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MEDICINAL PLANTS

Clonal fidelity and phytochemical analysis of *in vitro* propagated *Kaempferia rotunda* Linn.—an endangered medicinal plant

Suprava Sahoo[1] · Jyotirmayee Lenka[1] · Basudeba Kar[1] · Sanghamitra Nayak[1]

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Abstract

Kaempferia rotunda Linn. (*Zingiberaceae*) is an important herb that has both therapeutic and cosmetic applications. An efficient protocol has been developed for *in vitro* propagation of *K. rotunda* using axillary bud explants from unsprouted rhizomes. Murashige and Skoog medium containing 3.0 mg L^{-1} 6-Benzyladenine (BA) in combination with 1.0 mg L^{-1} indoleacetic acid (IAA) was found to be optimum for the regeneration, multiplication, and *in vitro* maintenance of plantlets. Two-yr-old *ex vitro* grown micropropagated plants were assessed for stable drug-yielding potential through the evaluation of essential oil contents, its phytoconstituents, and antioxidant activity. Gas chromatography and mass spectroscopy (GCMS) analysis of essential oil of rhizome showed the presence of benzoic acid (61.34% and 58.27%), bornyl ester (15.11% and 14.66%), zingiberene (5.15% and 5.74%), and camphor (3.72% and 3.82%) in both micropropagated and conventionally grown *K. rotunda*, respectively. Methanolic extracts and essential oils of the rhizome of both plants possess almost the same antioxidant activity as revealed from DPPH free radical scavenging assay. Micropropagated *K. rotunda* also proved to be genetically stable as revealed by RAPD and ISSR-based molecular profiling. Thus, this study concluded that micropropagation of *Kaempferia rotunda,* an endangered medicinal plant, can be recommended for large-scale commercial production of true-to-type plantlets with stable drug-yielding potential.

Keywords *Kaempferia rotunda* · Tissue culture · Phytomedicinal yield · Molecular profiling

Introduction

Kaempferia rotunda Linn. commonly known as bhuichampa (Hindi) or blackhorm/Peacock ginger (English) belonging to the family *Zingiberaceae*, is a fragrant and rare medicinal herb with rhizomatous root stalks found in various parts of India (Nair 2004). The plant is well known for its different remedial properties (Sereena *et al.* 2011) and is used as one of the traditional cuisine ingredients (Mustafaanand 2014). The leaves and rhizomes are used as vegetables or as a food flavoring spice and are also used in cosmetic powders. The plants are quite effective in the treatment of high blood sugar in diabetic patients (Sereena *et al.* 2011). The plant is used for treating inflammations, gastropathy, wounds, blood clots, tumors, and cancerous swellings (Udayan and Balachandran 2009). *K. rotunda* is enriched with different volatile bioactive constituents like α-pinene, camphene, β-pinene, camphor, linalool oxides, bornyl acetate, benzyl benzoate, and *n*-pentadecane (Table 1) (Xu *et al.* 2012; Ajay 2014).

Increasing unscientific and large-scale drug collection has led to this plant being rooted out of the environment and being considered an endangered species (Rao *et al.* 2003; Mustafaanand 2014). Conventional propagation is also season dependent, and some soil pathogens have a negative impact on the quality of the plant (Mustafaanand 2014). Furthermore, the commercial requirements of the plant and its phytoconstituents for multifarious use have led to the overexploitation and indefensible harvesting of *K. rotunda*, a species whose natural habitat has seen a decline in population over the years (Chen *et al.* 2016). For these reasons, plant tissue culture is preferred over traditional vegetative propagation methods such as splitting rhizomes, which are not sufficient to meet the ever-increasing demand for this plant. Moreover, it is extremely difficult to conserve and store germplasm that is disease-free and of high quality (Nayak 2002; Chan and Thong 2004; Singh *et al.* 2011).

✉ Suprava Sahoo
supi.sos2000@gmail.com

[1] Centre for Biotechnology, Siksha O Anusandhan (Deemed to Be University), Kalinga Nagar, Ghatikia, Bhubaneswar 751003, Odisha, India

Table 1. Major constituents of *Kaempferia rotunda* Linn. reported in various literatures

Compound	Chemical formula	Reference
Benzyl benzoate	$C_{14}H_{12}O_2$	Woerdenbag *et al.* 2004; Sirat *et al.* 2005
n-pentadecane	$C_{15}H_{32}$	Woerdenbag *et al.* 2004; Sirat *et al.* 2005
Camphene	$C_{10}H_{16}$	Woerdenbag *et al.* 2004; Xu *et al.* 2012; Ajay 2014
Camphor	$C_{10}H_{16}O$	Sirat *et al.* 2005; Xu *et al.* 2012
β-pinene	$C_{10}H_{16}$	Xu *et al.* 2012
α-pinene	$C_{10}H_{16}$	Xu *et al.* 2012; Ajay 2014
Linalool oxides	$C_{10}H_{18}O_2$	Xu *et al.* 2012
Endo-borneol	$C_{10}H_{18}O$	Ajay 2014
Dehydroisoandrosterone acetate	$C_{19}H_{28}O_2$	Ajay 2014
Naphthalene, decahydro-1,1,4a-trimethyl-6-methylene-5-(3-methylene-4-pentenyl) [4aS-(4aα,5β,8aα)]	$C_{20}H_{32}$	Ajay 2014
β- phellandrene	$C_{10}H_{16}$	Ajay 2014

These problems can be evaded by biotechnology through plant tissue culture. Despite seasonal changes and weather throughout the year, it is possible to produce countless plantlets from a single explant within a short span of time and limited space under controlled conditions (Amoo *et al.*. 2012). Several plant species can now be propagated and conserved by *in vitro* culture methods in a safe and sustainable way using clonal propagation techniques as an alternative platform. (Behera *et al.* 2018a; Jena *et al.* 2018; Sahoo *et al.* 2020).

Plants regenerated through tissue culture continue to face the challenge of somaclonal variation (Nayak *et al.* 2011). The key component of the commercial micropropagation of *K. rotunda* is maintaining its genetic and phytochemical stability to preserve its original properties. Moreover, it is imperative to assess the genetic fidelity and drug-yielding potential of tissue culture-derived plants. A tissue culture technique can only be commercially utilized when the genetic stability of *in vitro* regenerants would be evaluated. With the advent of DNA marker technology, systematic sampling and analysis of germplasm have become a common practice in recent years (Purohit *et al.* 2017). A wide variety of DNA polymorphism detection methods are available, for example, RFLP (restriction fragment length polymorphism), AFLP (amplified fragment length polymorphism), ISSR (inter simple sequence repeats), and SSR (simple sequence repeats)), but RAPD (random amplified polymorphic DNA) has been used extensively for clonal integrity, the detection of genetic, and somaclonal variations (Agnihotri *et al.* 2009). In general, ISSR markers are used because they have a comparative advantage over RAPD, SSR, and AFLP markers (Giri *et al.* 2012; Virk *et al.* 2000). For this, molecular markers like RAPD and ISSR are generally preferred as they are polymorphic, reproducible, and informative and are commonly used to estimate plant genetic stability (Jena *et al.* 2020). Moreover, several studies are available on the genetic fidelity and phytochemical analysis of other plants of the *Zingiberaceae* family (Mohanty *et al.* 2011; Behera *et al.* 2018b; Jena *et al.* 2018).

Few reports are there on *in vitro* regeneration of *K. rotunda* (Geetha *et al.* 1997; Chirangini *et al.* 2005) but no report is yet available on the genetic and phytochemical stability of micropropagated *K. rotunda* to the best of the present authors' knowledge. The present investigation aimed at developing a reproducible micropropagation protocol for *K. rotunda* and assessing the genetic fidelity of regenerated plants using RAPD and ISSR markers. Phytochemical analysis of the rhizomes of micropropagated plants was also performed by GC–MS and antioxidant activity.

Materials and methods

Plant materials The rhizomes of *K. rotunda* were collected from Kalimpong (27.0594° N, 88.4695° E), West Bengal, and identified by Dr. P. C. Panda, Principal Scientist, Regional Plant Resource Centre (RPRC), Bhubaneswar. The collected rhizomes were planted in the medicinal garden of the Centre for Biotechnology, Siksha O Anusandhan Deemed to be University, Bhubaneswar.

Establishment of *in vitro* culture For the tissue culture experiment, rhizomatic buds were collected and washed thoroughly in tap water in order to remove soil and sand adhered to the rhizomes. The cleaned rhizomes were then washed with liquid detergent (Tween-20, HiMedia, Mumbai, India) for 5 to 8 min. After thorough washing with distilled water, young sprouting buds were used as explant for culture establishment. The explants were surface sterilized with 0.1% mercuric chloride solution (Merck, Mumbai, India) for 3 to 6 min followed by repeated washing with distilled water under aseptic conditions to remove traces of surface sterilant. Then,

the sterilized buds were inoculated on Murashige and Skoog (MS; Murashige and Skoog 1962) medium supplemented with different concentrations and combinations of 1.0 to 3.0 mg L^{-1} benzyladenine (BA; Sigma-Aldrich, St. Louis, MO), 1.0 to 3.0 mg L^{-1} kinetin (Kn) (Sigma-Aldrich), 0.5 to 1.0 mg L^{-1} naphthaleneacetic acid (NAA) (Sigma-Aldrich), 0.5 to 1.0 mg L^{-1} indoleacetic acid (Sigma-Aldrich), 50.0 to 100.0 mg L^{-1} adenine sulphate (Ads) (Sigma-Aldrich), and 30.0 g L^{-1} of sucrose (HiMedia). Media pH was adjusted to 5.7 ± 0.1 and gelled with 0.8% *(w/v)* agar (HiMedia) and autoclaved at 121 °C for 20 min. The culture tubes were kept at 25 ± 1 °C under white fluorescent light with a photoperiod of 16:8 h light/dark cycles in a culture room. The plantlets were subjected to initiation media for the first 2 mo and then into the multiplication media. The regenerated plantlets were sub-cultured to the fresh medium at every 75-d interval. The data on responsive explants were noted after every 2 mo of inoculation.

***Ex vitro* establishment of micropropagated plants** The acclimatization of *K. rotunda* plantlets cultured *in vitro* for 2 yr was performed by taking the plantlets from the tubes, cleaning them properly, and planting them in soil, cow dung, and sand mixture in 1:1:1 ratio and keeping them in the greenhouse. The pots were transferred to the field after 1 mo for complete growth. *In vitro* propagated plants (IVP) and conventionally propagated (CP) plants were compared for different molecular and biochemical characteristics after maturity.

Genetic fidelity analysis Micropropagated plants were studied using 15 ISSR and 25 RAPD primers to determine genetic equality. A step-by-step procedure was followed for extracting genomic DNA from the leaves of CP and IVP hardened *K. rotunda* (annually for 2 yr) using the Doyle and Doyle method (1990). Following RNaseA (GeNei, Bengaluru, India) purification, quantitative and qualitative analysis of total genomic DNA was performed with a spectrophotometer (Thermo Scientific, Waltham, MA) and agarose gel (HiMedia), respectively. The PCR analysis was performed with a mixture consisting of 25.0 ng genomic DNA, 200.0 mM dNTPs (Sigma-Aldrich), 1X assay buffer (with 15.0 mM $MgCl_2$) (SRL, Gurgaon, India), 0.5 U of Taq polymerase (GeNei), and 5.0 pM primers (IDT, Coralville, IA). The PCR program was set as 5 min of initial denaturation at 94 °C following 42 cycles of 1 min denaturation at 94 °C, 45 s of annealing at a particular temperature of primer, and 1 min extension at 72 °C along with final extension at 72 °C for 7 min in a Veriti thermal cycler (Applied Biosystems, Foster City, CA). The amplified DNA was run in 1.5% agarose gel (having 0.5 µg mL^{-1} EtBr). A 100 bp plus DNA ladder was used to estimate the size of the amplification products. To check the reproducibility, all the PCR reactions were performed twice.

Essential oil yield and GC–MS analysis The plants after 2 yr of complete growth in *ex vitro* conditions were taken for oil extraction and GC–MS analysis. The essential oils (EO) from both CP and IVP *K. rotunda* rhizomes were extracted in triplicates *via* hydro-distillation in a Clevenger-type apparatus (Borosil, Mumbai, India). The percentage of the oil yield on a fresh weight basis *(v/w)* was recorded and the oils were dehydrated using anhydrous Na_2SO_4 (Sigma-Aldrich) and stored at − 4 °C until further use.

The chemical profiling of rhizome essential oils was performed using GC–MS. One µL of sample oil was injected into a 6890 series instrument (Agilent Technologies, Palo Alto, Santa Clara, CA) equipped with HP-5 fused silica capillary column using Helium as the carrier gas. The rest of the procedure was carried out by referring to the protocol of Sahoo *et al.* (2020). The detecting compound's mass spectra were compared with the in-house MIST/EPA/NIH mass spectra library (NIST 11). By using straight chain n-alkanes (Sigma Aldrich) under identical operating conditions, the retention index (RI) was determined. Then, the compounds were identified from the NIST library, matching the spectra, and doing an RI comparison from Adams 2007. Ten plants from each group (CP and IVP) were randomly selected for GC–MS analysis.

Preparation of rhizome and leaf extract A comparison was conducted for the total flavonoid and phenolic content as well as the antioxidant activity of the IVP and CP plants. Rhizomes and leaves were then taken from each group (both CP and IVP) for solvent extraction. After air-drying, samples were powdered, subjected to soxhlet extraction (Borosil) for 12 h using methanol (HiMedia), and then filtered, concentrated with rotary evaporators, and stored in the refrigerator until needed.

Evaluation of total phenolic and flavonoid content The Folin-Ciocalteu method was used as described by Sahoo *et al.* (2013) for determining the total phenolic content (TPC) of IVP and CP *K. rotunda* extracts using Gallic acid (Sigma-Aldrich) as standard. Likewise, total flavonoid content (TFC) was determined by aluminium chloride colorimetric method (Sahoo *et al.* 2013) of both plant extracts, and it was done in triplicates. TFC was calculated from the calibration curve of Quercetin (Sigma-Aldrich) and expressed as mg Quercetin equivalent g^{-1} of the extract.

Antioxidant activity DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was used to analyze the radical scavenging activity of both the rhizome EO and extract of *K. rotunda* (both CP and IVP). The analysis was carried out in triplicates using the positive control (ascorbic acid) (Sigma-Aldrich) (Sahoo *et al.* 2014). The methanolic solution of essential oils (1.0, 5.0, 10.0, 20.0, and 30.0 µg mL^{-1}) was mixed with 1.0 mL

of 0.1 mM DPPH (Sigma-Aldrich), then the reaction mixtures were kept in dark for 30 min at room temperature; and finally, absorbance was recorded at 517 nm using UV–visible spectrophotometer (Thermo Scientific). The IC_{50} value of EO was noted.

Statistical analysis Every data set was calculated based on means and standard deviations. All data were analyzed using one-way analysis of variance (ANOVA) in Minitab 17 statistical software (Minitab Inc., State College, PA). After performing an ANOVA, the means were further separated through Tukey's HSD test at $p < 0.5$.

Results and discussion

Establishment of *in vitro* culture Sprouted rhizomatous buds of *K. rotunda* were taken as an explant for *in vitro* plant regeneration. They responded by breaking their outer thick sheath and forming shoot primordium in 7 to 12 d (Fig. 1*a* and *b*) on MS media with 1.0 to 3.0 mg L^{-1} BA, 0.5 to 1.0 mg L^{-1} IAA, 0.5 to 1.0 mg L^{-1} NAA, 1.0 to 3.0 mg L^{-1} Kn, and 50.0 to 100.0 mg L^{-1} Ads (Table 2). The highest percentage of explants forming shoots, including 95.82 ± 1.22 (Table 2), was found in media containing 3.0 mg L^{-1} BA and 0.5 mg L^{-1} IAA. Media containing 3.0 mg L^{-1} BA and 1.0 mg L^{-1} IAA were found to be optimum for *in vitro* shoot multiplication with 84.4 ± 2.1 percentage and was also effective for the highest number of shoots (15.3 ± 1.91) (Fig. 1*c*, *d*, and *e*). This hormonal combination for multiple shoot initiation was also found to be effective in other species of the *Zingiberaceae* family (Bejoy *et al.* 2006; Parida *et al.* 2010; Mohanty *et al.* 2010). Chirangini *et al.* (2005) also reported multiple shoot development in *K. rotunda* when the explants were inoculated with different hormonal combinations of NAA and BAP. According to Geetha *et al.* (1997), the IVP *K. rotunda* plantlets sprouted within 10 d on initiation media containing 0.5 mg L^{-1} Kn and 1.5% sucrose solidified on 0.7% agar, then the established explants were subjected to multiplication media supplemented with either 1.0 mg L^{-1} BA or Kn, which was found to be equally successful for multiple shoot production (Geetha *et al.* 1997). Additionally, among the cytokinin-auxin hormonal combinations used by them, 1.0 mg L^{-1} BA and 0.5 mg L^{-1} NAA showed optimum growth and produced 6 to 7 harvestable shoots (Geetha *et al.* 1997). In the current study, 3.0 mg L^{-1} BA and 1.0 mg L^{-1} IAA were found to be optimum for both shoot multiplication and rooting in *K. rotunda*. Several species of *Zingiberaceae* have reported the effectiveness of BA for shoot regeneration (Parida *et al.* 2010; Rakkimuthu *et al.* 2011).

***Ex vitro* establishment of micropropagated plants** Healthy plantlets having well-developed roots and shoots were removed from culture media and transferred successfully to pots with sterilised soil, cow dung, and sand at a 1:1:1 proportion (Fig. 1(*f*)). These pots were kept under laboratory conditions for 20 to 30 d; and after 30 d, 100 plantlets were then shifted to the greenhouse for *ex vitro* acclimatization. After one mo, the acclimatized plants were transferred to the field condition for establishment, which showed a 90.00% survival rate (Fig. 1(*g*)).

Genetic fidelity analysis A successful *in vitro* propagation method is dependent on plant regeneration and propagation of genetically stable plantlets (Jena *et al.* 2020). IVP plants can show different somaclonal or epigenetic patterns depending on their source of explants, method of regeneration, amount of plant growth substances, particularly

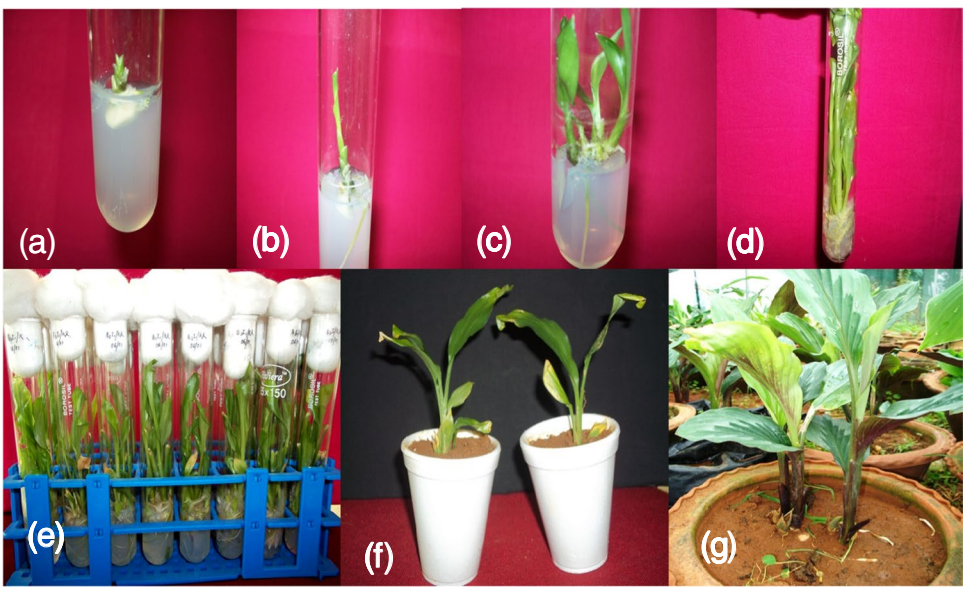

Figure 1. Establishment of tissue culture in *Kaempferia rotunda* Linn.: (*a–e*) explants showing shoot and root initiation and multiplication from rhizome bud on Murashige and Skoog (MS) medium containing 3.0 mg L^{-1} benzyladenine (BA) and 1.0 mg L^{-1} indoleacetic acid (IAA) (*a*) and (*b*) Rhizome explant showing shoot initiation after 7 and 12 d respectively; (*c*) Regeneration of shoots after 20 d; (*d*) Elongation of shoots and roots after subculturing in same medium; (*e*) *in vitro* multiplication of plantlets; (*f*) Potted plants; (*g*) Micropropagated plants of *K. rotunda* growing under field conditions after 2 yr.

Table 2. Effect of different growth regulators on *in vitro* shoot initiation and shoot multiplication of *Kaempferia rotunda* Linn. on Murashige and Skoog (MS) medium

MS medium plus growth regulator (mg L^{-1})	Percentage of shoot initiation (mean ± SD)	Percentage of shoot multiplication (mean ± SD)	Number of shoots per explant (mean ± SD)	Number of roots per explant (mean ± SD)
PGR-free	24.8 ± 0.2 c	10.4 ± 0.2 b	3 ± 2.0 a	1 ± 0.6 a
BA 1	53.6 ± 1.05 h	15.6 ± 1.95 h	6.3 ± 2.06 c	5.2 ± 1.80 b
BA 1 and IAA 0.5	70.78 ± 2.06 de	16.5 ± 1.61 h	6.8 ± 2.60 bc	7.6 ± 2.10 ab
BA 2 and IAA 0.5	61.53 ± 2.05 g	30.6 ± 1.95 g	8.74 ± 2.29 bc	7.9 ± 2.34 ab
BA 3	76.13 ± 2.11 c	73.22 ± 1.74 cd	10.11 ± 2.01 abc	6.6 ± .91 ab
BA 3 and IAA 0.5	**95.82 ± 1.22** a	77.0 ± 1.53 bc	10.5 ± 1.95 abc	8.8 ± 2.00 ab
BA 3 and IAA 1	86.91 ± 2.23 b	**84.4 ± 2.1** a	**15.3 ± 1.91** a	10.5 ± 2.02 ab
Kn 3 and NAA 0.5	71.87 ± 1.99 cde	35.2 ± 1.6 g	8.8 ± 2.53 bc	7.7 ± 2.08 ab
Kn 3 and NAA 1	74.78 ± 1.87 cd	47.0 ± 1.94 f	7.9 ± 1.97 bc	**11.4 ± 0.2** a
BA 3, IAA 1, and Ads 50	74.55 ± 2.27 cd	71.4 ± 2.0 d	9.1 ± 2.20 bc	5.74 ± 2.09 ab
BA 3, IAA 1, and Ads 100	73.84 ± 1.77 cd	75.2 ± 1.91 bcd	12.7 ± 1.78 ab	9.55 ± 2.14 ab
BA 3, Kn 1, and NAA 0.5	67.85 ± 0.92 ef	44.2 ± 1.78 f	8.55 ± 2.46 bc	7.5 ± 2.10 ab
BA 3, Kn 1, and NAA1	60.36 ± 1.5 g	48.0 ± 2.29 f	7.1 ± 1.95 bc	10.4 ± 2.07 ab
BA 3, Kn 2, and IAA 0.5	75.48 ± 0.9 cd	78.22 ± 0.998bc	11.71 ± 0.2 abc	8.64 ± 2.19 ab
BA 3, Kn 2, and IAA 1	77.53 ± 1.5 c	79.50 ± 2.1ab	12.5 ± 1.88 ab	9.98 ± 2.30 ab
BA 3, Kn 3, and NAA 1	63.84 ± 1.87 fg	49.6 ± 2.22 f	10.7 ± 2.19 abc	10.5 ± 2.00 ab
BA 3, Kn 3, and IAA 1	64.97 ± 2.13 fg	56.2 ± 2.17 e	10.2 ± 0.2 abc	9.66 ± 0.22 ab

Each treatment included 20 explants and was repeated three times. Data are shown as mean ± standard deviation (SD) where $n = 3$. Mean with different *letter* in a *column* were significantly different according to Tukey's HSD test at $p < 0.05$. *BA*, benzyladenine; *Kn*, kinetin; *IAA*, indole 3-acetic acid; *NAA*, α-naphthaleneacetic acid

synthetic ones, and duration of culture (Das Bhowmik *et al.* 2016). Genetic analysis of the regenerants is, therefore, necessary to authenticate their clonal stability for commercial purposes. ISSR and RAPD markers were selected as they are cost-effective, non-radioactive, and do not require prior sequence information to amplify DNA (Behera *et al.* 2018a). ISSR and RAPD banding patterns of *in vitro* regenerated *K. rotunda* plants were compared with CP plants at 6-mo intervals up to 2 yr. The analysis included 66 regenerants taking a minimum of 20 plants each time, and the experiments were repeated three times. Among 15 different ISSR primers screened, 10 responded to the amplification of genomic DNA and produced 62 bands ranging from 3 to 10 with an average of 6.2 bands per primer. The DNA bands observed in 66 plantlets were highly monomorphic in nature producing 4092 bands [(no. of bands with all primers) X (no. of plantlets analyzed)]. The highest number of monomorphic bands (10) were observed in primer (GACA)4 (range 400 to 1800 bp) and the lowest number of monomorphic bands (3) in primer (GTGC)4 (range 380 to 1250 bp) and primer (AGG)6 (range 350 to 850 bp). Fifteen RAPD primers generated 47 distinct and scorable bands ranging from 300 to 1900 bp with 5.2 bands per primer on average. There were 3102 bands [(no. of bands with all primers) X (no. of plantlets analyzed)] generated, primer A10 produced the highest number of bands (8 bands from 480 to 1400 bp), and primer N6 (900 bp) generated the lowest. ISSR and RAPD banding pattern were shown in Fig. 2*b* and *c* with T(GA)9 and D20 primers, respectively. After 2 yr of culture, no polymorphisms were found in the micropropagated plants of *K. rotunda* by RAPD and ISSR markers, and the analysis showed a profile similar to the control indicating that genetic variation did not occur *in vitro*.

The genetic stability analysis of *in vitro* raised *K. rotunda* was not available to the best of the present authors' knowledge, so the present study focused on the fidelity analysis of the regenerated plants using RAPD and ISSR markers. The result obtained showed a profile similar to the control plant indicating that *in vitro* regenerants were true-to-type clones. *K. rotunda* clones have been shown to be genetically stable

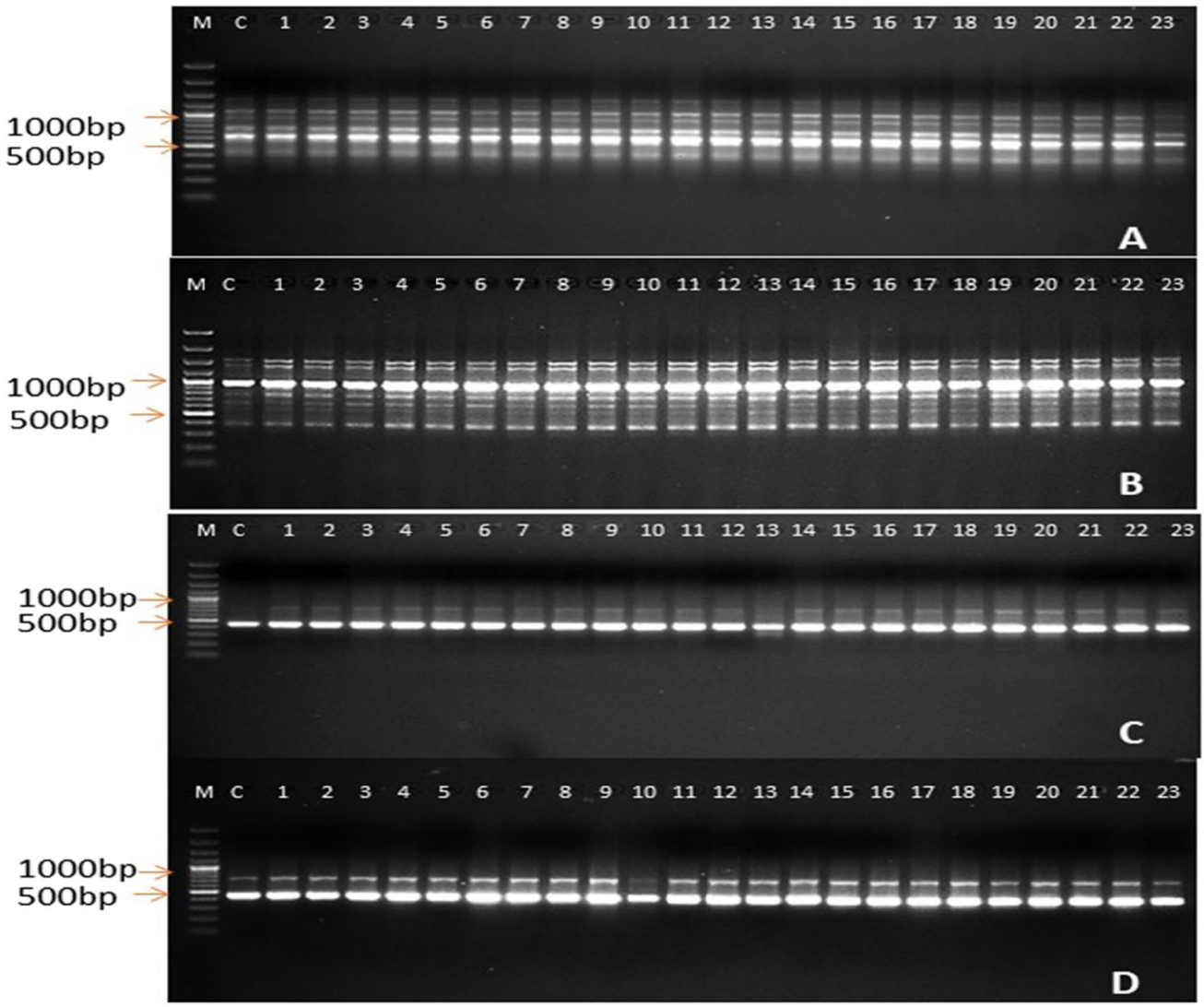

Figure 2. ISSR (inter simple sequence repeats) banding pattern with primer T(GA)9 (400–1500 bp) (*A*) and primer (GA)9 T (400–1450 bp) (*B*), RAPD (Random Amplified Polymorphic DNA) banding pattern with primer D20 (450–750 bp) (*C*), and primer D18 (400–1550 bp) (*D*) (M:Marker, C:Control, *Lane 1–23*:micropropagated plants). The genetic fidelity of micropropagated *Kaempferia rotunda* Linn. was analysed at every 6-mo intervals for up to 2 yr of culture.

Table 3. ISSR (inter simple sequence repeats) banding pattern of micropropagated and field-grown mother plants of *Kaempferia rotunda* Linn

Primer	Sequence	Total bands	Melting temperature of primers	Range of amplicons [bp]
(CAA)5	CAACAACAACAACAA	7	40°	450–1300
(GGA)4	GGAGGAGGAGGA	4	40°	300–1250
(GA)9 T	GAGAGAGAGAGAGAGAGAT	8	56°	400–1450
(GTGC)4	GTGCGTGCGTGCGTGC	3	56°	380–1250
(GTG)5	GTGGTGGTGGTGGTG	5	50°	400–1100
(GAC)5	GACGACGACGACGAC	9	50°	450–1400
(AGG)6	AGGAGGAGGAGGAGGAGG	3	60°	350–850
(GACA)4	GACAGACAGACAGACA	10	48°	400–1800
T(GA)9	TGAGAGAGAGAGAGAGAGA	6	56°	400–1500
ISSR 15	TATATATATATATATAG	7	36°	390–1350
Total			**62**	

Table 4. RAPD (random amplified polymorphic DNA) banding pattern of micropropagated and field-grown mother plants of *Kaempferia rotunda* Linn

Primer	Sequence	Total bands	Melting temperature of primers	Range of amplicons [bp]
A10	GTGATC GCAG	8	28°	480–1400
A11	CAATCG CCGT	7	32°	300–1700
A18	AGGTGA CCGT	5	32°	1050
D8	GTGTGC CCCA	4	34°	450–1200
A20	GTTGCG ATCC	7	32°	550–1300
D18	GAGAGC CAAC	6	32°	400–1550
D20	ACCCGG TCAC	2	34°	450–750
N6	GAGACG CACA	1	32°	900
N16	AAGCGA CCTG	7	28°	500–1900
Total			**47**	

for the first time in the present report. Moreover, combining two types of markers amplifying different regions of the genome has proved to be a precise way to evaluate genetic stability (Ray *et al.* 2006; Venkatachalam *et al.* 2007). Panda *et al.* (2007) and Tyagi *et al.* (2007) also studied genetic stability through RAPD markers of *in vitro* grown turmeric plants. The genetic stability of micropropagated plants was shown by 10 ISSR and 9 RAPD markers in the present study (Tables 3 and 4), which is in close agreement with the results of Mohanty *et al.* (2010) in *Curcuma caesia*. The genetic integrity using ISSR and RAPD markers was studied in many species of the *Zingiberaceae* family like *A. calcarata* (Das Bhowmik *et al.* 2016), *A. galanga* (Sahoo *et al.* 2020), *A. subulatum* (Purohit *et al.* 2017), *C. caesia* (Mohanty *et al.* 2010), *C. longa* (Pittampalli *et al.* 2022), and *C. zedoaria* (Jena *et al.* 2020). Similarly, Mohanty *et al.* (2008) reported that micropropagated ginger with over 2 yr of cultivation did not exhibit a decrease in genetic stability, which is in close agreement with the present study's findings.

GC–MS analysis The biochemical stability of essential oil from the rhizome of both the CP and IVP plants was estimated by analyzing chemical constituents through GC–MS. The essential oil yield from CP and IVP plants obtained through hydrodistillation using the Clevenger apparatus was 0.15% and 0.2% (*v/w*), respectively. Significantly, a total of 9 phytoconstituents were identified in CP and IVP plants with an area percentage of 96.81% and 98.00%, respectively, of the total peak area. The maximum peak area was found to be of benzoic acid (58.27 ± 0.45%; 61.34 ± 0.49%), followed by bornyl ester (14.66 ± 0.36%; 15.11 ± 0.39%) and zingiberene (5.74 ± 0.35%; 5.15 ± 0.33%) in both CP and IVP plants, respectively (Table 5) (Fig. 3). The phytochemical composition of *in vitro* propagated *K. rotunda* was comparably stable and similar to that of the corresponding CP plants. The present study's findings are in close agreement with the studies of Woerdenbag *et al.* (2004), Jamalluddin (2014), and Feng (2009), who reported benzyl benzoate as the chief component. Additionally, contrary to the present study's report, Sirat *et al.* (2005) and Ajay (2014) demonstrated pentadecane and endo-borneol, respectively, as the major component. Furthermore, the EO yields and constituents of CP and IVP plants are quite similar. A previous study by Jena et al (2020) also found a similarity in chemical composition in leaf and rhizome oil of IVP and CP plants

Table 5. Chemical composition of rhizome essential oil of *Kaempferia rotunda* Linn

Compound name	Chemical formulae	Area % mean ± SD mother plant (CP)	Area % mean ± SD *in vitro* propagated plant (IVP)	Retention time
β –Myrcene	$C_{10}H_{16}$	3.89 ± 0.15 d	3.56 ± 0.15 d	4.658
Camphor	$C_{10}H_{16}O$	3.82 ± 0.2 d	3.72 ± 0.01 d	9.026
Bornyl ester	$C_{12}H_{20}O_2$	14.66 ± 0.36 b	15.11 ± 0.39 b	14.489
Curcumene	$C_{15}H_{22}$	1.80 ± 0.29 f	1.2 ± 0.2 f	22.139
Zingiberene	$C_{15}H_{24}$	5.74 ± 0.35 c	5.15 ± 0.33 c	22.701
Pentadecane	$C_{15}H_{32}$	2.00 ± 0.26 ef	1.97 ± 0.24 ef	22.976
Amorphene	$C_{15}H_{24}$	3.85 ± 0.21 d	3.17 ± 0.15 d	23.234
β- Sesquiphellandrene	$C_{15}H_{24}$	2.78 ± 0.2 e	2.78 ± 0.2 e	23.716
Benzoic acid	C_6H_5COOH	**58.27 ± 0.45** a	61.34 ± 0.49 a	32.939

Data are shown as mean ± standard deviation (SD) where $n = 3$. Mean with different *letter* in a *column* were significantly different according to Tukey's HSD test at $p < 0.05$. Rhizome essential oils were extracted from both mother plants and *in-vitro* grown plants of *K. rotunda*

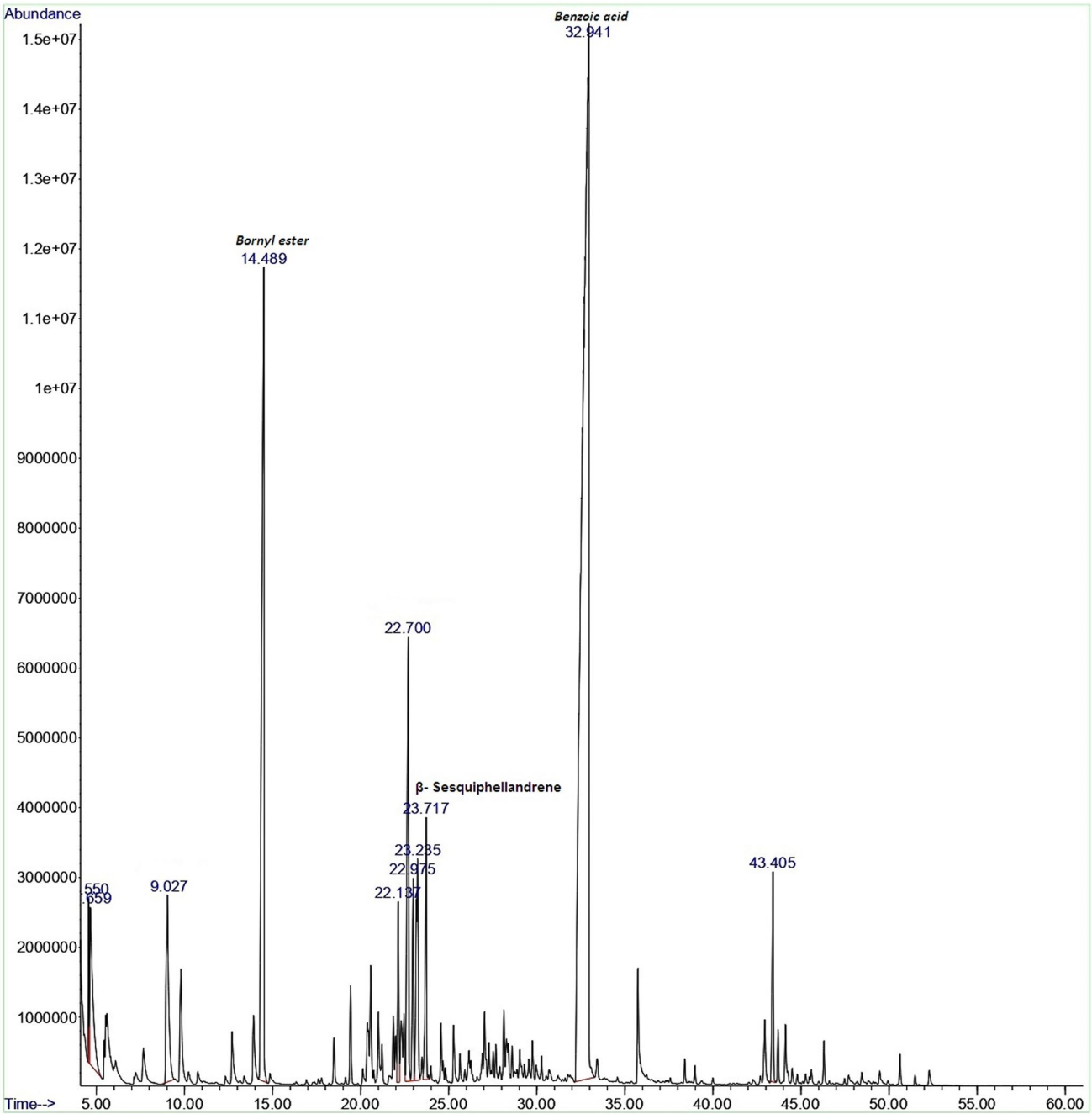

Figure 3. Gas chromatography–mass spectrometry chromatogram of *Kaempferia rotunda* Linn. rhizome oil detecting various volatile constituents.

of *Curcuma zedoaria*, which supports this present study's result. Likewise, leaf and rhizome oil both from CP and its IVP *C. longa* plants have homogeneous chemical compositions (Nayak et al. 2011; Singh *et al.* 2011). The yield of essential oils and benzoic acid contents as well as other identified chemical compounds in oils were not significantly different between CP and IVP plants of *K. rotunda*, which evidences their stability in terms of drug-yielding potential. Prior to supplying tissue culture plants to markets, farmers, or industrial users, the phytochemical composition must be determined.

Total flavonoid and phenolic contents The phenols and flavonoids of plants are the secondary metabolites responsible for a variety of pharmacological effects mainly as natural antioxidants in terms of their ability as radical scavengers (Wink 2015). In this present study, the extracts of both CP and IVP plants of *K. rotunda* were subjected

to TPC and TFC evaluation, and they possessed an appreciable amount of phenolic and flavonoid content. The maximum amount of phenolics was found in the IVP leaf (51.38 ± 1.03), followed by the CP leaf (48.23 ± 0.25), IVP rhizome (32.83 ± 0.15), and CP rhizome (30.5 ± 0.23) mg (gallic acid equivalent) of the extract (Table 6). Correspondingly, high flavonoid content was possessed by IVP leaf and rhizome, including 65.83 ± 1.17 and 38.81 ± 0.62 mg quercetin equivalent g^{-1} of extract, respectively (Table 6). The above findings indicate that as compared to rhizomes, leaves were found to contain significantly more phenolic and flavonoid content. However, the TPC and TFC values of CP and IVP plants had no noteworthy differences. Parenthetically, it was observed that the micropropagated plants displayed slightly higher TPC and TFC values as compared to the source plants. A similar finding has been made in the reports by Behera *et al.* (2019) and Sahoo *et al.* (2020).

Antioxidant activity Free radical scavenging activity was measured using a DPPH assay that showed noticeable antioxidant activity in the samples when compared with Ascorbic acid, which was taken as standard. DPPH free radical assay is an easy, rapid, and sensitive method for measuring the antioxidant activity of a specific compound or plant extract (Sahoo *et al.* 2014). The obtained results revealed that higher DPPH scavenging activity was found in essential oil in contrast to plant extract. The IC_{50} values of ascorbic acid and rhizome oil of CP and IVP plants were found at a concentration of 5.29 µg mL^{-1}, 25.45 µg mL^{-1}, and 9.98 µg mL^{-1}, respectively. Parenthetically, at a concentration of 28.98 µg mL^{-1} and 25.96 µg mL^{-1}, the IC_{50} values of CP and IVP rhizome extracts were observed while the leaf extracts of CP and IVP plants recorded the IC_{50} value at a concentration of 33.42 µg mL^{-1} and 22.44 µg mL^{-1}. It was found that *K. rotunda* had a dose-dependent DPPH activity (Fig. 4(*A*), (*B*)). The rhizome oils of IVP plants showed higher antioxidant activity almost equivalent to the standard taken. It was particularly noted that there is a variation in antioxidant activity among different plant parts (like leaves and rhizomes) as well as different samples (oils and extracts); but from the analysis of oils, the antioxidant potential of IVP plants of both the samples was higher to that of CP plants. There has been a similar finding in reports published by Behera *et al.* (2022) on *C. amada* and Sahoo *et al.* (2020) on *A. galanga*. Prior to using IVP medicinal plants as a replacement source for mother plants, the antioxidant activity must be validated.

Table 6. Total phenolic content (TPC) and total flavonoid content (TFC) of both conventionally propagated and *in vitro* propagated plants of *Kaempferia rotunda* Linn

Plant parts extract	TPC	TFC
Conventionally propagated leaf	48.23 ± 0.25 a	58.94 ± 0.36 a
In vitro propagated leaf	51.38 ± 1.03 a	65.83 ± 1.17 a
Conventionally propagated rhizome	30.5 ± 0.23 b	32.15 ± 0.32 b
In vitro propagated rhizome	32.83 ± 0.15 b	38.81 ± 0.62 b

Data are shown as Mean ± standard deviation (SD) where $n = 3$. Mean with different *letter* in a *column* were significantly different according to Tukey's HSD test at $p < 0.05$

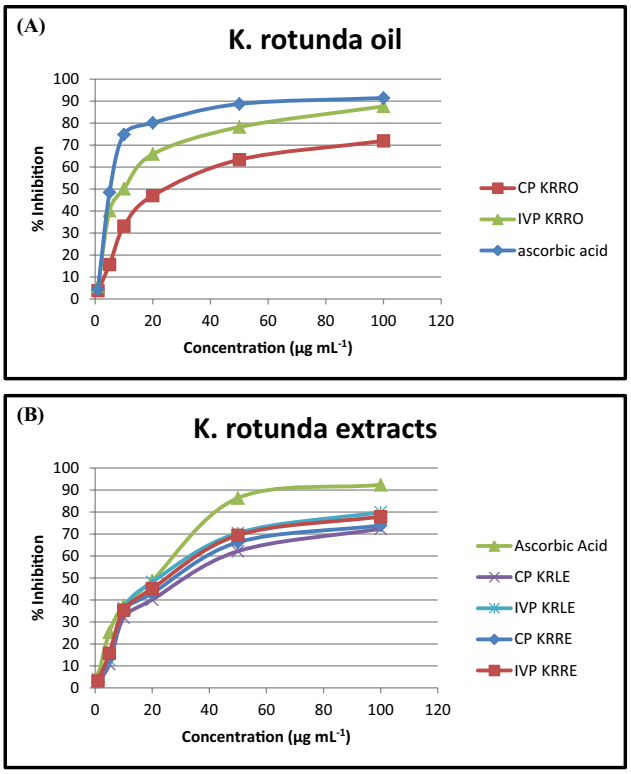

Figure 4. DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity of essential oils (*A*) and methanolic extracts (*B*) of both CP and IVP *Kaempferia rotunda* Linn.

Conclusion

An efficient *in vitro* propagation protocol has been established for *K. rotunda* using axillary buds as an explant. Among various MS media containing different hormonal concentrations tried, media having 3.0 mg L^{-1} BA with 1.0 mg L^{-1} IAA were found to be optimum for shoot multiplication. Genetic fidelity was confirmed in micropropagated plants using ISSR and RAPD prior to the production of uniform regenerants for commercial planting. The phytochemical uniformity of the IVP plants was proved using GC–MS analysis as well as antioxidant activity. Thus, this protocol can be useful for producing large numbers of stable plants, which can be conserved and used for commercial purposes in the food, beverage, and pharmaceutical industries.

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Declarations

Conflict of interest The authors declare no competing interests.

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