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Phytochemical fidelity and therapeutic activity of micropropagated *Curcuma amada* Roxb.: A valuable medicinal herb

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ABSTRACT

Curcuma amada Roxb. is a valuable medicinal herb with food, economic, pharmaceutical, and industrial value. This plant is commonly propagated through rhizome which is season dependent and slow process. Moreover, rhizomes are easily infected by different soil-borne pathogen, which deteriorates the quality and quantity of production. Hence, this study was carried out to develop one step plant regeneration protocol using rhizomatic bud on meta-Topolin (mT) supplemented medium. About 600 plants were produced on Murashige and Skoog (1962) (MS) medium added with 3.0 mg/L mT after twelve weeks of culture. Well rooted plants were successfully hardened (95%) and subsequently all the plants were established in the field. Quantitative phytochemical analysis and assessment of chemical composition of essential oil by Gas Chromatography - Mass Spectroscopy (GC-MS) confirmed the biochemical fidelity of the field-established tissue culture plants. Robust therapeutic activities such as antioxidant, anticancer, and antimicrobial activities were found in the rhizome extract and essential oil of the field-established tissue culture plants. It was concluded that tissue cultured plants are at par with the mother plant in terms of phytochemicals composition and therapeutic activity. Thus, this protocol could be useful for the production of a large number of plants aiming for its conservation, propagation as well as sustainable utilization in food, beverage, and pharmaceutical industries.

1. Introduction

Curcuma amada Roxb. is a valuable aromatic, medicinal plant of Zingiberaceae family. This plant is popular as ‘Amba ada’ or ‘Mango ginger’ (Sutar et al., 2020) mainly because its rhizome has a raw mango flavor (Tamta et al., 2016; Akter et al., 2019). This plant has medicinal, aromatic, food value as well as industrial demand. It is used in Indian traditional systems of medicine including Ayurveda and Unani from ancient time to cure several ailments such as tumor, cancer, alexiteric, bronchitis, asthma, antipyretic, diuretic, skin diseases, hiccough, and inflammation due to injuries (Rajkumari and Sanatombi, 2017; Sutar et al., 2020). The rhizome along with common salt is used to cure cold and cough (Kapoor, 1990). The rhizome is also used for healing of wounds, cut and itching as well as purification of blood (Kapoor, 1990; Srivastava et al., 2001). The leaf of this plant is used for contusions and sprains (Rao et al., 1989). The paste prepared by mixing *C. amada* whole plant and pepper is used for the treatment of piles (Kapoor, 1990). This plant possessed a number of therapeutic activities including antimicrobial, anti-inflammatory, analgesic, anticancer, anti-hyperglyceridemic, antioxidant activity, etc. (Tamta et al., 2016; Rajkumari and Sanatombi, 2017; Nag et al., 2021). The aforesaid therapeutic activities may be shown due to the presence of diverse bioactive compounds including curcumin, demethoxy curcumin, bis-demethoxy curcumin, phenol, terpenoids, and β-mycerene (Policegoudra et al., 2011). The essential oil of

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its rhizome has possessed various medicinal properties and therapeutic activities (Gupta, 2001). Besides, the rhizome of this plant is used as a crucial ingredient for the preparation of pickles, candies, sauces, curries, and salads (Gupta, 2001; Tamta et al., 2016). In Odisha (India), its rhizome is used as a flavoring agent for preparation of different food items like vada, dahi vada, chutney, curd water rice, pickles, raita, chat, etc. (Sutar et al., 2020). *C. amada* has been commonly used as flavoring agent in food and beverage industries due to the high amount of β-mycerene in its rhizome essential oil (Tamta et al., 2016; Surendran et al., 2021).

This plant is commonly propagated through vegetative method using rhizomes, which is a very slow growth process and season dependent (Raju et al., 2013). The rhizome quality is deteriorated due to attacks by various soil-borne pathogens like bacteria and fungus. These pathogens are also transmitted to the next generation in vegetative propagation through rhizome (Balachandran, 1990; Prakash et al., 2004). Thus, this traditional method of propagation is not suitable to produce a large quantity of plants around the year. These problems are sorted out by using the plant tissue culture technique. A number of reports are available on the development of micropropagation protocol for *C. amada* through meristem culture using rhizome explant (Prakash et al., 2004; Das et al., 2010; Ferdous et al., 2012; Bhattacharya and Chakraborty, 2015), callus culture using leaf sheath (Prakash et al., 2004) and synthetic seed using micro shoot and somatic embryo (Banerjee et al., 2012; Raju et al., 2013, 2016). The genetic fidelity analysis of *C. amada* micropropagated plant has been reported by Prakash et al. (2004) and Das et al. (2010). However, reports on phytochemical analysis and therapeutic activity of field established tissue culture plants of *C. amada* are very scanty. In this study, attempt has been made to develop micropropagation protocol of *C. amada* using rhizomatic bud explant. Phytochemical analysis and chemical composition of essential oil of field-established tissue culture plants vis-à-vis mother plant was carried out. Most importantly, assessment of pharmacologically important parameters including antioxidant, anticancer, and antimicrobial activities of field-established tissue culture plants were tested.

2. Materials and methods

2.1. Collection and preparation of plant materials

Curcuma amada plants were collected from Medicinal Plant Knowledge Centre (MPKC), Bhubaneswar, Odisha, India and planted in medicinal plant garden, Department of Botany, Ravenshaw University, Cuttack, Odisha, India. For the tissue culture experiment, young rhizomatic buds were collected from one year old plant of *C. amada* and were washed in tap water to remove the sand and soil adhered to the rhizome. The rhizomatic buds were washed with 1.0% liquid detergent Tween 20 for 10 min and treated with 2.0% fungicide (Bavistin) for 5 min and rinsed four times with double distilled water. For surface sterilization, rhizomatic buds were treated with 0.1% mercury chloride for 7 min followed by rinsing with autoclaved double distilled water four times. These sterilized rhizomatic buds were used for the tissue culture experiments.

2.2. Culture media for shoot proliferation and plantlet regeneration

The sterilized rhizomatic buds were inoculated on Murashige and Skoog (1962) (MS) medium and MS medium added with different concentrations (1.0–5.0 mg/L) of N^6-Benzyladenine (BA), meta-Topolin (mT), Zeatin (Z), and trans-Zeatin riboside (ZR) for multiple shoots proliferation or plantlet regeneration. Each culture medium consisted of 3.0% sucrose, 0.6% agar and the pH were adjusted to 5.8 ± 0.1 before autoclave of the medium.

For large number shoots or plantlets production within a short time period, *in vitro* shoots were harvested and cut into small pieces and inoculated on the optimum shoot proliferation or plantlet regeneration medium. All the cultures were maintained in the culture room (at 25 ± 1 °C; 16 hrs light/8 hrs dark; and 3000 lux light intensity).

2.3. Acclimatization of in vitro plantlets

After multiple shoots proliferation and root development on the same culture medium, the cluster of plantlets were taken out and washed properly to remove the solid medium from roots. Then each individual shoot having roots was detached from a cluster of shoots. Each plantlet was potted in a small pot containing sterile garden soil, tap water was given as per requirement, covered with a polyethylene bag and kept inside the culture room condition for one week. Then plants were transferred to shade condition for two weeks. After three weeks of planting, the polyethylene bag was removed and plants were kept in shade condition for one more week. Finally, acclimatized plants were planted in the field and used for further study.

2.4. Phytochemical analysis

Rhizomes of mother plant and ten randomly selected field-established tissue culture plants were collected, washed properly, sliced into small pieces and dehydrated under shade condition to get a constant weight. The dried rhizome samples were powdered separately and used for phytochemical analysis. The quantitative phytochemical analysis of rhizome for tannins, saponins, phenolic, alkaloids, and flavonoids quantity was estimated using the procedure described by Sutar et al. (2020).

2.5. Chemical composition of essential oil

Rhizomes of mother plant and three randomly selected field-established tissue culture plants (out of the ten plants selected for phytochemical analysis) were collected, washed, cut into small pieces and used for essential oil extraction separately. Essential oil of rhizome was extracted through Clevenger's apparatus as reported earlier by Guenther (1972). The essential oil was collected and dehydrated by using Na_2SO_4 and stored at 4 °C for the Gas Chromatography-Mass Spectroscopy (GC-MS) analysis, antioxidant activity, antimicrobial activity, and anticancer activity.

The chemical composition of rhizome essential oil of *C. amada* was analyzed by GC-MS using 1 μL of diluted essential oil (oil: hexane; 1:10). The components of essential oil were identified through a 7980 A gas chromatography system (Agilent Technologies, USA) with HP5-MS column (dimension 30 m × 320 μm × 0.25 μm). The program conditions include: 2 min initial hold at 40 °C, then increased from 40 °C to 125 °C gradually with an increment of 3 °C/min, 3 min hold at 125 °C, then the temperature was risen to 250 °C with an increment of 3 °C/min followed by hold at 250 °C for 3 min, further increased in temperature up to 300 °C at 10 °C/ min followed by hold at 300 °C for 3 min. MS was conducted at 250 °C as a transfer line and ion source temperature, 150 °C quadrupole temperature, 70 eV ionization potential and 50–550 at. mass units (amu) scan range. For identification of mass spectra, 5977 A mass selective detector was used. Helium in a split ratio of 10:1 and a flow rate of 1 mL/min was used as the carrier gas. Data were processed by the MSD Chem-station F.01.01.2317 software. Version 2.0 g of the NIST/EPA/NIH mass spectral library was used for compound identification (Agilent Technologies, USA). Essential oil samples were analyzed in three biological replicates and data were represented as mean ± standard deviation.

2.6. Extract preparation

For antioxidant, antimicrobial, and anticancer activity studies the rhizome samples of mother plant vis-à-vis field-established tissue culture plants (three out of the ten randomly selected plants for phytochemical analysis) of *C. amada* were used individually. The extracts were prepared in a methanol solvent system. Rhizome sample and solvent were taken in 1:10 ratio and extracts were prepared using Advanced Micro Wave Digestion System (Milestone, Italy) at 40 °C for 1 h with ramping time 15 min. The extracts were filtered using Whatman no. 1 filter paper, dried to get constant weight, and stored at 4 °C for future use.

2.7. Antioxidant activity

2, 2′- diphenyl-1-picrylhydrazyl (DPPH) scavenging activity of methanol extract and essential oil of rhizome was estimated by the standard method as reported earlier (Behera et al., 2018a) with a minor change. Different concentrations (10–100 μg/ mL) of the sample were prepared by diluting in methanol. Then 1 mL of different concentrations of methanolic extract was mixed with 1 mL of DPPH (0.15 mM in methanol). Similarly, 1 mL of essential oil was taken and mixed with 1 mL of DPPH (0.15 mM in methanol). For the control 1 mL DPPH was taken and mixed with 1 mL methanol. These mixture samples were left in dark condition at room temperature for 30 min and the absorbance was taken at 517 nm. The ascorbic acid was used as positive control and methanol was used as blank.

2.8. Anticancer activity

To measure the anticancer activity of methanolic rhizome extract and rhizome essential oil obtained from the mother plant and field-established tissue culture plants (three of the ten plants selected for phytochemical analysis) cell proliferation assay and apoptosis assay were carried out. Three cancer cell lines such as cervical cancer cell (HeLa), liver cancer cell (HepG2), and breast cancer cell (MCF7), obtained from NCCS Pune were used to measure the anticancer activity using the methods described previously (Meher et al., 2021; Das et al., 2021). The apoptosis assay was carried out using the IC_{50} values of plant extract and essential oil against HeLa cell line. The apoptotic cells were identified under fluorescence microscopy (Nikon Eclipe Ts2R-FL) based on changes of morphological features like nuclear condensation, formation of membrane blebs as well as apoptotic bodies appearance using 4,6-diamidino-2-phenylindole (DAPI), Ethidium bromide (EtBr), Acridine orange (AO), and Propidium iodide (PI) staining at 72 hrs of post treatment.

2.9. Antimicrobial activity

Two gram-positive bacteria namely *Streptococcus pyogenes* (MTCC 1926), *Streptococcus mutans* (MTCC 497) and three gram-negative bacteria such as *Salmonella typhii* (MTCC 1252), *Shigella flexneri* (MTCC 1457), and *Vibrio cholerae* (MTCC 0139) obtained from the Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India. Each bacterial strains were cultured on nutrient agar medium (Hi-media, India) and the pure cultures were maintained at 4 °C. Each strain was subjected to a regular subculture for maintaining its viability. These bacterial strains were used to test the antimicrobial activity of the rhizome methanol extract and essential oil of both the mother plant and tissue cultured plant by agar well diffusion method as described by Moharana et al. (2014). Briefly, about 10 μL/well essential oil, 5 mg/well plant extract and 10 μg/well kanamycin were loaded separately in each petri dish. Petri dishes without samples or antibiotics were taken as a negative control whereas plates with Kanamycin loaded wells were used as a positive control. Diameter of the zone of inhibition was measured after 24 h incubation of the cultured petri dish at 37 °C. All these experiments were performed in triplicates and data was represented in mean ± standard deviation.

2.10. Data analysis

In this study, all experiments were repeated three times. Multiple shoot proliferation or plantlet formation data was taken after six weeks of cultured. One explant was inoculated per culture vessel with twelve replicas. All data were subjected to Analysis of Variance for a Completely Randomized Design. The significant difference among the means with standard deviation in a column was done by Duncan's new multiple range test (Gomez and Gomez, 1984). Phytochemical analysis data were analyzed by *t*-test.

3. Results

3.1. Multiple shoot proliferation and plantlet regeneration

Rhizome buds were inoculated on MS medium and MS medium added with different concentrations of BA, mT, Z, and ZR for multiple shoot proliferation (Fig. 1A). Shoot buds were initiated after one week of culture (Fig. 1B). The percentage of explant responded for multiple shoot proliferation varies with respect to different growth regulators (Table 1). Simultaneously shoot and root formation was observed on each of the culture medium. On MS basal medium about 47.2% of explants were responded for shoot initiation. One shoot was formed per explant with length 4.5 cm, whereas, on average 5.3 roots were formed with an average length of 3.5 cm (Fig. 1C). In contrast, when rhizomatic buds were inoculated on MS medium with different growth regulators, the percentage of explants responsed for shoot proliferation and a number of shoots proliferations per explant were increases. About 66.7% of explants responded on MS medium added with 3.0 mg/L BA. An average of 4.5 numbers of shoots formed per explant with shoot length 5.2 cm and 15.8 roots were formed per explant with root length 4.8 cm (Fig. 1D). MS medium supplemented with mT (3.0 mg/L) was found to be the best medium among all the tested media (Table 1) with a maximum number of shoots and roots. On this medium highest percent of explants (88.9%) responded for multiple shoots proliferation with an average of 19.8 shoots and 53.2 roots after six weeks of culture (Fig. 1E & F), whereas an average of 10.5 shoots per explant were observed on MS medium augmented with 2.0 mg/L ZR (Fig. 1G).

After medium optimization, in a quest for large-scale shoot multiplication or plantlet regeneration within a short period of time from a single explant, shoot segments (40 shoot segments obtained from 20 shoots) were inoculated on the optimum culture medium (MS + 3.0 mg/L mT). About 15 shoots and 46 roots per shoot segment were produced simultaneously (Fig. 1H). In this process, we could able to produce about 600 shoots or plantlets with roots after twelve weeks of culture using a single rhizomatic bud explant.

3.2. Acclimatization

Cultures (cluster of shoots with roots) were taken out from culture vessels, washed properly, and shoots were separated from the cluster with each shoot having approximately 4–5 roots per shoot (Fig. 1I). These plantlets were planted in small pots containing sterilized garden soil. About 95% of plants were successfully acclimatized after four weeks (Fig. 1J) and all the plants acclimatized in pots were established in the field under full sun (Fig. 1K).

3.3. Phytochemical analysis

Phytochemical analysis was performed to compare alkaloids, flavonoids, phenolic, tannins, and saponins in rhizomes of the mother plant and field-established tissue culture plants. The phytochemicals viz. alkaloids, flavonoids, phenolics, and saponins were found to be in similar quantity among mother plant and field-established tissue culture plants. In contrast, tannins content was higher (122.98 mg/g) in mother plant rhizome compared with field-established tissue culture plants rhizome (118.56 mg/g) (Table 2).

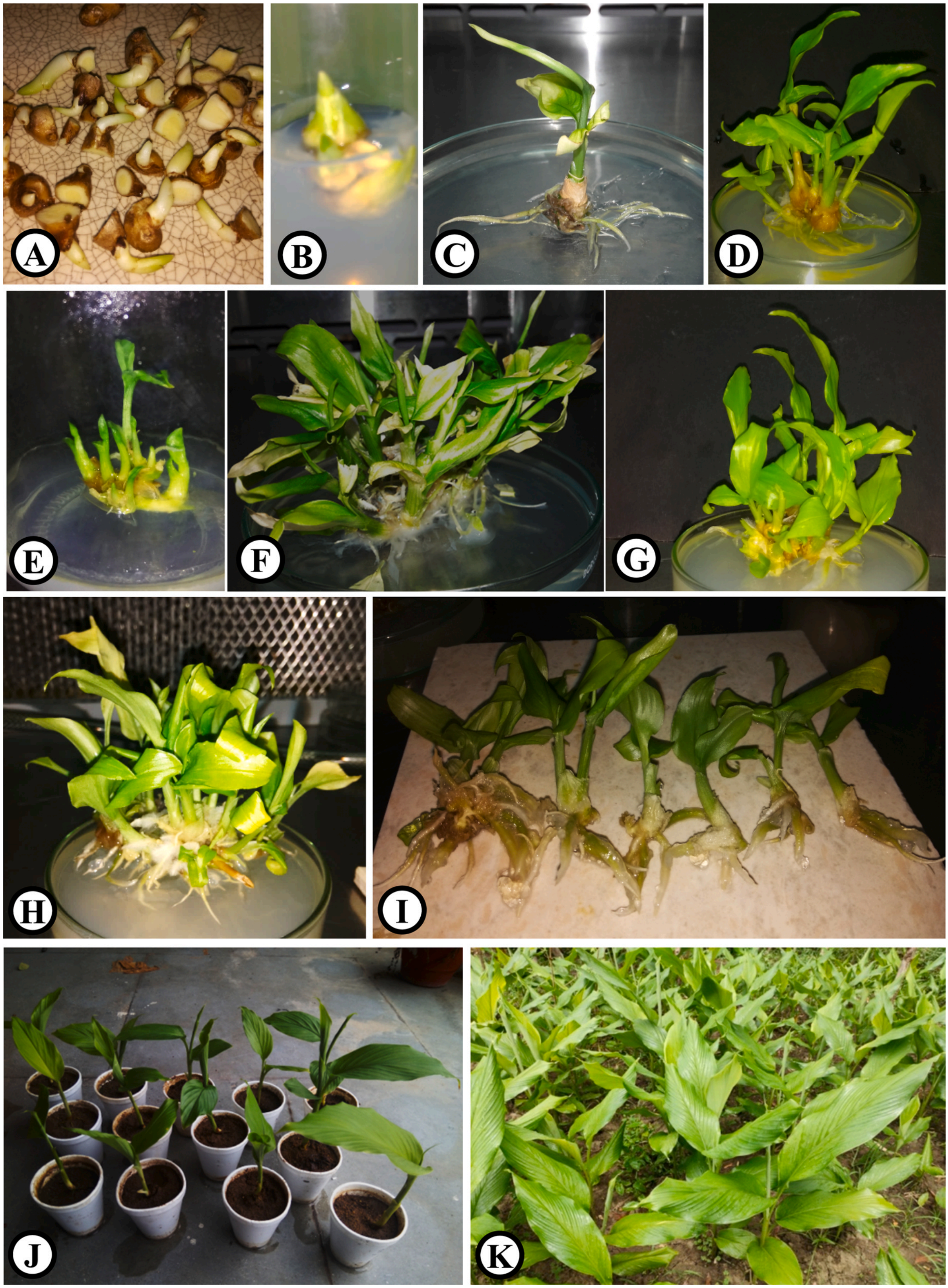

Fig. 1. Micropropagation of *C. amada* using rhizome bud: (A) Rhizomatic buds; (B) Shoot bud initiation from rhizomatic bud; (C) Shoot formation on MS medium; (D) Multiple shoots proliferation from rhizomatic bud on MS medium added with 3.0 mg/L BA; (E & F) Multiple shoot initiation and proliferation from rhizomatic bud on MS medium added with 3.0 mg/L mT; (G) Multiple shoot proliferation on MS medium added with 2.0 mg/L ZR; (H) Multiple shoot proliferation from *in vitro* shoot segment on MS medium added with 3.0 mg/L mT; (I) *In vitro* shoot having roots separated from cluster of shoot culture; (J) Acclimatization of *in vitro* regenerated plantlets on small pot containing sterilised garden soil; (K) *In vitro* regenerated plants established in the field.

Table 1
Influence of plant growth regulators on multiple shoot and plantlet formation from rhizomatic bud explant of *C. amada*.

MS medium with plant growth regulators (mg/L)				Response (%)	Shoots/ explant	Mean shoot length (cm)	Roots/ explant	Mean root length (cm)
BA	mT	Z	ZR					
0.0	–	–	–	47.2 ± 1.4^{m}	1.0 ± 0.0^{r}	4.5 ± 0.3^{cd}	$5.3 \pm 0.^{3s}$	$3.5 \pm 0.2^{e\text{-}g}$
1.0	–	–	–	55.6 ± 1.0^{l}	$3.8 \pm 0.3^{n\text{-}p}$	4.5 ± 0.2^{cd}	12.4 ± 0.7^{p}	4.2 ± 0.1^{bc}
2.0	–	–	–	61.1 ± 0.6^{j}	4.0 ± 0.0^{no}	$4.8 \pm 0.5^{a\text{-}c}$	$14.0 \pm 1.0^{\circ}$	4.5 ± 0.2^{ab}
3.0	–	–	–	66.7 ± 1.2^{h}	$4.5 \pm 0.5^{l\text{-}n}$	5.2 ± 0.3^{a}	15.8 ± 0.8^{l}	4.8 ± 0.2^{a}
4.0	–	–	–	61.1 ± 0.8^{j}	3.0 ± 0.0^{pq}	5.0 ± 0.3^{ab}	12.0 ± 0.5^{pq}	4.2 ± 0.2^{bc}
5.0	–	–	–	58.3 ± 0.6^{k}	3.0 ± 0.0^{pq}	4.5 ± 0.5^{cd}	10.5 ± 0.5^{r}	$3.6 \pm 0.2^{d\text{-}f}$
–	1.0	–	–	63.8 ± 0.7^{i}	9.5 ± 0.5^{fg}	4.0 ± 0.3^{ef}	32.3 ± 1.5^{e}	$3.5 \pm 0.3^{e\text{-}g}$
–	2.0	–	–	77.8 ± 1.4^{d}	14.6 ± 1.0^{c}	4.5 ± 0.6^{cd}	39.8 ± 1.0^{c}	$3.5 \pm 0.2^{e\text{-}g}$
–	3.0	–	–	88.9 ± 1.6^{a}	19.8 ± 1.4^{a}	5.0 ± 0.3^{ab}	53.2 ± 2.1^{a}	4.0 ± 0.3^{cd}
–	4.0	–	–	83.3 ± 0.9^{b}	16.0 ± 0.5^{b}	5.0 ± 0.2^{ab}	42.6 ± 1.0^{b}	4.2 ± 0.2^{bc}
–	5.0	–	–	83.3 ± 0.5^{b}	12.5 ± 0.9^{d}	$4.8 \pm 0.3^{a\text{-}c}$	36.5 ± 0.5^{d}	4.0 ± 0.2^{cd}
–	–	1.0	–	55.6 ± 0.5^{l}	$4.5 \pm 0.5^{l\text{-}n}$	3.5 ± 0.2^{gh}	15.5 ± 0.4^{lm}	$3.5 \pm 0.3^{e\text{-}g}$
–	–	2.0	–	63.8 ± 0.9^{i}	$5.0 \pm 0.0^{k\text{-}m}$	4.1 ± 0.4^{de}	18.2 ± 0.6^{j}	$3.8 \pm 0.5^{c\text{-}e}$
–	–	3.0	–	69.5 ± 0.9^{g}	6.5 ± 0.4^{j}	$4.8 \pm 0.3^{a\text{-}c}$	20.5 ± 0.9^{i}	4.0 ± 0.1^{cd}
–	–	4.0	–	61.1 ± 0.4^{j}	5.3 ± 0.5^{kl}	4.5 ± 0.0^{cd}	15.5 ± 0.5^{lm}	4.0 ± 0.0^{cd}
–	–	5.0	–	58.3 ± 0.5^{k}	$3.8 \pm 0.3^{n\text{-}p}$	$4.8 \pm 0.4^{a\text{-}c}$	12.4 ± 0.7^{p}	$3.5 \pm 0.3^{e\text{-}g}$
–	–		1.0	72.2 ± 0.7^{f}	5.8 ± 0.8^{jk}	4.5 ± 0.3^{cd}	22.0 ± 0.9^{h}	$3.8 \pm 0.4^{c\text{-}e}$
–	–		2.0	83.3 ± 0.6^{b}	10.5 ± 0.9^{e}	$4.8 \pm 0.5^{a\text{-}c}$	25.6 ± 1.0^{f}	$3.5 \pm 0.2^{e\text{-}g}$
–	–		3.0	80.5 ± 0.5^{c}	10.0 ± 0.5^{ef}	4.5 ± 0.2^{cd}	23.8 ± 0.8^{g}	$3.5 \pm 0.1^{e\text{-}g}$
–	–		4.0	75.0 ± 0.9^{e}	$8.7 \pm 0.3^{g\text{-}i}$	$3.8 \pm 0.3^{e\text{-}g}$	22.0 ± 1.7^{h}	$3.2 \pm 0.2^{f\text{-}h}$
–	–		5.0	77.8 ± 0.8^{d}	9.0 ± 0.6^{gh}	3.0 ± 0.0^{i}	17.5 ± 0.9^{jk}	2.5 ± 0.3^{i}

All data represented in means with standard deviation (SD). In each column, different letters in superscripts specify statistically significant difference between the means ($P \leq 0.05$; Duncan's new multiple range test)

Table 2
Quantitative phytochemicals analysis of mother plant vis-à-vis tissue culture plant rhizome of *C. amada*.

Phytochemicals	Mother plant	Tissue culture plant
Alkaloids (mg/g DW)	12.61 ± 0.43^{a}	12.87 ± 0.62^{a}
Flavonoids (mg/g DW)	11.10 ± 0.60^{a}	10.90 ± 0.45^{a}
Phenolics (mg GAE/g DW)	8.43 ± 0.58^{a}	8.20 ± 0.70^{a}
Tannins (mg TAE/g DW)	122.98 ± 2.3^{a}	118.56 ± 1.52^{b}
Saponins (mg/g DW)	31.70 ± 1.00^{a}	31.36 ± 0.84^{a}

All data represented in means with standard deviation (SD). In each row different letters in superscripts specify statistically significant difference between the means ($P \leq 0.05$; *t*-test). DW: dry weight, GAE: gallic acid equivalent, TAE: tannic acid equivalent.

3.4. Chemical composition of essential oil

The chemical composition of essential oil from the rhizome of both the mother plant and field-established tissue culture plants was analyzed by GC-MS (Table 3). Eighteen compounds were identified in the mother plant with an area percentage of 99.23%, whereas, sixteen compounds were identified in tissue cultured plant with 99.85% of area percentage. Major compounds identified were β-Myrcene, β-Ocimene, β-Pinene, α-Pinene, and Caryophyllene from both mother plant and field-established tissue culture plants (Table 3). Out of all identified compounds present in the essential oil, β-Myrcene was found to be in the highest percentage in both the mother plant (90.03%) and in the field-established tissue culture plants (90.67%).

3.5. Antioxidant activity

Antioxidant activity of the methanolic extracts and essential oils of the rhizomes of mother plants and field-established tissue culture plants was determined in terms of DPPH scavenging activity. Higher DPPH scavenging activity was found in essential oil in comparison to methanol extract (Fig. 2A and B). The IC_{50} value of ascorbic acid was found at a concentration of 23.6 μg/mL. While, the IC_{50} values of rhizome extract of mother plants and tissue culture plants were recorded at the concentration of 46.1 μg/mL and 48.3 μg/mL, respectively. Parenthetically, the IC_{50} values of essential oil of mother plants and tissue culture plants were shown at the concentration of 34.7 μg/mL and 35.6 μg/mL, respectively. Field-established tissue culture plants retained a similar level of antioxidant activity in comparison to mother plant.

Table 3
Chemical composition of *C. amada* rhizome essential oil.

Sl. No.	Name of compounds	RT value	Area (%)	
			Mother plant	Tissue culture plant
1	α-Pinene	12.16	0.52 ± 0.00	0.54 ± 0.00
2	(-)-β-Pinene	14.10	3.07 ± 0.03	3.14 ± 0.01
3	β-Myrcene	15.04	90.03 ± 0.88	90.67 ± 0.02
4	D-Limonene	16.57	0.12 ± 0.02	0.09 ± 0.00
5	Eucalyptol	16.65	0.04 ± 0.03	ND
6	Trans-β-Ocimene	17.04	0.17 ± 0.00	0.18 ± 0.00
7	β-Ocimene	17.55	3.51 ± 0.04	3.59 ± 0.01
8	Citronellol	17.70	0.07 ± 0.00	0.07 ± 0.00
9	Bicycle [3.1.0] hexane, 6-methylene	19.63	ND	0.02 ± 0.02
10	β-Linalool	19.95	0.09 ± 0.00	0.09 ± 0.00
11	Perillen	20.02	0.25 ± 0.00	0.25 ± 0.00
12	Allo-Ocimene	21.36	0.16 ± 0.00	0.16 ± 0.00
13	Caryophyllene	35.17	0.47 ± 0.00	0.48 ± 0.00
14	Y-Elemene	35.90	0.11 ± 0.00	0.12 ± 0.00
15	Cycloheptasiloxane, tetradecamethyl-	39.45	0.12 ± 0.08	ND
16	Nerolidol 2	42.03	0.08 ± 0.00	0.08 ± 0.00
17	(-)-Epinephrine, tris (trimethylsilyl) ether	46.71	0.04 ± 0.06	ND
18	2,6,11,15-Tetramethyl-hexadeca-2,6,8,10,14-pent	56.55	0.28 ± 0.00	0.29 ± 0.00
19	(E, E)– 7,11,15-Trimethyl-3-methylene-hexadeca-1	57.65	0.10 ± 0.00	0.10 ± 0.00

ND: Not Detected; RT: Retention Time; All data represented in means with standard deviation (SD).

3.6. Anticancer activity

Anticancer activity of methanolic extract and essential oil of rhizomes from the field-established tissue culture plants and the mother plant was investigated against three different cancer cell lines viz. HeLa cell, HepG2 cell, and MCF7 cell. The rate of cell viability (%) varies among cell lines of different tissue origin with respect to methanolic extract and essential oil of rhizome (Fig. 3A–F). The anticancer activity

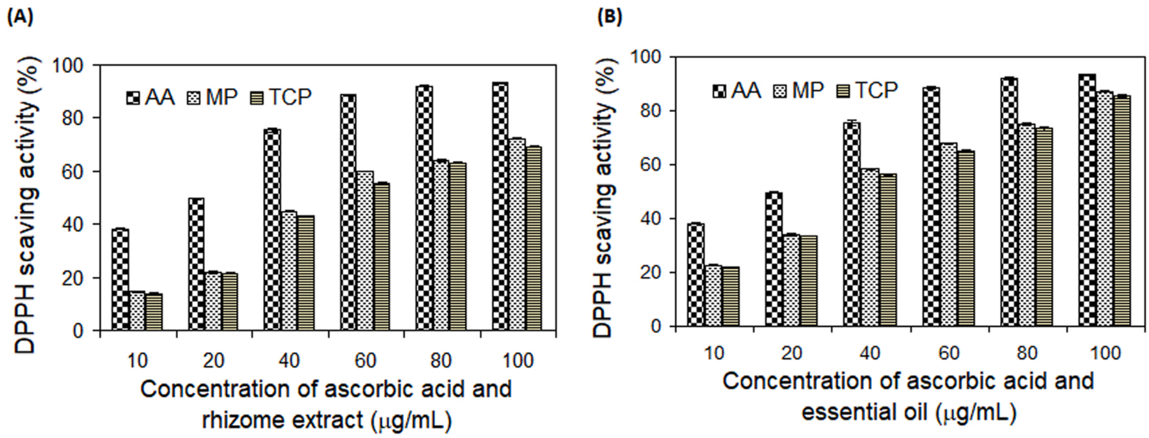

Fig. 2. DPPH scavenging activity (%) of *C. amada*: (A) Rhizome methanol extract; (B) Rhizome essential oil. AA: Ascorbic acid; MP: Mother plant; TCP: Tissue culture plant established in field condition. No statistically significant variation was found for the antioxidant activity between the MP and TCP at $p \leq 0.01$. All data represented in means ± standard deviation.

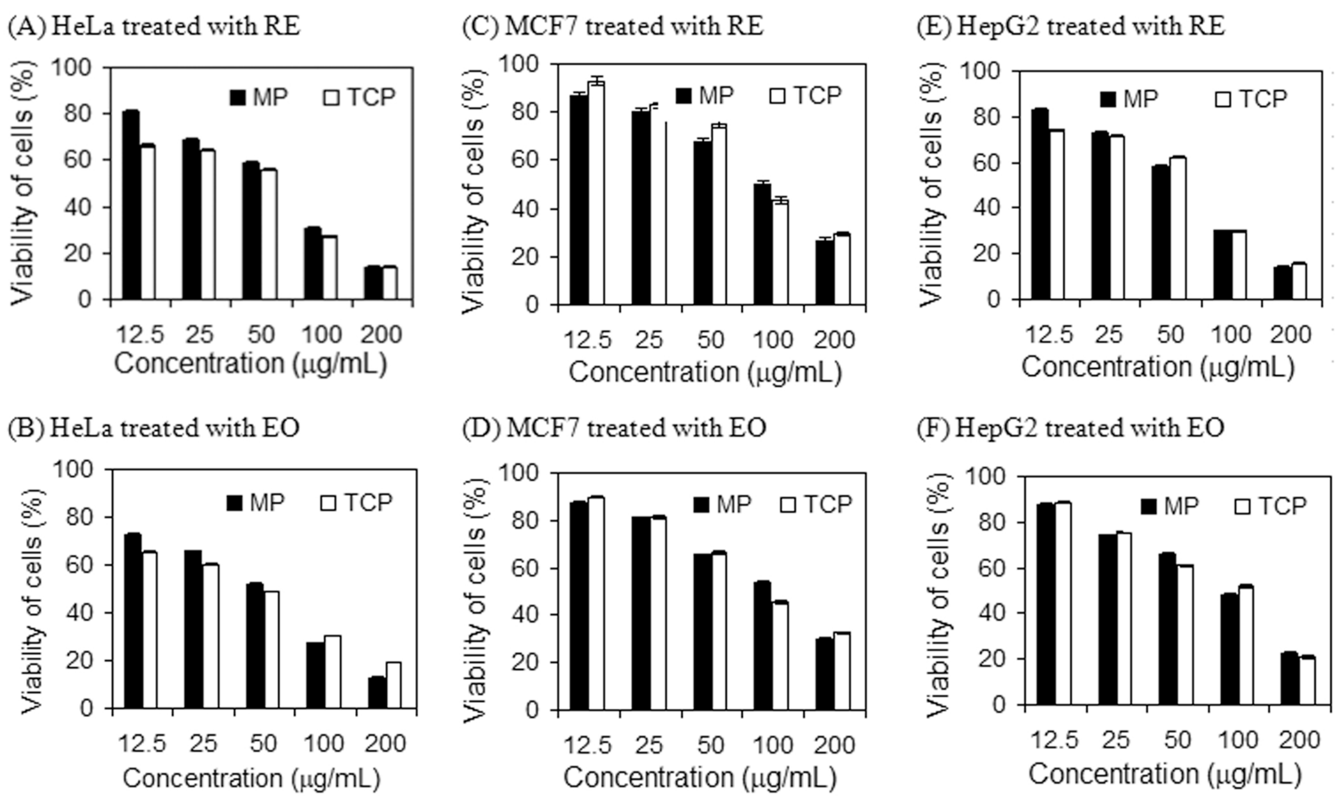

Fig. 3. Anti-proliferation activity (%) of *C. amada* rhizome methanol extract and essential oil against cell lines of different tissue origin viz. HeLa, MCF7, and HepG2 cell lines at 72 h of treatment. MP: Mother plant; TCP: Tissue culture plant; EO: Essential oil; RE: Rhizome extract. The difference in anti-proliferation activity between the MP and TCP was found to be not statistically significant at $p \leq 0.01$. All data represented in means ± standard deviation.

expressed in terms of IC_{50} value of both essential oils and methanol extracts against aforesaid cell lines is collated in Table 4. It was found that the field-established tissue culture plants retained the anticancer activity of similar to mother plant. The apoptosis assay was also performed by inducing apoptosis to cancer cell line, HeLa with treatment of IC_{50} concentration of methanolic extracts and essential oils of rhizomes of mother plant and filed-established tissue culture plants. Apoptotic cells were visualized using different fluorescent dyes such as DAPI, EtBr, AO, and PI staining under the inverted fluorescence microscope. The various morphological changes such as nuclear condensation, formation of membrane blebs as well as appearance of apoptotic bodies (Fig. 4) indicated apoptosis cells. The induction of apoptosis with the treatment of methanol extracts and essential oils of mother plant and field-established tissue culture plants was found to be statistically significant ($p \leq 0.01$) in comparison with untreated cells (Fig. 5A–D). The induction of apoptosis was not statistically significant between the mother plant and field-established tissue culture plants.

3.7. Antimicrobial activity

Antibacterial activity of rhizome extract and essential oil of mother plant vis-à-vis tissue culture plants of *C. amada* was tested against

Table 4
Anticancer activity of rhizome methanol extract and essential oil of *C. amada*.

Plant extract / essential oil		Cancer cell lines (IC_{50} value in ug/mL)		
		MCF7	HeLa	HepG2
Essential oil	Mother plant	110.3 ± 3.5^{a}	76.6 ± 1.9^{c}	72.3 ± 4.2^{d}
	Tissue culture plant	98.2 ± 3.2^{b}	66.5 ± 3.8^{d}	90.2 ± 3.6^{c}
Methanol extract	Mother plant	98.2 ± 2.7^{b}	81.5 ± 3.0^{b}	103.7 ± 4.6^{a}
	Tissue culture plant	86.1 ± 2.3^{c}	90.5 ± 2.9^{a}	95.1 ± 3.3^{b}

All data represented in means with standard deviation (SD). In each column, different letters in superscripts specify statistically significant difference between the means ($P \leq 0.05$; Duncan's new multiple range test)

different pathogenic bacteria including two gram-positive bacteria namely *S. pyogenes* and *S. mutans* as well as three gram-negative bacteria such as *S. typhii*, *S. flexneri,* and *V. cholerae*. Both essential oils and methanolic extracts of rhizome of the mother plant as well as field-established tissue culture plants showed comparatively less antibacterial activity compared with kanamycin (Table 5). It was also observed that both rhizome extract and essential oil between the mother plant and field-established tissue culture plants have similar antibacterial activity.

4. Discussion

Zingiberaceae plants are commonly grown through rhizomes due to lack of seed or scanty seed production as well as low viability of seeds (Raju et al., 2013). Further, the rhizomes are easily infected by different soil-borne pathogen, which are inherited generation after generation through vegetative propagation by rhizome (Prakash et al., 2004). To solve this problem, biotechnological approaches are required for the production of healthy plants around the year. In this study, tissue culture protocol was developed using rhizomatic bud explant to produce a large number of plants for *C. amada*. A number of tissue culture plant regeneration protocols have been developed in Zingiberaceae plants using rhizome bud or rhizome explant (Salvi et al., 2002; Prathanturarug et al., 2003; Prakash et al., 2004; Bejoy et al., 2006; Das et al., 2010; Ferdous et al., 2012; Senarath et al., 2017; Behera et al., 2018b).

Rhizomatic buds of *C. amada* were inoculated on MS basal medium, but it was found that MS basal medium without growth regulators is not sufficient for the production of multiple shoots or plantlets. Thus, MS medium was supplemented with different types and concentrations of cytokinins i.e., BA, mT, Z, and ZR for shoot multiplication and plantlet regeneration from the rhizomatic buds. It was found that mT supplemented medium was suitable medium for multiple shoot proliferation and plantlet regeneration of *C. amada*. Highest number of shoots (19.8) with 53.2 roots per explant were obtained on MS medium + 3.0 mg/L mT after six weeks of culture. This finding was corroborated with the previous report that mT supplemented medium is better for shoot proliferation or plantlet regeneration in different plant species including *Curcuma longa* (Salvi et al., 2002), *Hedychium coronarium* (Behera et al., 2019), *Prunus* stock (Gentile et al., 2014), *Pterocarpus marsupium* (Ahmad and Anis, 2019), and *Withania somnifera* (Kaur et al., 2021). The positive response of mT, a naturally occurring aromatic cytokinin, in comparison with BA or other cytokinins and its analogues are probably due to its lower toxicity (Valero-Aracama et al., 2010). Besides, the presence of a hydroxyl group in mT gives the structural advantage to mT over BA. It can easily undergo O-glucosylation to form storage forms of cytokinin i.e., O-glucoside metabolites, that are steady under certain situation and quickly converted into potential cytokinin whenever needed (Werbrouck et al., 1996; Bairu et al., 2011; Aremu et al., 2012). Moreover, simultaneously multiple shoot and root formation was found in the different tested medium used in the study. Similar results have been reported in other Zingiberaceae species i.e., *Alpinia galanga* (Singh et al., 2014), *Hedychium coronarium* (Mohanty et al., 2013; Behera et al., 2018b, 2019), and *Zingiber officinale* (Jain et al., 2018). For the production of a large number of shoots/ plantlets, *in vitro* shoot segments were further cultured on the optimum medium (MS + 3.0 mg/L mT) and ultimately using this protocol about 600 plantlets were obtained after twelve weeks. *In vitro* regenerated plants were successfully acclimatized (95%) and subsequently planted in the field with zero mortality. This acclimatization was comparatively better than the previous reports [70%, Prakash et al. (2004) and 80%, Ferdous et al. (2012)].

Evaluation of phytochemical composition and concentration of tissue culture plants is essential before supplying to market, farmers or industrial utilization. Thus, in this study, phytochemicals such as tannins, saponins, flavonoids, phenolics, and alkaloids were quantified in field-established tissue culture plants. No remarkable difference in phytochemical composition and concentration between the mother plant and field-established tissue culture plants has been noticed. These results are in agreement to the work reported by Behera et al. (2018b; 2019) on *H. coronarium*. Tannins, flavoinds, and phenolics compounds possess different biological activity including antifungal, antioxidant, anti-inflammatory, anti-cancer, and anti-diabetic activity (Sutar et al., 2020). Thus, the biological or therapeutic activity of this plant may be attributed to the presence of the above-mentioned phytochemicals.

The chemical composition of rhizome essential oil of the mother plant and field-established tissue culture plants was also analyzed by GC-MS. No significant difference between the mother plant and field-established tissue culture plants in the constituents of essential oil has been seen. This study found that β-Myrcene was a major compound in rhizome essential oil of *C. amada* (90.03% in the mother plant and 90.67% in the field-established tissue culture plants). In contrast, 88.6%, 80.54%, and 40.2% of β-Myrcene have been reported in the mother plants of *C. amada* by Choudhury et al. (1996), Singh et al. (2003), and Tamta et al. (2016) respectively. Accumulation of metabolites are influenced by a number of factors including geographical location, genotypes, age of the plants, growth condition and production practice etc., which may be the possible reason for quantitative variation of same metabolites in the mother plants of *C. amada* as reported by various workers as mentioned above. β-Myrcene is a monoterpene compound and is commonly used as a flavouring and aromatic agent in the food and beverage industries (Surendran et al., 2021). Besides, β-Myrcene has a number of therapeutic activities such as anxiolytic, sedative, antidiabetic, antibacterial, anticancer, antioxidant, anti-ageing, anti-inflammatory, and analgesic activities (Inoue et al., 2004; Ojeda-Sana et al., 2013; Rufino et al., 2015; Bai and Tang, 2020; Surendran et al., 2021).

The validation of therapeutic activities of tissue culture medicinal plants is imperative before it is used as an alternative source of mother plant. Thus, different therapeutic activities viz. antioxidant activity, anticancer activity as well as antimicrobial activity was studied in field-established tissue culture plants vis-à-vis mother plant. The antioxidant activity was evaluated by DPPH assay, which is comparatively an easy and rapid one, for estimating free radical scavenging activity of rhizome extract and essential oil. The DPPH assay is widely used for the estimation of free radical scavenging activities of antioxidant due to the presence of its stable free radical (Sanchez-Moreno, 2002). In the radical form, DPPH molecule had an absorbance at 517 nm, which is disappeared after accepting hydrogen radical or an electron from an antioxidant compound and become a stable diamagnetic molecule (Tamta et al., 2016). This study revealed that, there is no significant difference in the antioxidant activity between field-established tissue culture plants and mother plant. Essential oil had shown more scavenging activity in comparison with rhizome extract. However, compared with ascorbic acid both the essential oil and extract of rhizome have shown less antioxidant activity. This is in agreement with the result of Tamta et al. (2016). The antioxidant activity of essential oil was more than the extracts, which may be due to the presence of the highest percentage (>90%) of β-Myrcene in essential oil (Ojeda-Sana et al., 2013). The anticancer activity of rhizome extract and essential oil of both mother

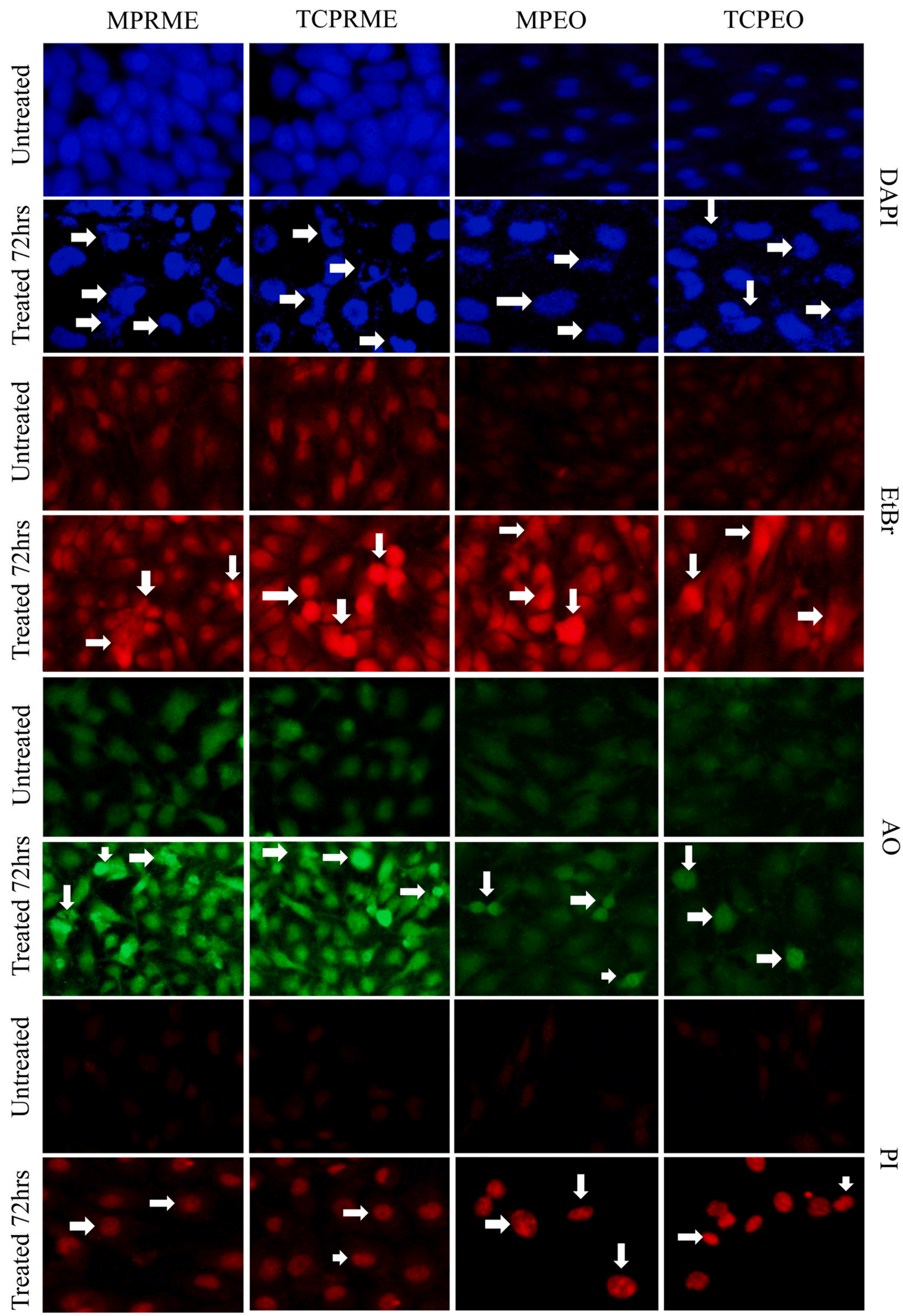

Fig. 4. Induction of apoptosis to HeLa cancer cell line by the rhizome methanolic extract and essential oil of *C. amada*, treated at IC_{50} concentration. The apoptotic cells are characterized by changes in morphological features i.e. chromatin condensation, blebbing of plasma membrane as well as apoptotic bodies (indicated by arrows). The changes in morphological characters are shown under fluorescence microscope after labeling with DAPI, EtBr, AO, and PI. The untreated control cells were also included for the comparison. MPRME: Mother plant rhizome methanol extract; TCPRME: Tissue culture plant rhizome methanol extract; MPEO: Mother plant essential oil; and TCPEO: Tissue culture plant essential oil.

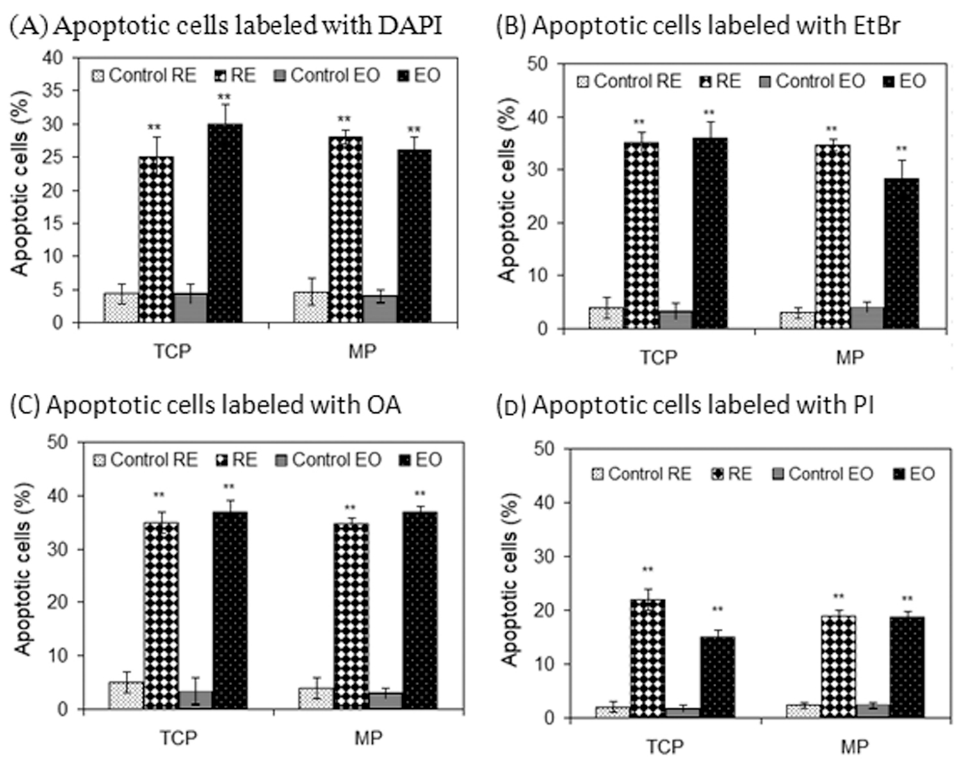

Fig. 5. Percentage of apoptotic cells after 72 h treatment with rhizome methanol extract and essential oil of mother plant and tissue culture plant in comparison to untreated cells (Control). Both the samples effectively induced apoptosis to HeLa cancer cell line compared to control and was found to be statistically significant at $p \leq 0.01$. Control RE: Control rhizome extract; RE: Rhizome extract; Control EO: Control essential oil; EO: Essential oil; TCP: Tissue culture plant; MP: Mother plant. All data represented in means ± standard deviation as bars.

Table 5
Antibacterial activity of rhizome extract and essential oil of *C. amada*.

Sample or antibiotic	Inhibition zone in diameter (mm)				
	Gram-positive bacteria		Gram-negative bacteria		
	S. pyogenes (MTCC 1926)	*S. mutans* (MTCC 497)	*S. typhii* (MTCC 1252)	*S. flexneri* (MTCC 1457)	*V. cholerae* (MTCC 0139)
MPEO (10 µL/well)	14.5 ± 1.2	15 ± 0.5	19 ± 1.0	18.7 ± 2.2	15 ± 0.5
TCPEO (10 µL/well)	14 ± 1.0	14 ± 1.1	18 ± 0.57	19 ± 0.28	15 ± 1.1
MPRME (5 mg/well)	17 ± 0.9	16 ± 2.0	18 ± 0.6	22 ± 0.8	17.5 ± 1.0
TCPRME (5 mg/well)	18 ± 1.5	16 ± 1.0	19 ± 1.6	22 ± 1.0	17 ± 0.57
Kanamycin (10 ug/ well)	24 ± 0.5	22 ± 1.1	19 ± 0.5	25 ± 1.5	20 ± 1.0

All data represented in means with standard deviation (SD). MPEO: Mother plant essential oil; TCPEO: Tissue culture plant essential oil; MPRME: Mother plant rhizome methanol extract; TCPRME: Tissue culture plant rhizome methanol extract.

plant and field-established tissue culture plants was screened against three cell lines i.e., HeLa cell, HepG2 cell, and the MCF7 cell of different tissue origin. The anticancer activity of field-established tissue culture plants rhizome extract and essential oil were shown more activity against MCF7 cell (IC_{50} value is 86.1 ug/mL) and HeLa cell (IC_{50} value is 66.5 ug/mL). In contrast, mother plant essential oil was more effective against HepG2 cell (IC_{50} value is 72.3 ug/mL) compared with field-established tissue culture plants. Previously, the anticancer activity of *C. amada* has been reported against human glioblastoma cell line (Ramachandran et al., 2015), and MCF7 and MDA-MB-231 cells (Jambunathan et al., 2014). The antimicrobial activity of rhizome extract and essential oil between field-established tissue culture plants and mother plant was found to be approximately similar against pathogenic bacteria viz. *S. pyogenes*, *S. mutans*, *S. typhii*, *S. flexneri*, and *V. cholera* due to the presences of phytochemicals like phenolic and β-Myrcene (Policegoudra et al., 2011; Sun et al., 2017; Rajkumari and Sanatombi, 2017). The finding of antimicrobial activity is in agreement with the result reported by Kaur et al. (2018).

5. Conclusion

Curcuma amada has copious antiqueue applications in a different region of the world due to its medicinal, economic, food as well as industrial value. This study presents for the first time a single step *in vitro* plant regeneration protocol using rhizomatic bud explant on mT supplemented medium. About 600 plants were regenerated from a single explant through shoot up-scaling method on MS medium augmented with 3.0 mg/L mT after twelve weeks. Phytochemical fidelity and therapeutic activity of *in vitro* plants was also assessed in comparison with the mother plant and was confirmed that field-established tissue culture plants were phyto-chemically and therapeutically stable compared with the mother plant. Thus, this protocol could be helpful for the production of a large number of stable plants aiming at their conversation, commercial utilization in food, beverage and pharmaceutical industries sustainably.

Author contributions

SB designed the study and carried out all experimental work with KM. SB and RKM performed the anti-cancer activity studies. SM helped in GC-MS data analysis. SKM and PKD collected plant and maintained in field. SKN supervised the work of SB and KM. SKN and PKN edited the final manuscript. Finally, all authors approved the manuscript for publication.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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