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Evaluation of yield, quality and antioxidant activity of essential oil of *in vitro* propagated *Kaempferia galanga* Linn.

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ABSTRACT

Objective: To determine chemical constituents and antioxidant properties of essential oil from rhizome of the medicinal plant, *Kaempferia galanga* (*K. galanga*) Linn. (Zingiberaceae) in conventionally propagated (CP) and *in vitro* propagated (IVP) plants. **Methods:** *In vitro* (micro) propagation of *K. galanga* was done by inoculating explants on to Murashige and Skoog agar medium, supplemented with suitable combinations of phytohormones; the regenerants were transferred to soil for further growth. Essential oil preparations of both CP and IVP rhizomes grown in soil, obtained by the hydro-distillation method were analyzed by gas chromatography-mass spectrometry. Antioxidant activities of essential oil samples were monitored. **Results:** Maximum numbers of regenerated shoots were found in the medium supplemented with 1 mg/L benzyl adenine and 0.5 mg/L indole-3-acetic acid. A total of 6 compounds were identified from rhizomes from CP and IVP plants that yielded 96.9% and 97.81% of the total oil contents, respectively. The major compound of rhizome oil identified from CP and IVP rhizomes was ethyl p-methoxy cinnamate in quantities, 82.01% and 71.77%, respectively, without any compositional variation. Antioxidant properties of essential oil preparations were assessed by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and hydrogen peroxide radical scavenging assays. Moreover, antioxidant activities of rhizome-oil from IVP plants were better than that of CP oil samples. **Conclusions:** As IVP rhizomes had better oil yield, those could be used for a large scale commercial propagation for sustainable use of essential oil. The principal chemical in the essential oil, ethyl p-methoxy cinnamate could help apothecary, for several ailments.

1. Introduction

Kaempferia galanga (*K. galanga*) Linn. (aromatic or sand ginger, family Zingiberaceae) is regarded as a cash plant with several medicinal properties, as many a pharmaceutical establishments require its rhizomes for the essential oil as well as for direct uses in the preparation of Ayurvedic drugs, perfumery, cosmetics, spices and a few more[1]. Further, bulky leaves of the plant are used for flavouring foodstuffs, preparing mouthwashes and a hair tonic, locally; leaves are antinociceptive and anti-ulcerative[2,3]. Antioxidant activity of the plant was too reported using the ferric reducing antioxidant power, the β-carotene bleaching and the oxygen radical capacity assay by a Malaysian variety of the plant[4]. Rhizomes are used for curing bronchitis, asthma, malaria, skin disease, wounds and spleenic disorders[5]. They are used for the preparation of decoction or powders, which are used for indigestion, cold, pectoral pain, abdominal pain, headache and toothache. Its alcoholic maceration has also been applied as liniment for rheumatism[6]. The essential oil from rhizome had been known having antimicrobial activities. Crude methanolic extract of *in vitro* propagated (IVP) rhizomes was screened for antibacterial activity against four bacteria, of undefined antibiotic sensitivity[7]. Antibacterial activity of the rhizome against bacteria, *Staphylococcus epidermidis (S. epidermidis)* and *Bacillus subtilis (B. subtilis)*, isolated from skin were demonstrated that rhizome-extracts were

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comparable to various foot skin deodorant formulations that are commercially available[8]. Antimalarial activity of the plant was demonstrated against the falciparum malarial parasite by rhizome-extracts[9].

The active constituents of galangal oil are ethyl p-methoxy cinnamate (EPMC), methylcinnamate and penta decane, etc., as reported[6,10]. EPMC from the rhizome extract was reported as highly cytotoxic to He La cells[6]. Crude rhizome extracts were used for the demonstration of antineoplastic activity against human colorectal adenocarcinoma, but extracts were non-toxic to normal Vero cell cultures, the study was based on cytotoxic activity that was monitored by the standard assays with 3-(4,5-dimethyl-2-thhiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and sulforhodamine B (SRB), against four cancerous cell lines[11]. EPMC was effective against the hepatic microsomal cytochrome P450s enzyme activities in mice that mostly induce liver disorders leading to cancer; this compound has antineoplastic activity[12]. This chemical was further reported as active against fibrosarcoma in mouse model induced by benzo(a)pyrene; the study demonstrated that the chemo preventive activity of the compound against fibrosarcoma was through the inhibition of COX-2, as known from *in silico* analysis[13].

Anti-inflammatory effect of the plant by plant derived EPMC were dose dependent in inhibiting carrageenan induced edema with an minimum inhibitory concentration (MIC) value of 10 mg/kg[14]. Hepatoprotective activities of freshly powdered rhizomes were demonstrated in carbon tetrachloride induced liver damage in rats[15]. The bioactive compound, EPMC isolated from ethanol extract of rhizome had a decreasing effect on micropthalmia associated transcription factor and tyrosinase levels in MSH-stimulated B16F10 cells, helping in immunoregulation. It also could be used as a skin lightening agent to treat hyperpigmentary disorders in man[16].

Larvicidal properties of the chloroform extract of rhizome were reported against the notorious vector of the dengue virus, *Aedes aegypti (Ae. aegypti)*[17]. In integrative agricultural management, the use of rhizome extract against the nematode, *Meloidogyne incognita (M. incognita)* reduced utilizations of nematicides, carbofuran, fosthiazate and metam sodium[18].

The price of the essential oil of the plant varies from US $ 600 to 700 per kg on the international market, and rhizomes as well as leaves are highly exploited by the local people and pharmaceutical industries[19]. The high price of the natural essential oils coupled with their limited availability has encouraged a search for substitutes. Low productivity, disease susceptibility and higher cost of seed rhizome production are the major constraints faced by growers. Furthermore, the collection of plant material for the extraction of drug is the major task, as this plant species has become endangered[20]. These problems can be alleviated through the application of a tissue culture technique, an efficient-the long-recognized tool for rapid multiplication of true-to-type genotypes that could be made available, commercially[21]. Despite intensive studies on the chemical composition of rhizome of *K. galanga*, only very few reports are available on the chemical constituents of the plant oil. The essential oil samples of IVP and conventionally propagated (CP) rhizomes were analyzed by gas chromatography-mass spectrometry for the quantitative estimation of chemicals. Further, there is no report on the antioxidant activity of essential oil of this endangered plant, a comparative evaluation of antioxidant activity of the essential oil samples of IVP and CP rhizomes were done. It is anticipated that apothecary would benefit from the principal chemical, EPMC, for further drug development, since the plant has holistic medicinal properties.

2. Materials and Methods

2.1. Plant material

K. galanga was grown in our garden. Young sprouting buds from rhizomes of 10 disease-free plants were used as explants for the initiation of the *in vitro* culture. Explants were cleaned with a liquid detergent and were treated with a seed-dresser, 0.1% mercuric chloride for 8 to 10 min, aseptically and those were cleaned with sterile distilled water to remove traces of mercuric chloride, prior to the culture initiation.

2.2. In vitro propagation and field transfer of regenerants

Explants were inoculated into autoclaved basal Murashige and Skoog (MS) agar medium[22], with varying combinations of benzyl adenine (BA) (1-5 mg/L), indole-3-acetic acid (IAA) (0.5-1.0 mg/L), naphthalene acetic acid (NAA) (0.5 mg/L) and adenine sulphate (Ads) (50-100 mg/L), for IVP (Table 1), with 30 g/L of sucrose; the pH of the medium was adjusted to 5.7, and 0.8% agar was used. Fifteen replicate explants were used for each treatment. Culture tubes containing the inoculated explants were kept under a bank of white fluorescent lights at temperature 24 ℃, in a culture room. IVP plants with well-developed shoot and roots were transferred to field for further growth and oil extraction was done from rhizomes.

2.3. Extraction and quantitative evaluation of essential oil

Essential oil was extracted by hydro-distillation of fresh rhizomes of CP and IVP plants with a Clevenger's apparatus. The total amount of oil in rhizomes was calculated by the following method. Fresh weight of rhizome oil yield (%) (v/w) = (volume of essential oils (mL)/ weight of raw rhizomes taken) ×100

The distilled rhizome essential oil was dried over anhydrous sodium sulphate and stored at −4 ℃ in air tight containers for further uses. Each essential oil extraction was run in triplicates for confirmation of oil yield.

2.4. Qualitative analysis of essential oil

The component identification of essential oil was achieved by the gas chromatography-mass spectrometry (GC-MS) analysis, using the 6890 series instrument (Agilent Technologies, Palo Alto, California), equipped with flame ion detector (FID) and a HP-5, fused silica capillary column (30 m x 0.25 mm) with internal diameter, film thickness, as 0.25 μm; the temperature was programmed from 50-240 ℃ at 4 ℃/min; from 240 ℃ to 270 ℃ at 15℃/min; held isothermal at 50 ℃ for 1 min and at 270 ℃ for 15 min. The temperatures of both auto-injector and detector were kept at 280℃; the sample injection volume was 1 μL and the split ratio was 100:1. The carrier gas was nitrogen at the flow rate of 1.2 mL/min. GC-MS (70eV) data were measured on the same gas chromatograph, coupled with MSD 5973. The MS source temperature was at 230 ℃, but the MS quadrapole temperature was at 150 ℃; the interface temperature was at 290 ℃; the mass scan was done at 20-600 amu; the carrier gas was helium at the flow rate, 1.0 mL/min. The retention index was calculated using a homologous series of *n*-alkanes C8-C18. Chemical constituents were identified by comparison of their mass spectra and retention indices with those of National Institute of Standards and Technology library data[23]. For the GC-MS evaluation, 10 plants from each group, IVP and CP were randomly selected.

2.5. DPPH free radical scavenging activity

Essential oil samples were individually assessed for their possible antioxidative activities by employing two complementary tests, DPPH free radical-scavenging and H_2O_2 scavenging assays. Radical scavenging activity of essential oil samples of both IVP and CP rhizomes were determined by a spectrophotometry, based on the reduction of a methanolic solution of DPPH[24]. Aliquots of 1 mL of varying concentrations of oils (1, 5, 10, 20, 50 and 100 μg/mL) in methanol were added to an aliquot 2 mL of 0.1 mM methanolic solution of DPPH, for each concentration. The mixture was shaken vigorously and left to stand at the room temperature for 30 min in dark. When DPPH was reduced, the change of colour from deep violet to light yellow occurs, which was measured at 517 nm on a UV/visible spectrophotometer (UV1, Thermo scientific). Absolute methanol was used as the base control. The DPPH solution was freshly prepared and stored in the dark. Tests were carried out in triplicates with ascorbic acid, as the standard antioxidant. Radical scavenging activity was expressed as 50% inhibition concentration (IC_{50}) of DPPH radical and was calculated by following equation,

$$IC_{50} = [(Acontrol - Atest)/Acontrol] \times 100$$

where, Acontrol was the absorbance of the control solution without the essential oil and Atest was the absorbance of oil and ascorbic acid. The antioxidant activity of each

Table 1

Effect of growth regulators on shoots and root *in vitro* multiplication of *K. galanga* with phytohormones.

MS medium + growth regulator (mg/L)	Shoot/explant numbers mean ± sd	Shoot length (cm) mean ± sd	Root/explant numbers mean ± sd	Root length (cm) mean ± sd
BA (1)	5.00±1.00	10.20±0.80	5.33±1.52	2.30±0.30
BA (2)	7.66±2.51	4.33±0.70	3.66±0.57	2.10±0.26
BA (3)	5.33±1.52	5.90±0.61	4.33±1.10	3.06±0.20
BA (1)+IAA (0.5)	11.33±1.52	10.80±0.32	6.33±1.52	2.76±0.32
BA (3)+IAA (0.5)	7.00±1.00	10.23±0.55	4.66±1.15	2.73±0.60
BA (3)+IAA (1)	6.66±1.52	8.40±0.40	5.33±1.15	1.90±0.40
BA (3)+IAA (0.5) +Ads (50)	5.66±1.52	5.46±0.41	3.66±0.57	2.10±0.26
BA (3)+IAA (0.5) +Ads (100)	6.66±1.52	5.90±0.30	4.33±1.10	1.80±0.40
BA (3)+IAA (1) +Ads (100)	8.00±1.00	6.63±0.40	5.33±1.15	2.10±0.26
BA (3)+NAA(0.5)	4.33±1.52	2.90±0.51	3.00±1.00	1.50±0.10

Note: Phytohormones, concentration in parenthesis: benzyl adenine (BA) (1-3 mg/L); indole-3-acetic acid (IAA) (0.5-1.0 mg/L); naphthalene acetic acid (NAA) (0.5 mg/L) and adenine sulphate (Ads) (50-100 mg/L).

sample was expressed in terms of IC_{50} (i.e., micromolar concentration required to inhibit DPPH radical formation by 50%), calculated from the graph after plotting inhibition percentage against sample concentration.

2.6. Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity of both CP and IVP oil samples were estimated, as detailed[24]. A stock of 4 mM solution of H_2O_2 was prepared in phosphate buffer saline (PBS, pH 7.4) solution. Oil samples in aliquots of 4 mL, at varying concentrations (1-100 μg/mL) were mixed with an aliquot of 0.6 mL of 4 mM H_2O_2 solution prepared in PBS, and the mixture was incubated for 10 min, for each sample. The absorbance of the solution was noted at 230 nm against a blank solution containing the essential oil in PBS without H_2O_2. Ascorbic acid was too used as the positive control. The amount of H_2O_2 radical inhibited by the oils was calculated using the following equation:

H_2O_2 radical scavenging activity% = [(Acontrol-Atest)/ Acontrol] x 100,

where, Acontrol was the absorbance of H_2O_2 radical + methanol; Atest was the absorbance of H_2O_2 radical+ sample or standard.

3. Results

3.1. In vitro propagation

Rhizome buds of CP plants were used as explants (Figure 1a) and inoculated to MS agar medium with different phytohormone combinations of BA (1-5 mg/L), IAA (0.5-1.0 mg/L), NAA (0.5 mg/L) and Ads (50-100 mg/L). The multiplication of shoot buds occurred in all hormone combinations in media, but the maximum multiplications of shoots (Figure 1b) were noted in MS supplemented with BA (1 mg/L) in combination with IAA (0.5 mg/L) (Table 1). After 90 days of culturing, fully grown IVP plantlets were transferred from *in vitro* condition (Figure 1c), as regenerants to pots containing soil, farmyard manure and sand at the ratio 1:1:1, respectively (Figure 1d). Subsequently, those were kept in a greenhouse for 30 more days for further growth. In field conditions, about 94% of plants survived successfully with normal growth (Figure 1e; Figure 1f). Rhizomes developed after 3-4 months of transferring of plantlets into pots and were subsequently harvested.

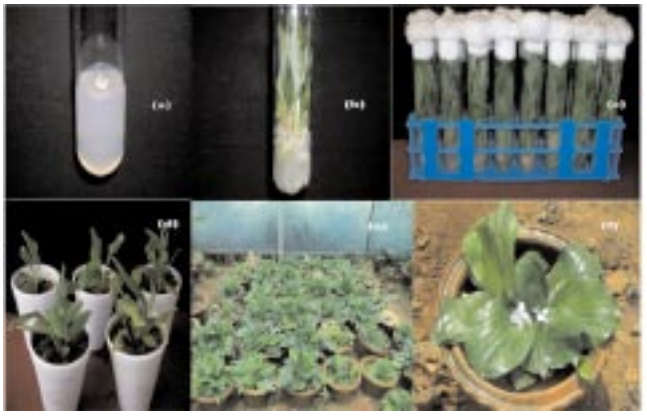

Figure 1. Different stages of *in vitro* propagation of *K. galanga*; 1a: Rhizome bud explant, 1b: Regenerated plants with shoots and roots; 1c: Multiplication of plantlets *in vitro* conditions; 1d: Potted plantlets in hardening stage; 1e: *In vitro* propagated plants growing under field condition; 1f: Flowering stage of *in vitro* propagated *K. galanga*.

3.2. Chemical composition of essential oil

The steam distillation of rhizomes yielded yellowish essential oils, which possessed the characteristic spicy-campherous odour. CP rhizomes yielded 0.6% and IVP rhizomes yielded 0.9% essential oil. A detailed chemical composition of the essential oil was assessed by GC-MS analysis. From chromatograms of essential oil samples of CP and IVP rhizomes, it was discernible that 6 major identified components, accounting for 97.81% and 96.9% of the total peak area in CP (Figure 2) and IVP (Figure 3) *K. galanga*, respectively were recorded. The result demonstrated the presence of EPMC with the maximum peak area (82.01%)

Table 2
Chemical constituents of essential oil of rhizomes from GC-MS analysis of CP and IVP *K. galanga*.

Compound name	Retention index	RT for CP derived oil (min)	RT for IVP derived oil (min)	Area % CP mean ± sd	Area % IVP mean ± sd
3-Carene	948.00	5.100	5.098	3.41±0.10	2.05±0.11
Eucalyptol	1 059.00	5.616	5.514	1.60±0.05	2.70±0.05
Ethyl cinnamate	1 373.00	21.917	21.996	9.69±0.10	18.14±0.61
Borneol	1 168.00	22.972	9.826	0.62±0.02	1.69±0.06
Pentadecane	256.12	29.618	23.013	0.57±0.07	1.46±0.08
Ethyl p-methoxy cinnamate	1 765.00	34.056	33.493	82.01±0.25	71.77±0.55

Note: RT, retention time; CP, conventionally propagated; IVP, *in vitro* propagated.

from CP rhizomes and (71.77%) from IVP rhizomes, followed by 5 chemicals, ethyl cinnamate, 3-carene, eucalyptol, borneol, pentadecane (Table 2).

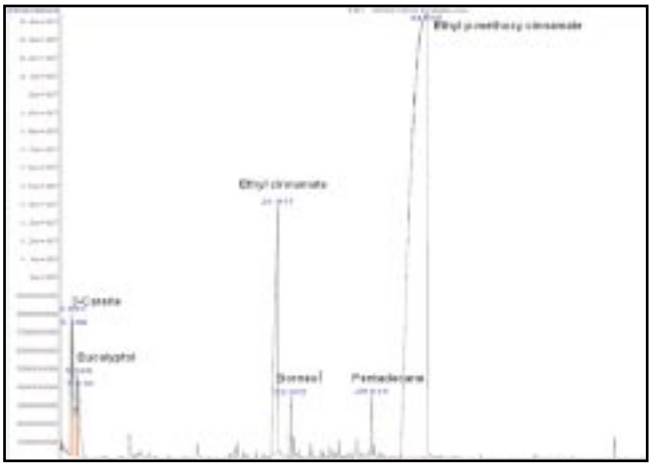

Figure 2. GC-MS spectrum of rhizome oil of conventionally propagated *K. galanga*.

3.3. Antioxidant activity

It was evident that increased antioxidative activity in DPPH free radical-scavenging assay was seen with increasing concentrations of oil samples (Figure 4), as a lower IC_{50} value indicated higher antioxidant activity. The DPPH scavenging activity of essential oil samples indicated a concentration dependent antioxidant activity against H_2O_2, with IC_{50} values, 6.6, 26, and 19.5 μg/mL for ascorbic acid, oil samples of CP and IVP rhizomes, respectively. Similarly, the H_2O_2 scavenging activity of essential oil samples indicated a concentration dependent antioxidant activity against H_2O_2, with IC_{50} values, 29, 24.5 and 21.5 μg/mL, for oil samples of CP and IVP rhizomes and ascorbic acid, respectively (Figure 5).

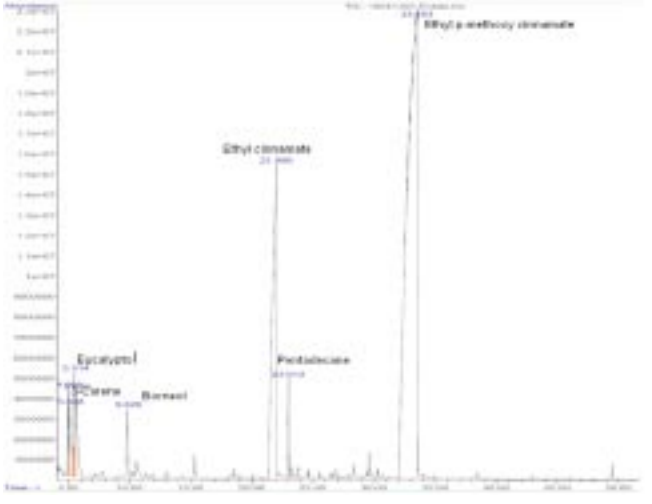

Figure 3. GC-MS spectrum of rhizome oil of *in vitro* propagated *K. galanga*.

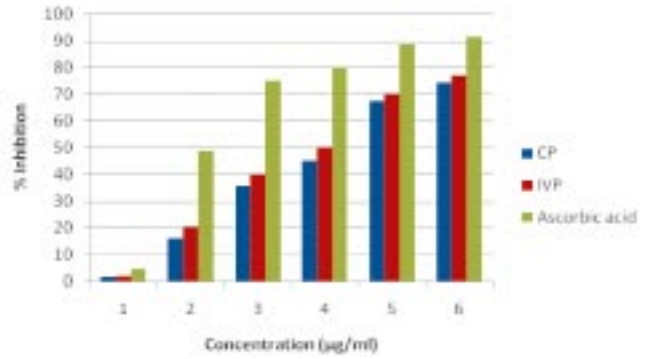

Figure 4. Radical scavenging activity of essential oil of both conventionally propagated (CP) and *in vitro* propagated (IVP) *K. galanga* rhizomes using DPPH; numbers in abscissa, 1 to 6 were 1, 5, 10, 20, 50 and 100 μg/mL oil concentrations.

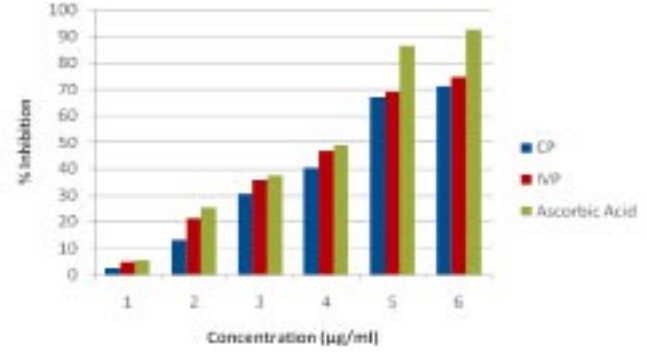

Figure 5. Hydrogen peroxide scavenging activity of essential oil of both conventionally propagated (CP) and *in vitro* propagated (IVP) *K. galanga*; numbers in abscissa, 1 to 6 were, 1, 5, 10, 20, 50 and 100 μg/mL oil concentrations.

4. Discussion

The maximum number of shoots was multiplied in MS agar supplemented with BA 1 mg/L in combination with IAA 0.5 mg/L. Additionally, IVP plantlets growing well in field conditions had a higher yield of essential oil, as well as peak areas in GC-MS analysis. Indeed, plant based secondary metabolites such as, essential oils are widely used in the pharmaceutical industries and are considered 'generally recognised as safe[25]. In addition to EPMC, the plant has triterpenoids, flavonoids and resins in chloroform extract and steroids, triterpenoids, alkaloids, flavonoids, carbohydrates, resins and proteins in the methanolic extract[26]. Two novel sulfonated diarylhepatonoid epimers, kaempsulfonic acid A(1) and B(2) were isolated from this plant[27]. But, no information is available in the literature on the comparative account of the chemical composition of essential oil of CP and IVP *K. galanga* rhizomes. In an earlier study, the compound ethyl p-methoxy cinnamate had 59.5% of the

total compounds detected in essential oil, followed by ethyl cinnamate, 3-carene, pentadecane, borneol, bornyl acetate, delta-selinene, camphor, alpha-piene and immidazole-5-carbonylvinyl-4-nitro, using another cultivar of the plant, *K. galanga* from south Odisha[6]. In this study, the values were 71.77% and 82.01% from CP and IVP *K. galanga* rhizomes, respectively with the local cultivar of central Odisha that needs molecular typing. EPMC had also been reported, as the major constituent of rhizome oil of *K. galanga* of different origins[10,28]. Our report is in close agreement with in *Curcuma longa*[29], another well-studied plant from Zingiberaceae, where the IVP clones were more uniform for their volatile constituents.

In this study, both IVP and CP sources of oil had remarkable antioxidant activities. In general, the essential oil obtained from IVP rhizomes showed a greater activity than that from CP rhizomes. The DPPH free radical scavenging activity of oil samples and that due to hydrogen peroxide of essential oil samples from IVP rhizomes, herein, were more than the activity level due to CP rhizomes; invariably reducing values due to ascorbic acid were the highest in each experiment. Antioxidants are compounds that neutralize chemically active products of metabolism such as, free reactive oxygen radicals, which can damage the body. Reactive oxygen species (ROS) include several metabolic by-products, hydrogen peroxide, hydroxyl radicals, nitric oxide, superoxide anions, peroxy nitrite and a few more that are potential enough to trigger degenerative processes in human body[30]. Compounds such as, phenolic acids, polyphenols and flavonoids are recognized as antioxidants, as they have the ability to scavenge free radical generating ROS. It has been documented that plant phenols neutralize free oxygen, eventually play a major role in the prevention of cancer, cardiovascular, neurogenerative diseases and a few more, with their potentiality to act as antioxidants[31]. Essential oils, despite their wide uses and fragrances, constitute an effective alternate to synthetic compounds produced by chemical industry without having any side effects[32]. Antioxidant activities of essential oils from aromatic plants are mainly attributed to inherent active compounds.

This study has shown that the yield of oil extracted from IVP plants is significantly higher than CP plants. Essential oils of both CP and IVP *K. galanga* displayed strong antioxidant properties, for which this plant is used as preventive agents from various diseases in diverse cultures. However the chemical profile and relative amount of compounds were very similar in both the oil samples. The result indicated that IVP *K. galanga*, with enhanced oil yield and uniform constituents, could be efficiently used for large scale commercial production of drug, thereby reducing the overexploitation of natural resources.

Conflicts of interests

The authors declare that they have no conflicts of interests.

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