**In vitro regeneration of *Piper longum* L. and comparative RP-HPLC analysis of piperine production of in vitro and in vivo grown plants**

Mousumi Chatterjee, Sabyasachi Chatterjee, Indrani Chandra

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**Abstract**

*Piper longum* L. is a well known spice plant belonging to the family Piperaceae with high pharmacognosy potential, but it is becoming threatened due to overexploitation. Thus, this investigation aims to standardize a cost effective protocol for in vitro propagation of this economically important plant. Internodal segments were used as explant for callogenesis in Murashige and Skoog medium with 3% sucrose and 0.8% agar, with NAA or 2,4 D. Optimum callus induction was observed in MS medium with 5.0 mg/L NAA. Calli were subcultured on shoot regeneration media containing different concentrations of cytokinin (KIN/BAP) along with 0.1 mg/L NAA. Best shoot regeneration was obtained on MS media supplemented with 2.0 mg/L KIN and 0.1 mg/L NAA. Induced shoots were rooted in either NAA or IBA and highest rooting was induced in MS medium enriched with 0.5 mg/L NAA. Rooted plantlets were acclimatized and 88% of hardened plants survived. Field emission scanning electron microscopic showed that regeneration from callus had occurred by somatic embryogenesis. A comparative study on identification and quantification of piperine (the chief alkaloid of the cultivar) were done from root and fruit of both in vitro and in vivo grown plants through Reverse Phase-High Performance Liquid Chromatography method. In vitro grown fruit was found to have the maximum amount of piperine.

**Key message**

For the first time, a comparative study in identification and quantification of piperine was done from both in vitro and in vivo grown *Piper longum* through RP-HPLC method.

**Keywords**

Pharmacognosy · In vitro propagation · Callogenesis · FE-SEM · RP-HPLC

**Abbreviations**

- **MS**: Murashige and Skoog
- **NAA**: 1-Naphthaleneacetic acid
- **2,4-D**: 2,4-Dichlorophenoxyacetic acid
- **KIN**: Kinetin
- **BAP**: N6-Benzylaminopurine
- **IBA**: Indole-3-butyric acid
- **mg/L**: Milligram/Litre
- **FE-SEM**: Field emission scanning electron microscope
- **RP-HPLC**: Reverse Phase-High Performance Liquid Chromatography
- **Rt**: Retention time
- **ANOVA**: Analysis of variance

**Introduction**

Archaeological evidences indicate the use of plants as medicine since 5000 years ago, including Egyptians, Mesopotamians, and ancient Indian Ayurveda. *Piper longum* L., the close relative of black pepper (*Piper nigrum*), commonly known as ‘Indian long pepper’ (India Biodiversity Portal), belongs to the family Piperaceae. This flowering vine is widely distributed in the tropical and sub tropical regions of the world (Kumar et al. 2016; Satyavati et al. 1987), where it is cultivated for its fruit which is used as spice from the fifth or sixth century BCE by Greeks and romans (www.gosumitup.com), while the ancient textbook of Ayurveda also refers its medicinal and dietary use.
This spice is generally used for seasoning and contains various bioactive compounds with a broad range of medicinal properties (Kumar et al. 2011; Yadav et al. 2020). A number of previous works (Kanaki et al. 2008; Kumar et al. 2011) have suggested that Piperine (C17H19O3N) is one of the major alkaloids, which is efficient to exhibit various pharmacological activities and responsible for its spiciness too (Kumar et al. 2011).

*P. longum* can be multiplied naturally via seed germination or by vegetative propagation. However, conventional seed germination cannot be easily accomplished due to the low availability of viable seeds, delayed and prolonged germination period (Pradhan 2015; Rani and Dantu 2012), while scanty rooting is a great constraint of vegetative propagation of the plant. The plant has been included in IUCN Red list of threatened medicinal plants of India under medicine for human and veterinary group (Gowthami et al. 2021). High economic value, rare commercial availability of long pepper (www.ayushveda.com) and its existential threat (Nair 2000) have created an immediate need for establishing suitable cost effective protocol for micropropagation.

In vitro callogenesis and indirect clonal propagation provide feasible and efficient methods of recovery of secondary metabolites throughout the year without seasonal variations (Isah et al. 2018). Huge amounts of callus can be induced for quantitative enhancement of potent bioactive compounds and their commercialization, and large numbers of plantlets can be produced in short span of time to meet up the increasing market demand of the plants. There is some available literature on in vitro propagation of *P. longum* (Bhat et al. 1992; Sarasan et al. 1993; Soniya and Das 2002; Parida and Dhal 2011; Rani and Dantu 2012; Pradhan 2015; Ravindaran et al. 2016; Sathelly 2016; Saravanan 2019; Prajapati et al. 2019). However, the indirect regeneration of the plant with a study of callus morphology was seldom documented, in particular with confirmation of the regeneration pathway through field emission scanning electron microscope (FE-SEM), even in literature, very less efforts have been found on standardization of efficient, cost effective protocol for indirect regeneration from callus. Moreover, there are no reports on the comparative profiling as well as quantification of the principal bioactive alkaloid Piperine, through Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) in parts of in vitro and in vivo grown plants. This research sets out to fill in those gaps.

**Materials and methods**

**Collection of explants**

The plant material (monoecious female) was collected in June 2019 from the Haripal (Located: 22° 49’ 53’’ N 88° 7’ 7’’ E; altitude: 16 m (52ft)) Hooghly District, W.B, India. Fresh rooted shoots of *P. longum* were collected and maintained in the garden of Dept. of Biotechnology, The University of Burdwan, WB, India. The material was confirmed by Botanical Survey of India, Kolkata (Supplementary Certificate 1: Voucher specimen no MC-01) and used as source of explants for the in vitro regeneration.

**Sterilization of explant and culture establishment for callus induction**

Tender twigs with 4–5 nodes were excised from stock plants on March to avoid mealy bug attack, and were washed thoroughly in running tap water for 10 min. Twigs were then treated with 70% (v/v) ethyl alcohol for 30 s, followed by immersing in 0.01% mild detergent, Tween-20 (Merck, USA) for 6 min prior to explant treatment with 0.1% (w/v) HgCl2 (Merck, USA) for 2 min. Finally, explants were rinsed with sterile distilled water (Milli-Q water system, Merck Millipore, USA) thrice to wash out all used chemicals and were blotted dry on sterile filter paper. All procedures were done in sterile conditions (Biosafety Cabinet A2, Biobase Inc., China). Internodal segments (approx. 1 cm long) were excised and inoculated in MS (Murashige and Skoog 1962) basal medium (Himedia, India), fortified with NAA (0.1 mg/L, 0.5 mg/L, 1.0 mg/L, 2.0 mg/L, 3.0 mg/L, 4.0 mg/L, 5.0 mg/L) and 2,4-D (0.1 mg/L, 0.5 mg/L, 1.0 mg/L, 2.0 mg/L, 3.0 mg/L, 4.0 mg/L, 5.0 mg/L, 2.0 mg/L, 3.0 mg/L, 4.0 mg/L, 5.0 mg/L). Cultures were incubated in a growth chamber (Thermo Fisher Scientific, USA) at 25 ± 2 °C with a 16 h photoperiod (2000 lx intensity) and 65% relative humidity. All media were done in ten replicates and the experiment was repeated thrice under the same cultural conditions. Data of callus induction frequency (%) and fresh weight were recorded after 28 days of culture.

**Shoot and root regeneration**

Induced calli with shoot buds were transferred in shoot regeneration medium containing 0.5 mg/L, 1.0 mg/L, 2.0 mg/L, 3.0 mg/L, 4.0 mg/L, 5.0 mg/L BAP or 0.5 mg/L, 1.0 mg/L, 2.0 mg/L, 3.0 mg/L, 4.0 mg/L, 5.0 mg/L KIN in combination with 0.1 mg/L NAA. After 25 days, data were recorded for statistical analysis and further, regenerated shootlets were transferred onto rooting media supplemented with 0.1–1.0 mg/L NAA or 0.1–1.0 mg/L IBA to study the effect of different auxins in rhizogenesis. After 10 days of culture in rooting media, data were collected for statistical analysis. Ten replicates were studied for each culture condition and experiments were repeated thrice under the same physical and chemical environment.
Hardening of in vitro grown plantlets

Complete plantlets were hardened in plastic bags with sterilized sand:soil (1:1) for the first 18 days, with supplementation of half strength MS nutrient broth and watering done with sterile distilled water (maintained at 25 ± 2 °C and 65% relative humidity). Thenceforth, acclimatized plantlets were transferred into a second plastic bag containing garden soil:sand:organic manure (1:1:1) and maintained for 10 more days. Finally, acclimatized plantlets were transferred into earthen pots and shifted to the polyhouse (Dept. of Biotechnology, BU, Pin: 713104, WB, India).

Sample preparation for FE-SEM

Regenerated callus (28 days old) was fixed in 2.5% solution of glutaraldehyde with Phosphate buffer 0.2 M (pH 7.0) at 4 °C for 4 h. The specimens were washed in the same buffer (20 min) and then dehydrated in increasing alcohol concentrations (20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%), followed by absolute ethanol, for 15 min in each solution. After the alcoholic dehydration, specimens were subjected to a series of absolute ethanol and pure iso-amyl acetate mixture (1:3, 2:2, 3:1 and then pure iso-amyl acetate) before Critical point drying (CPD) and then dried to critical point with liquid CO₂. Then, stub was prepared by coating with gold sputter for 40 s and observed under FE-SEM (Carl Zeiss Gemini 300).

Statistical analysis

All experiments included ten replicates and were repeated thrice. All collected data (fresh callus weight, number of shoots per explant, shoot length, number of leaves per plantlets and number of roots per plantlets) were recorded after regular intervals as outlined above. The mean ± SE of all data were statistically evaluated by one way analysis of variance (ANOVA) followed by Duncan’s multiple range test at p < 0.05, performed by the IBM SPSS software.

Sample concentration = \( \frac{\text{Sample area}}{\text{Mean standard area}} \times \frac{\text{Standard weight}}{\text{Standard dilution}} \times \frac{\text{Sample dilution}}{\text{Sample weight}} \)

Sample preparation and analysis of piperine through RP-HPLC

Piperine was extracted and samples were prepared with some modification as reported by Santosh et al. (2005). Fresh in vitro cultured callus, roots and matured dry fruits were collected from both in vitro and in vivo grown plants, washed thoroughly under tap water and shed dried for 72 h. Dried samples were then crushed and ground into fine powder by using porcelain mortar and pestle. Each powdered sample (10 × 10³ mg) was refluxed with 500 ml of HPLC grade methanol (Merck, USA) for 5 h in Soxhlet apparatus. The sample-solvent mixture was then allowed to cool and filtered to remove solvent. Reflux was performed again by using fresh methanol (500 ml) for another 4 h. The methanolic extract was then concentrated in a rotary evaporator (Biobase Inc, China) at 70 °C followed by lyophilizer drying (Eyela Inc, Japan). Each sample (1 mg) was resuspended in 20 ml of 100% HPLC grade methanol (Merck, USA), homogenized for 10 min and subjected to ultrasonication for 45 min. Extracts were then filtered subsequently by using Whatman No1 filter paper (Merck,USA) and membrane polytetrafluoroethylene syringe filter (PTFE with 0.22 µm porocity; Himedia, India). Chromatographic analysis was done by using a RP-HPLC system (equipped with 5160 quaterary gradient pump and 5420 UV–Vis detector, Model: Chromster, Make: Hitachi Corporation, Japan). The isocratic solvent system of methanol and water (80:20) was used at a flow rate of 1 ml/min. The detecting wave length and total run time was set at 343 nm and 20 min respectively. The sample was injected manually in a manual injector port (20 µl for each sample). The reverse phase C₁₈ packed column (5C₁₈-MS-II, 4.6ID × 250 mm, COSMOSIL) was used for the HPLC analysis. Chromatograms were obtained from individual running of prepared samples (in vivo and in vitro root and fruit) and standard piperine (Sigma Aldrich, USA, Catalogue No. 75047-50 mg) were analyzed. The following formula was used (Siva et al. 2015) for quantification of piperine.

\[ \text{Sample concentration} = \frac{\text{Sample area}}{\text{Mean standard area}} \times \frac{\text{Standard weight}}{\text{Standard dilution}} \times \frac{\text{Sample dilution}}{\text{Sample weight}} \]
Results and discussion

Induction of callus

Callus induction was obtained in MS media fortified with variable concentrations of NAA (0.1 mg/L, 0.5 mg/L, 1.0 mg/L, 2.0 mg/L, 3.0 mg/L, 4.0 mg/L, 5.0 mg/L) or 2,4D (0.1 mg/L, 0.5 mg/L, 1.0 mg/L, 2.0 mg/L, 3.0 mg/L, 4.0 mg/L, 5.0 mg/L). After 28 days, three different types of callus were observed: compact, loosely clustered friable, transparent (Supplementary Table 1).

Callus induction frequency was better (Fig. 2a, b) in both the ranges of higher (3.0–5.0 mg/L) and lower (0.1–2.0 mg/L) concentrations of NAA rather than with 2,4-D. Callus induction frequency was maximum (90–98%) in 0.1–5.0 mg/L NAA with green, regular, compact, organogenic callus (Fig. 1a; Supplementary Table 1). This type of calli were called embryogenic calli as in a previous report (Na et al. 2007). Conversely, callus induction was reduced (25–38%) to moderate (45–50%) in media with of 2,4-D, and such calli were brownish, often transparent (Supplementary Fig. 1; Table 1), and non embryogenic (Na et al. 2007). Hence, NAA was more efficient for induction of embryogenic callus in comparison to 2,4 D in *P. longum*.

It had been observed that fresh weight of callus was directly proportional with NAA concentration. Highest mass of callus was obtained in MS media supplemented with 5.0 mg/L of NAA (Supplementary Table 1; Fig. 2) but mean difference was significantly different in MS medium containing 2,4 D when tested p < 0.05 level during one way ANOVA followed by Duncan’s multiple range test.

In previous report (Sarasan et al. 1993), profuse nodular callusing was found at 2.0 mg/L 2,4-D + 1.0 mg/L BA. Sathelly et al. (2016) observed appearance of globular compact callus in MS medium with a combination of auxin and cytokinin (1.0 mg/L IAA + 1.0 mg/L BAP) but in current study, callus was induced only in presence of auxin (2,4-D/NAA). Among those two used auxin, NAA proved to be more efficient. 2,4-D showed ill response (Supplementary

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Fig. 1 Stereomicrograph of tissue culture stages of *Piper longum* L. a Embryogenic callus in MS media containing NAA (5.0 mg/L). b Shoot regeneration in MS media containing 2 mg/L Kinetin + 0.1 mg/L NAA. c root induction(0.5 mg/L). d Hardened plantlets. e Flowering and fruiting in in vitro grown plant

Fig. 2 Callus weight (g) at different concentrations (mg/L) of a NAA and b 2,4-D
In vitro shoot regeneration

Observations were done for evaluating shoot number, shoot length, leaf number. The best result was observed at 2.0 mg/L Kinetin + 0.1 mg/L NAA concentrations in MS media (Table 1; Fig. 1b; Supplementary Fig. 2b) which was found to elicit optimal response with an average 18.46 ± 0.51 shoot bud induction, 8.06 ± 0.24 cm shoot length and 33.06 ± 0.38 leaf number (Table 1). It was also observed that ascending PGR concentration induced yellowish green colored succulent leaf with stunted shoot growth that seemed phenotypically different from in vivo grown plant.

In previous report (Sathelly et al. 2016), it was stated that media with individual kinetin showed lowest response and the combination of two cytokinin (BAP and Kinetin) showed highest percentage of shoot induction. But in this study, proved that only one cytokinin (KIN) is efficient to induce maximum number of shoot per explant which is also cost effective.

In vitro root induction

Initiation of rooting was started after 10 days on medium containing NAA. Control culture showed delayed rooting. Response of root inducing growth regulators were tabulated (Table 2). Though all cultures induced root in presence of auxin but best root induction was observed at 0.5 mg/L NAA (Fig. 1c; Supplementary Fig. 3) with an effective mean root number 33.33 ± 0.42. The control culture also induced root in a longer time, 45 days.

Data on root induction was recorded in Table 2, which do not imply similarity with previously reported works (Sathelly et al. 2016; Prajapati et al. 2019). In those literature, root induction was found on MS medium fortified with 1 mg/L NAA and 1.0 mg/L IBA respectively but in this present study, maximum number of roots were generated in MS media enriched with 0.5 mg/L NAA alone.

Hardening of plantlets

Primary hardening, followed by secondary hardening (Fig. 1d) was accomplished with 88% of success rate. After proper maintenance, growth and development of the plants were monitored carefully. After 4 months of accomplishment of hardening, flowering of the in vitro grown plant was observed in the month of July and August and fruiting started within a month, on September(Fig. 1e). So, no changes were found in the reproductive and harvesting

### Table 1 Response of PGRs for shoot regeneration after 25 days

| NAA (mg/l) | BAP (mg/L) | KIN (mg/L) | Number of shoots/explant (Mean ± SE) | Shoot length (cm) (Mean ± SE) | Number of leaf (Mean ± SE) |
|------------|------------|------------|-------------------------------------|------------------------------|-----------------------------|
| 0.1        | 0.5        | –          | 09.40 ± 0.42ef                      | 3.33 ± 0.10f                 | 15.93 ± 0.94b               |
| 0.1        | 1.0        | –          | 15.00 ± 0.04ab                      | 4.79 ± 0.17e                 | 25.53 ± 0.23cd              |
| 0.1        | 2.0        | –          | 12.73 ± 0.37cd                      | 3.20 ± 0.14d                 | 19.13 ± 0.43cf              |
| 0.1        | 3.0        | –          | 10.46 ± 0.24d                       | 3.03 ± 0.14d                 | 17.80 ± 0.30gb              |
| 0.1        | 4.0        | –          | 09.13 ± 0.31f                       | 2.72 ± 0.15d                 | 16.26 ± 0.85gb              |
| 0.1        | 5.0        | –          | 08.60 ± 0.50f                       | 2.35 ± 0.13d                 | 15.26 ± 0.77j               |
| 0.1        | –          | 0.5        | 10.06 ± 0.31d                       | 4.40 ± 0.26f                 | 18.53 ± 0.83ef              |
| 0.1        | –          | 1.0        | 12.26 ± 0.37d                       | 5.46 ± 0.39d                 | 22.80 ± 0.63c               |
| 0.1        | –          | 2.0        | 18.46 ± 0.51f                       | 8.06 ± 0.24a                 | 33.06 ± 0.38d              |
| 0.1        | –          | 3.0        | 15.60 ± 0.50f                       | 6.46 ± 0.15d                 | 29.33 ± 0.22b               |
| 0.1        | –          | 4.0        | 14.06 ± 0.44f                       | 5.53 ± 0.14d                 | 27.80 ± 0.92c               |
| 0.1        | –          | 5.0        | 11.06 ± 0.37f                       | 4.80 ± 0.21e                 | 24.60 ± 0.51cd              |

### Table 2 Response of root induction at various concentrations of NAA and IBA after 10 days

| NAA (mg/L) | IBA (mg/L) | Number of roots (Mean ± SE) |
|------------|------------|-----------------------------|
| 0.1        | –          | 14.20 ± 0.37c               |
| 0.5        | –          | 33.33 ± 0.42a               |
| 1.0        | –          | 25.00 ± 0.67b               |
| –          | 0.1        | 09.40 ± 0.40ef              |
| –          | 0.5        | 11.20 ± 0.37de              |
| –          | 1.0        | 08.46 ± 0.24ef              |

Values were represented by (Mean ± SE) of ten replicates per treatment with three times repetition. Values denoted by same letter were not significantly different (p < 0.05) using Duncan’s multiple range test. Here S.E represents standard error of mean.
season (India Biodiversity Portal; Vikaspedia) of in vitro propagated plants.

Few literatures of clonal propagation of this cultivar were reported previously, but all studies were documented only upto the direct or indirect propagation and no one had tried to observe the season of flowering and fruiting of the in vitro grown plants. From this study it had been demonstrated that profuse amount of *P. longum* plantlets can be obtained by following this cost effective process.

**SEM analysis**

Under the SEM, embryogenic calli showed various stages of somatic embryos such as heart (HS) and globular (GS) shaped structures (Fig. 3a) and scutellum (SC) associated with coleoptile (CO) (Fig. 3b) over the surface of 28 days old regenerated callus tissue.

Very few studies on callus induction of *P. longum* were documented previously but morphology was not well studied. In this attempt, the morphology of embryogenic callus was observed under Scanning Electron Microscope (Carl Zeiss Gemini 300). This study was confirmed through literature of SEM analysis in different plants (Chaudhury and Qu 2000; Cabral et al. 2011; Sivanesan et al. 2015).

**HPLC analysis of piperine**

Chromatograms (Fig. 4) obtained from HPLC analysis clearly indicated the noteworthy differences in piperine content among different plant parts grown under in vitro and in vivo conditions. Piperine was identified by comparing retention times in chromatograms of test samples with standard piperine (Rt − 5.963 min). Retention times of in vitro grown dry fruit (Rt − 5.964 min), in vivo grown dry fruit (Rt − 5.968 min), in vitro grown plant’s root (Rt − 5.957 min), in vivo grown plant’s root (Rt − 5.972 min) and in vitro grown callus tissue extract (Rt − 5.967 min) were used to calculate quantity of the piperine following the formula proposed by Shiva et al. (2015). Piperine content were 8.802 µg/ml, 0.650 µg/ml, 0.639 µg/ml, 0.038 µg/ml, 0.476 µg/ml respectively. Among all those samples, highest quantity of piperine was found in dry fruits of in vitro grown *P. longum* plant.

Such quantitative enhancement of chief alkaloid piperine (Kanaki et al. 2008; Kumar et al. 2011) in fruit of in vitro grown *P. longum* might be due to a cumulative effect of suitable plant growth promoters like kinetin and NAA on enzymetic activities involving in the biosynthetic pathway of piperine. The stress effect of growth temperature (25 ± 2 °C), which is quite less in comparison to the required temperature (30 ± 2 °C) for in vivo growth of the cultivar (agritech.tnau.ac.in) might be one of the reason behind the quantitative enhancement of piperine.

Few existing literatures (Rajopadhye et al. 2012; Khound et al. 2017) confirmed the presence of piperine in different plant parts, predominantly in root and fruit of naturally grown *P. longum* plant with different genotype. To the best of our knowledge, this is the first report on piperine content obtained from in vitro plants.

Quantitative enhancement of pharmacologically important piperine in in vitro plant counteract the cost of standardizing the protocol for its growth.

**Conclusion**

The novel finding of this study was enhancement of piperine content in the *P. longum* grown under in vitro condition. It was proved that in vitro grown fruits produce more quantity of piperine in comparison to fruits of in vivo grown plant. Hence, isolation and enhancement of secondary metabolite like piperine, in culture conditions, will lead to commercialization of highly valuable micropropagated plantlets and that may be considered as source of plant based natural

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Fig. 3  Micrograph of Scanning Electron Microscope (Carl Zeiss) of a embryogenic callus(Magnified at 400x with 20 µm aperture) showed various stages of somatic embryos such as a globular (GS), heart shaped (HS) structure (marked by arrow), b scutellum (SC) associated with coleoptile (CO) with 300KX with 3 µm aperture size
drug for many pharmacological formulations. Overall, the study revealed a good synergistic effect with best significant response for standardizing a cost effective protocol for micropropagation of *P. longum*, and its conservation. Thus, our finding may encourage cultivation of this economically important plant and provide a promising option for socioeconomic development of Indian farmers.

**Supplementary Information**  The online version contains supplementary material available at https://doi.org/10.1007/s11240-022-02237-0.

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**Author contributions**  The experiment was designed by MC and Dr. IC. Collection of plant, sample preparation and whole research work was executed and statistically analyzed by MC. Dr. SC monitored the research significantly. This experimental work is a part of Ph.D. thesis of MC. All authors read the manuscript, revised critically and approved the final version.

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**Fig. 4**  HPLC chromatograms of standard Piperine and Piperine present in various in vitro and in vivo grown plant extracts of *Piper longum* L.  
- **a** Chromatogram of standard piperine, **b** Chromatogram of callus extract, **c** Chromatogram of in vitro root extract, **d** Chromatogram of in vivo root, **e** Chromatogram of in vitro fruit extract, **f** Chromatogram of in vivo fruit extract.
Data availability  Authors certify that the work contained in this paper is original, and has not been submitted to any other journal for publication.

Declarations  

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

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