Sigma-Aldrich®). *O*-coumaric acid was used as an internal standard at 0.05 mg mL^{-1} final concentration in the extract.

Statistical analysis Experiments were conducted in a completely randomized design and repeated twice. The statistical comparison was conducted using version 8.5 OriginPro software (OriginLab® Corporation, Northampton, MA). Duncan's multiple range test was conducted with Windows v7.5.1. SPSS® software platform (IBM, Chicago, IL) was used to determine the significance at $P < 0.05$. All figures were generated using version 8.5 OriginPro software.

Table 2. Effect of different concentrations of PGRs on biomass and antioxidant phenolics and flavonoid accumulation in callus culture of *Ipomoea turbinata* Lagasca and Segura

PGR	Concentration (mg L ⁻¹)	FW (g L ⁻¹)			DW (g L ⁻¹)			TPC (mg g ⁻¹ DW)			TFC (mg g ⁻¹ DW)			FRSA (%)		
		Stem	Leaf	Root	Stem	Leaf	Root	Stem	Leaf	Root	Stem	Leaf	Root	Stem	Leaf	Root
TDZ	0.1	23.38 ± 1.6 ^{cd}	24.33 ± 1.8 ^{cd}	23.42 ± 1.7 ^d	2.01 ± 0.1 ^{cd}	2.21 ± 0.1 ^{cd}	2.11 ± 0.2 ^d	4.4 ± 0.3 ^{cd}	4.71 ± 0.3 ^{cd}	4.88 ± 0.3 ^c	0.829 ± 0.02 ^{ab}	0.831 ± 0.04 ^{ab}	0.876 ± 0.07 ^c	25.8 ± 1.8 ^{de}	29.6 ± 1.9	28.3 ± 1.8 ^{cd}
	0.5	36.73 ± 2.1 ^{cd}	39 ± 2.2 ^{de}	34.43 ± 2.0 ^c	2.90 ± 0.2 ^c	3.02 ± 0.2 ^c	2.86 ± 0.2 ^{cd}	4.07 ± 0.3 ^d	4.2 ± 0.3 ^d	4.1 ± 0.3 ^{cd}	0.853 ± 0.05 ^{ab}	0.853 ± 0.05 ^{ab}	1.025 ± 0.09 ^b	37.3 ± 2.1 ^{cd}	40.09 ± 1.8	39.7 ± 2.0 ^c
	1.0	56.57 ± 3.2 ^{de}	58.4 ± 3.1 ^b	55.33 ± 3.0 ^c	6 ± 0.4 ^d	6.06 ± 0.5 ^d	5.97 ± 0.5 ^d	8.16 ± 0.5 ^d	8.94 ± 0.6 ^d	8.86 ± 0.8 ^d	1.063 ± 0.09 ^a	1.025 ± 0.02 ^a	1.075 ± 0.08 ^b	58.3 ± 0.8 ^{ab}	61.9 ± 3.2 ^a	59 ± 2.9 ^b
	5.0	58.90 ± 2.9 ^d	61.4 ± 3.1 ^a	56.19 ± 3.0 ^d	6.08 ± 0.5 ^a	6.3 ± 0.5 ^d	6.01 ± 0.4 ^d	8.39 ± 0.6 ^d	9.04 ± 0.8 ^d	8.56 ± 0.6 ^d	1.011 ± 0.08 ^{ab}	1.162 ± 0.09 ^a	1.108 ± 0.09 ^{ab}	60.8 ± 3.0 ^d	62.6 ± 3.2 ^a	59.1 ± 3.0 ^b
	10.0	43.66 ± 2.2 ^{bc}	40.06 ± 2.1 ^d	42.47 ± 2.4 ^d	3.47 ± 0.4 ^{bc}	3.17 ± 0.3 ^{bc}	3.47 ± 0.3 ^c	2.59 ± 0.2 ^{cd}	2.6 ± 0.1 ^{cd}	2.85 ± 0.2 ^c	0.308 ± 0.05 ^d	0.245 ± 0.01 ^c	0.329 ± 0.02 ^d	39.6 ± 2.2 ^{bc}	35.9 ± 2.2 ^{bc}	66.9 ± 3.2 ^a
TDZ + NAA	0.1 + 1.0	51.71 ± 2.8 ^d	47.02 ± 2.6 ^d	47.8 ± 2.5 ^{ab}	4.29 ± 0.4 ^b	4.02 ± 0.3 ^{bc}	4.18 ± 0.3 ^{bc}	7.03 ± 0.5 ^b	6.38 ± 0.4 ^b	6.85 ± 0.4 ^b	1.118 ± 0.1 ^a	1.056 ± 0.09 ^a	1.212 ± 0.1 ^{ab}	40.1 ± 1.2 ^c	46.2 ± 2.5 ^{bc}	41 ± 2.1 ^c
	0.5 + 1.0	41.95 ± 2.5 ^c	36.4 ± 2.0 ^{de}	38.95 ± 2.1 ^{bc}	3.95 ± 0.3 ^{bc}	3.73 ± 0.2 ^{bc}	3.83 ± 0.2 ^c	6.19 ± 0.4 ^{bc}	5.71 ± 0.3 ^{bc}	6.34 ± 0.6 ^{bc}	1.011 ± 0.08 ^{ab}	1.097 ± 0.08 ^{ab}	1.139 ± 0.09 ^{ab}	49.1 ± 2.4 ^b	41.3 ± 2.2 ^{bc}	40.9 ± 2.5 ^c
	1.0 + 1.0	38.47 ± 2.0 ^{cd}	31.71 ± 2.3 ^c	33.09 ± 2.5 ^c	2.65 ± 0.2 ^{cd}	2.31 ± 0.2 ^{cd}	2.55 ± 0.2 ^{cd}	6.02 ± 0.5 ^c	5.23 ± 0.4 ^c	6.26 ± 0.5 ^{bc}	0.729 ± 0.06 ^b	0.708 ± 0.03 ^{bc}	0.908 ± 0.08 ^{bc}	31.6 ± 1.8 ^d	48.9 ± 2.6 ^b	38.6 ± 2.1 ^{cd}
	5.0 + 1.0	31.38 ± 2.0 ^d	28.52 ± 2.1 ^{cd}	28.7 ± 2.4 ^{cd}	2.52 ± 0.2 ^{cd}	2.03 ± 0.1 ^d	2.25 ± 0.2 ^{cd}	4.14 ± 0.4 ^{cd}	3.42 ± 0.2 ^c	4.28 ± 0.5 ^{cd}	0.81 ± 0.03 ^{ab}	0.821 ± 0.05 ^b	0.92 ± 0.06 ^{bc}	32 ± 1.9 ^d	32.9 ± 2.1 ^c	35.6 ± 2.3 ^{cd}
	10.0 + 1.0	29.38 ± 2.2 ^d	25.52 ± 2.1 ^{cd}	26.7 ± 2.3 ^{cd}	1.42 ± 0.1 ^d	1.63 ± 0.2 ^{de}	1.75 ± 0.1 ^{de}	1.44 ± 0.1 ^{de}	2.42 ± 0.2 ^d	2.28 ± 0.3 ^{cd}	0.71 ± 0.03 ^b	0.621 ± 0.05 ^{bc}	0.72 ± 0.05 ^{cd}	27 ± 1.6 ^{de}	29.9 ± 1.7 ^{cd}	29.6 ± 1.8 ^{cd}
NAA	1.0	17.18 ± 1.8 ^{cd}	16.31 ± 1.5 ^d	15.92 ± 1.4 ^{de}	2.11 ± 0.2 ^{cd}	2.18 ± 0.2 ^{cd}	2.23 ± 0.1 ^{cd}	3.4 ± 0.1 ^e	3.71 ± 0.3 ^{de}	3.88 ± 0.2 ^d	0.629 ± 0.05 ^{bc}	0.531 ± 0.04 ^{bc}	0.676 ± 0.04 ^{cd}	21.3 ± 1.1 ^{de}	25.7 ± 1.4 ^{de}	25.3 ± 1.9 ^d
	2.0	17.71 ± 1.4 ^{cd}	18.51 ± 1.6 ^d	17.43 ± 1.6 ^{de}	1.90 ± 0.1 ^d	1.02 ± 0.1 ^e	1.86 ± 0.1 ^{de}	2.07 ± 0.1 ^f	2.2 ± 0.2 ^f	2.1 ± 0.2 ^{ef}	0.554 ± 0.06 ^{cd}	0.525 ± 0.02 ^a	1.525 ± 0.02 ^a	20.3 ± 1.0 ^d	20.09 ± 1.3 ^d	21.7 ± 1.4 ^{de}
	3.0	15.31 ± 1.3 ^c	14.54 ± 1.4 ^d	14.99 ± 1.3 ^c	1.57 ± 0.2 ^{de}	1.07 ± 0.1 ^e	1.28 ± 0.1 ^{de}	2.41 ± 0.1 ^{ef}	2.23 ± 0.2 ^f	2.25 ± 0.2 ^{ef}	0.513 ± 0.04 ^d	0.526 ± 0.04 ^d	0.525 ± 0.04 ^{cd}	20.4 ± 1.9 ^{cd}	20.9 ± 1.3 ^d	20.6 ± 1.2 ^{de}
	4.0	13.48 ± 1.3 ^c	14.73 ± 1.2 ^d	13.42 ± 1.2 ^c	1.01 ± 0.1 ^e	1.16 ± 0.1 ^{de}	1.13 ± 0.1 ^{de}	1.53 ± 0.1 ^{fg}	1.17 ± 0.1 ^{fg}	1.38 ± 0.1 ^f	0.423 ± 0.04 ^{cd}	0.433 ± 0.03 ^d	0.475 ± 0.03 ^{cd}	18.3 ± 1.7 ^{ef}	19.6 ± 1.2 ^d	18.6 ± 1.1 ^{de}
	5.0	13.28 ± 1.2 ^c	13.11 ± 1.3 ^f	13.09 ± 1.5 ^c	1.14 ± 0.1 ^e	1.08 ± 0.1 ^e	1.1 ± 0.1 ^e	1.4 ^{de} ± 0.1	1.27 ± 0.1 ^{fg}	1.23 ± 0.1 ^g	0.419 ± 0.02 ^{cd}	0.421 ± 0.02 ^d	0.476 ± 0.03 ^{cd}	15.3 ± 1.1 ^e	14.7 ± 1.1 ^e	14.3 ± 1.0 ^e

TDZ thidiazuron, NAA α -naphthaleneacetic acid, FW fresh weight, DW dry weight, FRSA free radical scavenging activity, PGR plant growth regulator, TPC total phenolic compounds, TFC total flavonoid compounds

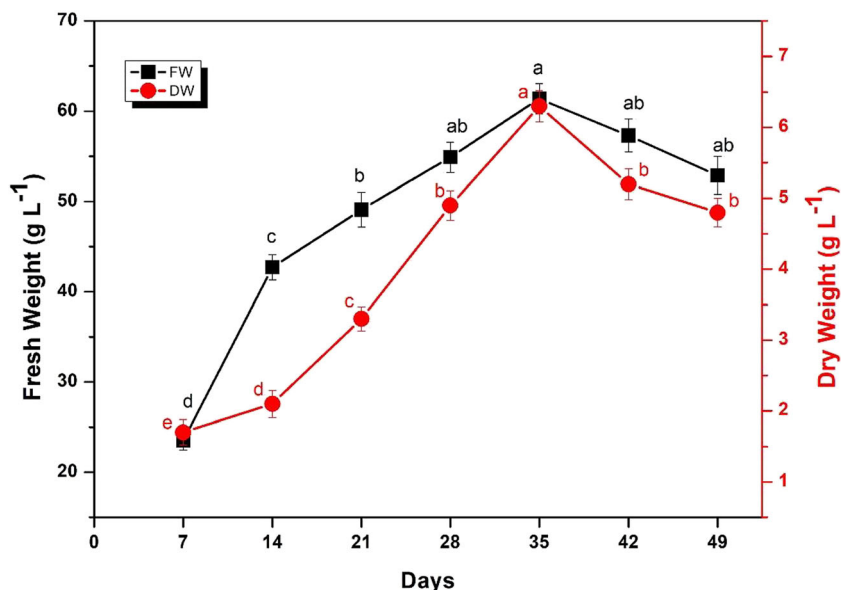
Values are means of three replicates \pm SD. Means with different letter/s are statistically different ($P < 0.05$)

Results and Discussion

Callus morphogenesis and biomass accumulation

Thidiazuron is reported to be a highly efficient growth regulator for callogenesis in various plant species (Prathanturug et al. 2005). In the current study, TDZ and NAA alone or in combination (almost all concentrations) successfully induced callogenesis. The maximum callus induction frequency (92%) was recorded in leaf-derived explants inoculated onto MS medium supplemented with 5.0 mg L⁻¹ TDZ (Table 1). No significant difference was observed in callogenesis of all the three types of explants, but the concentration and type of PGR affected the morphology of callus. Moreover, the type of explant also played a role in morphological variations in callus culture (Table 1). However, 5.0 mg L⁻¹ TDZ was found to be highly efficient for callus organogenesis (Fig. 1). Generally, from leaf-derived callus, higher FW and DW accumulation occurred in cultures treated with TDZ alone compared to NAA alone or in combination. Furthermore, the color of the resultant callus was green (Table 1). Thidiazuron has a potential role in the production of purine cytokinins to improve cell growth (Arinaitwe et al. 2000). Accordingly, TDZ stimulated biomass accumulation and other related growth parameters in callus cultures of *I. turbinata*. From all the three types of explants, NAA alone or in combination with higher concentrations of TDZ, displayed lower callogenesis compared to TDZ alone. However, auxins have been reported to be potential candidates in callogenesis in a number of plant species (Sané et al. 2012). No previous report exists on the effect of NAA or combined treatment of NAA and TDZ, in tissue culture responses in *I. turbinata*. However, Alam et al. (2010) reported the potential role of TDZ in callus induction in *Ipomoea batatas* (L.) Lam. The callus-inducing ability of TDZ in combination with NAA has been reported by Cheruvathur et al. (2015) in *Ipomoea marginata* (Desr.) Verdc. The MS medium devoid of any PGR did not favor any callogenesis. Conclusively, leaf explants exhibited a higher callogenetic response (92%), than stem-derived (90%) and root-derived (85%) explants along with maximal FW (61.4 g L⁻¹) and DW (6.3 g L⁻¹) accumulation (Table 2). The divergence in the response of explants of the same plant species to the same hormonal treatment is probably due to the particular biochemical and physiological potential of different tissues. Usually, a number of parameters are involved in callus formation such as the type of explant, concentration, nature of PGR, plant genotype and *in vitro* growth-promoting conditions (Mathur and Shekhawat 2013). Thidiazuron in lower and higher concentrations than the optimum concentration poorly promoted callus formation (Table 1). These results are in agreement with results reported by Ali and Abbasi (2014) and Khan et al. (2016) for the *Artemisia absinthium* L. and *Fagonia indica* Burm.f., respectively.

Figure 2. Growth dynamics in callus culture of *Ipomoea turbinata* Lagasca and Segura. FW = fresh weight and DW = dry weight. Values are means of three replicates ± SD. Means with different letters are statistically different ($P < 0.05$).



To determine the trends in growth kinetics and biomass accumulation, data were recorded at a 7-d interval for a total of 49 d. The growth curves of callus cultures of *I. turbinata* grown at 5.0 mg L⁻¹ TDZ showed a 7-d lag phase, 21-d exponential phase starting from day 14 to day 35, followed by a 7-d stationary phase from day 35 to day 42. From day 42 onward, a death phase was observed (Fig. 2).

Phenolic and flavonoid accumulation in callus cultures

Phenolic compounds are synthesized by plants as secondary metabolites to scavenge reactive oxygen species (ROS) and possess other numerous biochemical and pharmacological properties (Kasote et al. 2015; Nadeem et al. 2018c). Maximum TPC (9.04 mg g⁻¹) and TFC (1.16 mg g⁻¹) were noted from leaf-derived callus (Figs. 3 and 4) grown on

5.0 mg L⁻¹ TDZ compared to stem-derived callus (TPC 8.39 mg g⁻¹ and TFC 1.01 mg g⁻¹) and root-derived callus (TPC 8.56 mg g⁻¹ and TFC 1.01 mg g⁻¹; Table 2). A TDZ-based enhanced TPC and TFC *in vitro* culture system was reported by Pourebab et al. (2015) and Khan et al. (2016). In the current study, the dependent behavior of explants on the TDZ concentration for increased FW and DW accumulation (Fig. 2) and higher TPC and TFC (Figs. 3 and 4) has also established the link with the role of TDZ in callus organogenesis. Thidiazuron at a mild concentration of about 5.0 mg L⁻¹ increased the biosynthesis of TPC and TFC in callus cultures, while it inhibited the accumulation of TPC and TFC at higher concentrations. A possible reason for the accumulation of secondary metabolites in this trend is that TDZ stimulates the production of ethylene

Figure 3. Total phenolic content (TPC) and total phenolic production (TPP) of thidiazuron-optimized callus culture of *Ipomoea turbinata* Lagasca and Segura at different days of growth. Values are means of three replicates ± SD. Means with different letters are statistically different ($P < 0.05$).

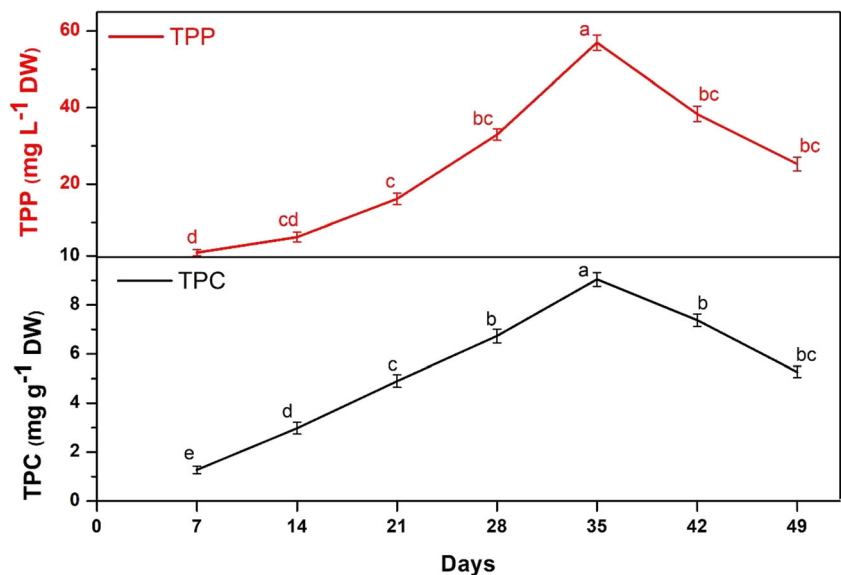
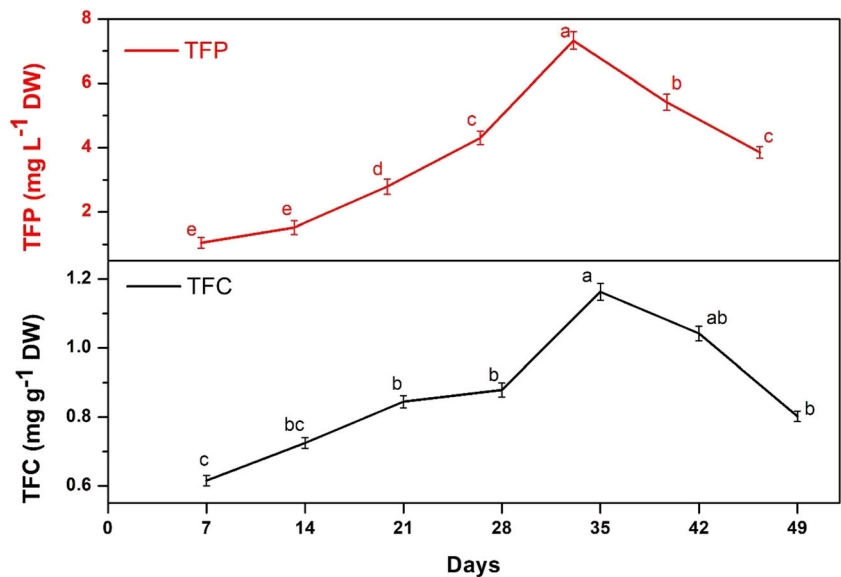


Figure 4. Total flavonoid content (TFC) and total flavonoid production (TFP) of thidiazuron-optimized callus culture of *Ipomoea turbinata* Lagasca and Segura at different days of growth. Values are means of three replicates \pm SD. Means with different letters are statistically different ($P < 0.05$).



at higher concentrations, and ethylene represses the biosynthesis of secondary metabolites (Shibli et al. 1997).

Antioxidant potential of callus cultures The profusion of ROS and other related free radicals induces oxidative stress in the body and is responsible for damaging the cells directly or indirectly. As a counter measure, a plant synthesizes different kinds of secondary metabolites, mainly phenolics and flavonoids, as antioxidants to scavenge these free radicals (Gill and Tuteja 2010). These metabolites can be employed in the body as an antidote for several disorders (Raskin et al. 2002). Guo et al. (2011) have reported that thidiazuron induces the expression of stress marker compounds such as abscisic acid, proline, and 4-aminobutyrate. When peanut seedlings were

cultured on TDZ-fortified media, the proline content was enhanced, which resulted in high oxidative stress. Moreover, TDZ also affected embryogenesis and organogenesis by inducing oxidative stress in plant cells (Younas et al. 2018).

To evaluate the presence of such antioxidants in callus, a DPPH-free radical scavenging assay (FRSA) was performed. In the current study, a high percentage of FRSA was observed in callus cultures (Fig. 5). In the cases of all three types of explants, an increasing trend was observed with increased TDZ concentrations. Maximum FRSA (62.6%) was observed in leaf-derived callus cultures (Table 2).

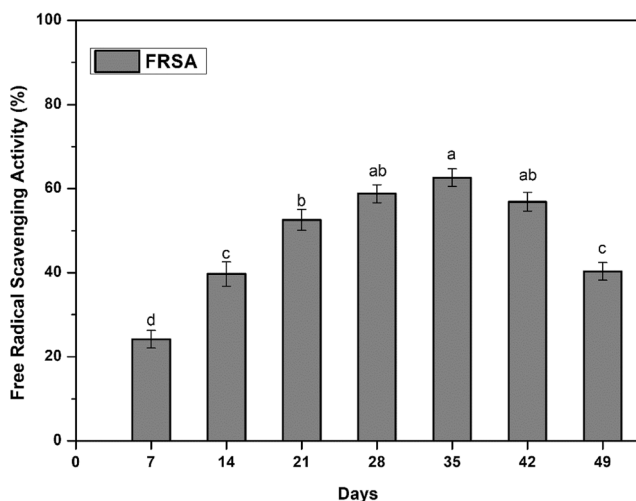


Figure 5. Percent (%) free radical scavenging activity in callus culture of *Ipomoea turbinata* Lagasca and Segura at different days of growth. FRSA, free radical scavenging assay. Values are means of three replicates \pm SD. Means with different letters are statistically different ($P < 0.05$).

RP-HPLC-assisted quantification of phenolics HPLC was used to identify and quantify the main phenolic acids and flavonoids accumulated in *I. turbinata* callus cultures. Due to unavailability of any reports on HPLC-based phytochemical profiles of *I. turbinata*, a number of reference compounds previously identified in other related species of genus *Ipomea* were tested (Páska et al. 2002; Lin et al. 2008; Oluyori et al. 2016; Khan et al. 2018). Chlorogenic acid, arctigenin, quercetin and kaempferol were the major compounds accumulated in *I. turbinata*. Upon evaluation of growth dynamics, it was noted that chlorogenic acid (13.48 mg g^{-1}), quercetin (6.19 mg g^{-1}) and kaempferol (5.48 mg g^{-1}) were accumulated in the highest amounts at day 28 after inoculation (14 d into exponential phase), while arctigenin was produced in the highest concentration (11.67 mg g^{-1}) at day 35 (end of exponential phase), as shown in Fig. 6a. Additional phenolic acids such as caffeic acid, ferulic acid, vanillic acid, protocatechuic acid, *p*-coumaric acid and *p*-hydroxybenzoic acid were also detected in lower concentrations (0.12 to 1.19 mg g^{-1}). The accumulation of phenolic acids such as *p*-coumaric acid and protocatechuic acid in the early and late stationary phase showed their role to alleviate nutrient depletion-induced stress

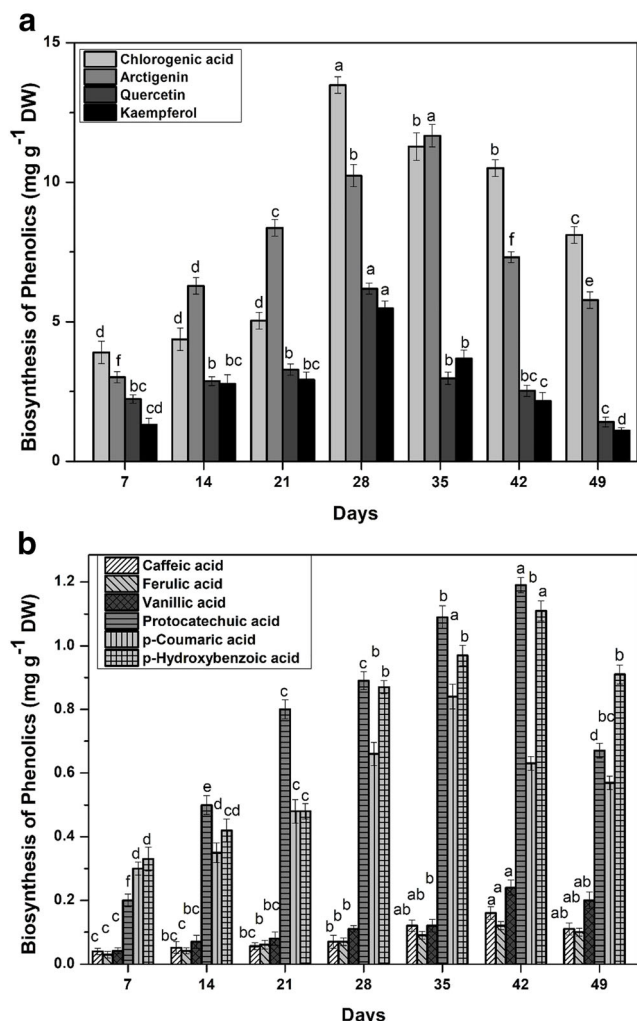


Figure 6. (a) Quantification of major phenolics in callus culture of *Ipomoea turbinata* Lagasca and Segura at different d of growth. Values are means of three replicates \pm SD. Means with *different letters* are statistically different ($P < 0.05$). (b) Quantification of minor phenolics in callus culture of *I. turbinata* at different growth phases. Values are means of three replicates \pm SD. Means with *different letters* are statistically different ($P < 0.05$).

(Fig. 6b). In accordance with the results on the total phenolic and flavonoid content, the leaf-derived callus cultures grown on MS medium supplemented with 5.0 mg L^{-1} TDZ accumulated higher amounts of these compounds than all other treatments (Table 2). Interestingly, arctigenin, a pharmacologically important dibenzylbutyralactone lignan, is produced in substantial amounts in callus cultures of *I. turbinata*. This is of particular interest when considering the biological activities of this compound such as anti-diabetic (Huang et al. 2012), anti-inflammatory (Zhao et al. 2009), antiviral (Hayashi et al. 2010), neuroprotective (Li et al. 2014) and anti-cancer (Han et al. 2016). Furthermore, other accumulated phenolics are not devoid of potent applications and could have pharmaceutical and industrially valued as well. This first report on the induction of improved biomass accumulation and production

of multipotent bioactive metabolites in callus cultures of *I. turbinata* showed the high potential of the multifunctional TDZ synthetic hormone as a stimulation tool for the production and future valuation of bioactive compounds in this species.

Conclusions

In the current study, a callus culture system for *I. turbinata* was established for the improved and feasible production of commercially important phenolic acids. Thidiazuron is an influential growth regulator for inducing callus production in *I. turbinata*, irrespective of the type of explant used. Thidiazuron has positively affected the biomass accumulation and biosynthesis of phenolic compounds. Thidiazuron at a low level can promote the cost efficient and easy production of these bioactive secondary metabolites. The system presented in this study is a step towards the establishment of a cell suspension culture and ultimate scale-up for industrial production of commercially important metabolites.

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Authors' contributions WA performed experiments of establishment of callus culture, optimization with TDZ, and analyses of TPC, TFC, and antioxidant activity. AZ and MN assisted WA with all of these experiments. BHA conceived idea, supervised research, and critically evaluated MS. CH contributed to HPLC analysis and critical revision of manuscript. MZ assisted with experiments and manuscript preparation.

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Compliance with ethical standards

Conflicts of interest The authors declare they have no conflicts of interest.

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