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MEDICINAL PLANTS

Effect of coconut water and cytokinins on rapid micropropagation of *Ranunculus wallichianus* Wight & Arnn—a rare and endemic medicinal plant of the Western Ghats, India

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Abstract

Ranunculus wallichianus is a rare medicinal plant endemic to Western Ghats, India. Nodal explants were inoculated on MS medium with 1.0 to 5.0 mg L^{-1} of 6-benzyladenine (BA), kinetin (KIN), or thidiazuron (TDZ) resulting in a low percentage of shoot regeneration. Coconut water (CW) was added to MS medium containing the above mentioned cytokinins to promote *in* vitro plantlet growth. Multiple shoots were regenerated on half-strength Murashige and Skoog (MS) medium containing 1.0 to 5.0 mg L^{-1} BA, KIN, or TDZ in combination with 5%, 10%, and 20% CW. The highest percentage of shoot multiplication was observed from cultures incubated on half-strength MS medium supplemented with 3.0 mg L^{-1} TDZ in combination with 10% CW. The cut ends of well-elongated shoots were transferred to medium containing 0.5 to 3.0 mg L^{-1} indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA) to induce *in vitro* root induction. Half-strength MS medium with 2.5 mg L^{-1} IBA and 250 mg L^{-1} activated charcoal shows high frequency of root formation. The well-rooted plantlets were transferred to pots for hardening with survival rate of 93% after 30 d. The present work indicates the addition of CW with the cytokinins in half-strength MS medium is suitable for rapid micropropagation of *R. wallichianus.*

Keywords Micropropagation · *Ranunculus wallichianus* · MS medium · cytokinin · coconut water · nodal explants

Introduction

Ranunculus wallichianus Wight & Arnn belonging to the family of Ranunculaceae is a rare and endemic medicinal plant of the Western Ghats, India, and is commonly called as Wallich Buttercup (Mathew 1996; Kumar 2004). The genus *Ranunculus* has more than 600 species throughout the world (Tamura 1993, 1995). In Asian countries, *Ranunculus* species are traditionally used to treat fever, rheumatism and asthma (Iqbal *et al*. 2011; Aslam *et al*. 2012) as well as pharmacological properties as antifungal (Mares 1987), antibacterial, anticancer, and antioxidant agents (Cao *et al*. 1992; Yin *et al*. 2008; Kaya *et al*. 2010; Akkol *et al*. 2012).

Being an endemic and over-used plant, *R. wallichianus* needs to be conserved and micropropagation is an effective tool to accomplish this (Fay 1992; Pathnaik and Chand 1996). Based on the previous literature, tissue culture studies have been reported in the *R. asiaticus* (Meynet and Duclos 1990; Pugliesi *et al*. 1992; Beruto and Debergh 1992, 2004; Beruto *et al*. 1996, 1999) and *R. lyallii* (Bicknell *et al*. 1996), although no *in vitro* culture studies of *R. wallichianus* have not been reported. The aim of the present study was to develop a protocol for producing *R. wallichianus* in large quantities from nodal explants by adding various concentrations of coconut water (CW) to the medium along with cytokinins to produce more shoots by micropropagation.

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Materials and methods

Collection of plants and surface sterilization The young and mature plants were collected from the Pampar Shola of Western Ghats, Tamilnadu, India. The explants were washed with running tap water for 30 min, followed by immersion in

Table 1. Effect of cytokinins on micropropagation of *Ranunculus wallichianus* Wight & Arnn using nodal explants cultured on half-strength Murashige and Skoog medium for 6 wk

Growth regulators (mg L^{-1})			Percent CW	Percent response	Number of shoots per explant	Shoot length (cm)
BA	KIN	TDZ				
1.0	–	–	–	43	2.1 ± 0.23^{hi}	2.6 ± 0.17^{ij}
2.0	–	–	–	52	2.2 ± 0.14^{hi}	2.9 ± 0.21^{hi}
3.0	–	–	–	60	3.4 ± 0.21^{fgh}	3.3 ± 0.13^{fgh}
4.0	–	–	–	58	2.7 ± 0.17^{gh}	3.0 ± 0.18^{gh}
5.0	–	–	–	51	2.3 ± 0.21^{hi}	2.8 ± 0.14^{hi}
–	1.0	–	–	39	1.3 ± 0.11^{j}	2.1 ± 0.16^{j}
–	2.0	–	–	46	1.9 ± 0.16^{ij}	2.4 ± 0.12^{ij}
–	3.0	–	–	45	2.1 ± 0.21^{hi}	2.5 ± 0.11^{ij}
–	4.0	–	–	37	1.6 ± 0.13^{ij}	2.3 ± 0.19^{ij}
–	5.0	–	–	33	1.2 ± 0.19^{j}	2.2 ± 0.21^{j}
–	–	1.0	–	67	3.2 ± 0.16^{fgh}	3.1 ± 0.24^{gh}
–	–	2.0	–	78	4.2 ± 0.28^{def}	3.4 ± 0.21^{fgh}
–	–	3.0	–	72	4.0 ± 0.15^{def}	3.8 ± 0.25^{efg}
–	–	4.0	–	63	3.7 ± 0.13^{efg}	3.5 ± 0.23^{fgh}
–	–	5.0	–	56	2.9 ± 0.27^{gh}	3.2 ± 0.12^{gh}
1.0	–	–	5	54	2.6 ± 0.19^{gh}	3.1 ± 0.21^{gh}
2.0	–	–	5	50	2.7 ± 0.13^{gh}	3.3 ± 0.18^{fgh}
3.0	–	–	5	53	3.1 ± 0.17^{fgh}	3.4 ± 0.15^{fgh}
4.0	–	–	5	62	3.7 ± 0.21^{efg}	3.5 ± 0.17^{fgh}
5.0	–	–	5	65	3.4 ± 0.23^{fgh}	3.2 ± 0.18^{gh}
–	1.0	–	5	36	1.6 ± 0.09^{ij}	2.5 ± 0.11^{ij}
–	2.0	–	5	43	1.8 ± 0.11^{ij}	2.7 ± 0.21^{hi}
–	3.0	–	5	47	2.1 ± 0.14^{gh}	2.8 ± 0.24^{hi}
–	4.0	–	5	45	2.2 ± 0.18^{gh}	3.0 ± 0.19^{gh}
–	5.0	–	5	47	1.9 ± 0.13^{ij}	2.2 ± 0.28^{j}
–	–	1.0	5	70	4.1 ± 0.17^{def}	4.0 ± 0.14^{def}
–	–	2.0	5	75	4.4 ± 0.12^{def}	4.2 ± 0.12^{def}
–	–	3.0	5	89	4.7 ± 0.21^{cde}	4.6 ± 0.27^{cde}
–	–	4.0	5	71	3.9 ± 0.25^{efg}	3.8 ± 0.12^{efg}
–	–	5.0	5	65	3.6 ± 0.18^{efg}	3.9 ± 0.13^{efg}
1.0	–	–	10	78	5.3 ± 0.13^{bcd}	5.4 ± 0.18^{bc}
2.0	–	–	10	79	5.6 ± 0.17^{bc}	5.3 ± 0.11^{bc}
3.0	–	–	10	80	5.4 ± 0.19^{bcd}	5.6 ± 0.13^{ab}
4.0	–	–	10	81	5.9 ± 0.25^{bc}	5.2 ± 0.21^{bc}
5.0	–	–	10	62	5.2 ± 0.18^{bcd}	4.9 ± 0.20^{bcd}
–	1.0	–	10	50	5.0 ± 0.25^{bcd}	4.0 ± 0.22^{def}
–	2.0	–	10	56	5.1 ± 0.13^{bcd}	3.8 ± 0.19^{efg}
–	3.0	–	10	45	4.9 ± 0.21^{cde}	4.2 ± 0.21^{def}
–	4.0	–	10	47	4.8 ± 0.18^{cde}	3.7 ± 0.16^{efg}
–	5.0	–	10	59	5.0 ± 0.21^{bcd}	4.6 ± 0.18^{cde}
–	–	1.0	10	82	5.8 ± 0.15^{bc}	5.9 ± 0.19^{ab}
–	–	2.0	10	91	6.3 ± 0.24^{ab}	6.1 ± 0.29^{a}
–	–	3.0	10	95	6.7 ± 0.26^{a}	6.3 ± 0.21^{a}
–	–	4.0	10	86	6.1 ± 0.11^{ab}	5.8 ± 0.23^{ab}
–	–	5.0	10	83	6.2 ± 0.23^{ab}	5.4 ± 0.15^{bc}
1.0	–	–	20	62	4.1 ± 0.14^{def}	4.2 ± 0.21^{def}

Table 1. (continued)

Growth regulators (mg L^{-1})			Percent CW	Percent response	Number of shoots per explant	Shoot length (cm)
BA	KIN	TDZ				
2.0	–	–	20	75	4.6 ± 0.18^{cde}	4.7 ± 0.23^{bcd}
3.0	–	–	20	70	4.3 ± 0.14^{def}	4.6 ± 0.19^{cde}
4.0	–	–	20	74	4.4 ± 0.12^{def}	4.5 ± 0.17^{cde}
5.0	–	–	20	66	4.0 ± 0.23^{def}	4.3 ± 0.16^{cde}
–	1.0	–	20	53	3.8 ± 0.13^{efg}	3.3 ± 0.18^{fgh}
–	2.0	–	20	52	3.5 ± 0.22^{efg}	3.1 ± 0.11^{gh}
–	3.0	–	20	67	4.2 ± 0.28^{def}	4.1 ± 0.17^{def}
–	4.0	–	20	61	3.9 ± 0.19^{efg}	3.7 ± 0.21^{efg}
–	5.0	–	20	54	3.7 ± 0.16^{efg}	3.6 ± 0.22^{fgh}
–	–	1.0	20	73	4.6 ± 0.13^{cde}	4.6 ± 0.24^{cde}
–	–	2.0	20	80	5.3 ± 0.21^{bcd}	4.9 ± 0.18^{bcd}
–	–	3.0	20	78	4.9 ± 0.18^{cde}	4.7 ± 0.13^{bcd}
–	–	4.0	20	86	5.2 ± 0.16^{bcd}	5.1 ± 0.15^{bc}
–	–	5.0	20	71	4.8 ± 0.19^{cde}	4.8 ± 0.12^{bcd}

Values represent means ± standard error. Means followed by the same *letter* within each *column* are not significantly different ($P = 0.05$) using Duncan's multiple range test

BA 6-Benzyladenine, *KIN* kinetin, *TDZ* thidiazuron, and *CW* coconut water

1% (w/v) Teepol solution (soap solution) (Reckitt Benckiser Pvt. Ltd., Himachal Pradesh, India) containing 5% Bavistin (fungicide) (Crystal Crop protection Pvt. Ltd., New Delhi, India) for 3 min. Explants were washed with distilled water six times and then with double-distilled water six times before surface sterilization with 0.1% (w/v) $HgCl_2$ (Hi-Media, Mumbai, India) for 3 min under aseptic conditions in a laminar air flow chamber (Atlantis Applications Engg. Pvt. Ltd., New Delhi, India). Finally explants were washed with sterile double distilled water five times to remove the traces of $HgCl_2$ (Dubey 1999).

Culture media and conditions Murashige and Skoog (MS; Murashige and Skoog 1962) medium containing 3% (w/v) sucrose (Hi-Media, India) and 0.8% (w/v) agar (Hi-Media, India) was used for the experiments. The pH of the medium was adjusted to 5.7 using 0.1 N NaOH (Hi-Media) and 0.1 N HCl (Spectrum Reagents and Chemicals Pvt. Ltd., Cochin, India) and autoclaved at 15 psi/121 °C for 15 min.

Extraction of coconut water CW was extracted from the mature fruit (Ghandi Market, Tiruchirappalli, India) by drilling holes through two of the micropyles under the sterile laminar air flow chamber (Nasib *et al.* 2008). Then the CW was passed through 0.45-μm sterile filters and heated at 80 to 100 °C for 10 min with continuous stirring to precipitate proteins, fats, and other materials. The precipitate was removed by filtration and the filtrate was stored at – 20 °C for future use (George 1993).

Multiple shoot induction Nodal explants were cultured on half-strength MS medium supplemented with 1.0 to 5.0 mg L^{-1} BA, KIN, or TDZ (Hi-Media) either individually or in combination with 5%, 10%, or 20% CW. The cultures were maintained at 25 ± 2 °C with 16 h photoperiod (50 μmol m^{-2} s^{-1} Photon Flux Density provided by white cool fluorescent tube light) and 8 h darkness and sub cultured at 10-day intervals. The number of shoots per explants and shoot length were recorded after 30 d of culture.

***In vitro* rooting and acclimatization of regenerated plants** *In vitro* propagated shoots exceeding 5 cm in length were excised and transferred to half-strength MS medium containing 250 mg L^{-1} activated charcoal with 0.5 to 3.0 mg L^{-1} indole-3-butyric acid (IBA) or 0.5 to 3.0 mg L^{-1} indole-3-acetic acid (IAA) for *in vitro* rooting. The percentage of rooting, mean number of roots per shoot, and mean shoot length was recorded after 2 wk transfer to rooting medium. Well-rooted plantlets were transferred to pots containing a mixture of sterilized red soil (TNB Analytical Laboratory Pvt. Ltd. Tiruchirappalli, India), vermiculite (Vermicompost yard, St. Joseph's College, Tiruchirappalli, India), and coconut husk (Agaram Trading Company, Tiruchirappalli, India) (1:1:1 v/v) combination for ex vitro acclimatization. The potted shoots were fertilized with quarter strength of M.S. liquid medium for 1 wk. After 4 wk, plantlets were transferred to pots containing garden soil and maintained in a greenhouse and eventually transferred to the field.

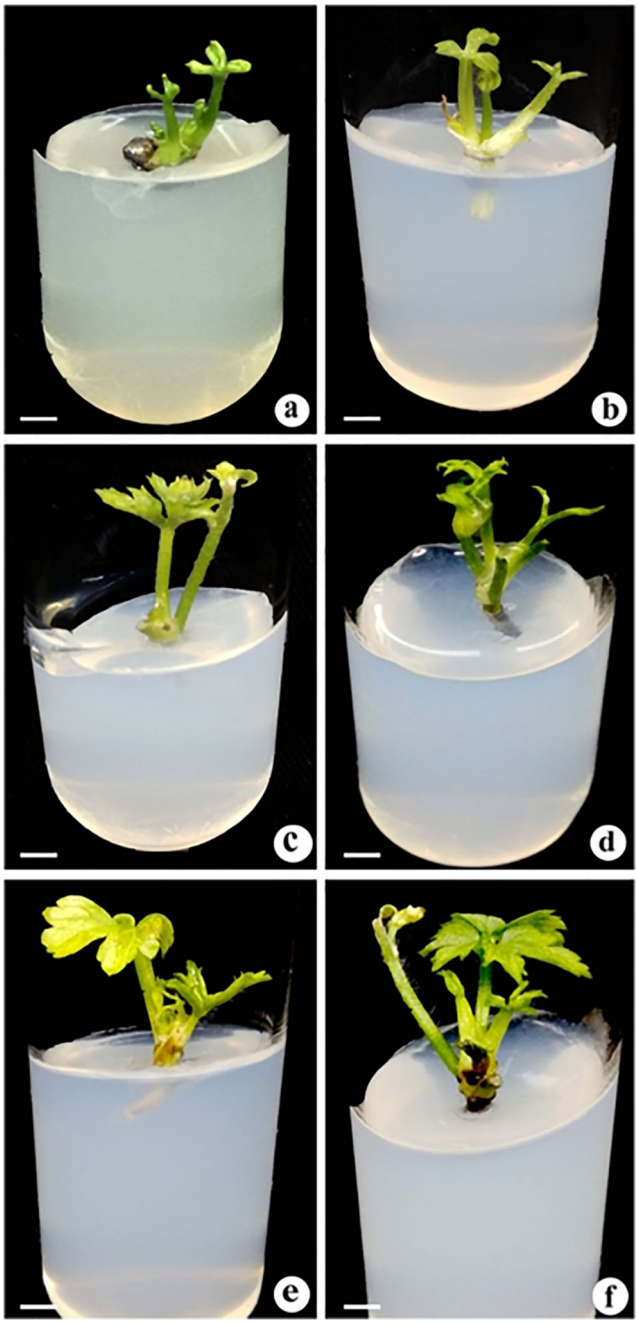

Fig. 1 *In vitro* shoots regeneration from nodal explants of *Ranunculus wallichianus* Wight & Arnn. (*a*) Shoot initiation on half-strength Murashige and Skoog (MS) medium supplemented with 3.0 mg L^{-1} of 6-benzyladenine (BA). (*b*) Shoot induction on half-strength MS medium containing 2.0 mg L^{-1} of thidiazuron (TDZ). (*c*) Shoot induction on half-strength MS medium with 4.0-mg L^{-1} kinetin (KIN) and 10% of coconut water (CW). (*d*) Multiple shoot induction on half-strength MS medium with 4.0 mg L^{-1} of BA and 10% of CW. (*e*) Multiple shoot initiation on half-strength MS medium with 3.0 mg L^{-1} of TDZ and 10% of CW after 2 wk. (*f*) Shoot multiplication on half-strength MS medium with 3.0 mg L^{-1} of TDZ and 10% of CW after 4 wk (*scale bar* 5 mm)

Statistical analysis Each experiment was repeated three times with 15 replicates, and the results were recorded after 6 wk culture. The mean number of shoots, shoot length, mean number of roots, and root length with standard error was calculated with Sigma plot statistical software version 17.0 (SPSS Inc., Chicago, IL). The treatment means were compared using Duncan's multiple range test (DMRT) at 5% probability level (Gomez and Gomez 1976).

Results and discussion

***In vitro* multiple shoot induction** Multiple shoots were produced from nodal explants cultured on half-strength MS medium supplemented with 1 to 5 mg L^{-1} BA, KIN or TDZ (Table 1). Of these cytokinins, 2 mg L^{-1} TDZ produced maximum rate (78%) of shoots, with an average of 4.2 ± 0.28 shoots per explant (Table 1, Fig. 1*b*). Several studies reported high frequency shoot regeneration with medium containing TDZ (Huetteman and Preece 1993; Tiwari *et al.* 2001; Faisal *et al.* 2005; Sujatha and Ranjitha Kumari 2007; Guo *et al.* 2011, 2012; Cheruvathur *et al.* 2013; Fatima *et al.* 2015; Vaidya *et al.* 2016). On the other hand, 3.0 mg L^{-1} BA and KIN produced shoots with 60% (3.4 ± 0.21 shoots per explant) (Fig. 1*a*) and 45% (2.1 ± 0.21 shoots per explant) efficiency, respectively. The highest average of shoot length was 3.4 ± 0.21 cm with 2 mg L^{-1} TDZ after 6 wk of culture. With 3 mg L^{-1} BA and KIN treatments, optimum average shoot length was 3.3 ± 0.13 and 2.5 ± 0.11 cm, respectively (Table 1). Meynet and Duclos (1990) reported similar results for direct regeneration of *R. asiaticus* on half-strength MS media supplemented with 2.2 μM BA in combination with 0.45 μM 2,4-D. Adventitious shoots of *R. asiaticus* were achieved by Pugliesi *et al.* (1992) using MS medium supplemented with a combination of 4.6 μM KIN and 5.4 μM NAA. The effective shoot proliferation in *R. lyallii* was demonstrated using half-strength MS basal medium supplemented with 0.2 mg L^{-1} BAP (Bicknell *et al.* 1996).

The effect of 5%, 10%, and 20% CW in combination with cytokinins on multiple shoot induction from nodal explants is shown in Table 1. Ninety-five percent of nodal explants cultured on medium containing 3 mg L^{-1} TDZ and 10% CW produced multiple shoots and averaged 6.7 ± 0.26 shoots per explant with an average shoot length of 6.3 ± 0.21 cm (Table 1: Fig. 1*e*, *f*). Roy and Banerjee (2002) documented that the combination of coconut water with various types of plant growth regulators enhances plant growth and development. Agampodi and Jayawardena (2009) and Tan *et al.* (2011) reported that plant growth regulators used in the plant tissue culture are naturally present in CW. The combination of CW and synthetic auxins are known to be more promotive of shoot multiplications in tissue culture (Loc *et al.* 2005). When utilizing BA, the maximum number of shoots (5.9 ± 0.25 shoots/explants with 5.2 ± 0.21 cm mean shoot length) was produced from nodal explants cultured on MS medium containing 4 mg L^{-1} BA and 10% CW (Table 1;

Table 2. Effect of auxins and 250 mg L^{-1} activated charcoal on root induction from *in vitro* raised shoots of *Ranunculus wallichianus* Wight & Arnn on half-strength Murashige and Skoog medium after 4 wk culture

Growth regulators (mg L^{-1})		Percent rooting	Number of roots per shoot	Root length (cm)
IAA	IBA			
0.5	–	49	1.9 ± 0.15^{d}	2.9 ± 0.19^{bc}
1.0	–	52	2.3 ± 0.19^{cd}	2.7 ± 0.21^{cd}
1.5	–	64	3.1 ± 0.21^{bc}	2.8 ± 0.21^{bc}
2.0	–	75	3.9 ± 0.18^{b}	3.3 ± 0.17^{ab}
2.5	–	71	3.9 ± 0.24^{b}	3.0 ± 0.19^{b}
3.0	–	63	3.4 ± 0.19^{bc}	2.7 ± 0.11^{cd}
–	0.5	66	2.7 ± 0.11^{cd}	2.4 ± 0.13^{d}
–	1.0	77	3.6 ± 0.13^{b}	2.8 ± 0.12^{bc}
–	1.5	80	3.8 ± 0.17^{b}	3.1 ± 0.25^{b}
–	2.0	89	4.4 ± 0.13^{ab}	3.5 ± 0.23^{a}
–	2.5	94	4.8 ± 0.23^{a}	3.7 ± 0.18^{a}
–	3.0	88	4.2 ± 0.14^{ab}	3.4 ± 0.22^{ab}

Values represent means ± standard error. Means followed by the same *letter* within each *column* are not significantly different ($P = 0.05$) using Duncan's multiple range test

IAA Indole-3-acetic acid and *IBA* indole-3-butyric acid

Fig. 1*d*), while KIN was much less effective for shoot induction (Table 1; Fig. 1*c*). The number of shoots per explant and the shoot length were inhibited at higher concentrations of CW (20%). Al-Khayri (2010) demonstrated that somatic embryogenesis of date palm (*Phoenix dactylifera* L.) was inhibited on media supplemented with 20% of CW. The morphological features and growth of *Calanthe* hybrids were decreased at high concentrations of CW (Baque *et al.* 2011). Our results suggest that 10% of CW with 3.0 mg L^{-1} TDZ is the optimal for rapid shoot production from nodal explants of *Ranunculus wallichianus*. A comparison of efficiency of hormones indicates that TDZ is superior to BA and KIN (Table 1; Fig. 1*e*, *f*).

***In vitro* rooting and acclimatization** Root initiation from well-elongated *in vitro* microshoots was observed after 7 d of culture on rooting medium and plantlets suitable for transplantation could be produced in 20 d. The high frequency (94% response) of root induction (4.8 ± 0.23 roots per shoot with 3.7 ± 0.18 cm root length) was noted on half-strength MS media containing 2.5-mg L^{-1} IBA with 250-mg L^{-1} activated charcoal (Table 2; Fig. 2*a*). A recent investigation by Choudhary *et al.* (2020) reported similar results on root induction in *Farsetia macrantha* with 2.0-mg L^{-1} IBA and 100-mg L^{-1} activated charcoal. For root induction, IBA with activated charcoal were also reported for *Hagenia abyssinica* (Feyissa *et al.* 2005) and *Cassia angustifolia* (Siddique and

Fig. 2 *In vitro* root induction and acclimatization of *Ranunculus wallichianus* Wight & Arnn. (*a*) Root induction on half-strength Murashige and Skoog (MS) medium with 2.5 mg L^{-1} of indole-3-butyric acid and 250 mg L^{-1} of activated charcoal after 20 d. (*Scale bar* 1 cm) (*b*) Hardened plantlet transferred to plastic pots for survival (*scale bar* 2 cm)

Anis 2007). In the present study, IBA was found to be more effective than IAA in terms of percent rooting and mean number of roots per shoot. After the sufficient root and shoot development, plantlets were successfully transferred to the field with a 93% survival rate (Fig. 2*b*).

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Authors' contributions PS and RT carry out the experiments and analyze the experimental data. PS prepared the manuscript. HDR gave the outline of the experimental design and edited the final version of the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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