MUTATIONAL BREEDING OF *CURCUMA LONGA* FOR MEDICINAL PURPOSES USING COLCHICINE AND GAMMA RAYS

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ABSTRACT

Mutational induction of *Curcuma longa* (Zingiberaceae) was performed to induce valuable clones for medicinal purposes. Two-month old *in vitro Curcuma longa* was treated with a chemical mutagen, colchicine, and a physical mutagen, gamma rays. Four concentrations of colchicine, 0 (control), 0.25, 0.5, and 0.75 % (w/v) with 4 duration treatments of 1, 2, 3, and 4 days were examined. For gamma rays, 5-intensity levels of gamma rays, 0 (control), 40, 50, 60, and 70 Gy were examined. The treated *Curcuma longa* (M1) was induced on shoot induction medium which contained 0.1 mg/l thidiazuron to obtain regenerated M2 plants, and then the induction was repeated until gaining of M3 generation plants. Numbers of vigorous M3 plants from colchicine and gamma rays experiments were selected for cultivation under greenhouse conditions. After 8 months, rhizomes were harvested. Thirty-seven colchicine-treated plants and sixteen gamma rays-treated plants were selected by phytomass criterion to further investigate chemical contents and cytological character.

Pulverised fingers of the colchicine-treated plants contained 2.00-6.20% (v/w) volatile oil while the control plants contained 4.75$\pm$0.34% (v/w). The gamma rays-treated plants contained 3.00-6.00% (v/w) volatile oil while the control plants contained 4.78$\pm$0.38% (v/w). In colchicine-treated plants, contents of curcumin, bisdemethoxycurcumin, and demethoxycurcumin were 1.24-4.28, 0.42-1.65, and 0.38-1.52% (w/w) respectively while those of the control plants were 3.45$\pm$0.21, 1.19$\pm$0.07, and 1.03$\pm$0.06% (w/w) respectively. In gamma rays-treated plants, contents of curcumin, bisdemethoxycurcumin, and demethoxycurcumin were 1.50-4.62, 0.56-1.74, and 0.24-1.79% (w/w) respectively while those of the control plants were 3.31$\pm$0.49, 1.29$\pm$0.12, and 0.98$\pm$0.17% (w/w) respectively. Three major constituents found in the volatile oil were *ar*-turmerone, turmerone, and curlone.

Eighteen of the plants treated with colchicine and four of the plants treated with gamma rays had greater yield production of total curcuminoids and volatile oil compared to the control plants.

It was only possible to establish the chromosome number in 20 plants, 10 colchicine-treated plants and 10 gamma rays-treated plants. All remained unchanged.

KEY WORDS: *CURCUMA LONGA* / MUTATIONAL INDUCTION / COLCHICINE / GAMMA RAYS / CURCUMINOIDS / VOLATILE OIL

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การปรับปรุงพันธุ์ขมิ้นชันเพื่อใช้ประโยชน์ทางยาโดยการกลายพันธุ์ด้วยโคลชิซินและรังสีแกมมา (MUTATIONAL BREEDING OF *CURCUMA LONGA* FOR MEDICINAL PURPOSES USING COLCHICINE AND GAMMA RAYS)

จารุวรรณ ดวงเนตร 4436783 PYPP/M

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บทคัดย่อ

การศึกษาการชักนำกลายพันธุ์ของขมิ้นชันด้วยสารก่อการกลายพันธุ์ คือ โคลชิซิน และวิธีการฉายรังสีแกมมา เพื่อให้ได้พันธุ์กลายที่มีคุณค่าในทางยา กล้าเพาะเลี้ยงขมิ้นชันอายุ 2 เดือนถูกกระตุ้นด้วยสารโคลชิซินที่ความเข้มข้น 0, 0.25, 0.50, และ 0.75% เป็นเวลา 1-4 วัน และรังสีแกมมาที่ระดับความเข้ม 0, 40, 50, 60, และ 70 เกรย์ หลังจากนั้นต้นแม่ (M1) ถูกนำมาชักนำยอดในอาหารเพาะเลี้ยงพื้นฐานที่มี thidiazuron เข้มข้น 0.1 มิลลิกรัมต่อลิตร จนกระทั่งได้ต้นลูกรุ่นที่ 2 (M2) และ 3 (M3) ต้น M3 ถูกคัดเลือกออกปลูกในเรือนกระจกเป็นเวลา 8 เดือน แล้วทำการเก็บเกี่ยวและคัดเลือกเหง้าเพื่อนำมาวิเคราะห์หาปริมาณสารสำคัญ ซึ่งผลการทดลองพบว่า ขมิ้นชันที่ผ่านการกระตุ้นด้วยโคลชิซินมีปริมาณน้ำมันหอมระเหยระหว่าง 2.00 ถึง 6.20% (v/w) ขณะที่กลุ่มควบคุมมีปริมาณ 4.75±0.34% (v/w) ส่วนขมิ้นชันที่ผ่านการกระตุ้นด้วยรังสีแกมมา มีความผันแปรอยู่ในช่วงระหว่าง 3.00 ถึง 6.00% (v/w)ขณะที่กลุ่มควบคุมมีปริมาณ 4.78±0.38% (v/w) ส่วนปริมาณสารกลุ่มเคอร์คูมินอยด์พบว่า curcumin, bisdemethoxycurcumin และ demethoxycurcumin ของต้น M3 ที่ผ่านการกระตุ้นด้วยโคลชิซินมีปริมาณ 1.24-4.28, 0.42-1.65 และ 0.38-1.52% (w/w) ตามลำดับ ในขณะที่กลุ่มควบคุมมีปริมาณ 3.45±0.21, 1.19±0.07, และ 1.03±0.06% (w/w) ตามลำดับ ส่วนขมิ้นชันที่ผ่านการกระตุ้นด้วยรังสีแกมมามีปริมาณ 1.50-4.62, 0.56-1.74 และ 0.24-1.79% (w/w) ตามลำดับ ในขณะที่กลุ่มควบคุมมีปริมาณ 3.31±0.49, 1.29±0.12 และ 0.98±0.17% (w/w) ตามลำดับ องค์ประกอบทางเคมีที่สำคัญในน้ำมันหอมระเหยของทั้งต้นทดสอบและต้นควบคุม คือ *ar*-turmerone, turmerone และ curlone นอกจากนี้พบว่ามีขมิ้นชันจากการชักนำด้วยโคลชิซิน 18 ต้น และจากการชักนำด้วยรังสีแกมมา 4 ต้น แสดงคุณสมบัติทางเคมีในเชิงปริมาณที่ดีกว่าต้นควบคุม

การตรวจสอบจำนวนโครโมโซมของต้นที่ผ่านการชักนำด้วยโคลชิซิน 10 ต้น และต้นที่ผ่านการชักนำด้วยรังสีแกมมา 10 ต้น ไม่พบการเปลี่ยนแปลงของจำนวนโครโมโซม

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ABBREVIATIONS

DPPH	1,1-Diphenyl-2-picrylhydrazyl
d.w.	Dry weight
EtOH	Ethanol
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectroscopy
HPLC	High performance liquid chromatography
IC_{50}	Inhibition concentration at 50%
MAO	Monoamine oxidize
MeOH	Methanol
MS	Murashige and Skoog (1962) medium
MW	Molecular weight
TDZ	Thidiazuron
LD_{50}	Lethal dose at 50%
LPS	Lipopolysaccharide
R^2	Correlation coefficient
RSD	Relative standard deviation
Rt	Retention time
S.E.	Standard error of mean
USP	United States Pharmacopoeia

CHAPTER I
INTRODUCTION

Curcuma longa (Zingiberaceae), turmeric, is one of domestic plants in Thailand. It is widely used as food and medicine. As traditional medicine, turmeric has been recommended as a practical remedy for healing of ulcers (1, 2). Extensive studies of turmeric found that volatile oil and curcuminoids were major compounds in rhizomes. The volatile oil itself showed anti-peptic activity (3). Aqueous and methanol extract of rhizome demonstrated significant decrease in gastric secretion (4). Moreover, methanol extract showed anti-inflammatory and anti-oxidation activities, and aqueous extract showed anti-depressant and immune surveillance activities (7-10). Clinical studies on the efficacy of turmeric for the treatment of gastric ulcer, peptic ulcer, and dyspepsia treatments were investigated. In all cases, turmeric demonstrated the effectiveness for symptomatic treatments without any toxicity (11-13). The Ministry of Public Health has recommended that turmeric can be used as herbal medicine for those diseases in National drug list. For medicinal purposes, Thai Herbal Pharmacopoeia has recommended that, in rhizome powder, the contents of curcuminoids should not less than 5%, and volatile oil should not less than 6% (5).

Mutational breeding in plants offers an opportunity to produce improving traits and create genetic variability. Combination of induced mutation technique with a variety *in vitro* culture methods can speed up mutational breeding programs (6). So far, induced mutation technique has been recommended to produce valuable traits which are high yield production, vigour, or resistance to pathogens and insects. Most of the prior works have been studied in agriculture and ornamental plants. In contrast, only limited work has been done to increase desired secondary metabolites in medicinal plants. In order to reach the destination, mutagens as colchicine and gamma ray are often shown their induced ability in many plant species such as *Digitalis obscura* (6), winged bean (7), potato (8), wheat (9), mungbean (10), *Artemisia annua* (11, 12), and *Scutellria baicalensis* (13). The effect of mutagens is not generally predictable, and each plant species must be examined individually. However, this is an alternative method to improve the quality of medicinal plants.

In this investigation, *in vitro Curcuma longa* were treated with colchicine and gamma rays. Effectiveness of both mutagens on induced mutation and the valuable mutants of *Curcuma longa* for medicinal purposes were evaluated. Curcuminoids and volatile oil contents were analysed by HPLC and GC-MS. The HPLC method for determination of curcumin, demethoxycurcumin, and bisdemethoxycurcumin was established. Cytological characteristic of the treated *Curcuma longa* was observed by using chromosome count technique.

CHAPTER II
LITERATURE REVIEW

1. *Curcuma longa* L.

Synonym: *Curcuma domestica* Val.

1.1 Botanical characteristics

Curcuma longa, turmeric, is generally known by Thais as ‘Khamin Chan’. However, it has difference names in difference parts of Thailand such as ‘Khamin’ (Central), ‘Khamin kaeng’, ‘Khaomin yok’, ‘Khamin-hua’, ‘Khee-min’, ‘Min’ (peninsular), ‘Taa-yo’ (Karen-Khamphaeng Phet), and ‘Sa-yo’ (Karen-Mae Hong Son) (14).

Turmeric is a perennial rhizomatous herb, having stout, fleshy, main rhizome nearly ovoid, about 3 cm in diameter and 4 cm long. Lateral rhizomes, slightly bent, 1 cm in diameter and 2-6 cm long. Rhizome flesh presents orange yellow color. Leaves emerge directly from underground stem with overlapping petioles, lanceolate, uniformly green, up to 30-50 cm long and 7-25 cm wide; apex acute and caudate with tapering base. Petiole and sheet are sparsely to densely pubescent. Blades are thin ellipse-shaped or elongate lance-shaped. Inflorescence (spike) is cylindrical, about 10-15 cm long and 5-7 cm in diameter, appearing with the leaves and developing in their centre. It consists of imbricate bracts, 5-6 cm long, pouch-like curved shape, white or white with light green color at upper half. Each bract subtends pale yellow flowers (except in the upper part). Bracteoles are up to 3.5 cm long. Flowers are about 5 cm long; calyx tubular, unilaterally split, unequally toothed; corolla white, tube funnel shaped, limb 3-lobed. Stamens lateral, petaloid, widely elliptical, longer than the anther; filament united to anther about the middle of the pollen sac, spurred at base. Ovary appears 3-locules (trilocular), each locule contains 2 ovules; style glabrous. The capsules are ellipsoid. Seeds are rare (1, 5).

Figure 1 Features of *Curcuma longa*. (A) Whole plant (Saralump P, Temsiririrkkul R, Chuakul W, Riewpaiboon A, Prathanturarug S, Charuchinda C, et al. Medicinal plants in Siri Ruckhachati Garden. Bangkok: Amarin printing; 1992.); (B) Cylindrical inflorescence and flowers; (C) Rhizome consists of main rhizome (Corm) and lateral rhizomes (fingers); (D) Main rhizome, fingers, and longitudinal section of finger with orange-yellow color inside. Scale bar: A = 10 cm, B-D = 1 cm.

1.2 Distribution, ecology, and cultivation

Turmeric is a domestic plant which is found throughout Thailand. However, its distribution can also be found throughout tropical and subtropical of the world such as in India, Nepal (2), Indonesia, Philippine (15), Brunei Darussalam, Jamaica, and Peru (16).

Turmeric grows well in rather hot climates with humidity at night, and prefers well-drain loam. Cultivation of turmeric should start in May. Before cultivation, land should be weeded, plowed, and left for 2 weeks. Then, the plots of 45x50 cm size are prepared by raising the soil to 25 cm above the ground. About 2-3 tons of manure per rai (1600 square meters) is applied on the land before planting. The 11-12 months old rhizomes are cut into small pieces, each piece containing 1-2 buds. The rhizomes should be planted 6-8 cm underneath the ground with spacing of 35x35 cm. Five to seven days after planting, the young shoots begin to develop. Irrigation may be needed if there is not enough rain. Weeding every month and applying of 2-3 tons per rai of fertilizer when turmeric is 2 and 4 months old are suggested to obtain good yields. After 7 months, the leaves become yellow, indicating the maturity of the rhizomes. However, rhizomes must leave in the ground until they are 9-10 months old before harvesting (1).

1.3 Ethnomedical uses

In Thailand, turmeric is well known for treatment of gastrointestinal illness such as ulcers, diarrhea, and dysentery. Externally for dermatitis, rashes, insect bites, and wounds healing (1, 17).

In India and Nepal, it has been used as household remedy. Its usage is recommended as carminative, stimulant, anthelmintic, for biliary disorders, hepatic disorders, diabetic wounds, rheumatism, and sinusitis (18, 19).

Traditional uses and recipes of turmeric in some countries are presented in table 1.

Table 1 Variety of medicinal purposes and their preparations of *Curcuma longa* in traditional medicine.

Medicinal purposes	Traditional preparation	Country	Reference
Peptic ulcers	Mix turmeric powder with honey and administrator orally 3 times a day.	Thailand	(1)
Diarrhea and dysentery	1. Bruise roast rhizome, add lime juice, and then squeeze the suspension. Take 1-2 cups of the liquid.	Thailand	(1)
	2. Bruise rhizome, add warm water within a ratio of 1:1. Take 2 teaspoonfuls of the suspension 3-4 times a day.	Thailand	(1)
Anthelmintic	Boil about 1 g of rhizome paste or powder in 400 ml of water, and add a little salt. Drink warm twice a day for 3-4 days.	Nepal	(20)
Treatment of ring worm and *Taenia versicolor*	Mix turmeric powder with rain water, and apply on infected area 2 times daily.	Thailand	(1)
Treatment of wounds	1. Boil turmeric powder with coconut oil or lard until yellow oil is obtained. Apply the obtained oil on the wounds.	Thailand	(1)
	2. Mix rhizome with a few milliliters of lime juice and alum or potassium nitrate. Apply on the wounds.	Thailand	(1)
Treatment of insect bites	1. Mix turmeric powder with water, and apply frequently on the area.	Thailand	(1)
	2. Scraps rhizome and rub on the affected area.	Thailand, Brunei Darussalam	(1, 21)

Table 1 Variety of medicinal purposes and their usage of *Curcuma longa* in traditional medicine (continued).

Medicinal purposes	Traditional preparation	Country	Reference
Treatment of skin rashes	Mix rhizome powder, juice from aerial root tips of *Pandanus tectorius*, and coconut oil or copra. Apply on rash area.	Kingdom of Tonga	(22)
Antiseptic of wounds	Mix rhizome powder with rapeseed oil (*Brassica napus* L. var. *napus* L.). Apply to cuts and wounds.	Nepal	(23)
Treatment of throat infection and expectorant	Decoction of rhizome powder and common salt (2 g of each) in 300-400 ml of water. Drink warm before bed-time for 3-4 days.	Nepal	(23)
Common cold, bronchitis, and cough	Mix rhizome powder and hot milk. Drink warm.	India, Oman	(18, 24)
Treatment of febrifuge, and anti-malarial	Inhalation and decoction of leaves.	Madagascar	(25)

1.4 Chemical studies

A great number of compounds have been identified as constituents in turmeric especially in rhizomes. Its rhizome contains phenolic compounds, terpenoids (26, 27), polysaccharide, and fatty acids (28, 29).

Phenolic compounds named curcuminoids are yellow pigments in the rhizomes. Total curcuminoids has been found 3-5% of rhizome (27). Three curcuminoids are isolated that are curcumin, demethoxycurcumin and bisdemethoxycurcumin. Curcumin, diferuloylmethane, is a main compound in between all three curcuminoids (30). Boiling curcumin with alkali gives vanillic acid and furfulic acids (16). Chemical structures of those three curcuminoids are shown in figure 2.

Terpenoids are constituents in volatile oil of turmeric (31). In rhizomes, volatile oil has been found 2-7% (27). Major compounds vary depending on forms of material, locations of cultivation and analysis methods. However, the majors have been declared including *ar*-turmerone, α-turmerone, β-turmerone, curlone, zingiberene, *ar*-curcumene, curcumene, curdione, α-phellandrene, germacrone, and β-sesquiphellandrene (5, 16).

Besides, miscellaneous terpenoids have been reported i.e., β-atlantone, β-biotol, β-bisabolene, cis-γ-bisabolene, 1-bisabolone, camphene, 3-carene, carvacrol, cariofilene, caryophyllene oxide, 8-*p*-cimenol, *p*-cimene, *p*-cimonene, 8-p-cimenol, 1,8-cineole, α-curcumene, γ-curcumene, *p*-cymene, β-caryophyllene, 2,5-dihydroxybisabola-3,10-diene, γ-elemene, α-felandrene, (*E,E*)-α-farnesene, germacrone-13-al, α-humulene, 4-hydroxybisabola-2,10-diene-9-one, isopulegol, limonene, 4-methoxy-5-hydroxy bisabola-2,10-diene-9-one, myrcene, myrcenone, α-pinene, β-pinene, procurcumadiol, sabinene, trans-sabinene hydrate, α-terpinene, γ-terpinene, terpinen-4-ol, α-terpineol, terpinolene, α-thujene, α-turmerol, β-turmerol, turmerone A, turmerone B, and α-zingiberene (16, 26, 27). In leaf, a diterpene, labda-8(17),12-diene-15,16-dial, has been reported (32). Chemical structures of some constituents in volatile oil represent in figure 3.

Polysaccharide, a glycan named ukonan is isolated from rhizomes (28).

Fatty acids are found in rhizomes i.e., tetradecanoic acids, *cis*–9-hexadecanoic acid, hexadecanoic acid, *cis*,*cis*–9,12-hexadecanoic acid, *cis* and *tran*-9-octadecenoic acid, octadecanoic and eicosanoic acid (33).

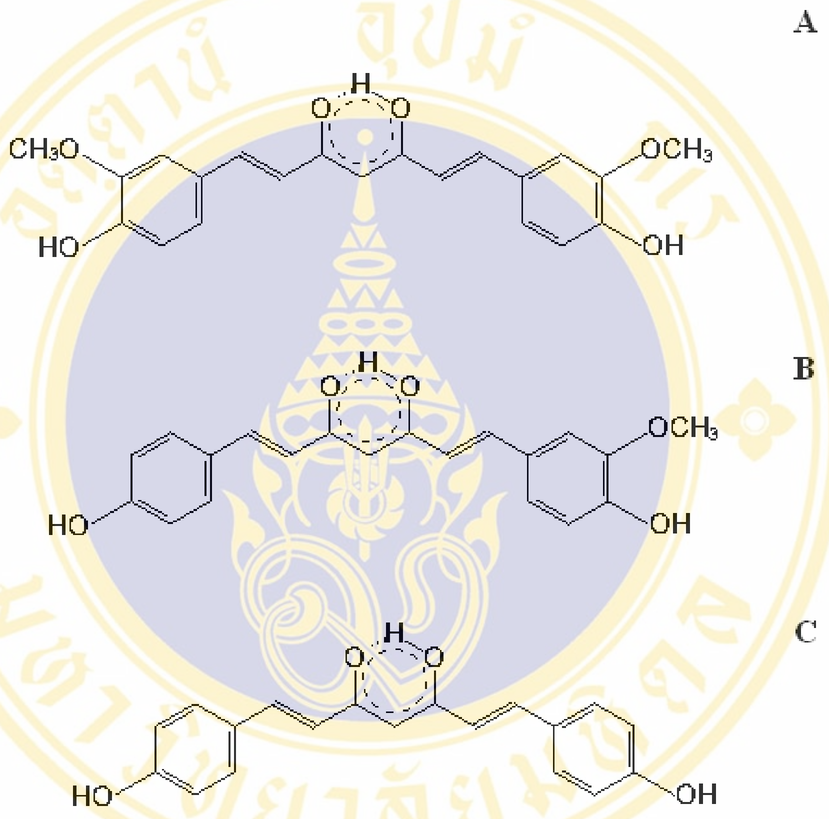


Figure 2 Structure of curcuminoids. (A) Curcumin; (B) Demethoxycurcumin; (C) Bisdemethoxycurcumin.

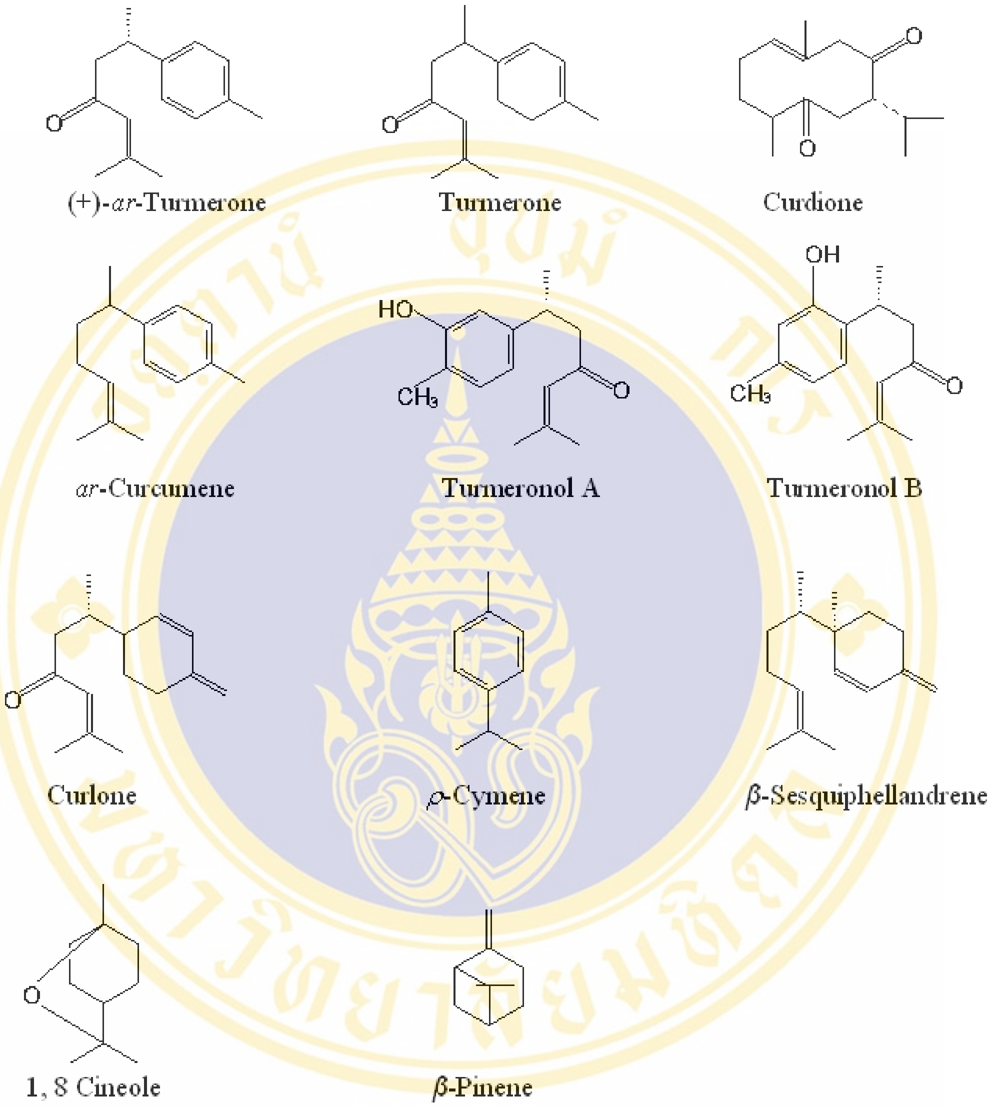
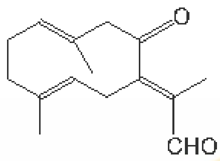
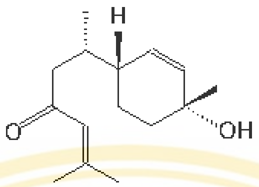


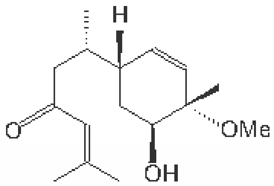
Figure 3 Structure of components found in volatile oil.



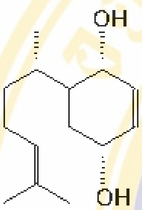
Germacrone-13-al



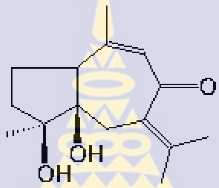
4-Hydroxybisabola-
-2, 10-diene-9-one



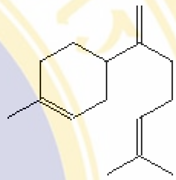
4-Methoxy-5-hydroxy
bisabola-2, 10-diene-9-one



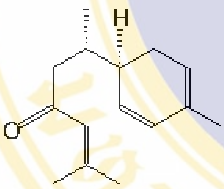
2, 5-Dihydroxybisabola-
3, 10-diene



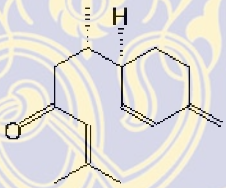
Procurcumadiol



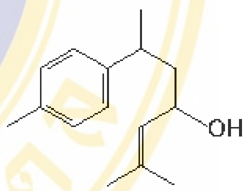
β-Bisabolene



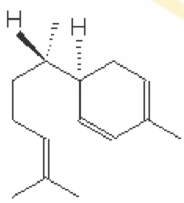
α-Turmerone



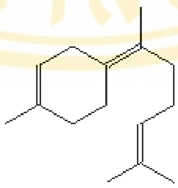
β-Turmerone



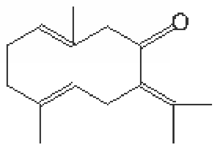
α-Turmerol



α-Zingiberene



cis-γ-Bisabolene



Germacrone

Figure 3 Structure of some components found in volatile oil (continued).

1.5 Biological studies

Biological activities of turmeric have been reported as present in table 2.

Table 2 Biological activities of turmeric.

Activity	Effect	Reference
Anti-ulcer	EtOH extract at doses of 500 mg/kg showed inhibitory effect to gastric secretion and protected gastro duodenal mucosa against the injuries caused by cyst destructive agents in rats.	(35)
Anti-inflammation	MeOH extract at concentration of 10 µg/ml showed 74% inhibition to prostaglandin E_2 production in LPS-induced mouse and 88.4% inhibition to nitric oxide formation in macrophages.	(21)
Anti-oxidation	MeOH extract at concentration of 47 µg/ml showed IC_{50} reduction of DPPH radical in *in vitro* model.	(36)
	Aqueous Extract at concentration of 50 µg/ml showed 50% inhibition of lipid peroxidation in brain tissues.	(37)
Anti-depressant	Aqueous extract at dose of 140-560 mg/kg showed inhibitory effect to monoamine oxidise A activity (MAO) in mice.	(38)
Immune surveillance	Aqueous extract at concentration of 700 ng/ml promoted adhesion of peripheral neutrophils to endothelial cells and activate nuclear transcription factor NF-*k*B. The studies carried out in human endothelial cells.	(39)
Anti-microbial	Essential oil at dilution of 1:40-1:320 showed growth inhibition of dermatophyte fungi.	(40)
	Essential oil, 15 µl volume, inhibited growth of pathogenic bacteria and fungi.	(30)
Anti-cancer	Curcumin inhibited chemical carcinogenesis in difference tissue sites in experimental animal models such as scavenging of superoxide anion and hydroxyl radical, inhibition of lipoxygenase and cyclooxygenase activities, TPA-induced ornithine decarboxylase (ODC) mRNA and activity, arachidonic acid metabolism, and formation of carcinogen-DNA adducts etc.	(41, 42)

Table 2 Biological activities of turmeric (continued).

Activity	Effect	Reference
Anti-venom	*ar*-Turmerone neutralized both hemorrhagic activity present in *Bothrops jararaca* venom, and lethal effect of *Crotalus durissus terrificus* venom in mice.	(43)
Anti-integrase activity	Curcumin at concentration of 40 µM showed IC_{50} to HIV-1 integrase.	(44)

1.6 Clinical studies

A randomized double-blind study was performed in 106 patients with acid dyspepsia, flatulent dyspepsia, or atonic dyspepsia. The result showed that turmeric powder at dose of 2 g/day for 7 days was effective for symptomatic treatment (17).

A controlled clinical trail of 50 patients with gastric ulcer was established. Turmeric powder was given orally at doses of 750 mg/day to the patients. The result showed that turmeric was effective for ulcer healing after 6 and 12 weeks of treatment (45).

A clinical trail of 45 patients with peptic ulcer demonstrated that turmeric powder at doses of 3g/day for 4, 8, and 12 weeks was effective for healing of ulcers with no toxicity appeared. Group of patients who had erosion, gastritis, and dyspepsia, and had no ulcers, the symptoms subsided in first and second week of treatment (46).

2. Plant chromosome and mutational breeding of plants *via* tissue culture

Each living plant cell typically contains genome, a complete set of genetic information, which determines the characteristic of the whole organism. Plant species are distinguished by having a characteristic basic number of nuclear chromosomes which is known in term of ploidy level. Diploid (2x) is a characteristic of having two fold of basic chromosome number. It basically presents during vegetative phase of growth, and plant cells normally have diploid.

Other whole multiples, such as haploid (x), triploid (3x), and tetraploid (4x) are commonly encountered. Organism whose somatic nuclei contain more than two haploid sets of chromosome are said to be “polyploidy”. On the other hand, the evidence of breaking of chromosomes into two or more fragments causes aneuploid, and the organism which its somatic nuclei bear such genome is called “aneuploidy” (47). Aneuploid causes either presenting of extra chromosomes, which is marked as 2x+1, 2x+2, or lacking of some copies of chromosomes, which is marked as 2x-1, 2x-2.

Mutational breeding in plants offers an opportunity to produce improving traits and create genetic variability. Potential of mutational breeding can be reinforced by tissue culture methods since the breeding program can be speeded up (6). Besides, tissue culture methods are benefit in several aspects. First of all, it offers a wide choice of plant material for treatment such as auxiliary bud, organ tissues, and cells. This means less risk of obtaining chimeric plants and higher probability for mutated cells to express the mutation in the phenotype. Tissue culture allows for the handling of large population for mutagenic treatment, selection, and cloning of selected mutants. It offers the possibility to rapidly execute the propagation cycles of subculture aimed to separate mutated from non-mutated sectors. Another advantage of this methodology is that high phytosanitary conditions are maintained throughout the whole process (48).

Induced mutation is proven to be an effective technique in order to produce valuable traits, for instance, high yield production, vigour, and resistance to pathogens and insects. This technique has been applied mostly in agriculture, ornamental, and other economically important plants such as winged bean (7), mungbean (10), pomegranate (49), wheat (9), potato (8), lotus (50), banana (51), cassava (52), and hop (53).

For medicinal purposes, very limited works have been done aim at overproducing of desired secondary metabolites in medicinal plants. A number of reports have been shown the success of induction in medicinal plants as follow.

Artemisia annua L. was treated with 0.25% (w/v) colchicine. An average artemisinin level in the tetraploid mutants was 38% higher than that of the wild-type (diploidy) while the average essential oil content of tetraploidy was 32% lower (11). The tetraploid clones inducing from hairy root tips of diploid *Artemisia annua* L. with colchicine at concentrations of 0.5%, 0.25%, 0.1% and 0.05% (w/v) produced up to six times more artemisinin than the diploid parents (12).

Digitalis obscura was irradiated with gamma ray at the intensity of 20-40 Gy. The derived variants corresponded to aneuploid changes and presented a high variability in their cardenolide production from 878 to 3291 µg/g dry weights while the wild T4 plant and control plants were 2946±206 and 2301±288 µg/g dry weight, respectively (6).

Calluses of *Scutellaria baicalensis* were treated in 0.2% (w/v) colchicine solution for 3- 24 h. At 12 h treatment showed optimum rate of tetraploid induction of 30%. The tetraploid mutants presented variability in baicalin production, however, 80% of the mutants showed up to 47.6% higher baicalin content than diploid parents (13).

Although the effect of induced mutations are not generally predictable and each plant species must be examined individually, however, the technique offers a chance to obtain the valuable mutants with an increased productivity of specific secondary metabolites.

CHAPTER III
MATERIALS AND METHODS

1. Materials

1.1 Plant materials

In vitro Curcuma longa clones (C12AC and C11AG) were selected from the culture stock of Tissue Culture Laboratory, Department of Pharmaceutical Botany, Faculty of Pharmacy, Mahidol University.

1.2 Materials used for mutational induction experiments

1.2.1 Chemicals

- Ammonium nitrate (NH_4NO_3)	(Fluka)
- Potassium nitrate (KNO_3)	(Merck)
- Calcium chloride dihydrate ($CaCl_2.2H_2O$)	(Merck)
- Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)	(Merck)
- Potassium dihydrogen phosphate (KH_2PO_4)	(Merck)
- Manganese(II) sulphate monohydrate ($MnSO_4.H_2O$)	(Merck)
- Zinc sulphate heptahydrate ($ZnSO_4.7H_2O$)	(Merck)
- Boric acid (H_3BO_3)	(Merck)
- Potassium iodide (KI)	(Merck)
- Sodium molybdate dihydrate ($Na_2MoO_4.2H_2O$)	(Merck)
- Copper(II) sulphate pentahydrate ($CuSO_4.5H_2O$)	(Merck)
- Cobalt(II) chloride hexahydrate ($CoCl_2.6H_2O$)	(Merck)
- Nicotinic acid ($C_6H_5NO_2$)	(Sigma)
- Pyridoxine hydrochloride ($C_8H_{11}NO_3.HCl$)	(Sigma)
- Thiamine hydrochloride ($C_{12}H_{17}ClN_4OS.HCl$)	(Sigma)
- Glycine ($C_2H_5NO_2$)	(Sigma)

- Iron(II) sulfate heptahydrate ($FeSO_4.7H_2O$)	(Merck)
- Sodium EDTA ($C_{10}H_{14}N_2Na_2O_8.2H_2O$)	(Merck)
- Myo-inositol ($C_6H_{12}O_6$)	(Sigma)
- Sucrose	(Merck)
- Agar gel	(Sigma)
- 95% Ethanol, commercial grade	
- Thidiazuron	(Fluka)
- Colchicine	(Fluka)

1.2.2 Apparatuses

- Balance model AG204	(Mettler teledo®)
- pH meter model MP220	(Mettler teledo®)
- Magnetic stirrer model AGIMATIC-E	(Selecta®)
- Autoclave model SS-325	(Tommy®)
- Laminar air flow cabinet model H1	(Clean ®)
- Rotary shaker	

1.3 Materials used for chemical analysis

1.3.1 Solvents

- Acetronitrile, HPLC grade	(Labscan)
- Glacial acetic acid, AR grade	(Labscan)
- Water, HPLC grade	(Labscan)
- Methanol, HPLC grade	(Labscan)
- Methanol, AR grade	(Labscan)
- *n*-Hexane, AR grade	(Labscan)
- Dichloromethane, AR grade	(Labsacn)

1.3.2 Reference standards

Standard curcumin, demethoxycurcumin, and bisdemethoxycurcumin were provided by Prof. Apichart Sooksamran, Department of Chemistry, Faculty of Science, Ramkhamhang University.

1.3.3 Apparatuses

- HPLC apparatus	
Vacuum degasser Series	(Perkin-Elmer®)
Pump series 200LC	(Perkin-Elmer®)
Diode array detector series 200	(Perkin-Elmer®)
Hypersil BDS-C18 column, 4.6mm x 250 mm	(BDS Hypersil®)
20 µl syringe loading sample injector model 7725i	(Rheodyne®)
- GC-MS	(Shimazu)
- UV spectrophotometer model 3000 Array	(Miltonroy®)
- Mill Type MM 200	(Retsch®)
- Ultrasonic bath	(Selecta®)
- Heating mantle	
- Rotary evaporator	(Eyela)

1.3.4 Miscellaneous

- Filter paper No.1	(Whatman®)
- Büchner funnel	
- Receiver glass tube	(Pyrex®)

1.4 Materials used for cytological observation

1.4.1 Chemicals

- *p*-Dichlorobenzene	(Fluka)
- Acetic acid	(Fluka)
- Hydrochloric acid	(Fluka)
- Potassium metabisulphite ($K_2S_2O_5$)	(Fluka)
- 95% Ethanol, commercial grade	
- Basic fuchsin	(Sigma)
- Orcein	(Sigma)

1.4.2 Apparatuses

- Microscope model CH30 RF200 (Olympus)
- Digital camera DP50 (Olympus)

1.4.3 Miscellaneous

- Filter paper No.1 (Whatman®)
- Filter paper No.42 (Whatman®)

2. Methods

2.1 Preparation of media for *in vitro* culture

2.1.1 Basic medium

The composition of basic medium based on MS medium is shown in table 3. A stock solution of microelements excluding $FeSO_4.7H_2O$ and Na_2EDTA was prepared at 100-fold concentration. Organic components (except myo-inositol) were prepared as 500 or 2,000-fold concentrates, and the solutions were stored at 4 ºC (in refrigerator).

A double concentrated MS stock solution without iron was prepared by dissolving macronutrients and myo-inositol one by one and adding the stock solution of microelements and organic compounds. After adjusting volume, the stock solution was divided into portions of 500 or 1,000 ml and stored at -20 ºC until required.

A solution of Fe-EDTA chelate (x100) was prepared by dissolving $FeSO_4.7H_2O$ (2.78 g/l) and Na_2EDTA (4.1 g/l) separately in water. The solutions were heated, mixed together, cooled, volume adjusted, and stored in the dark at 4 ºC.

Media were prepared by dissolving sucrose (3%) in water, adding the MS stock solution, and the Fe-EDTA solution. Agargel® (0.55%) was used as a gelling agent. Then, the media was adjusted pH to 5.8 and dispensed to the bottle before autoclaving.

2.1.2 Shoot induction medium

Thidiazuron (TDZ), a plant growth regulator, was brought into solution in a few drops of 1N NaOH. The volume was adjusted by water. The stock solution was kept at 4 ºC until required but not longer than 1 month.

The shoot induction medium, which contained 0.1 mg/l TDZ, was prepared based on basic medium preparation, and aliquot of TDZ stock solution was added before the gelling agent. The medium was adjusted pH to 5.8 and dispensed to the bottle before autoclaving.

For the experiment, 4 media were prepared as liquid media which contained all basic medium composition except gelling agent.

Table 3 Composition of MS medium.

Compounds	mg/l
Macroelements:	
Ammonium nitrate (NH_4NO_3)	1,650
Potassium nitrate (KNO_3)	1,900
Calcium chloride dihydrate ($CaCl_2.2H_2O$)	440
Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$)	370
Potassium dihydrogen phosphate (KH_2PO_4)	170
Microelements:	
Iron (II) sulfate heptahydrate ($FeSO_4.7\ H_2O$)	27.8
Sodium EDTA ($C_{10}H_{14}N_2Na_2O_8.2H_2O$)	41.0
Boric acid (H_3BO_3)	6.2
Manganese (II) sulfate monohydrate ($MnSO_4.H_2O$)	16.9
Zinc sulfate heptahydrate ($ZnSO_4.7H_2O$)	8.6
Potassium iodide (KI)	0.83
Sodium molybdate dihydrate ($Na_2MoO_4.2H_2O$)	0.25
Copper (II) sulfate pentahydrate ($CuSO_4.5H_2O$)	0.025
Cobalt (II) chloride hexahydrate ($CoCl_2.6H_2O$)	0.025
Organic substances:	
Myo-inositol ($C_6H_{12}O_6$)	100
Glycine ($C_2H_5NO_2$)	2.0
Nicotinic acid ($C_6H_5NO_2$)	0.5
Pyridoxine hydrochloride ($C_8H_{11}NO_3.HCl$)	0.5
Thiamine hydrochloride ($C_{12}H_{17}ClN_4OS.HCl$)	0.1

2.2 Preparation of plant materials

Initiative *Curcuma longa* clones were regenerated by using solid MS medium supplemented with 0.1 mg/l thidiazuron (TDZ), which was used as shoot induction medium. Before inoculation, leaves and roots were trimmed off remaining only the terminal buds of about 0.5 cm long. The terminal buds were inoculated vertically on the shoot induction medium, and incubated under controlled condition of 25-28^{o}C, 16/8-h (light/dark) photoperiod with light intensity of 37.5 µmol.m^{-1}.s^{-1}. After 4 weeks, tiny regenerated shoots appeared, and then they were transferred into MS basic medium for another 4 weeks. The regenerated plantlets were dissected from the cluster and separately cultured on the same medium for 8 weeks. This propagation's cycle took 16 weeks each, and it was repeated until the number of plant materials was sufficient for the experiments.

2.3 Mutational induction by colchicine

Effect of colchicine on *Curcuma longa* was studied at three degree of colchicine concentration. Colchicine treatment media were prepared by addition 0.25, 0.50, and 0.75 % (w/v) of colchicine into each 30 ml volume of liquid MS medium containing in 50 ml-Erlenmeyer flasks.

Two-month old terminal buds, 0.3-0.4 cm long, were inoculated into each treatment medium. The liquid MS medium without colchicine was used as control. After inoculation, all the treatment flasks were horizontally shake on rotary shaker for 1, 2, 3, and 4 days. This step, the treated shoots were marked as M1 plants.

After 1-4 days of treatment, the shoots were rinsed 3 times with sterile water, and cultured on shoot induction medium for 4 weeks to obtain M2 generation plants. Then, they were transferred into solid MS medium to culture for 6 weeks. The M2 plants were further induced in the same way until obtaining M3 generation plant.

During the process the treated plants were given the codes in order to identify the plants from the experiment. Sixteen treatments of colchicine mutational induction summarize, and treatment codes are explained in table 4.

Table 4 Treatments of colchicine induction experiment.

% (w/v) Colchicine	Treated duration (day)			
0.00 [control]	1 (C01)	2 (C02)	3 (C03)	4 (C04)
0.25	1 (C11)	2 (C12)	3 (C13)	4 (C14)
0.50	1 (C21)	2 (C22)	3 (C23)	4 (C24)
0.75	1 (C31)	2 (C32)	3 (C33)	4 (C34)

2.4 Mutational induction by gamma rays

In this investigation, irradiation of gamma rays, using ^{60}Co gamma source, was facilitated by the Office of Atoms for Peace, Ministry of Science and Technology.

Two-month old *Curcuma longa* was acute irradiated with broad range intensity of gamma rays, 0, 20, 40, 60, 80, and 100 Gy. After irradiation, the plants were subcultured into solidified MS medium, and incubated at the same controlled conditions as mentioned in 2.2. Lethality rate was observed every 15 days until the day sixtieth. The LD_{50} intensity was obtained by plotting between intensity and survival rate. The appropriated intensity for performing mutational induction in *Curcuma longa* was estimated from the LD_{50} intensity.

Four intensities of gamma rays, 40, 50, 60, and 70 Gy, were chosen to study in this experiment. Two-month old *Curcuma longa* was acute irradiated with those intensities, 50 plants each. After irradiation, M1 plants were subcultured into shoot induction medium to obtain M2 regenerated plants. Then, they were transferred into solidified MS medium. The M2 plants were further induced in the same way until obtaining M3 generation plant. Unirradiated plants were control, and were parallel proceeded as same as the treated plants.

During the process the treated plants were given the codes. The treatment codes are explained in table 5.

Table 5 Treatments of gamma rays induction experiment.

Intensity (Gy)	Treatment code
0 [control]	G0
40	G40
50	G50
60	G60
70	G70

2.5 Cultivation and harvest

The vessels containing experimented M3 plants from colchicine and gamma rays experiments were exposed to room temperature for 1 week, and then the plants were brought into acclimatised trays containing sand and rice shell ash (1:1), and maintained in acclimatised chamber with irrigation throughout day time for 2 weeks.

This stage, 2 selection criteria were performed in order to select the potential clones derived from both colchicine and gamma rays experiments.

Selection criterion 1: According to survival ability, numbers of vigorous M3 plants from both mutational induction experiments were selected to transplant into pots containing agriculture soil under greenhouse conditions.

Morphological characteristics of the plants were recorded, and abnormal characters were also noted.

After 8 months of cultivation, their rhizomes were harvested. Fingers of each cultivated plants were separated from their corms, cut into thin and small pieces, and dried in hot air oven at 50-60 ºC for 48 h. Data of fresh and dry weight were recorded.

Selection criterion 2: According to phytomass production, dry weight of fingers of each plant was evaluated comparing to that of average value of the control plants. The plants representing superior in dry weight (based on the average value) were chosen to chemically analyze and cytological observe.

2.6 Chemical analysis of *Curcuma longa*

Before all analyses, dried fingers were pulverized by using mill (Restch®), and sieved through the sieve No.40 to obtain homogeneous powders.

2.6.1 Quantitative determination of curcumin, demethoxycurcumin, and bisdemethoxycurcumin by HPLC

2.6.1.1 Extraction methods

The extraction methods were modified from the method of Thai Herbal Pharmacopoeia 1995 (54). Total curcuminoids content of the extract was detected by UV spectrophotometer, and calculated the amount from a linear regression of calibration curve of curcumin.

Calibration curve of curcumin

Stock solution of curcumin was prepared in methanol at concentration of 0.46 mg/ml. Five concentrations of curcumin were prepared from the stock solution in range of 0.92-3.68 µg/ml, and the absorbance of each solution was detected by UV spectrophotometer at wavelength of 420 nm. The calibration curve was drawn and evaluated by using linear regression.

Determination of total curcuminoids in the test extracts

Before detecting all the test extracts by UV spectrophotometer, 1 ml of the each extract was diluted with methanol into 50 ml-volumetric flask. The absorbance of each replica of the test extracts was detected by UV spectrophotometer at wavelength of 420 nm triplicate.

To make exhaustible extraction, effect of number and duration of extraction, and volume of methanol were examined in experiment A, B, and C.

Experiment A was to examine appropriate number of extraction.

Experiment A1-A4: A 300-mg amount of sample powder was sonicated with 10 ml of methanol (AR grade) in an ultrasonic bath for 0.5 h, 1-4 times. The mixture was filtered under vacuum over a Büchner funnel with filter paper (Whatman No.1). One ml of the filtrate was transferred into a 25-ml volumetric flask, and volume adjusted with methanol.

Experiment A1, the powder was extracted for 1 time. Experiment A2, A3, and A4, residue from the first extraction was further extracted with 10 ml of methanol in the same way for another 1, 2, and 3 time respectively.

Experiment B was to examine appropriate duration of extraction.

Experiment B1-B3: A 300 mg amount of sample powder was sonicated with 10 ml of methanol (AR grade) in an ultrasonic bath for 1 h, 1-3 times. After filtration, the extract solution was prepared in the same way as experiment A. Experiment B1, B2, and B3, the powder was extracted 1, 2, and 3 times respectively.

Experiment C was to examine appropriate volume of methanol.

Experiment C1-C4: A 300-mg amount of sample powder was sonicated with 10, 15, 20, and 30 ml of methanol (AR grade) in an ultrasonic bath for 0.5 h, 3 times. After filtration, the extract solution was prepared in the same way as experiment A. Experiment C1, C2, and C3, the powder was extracted 1, 2, and 3 times respectively.

All extraction experiments are summarized in table 6.

Table 6 Experiments on extraction methods of *Curcuma longa*.

Experiment		Volume of methanol (ml)	Duration of extraction (h)	Number of extraction (time)	Replication
A	A1	10	0.5	1	3
	A2	10	0.5	2	3
	A3	10	0.5	3	3
	A4	10	0.5	4	3
B	B1	10	1	1	3
	B2	10	1	2	3
	B3	10	1	3	3
C	C1	10	0.5	3	3
	C2	15	0.5	3	3
	C3	20	0.5	3	3
	C4	30	0.5	3	3

2.6.1.2 HPLC method

All analyses were carried out with Perkin-Elmer HPLC system consisting of a Perkin-Elmer series 200LC pump, a Perkin-Elmer series 200 vacuum degasser, a Perkin-Elmer series 200 diode array detector, and a 20 µl Rhedyne® model 7725i manual sample injector. A Hypersil BDS-C18 column (4.6 mm x 250 mm) and a 50 µl Microliter® syringe were used. Solvents were filtered by using 0.45 µm nylon membrane filters. The mobile phase consisted of 2% acetic acid and acetronitrile (55:45). The separation was carried out using an isocratic elution (0-20 minutes) with a flow rate of 1 ml/min, and detected at 425 nm.

Each plant extract was filtered through 0.45 µm polypropylene syringe filter (OrangeSci®). A 20 µl volume of the extract was injected into the HPLC system.

Method validation

System suitability test

To verify the stability of this chromatographic system, the system suitability test was performed by five consecutive injections of 300 µg/ml curcuminoids. The relative standard deviation (RSD) of relative peak area and retention times of curcumin, demethoxycurcumin, and bisdemethoxycurcumin was evaluated.

RSD value was calculated by:

$$RSD = \frac{S}{\bar{X}} \times 100$$

S = standard deviation

$\bar{X}$ = mean value of measurement

Linearity and range

Linearity test was established at various concentrations of the three standards, curcumin at concentration ranges of 5-70 µg/ml, demethoxycurcumin at concentration ranges of 1.4-70 µg/ml, and bisdemethoxycurcumin at concentration ranges of 1.5-50 µg/ml. The relation of relative peak area and concentration was plotted. Linear regression equation and correlation coefficient (R^2) were calculated. These values indicated linearity in the examined concentration range.

Accuracy and precision

Accuracy was evaluated as the percentage of recoveries over the spiked values of each 3 concentrations (3 replications each) of curcumin at 20, 40, and 70 µg/ml, demethoxycurcumin at 28, 56, and 70 µg/ml, and bisdemethoxycurcumin at 15, 30, and 50 µg/ml.

Precision was expressed by the relative standard deviation (RSD) of the percentage of recovery and retention time.

2.6.1.3 Determination of curcumin, demethoxycurcumin, and bisdemethoxycurcumin contents by HPLC

Pulverized fingers were extracted following the extraction method A3. The extract was made half dilution before injection into HPLC system. The curcumin, demethoxycurcumin, and bisdemethoxycurcumin contents were calculated from the calibration curve which derived from the step of linearity test of method validation.

2.6.2 Determination of volatile oil content

Volatile oil of fingers powder was determined by following the protocol of Thai Herbal Pharmacopoeia 1995 (54). The pulverized finger with accurately weight of about 5 g was distilled with 50 ml distilled water in 500-ml round bottom flask. The powder was distilled at a rate of 2 to 3 ml per minute for 5 hours. The content of volatile oil was calculated to percentage of volume of the oil per weight of the powder.

The constituents in the volatile oil of the samples were investigated by GC-MS.

<u>Qualitative determination of volatile oil by GC-MS</u>

The oil samples were analysed using Shimazu GC MS-QP2010 coupled with mass spectrophotometer as a detector. The volatile oil was prepared in hexane:

dichloromethane (95:5) and injected onto DB-1 gas chromatographic column (dimension: 30 m, 0.25 mm i.d., film thickness = 0.25 µm), which was perfused with helium gas and operated with split ratio of 50. The GC conditions were: oven temperature, 50 ºC for 5 minutes, then programmed from 50-200 ºC at 100 ºC/m, subsequently isothermal at 200 ºC for 5 minutes. Major constituents in the volatile oil were reported.

2.7 Cytological observation

Young roots and/or shoots of spur buds which regenerated from rhizome were used as materials for this study. Root tips and/or shoot tips were pretreated with *p*-dichlorobenzene solution for 6 h and hydrolysed in 1N HCl at 60ºC for 7 minutes. The slides were prepared by Feulgen squash method by soaking the tissues in Schiff's reagent for 30 minutes, and squashing with aceto-orcein dye on microscopic slide (55). Chromosome numbers of at least 10 cells of each plant were determined. The cells were observed under microscope with 1000x magnified.

CHAPTER IV
RESULTS AND DISCUSSION

1. Preparation of plant materials

The initiative *Curcuma longa* clone C12AC and C11AG were propagated, and the propagation's cycle was repeated until obtaining 494 plantlets of clone C12AC for colchicine experiment and 550 plantlets of clone C11AG for gamma rays experiment. Two-month old plants of both clones were used in mutational induction experiments.

2. Mutational induction of *Curcuma longa*

2.1 Mutational induction by colchicine

Colchicine-treated *Curcuma longa* showed frequency of lethality after they were cultured in shoot induction medium containing 0.1 mg/l TDZ for 4 weeks, and then transferred into MS medium for 4 weeks (figure 4). Lethality of 1 and 2 days treatment with 0, 0.25, 0.50, and 0.75% colchicine represented concentration dependence. The higher concentration the plants obtained the higher frequency of lethality they presented. Other two duration treatments of colchicine, 3-4 days, also showed concentration dependence at 0-0.50% colchicine, and, however, treatment of 0.75% colchicine represented slightly smaller lethality frequency than that of 0.5% colchicine.

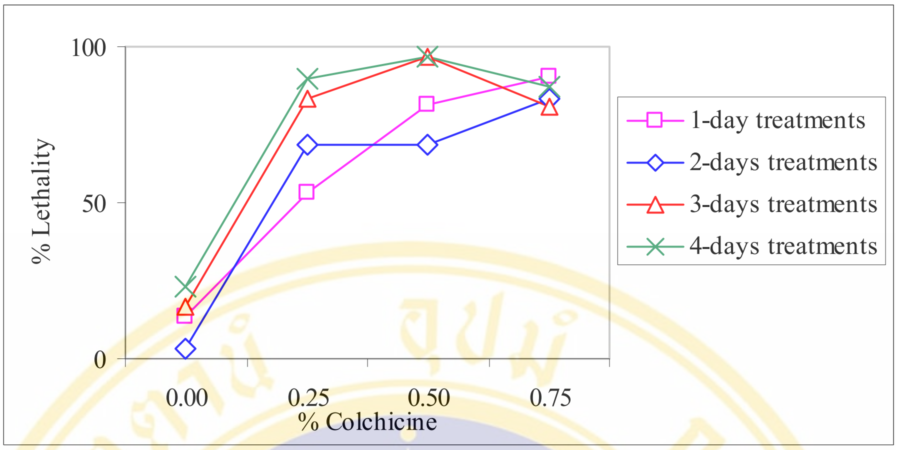


Figure 4 Frequency of lethality of colchicine-treated *Curcuma longa* after cultured in shoot induction medium for 4 weeks and in MS medium for 4 weeks. (All data are shown in appendix, table 19.)

Frequency of shoot response and the shoot regeneration rate are shown in figure 5 and 6. The frequency of shoot response and shoot regeneration rates decreased when the concentration of colchicine and treated duration increased.

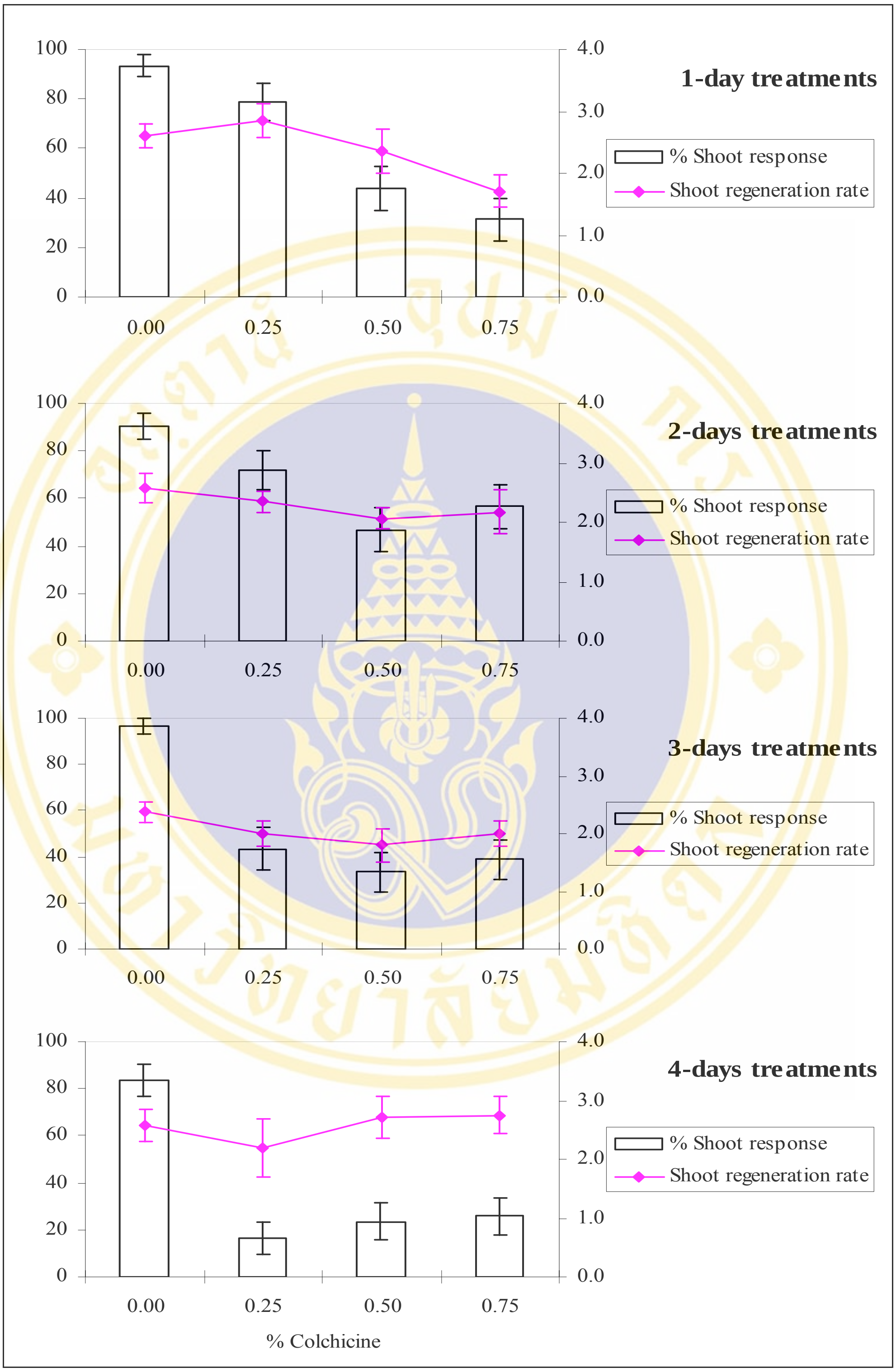


Figure 5 Frequency of shoot response and shoot regeneration rate of colchicine-treated *Curcuma longa* after incubating in shoot induction medium for 4 weeks and in MS medium for 4 weeks. Vertical line indicates S.E. (All data are shown in appendix, table 20.)

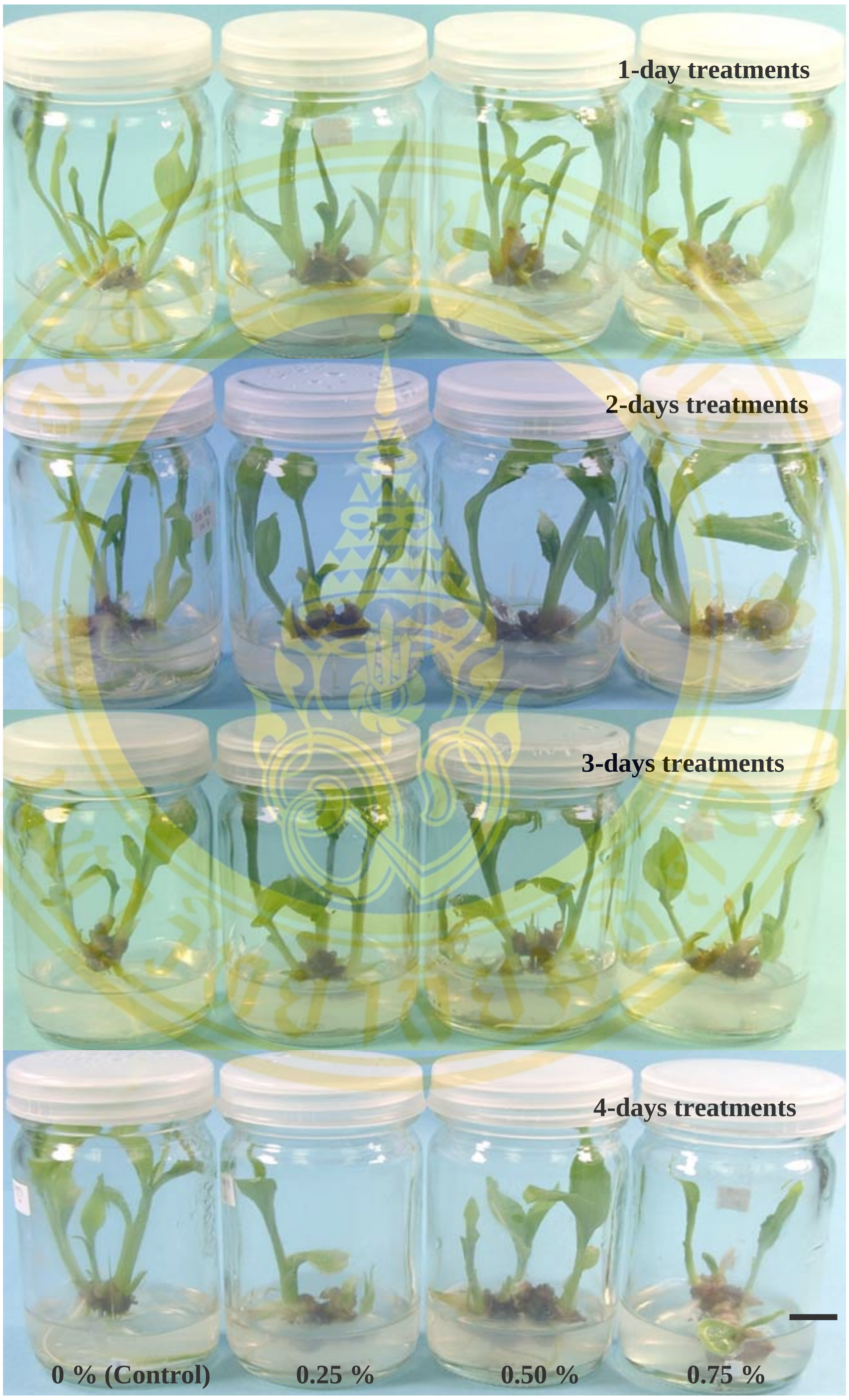


Figure 6 Colchicine-treated *Curcuma longa* after incubating in shoot induction medium for 4 weeks and in MS medium for 4 weeks. Scale bar = 1 cm.

2.2 Mutational induction by gamma rays

Observation of LD_{50} intensity

Two-month old *Curcuma longa* were irradiated with gamma rays at intensities of 0, 20, 40, 60, 80, and 100 Gy. Figure 7 demonstrates the frequency of lethality. At the day sixtieth after irradiation, the plants of 40 Gy and 60 Gy treatments showed 2 and 60% lethality respectively while that of 80 and 100 Gy treatments showed 100 % lethality. The relation plot between % lethality and gamma rays intensity was used to estimate that the LD_{50} intensity of *Curcuma longa* was 58 Gy.

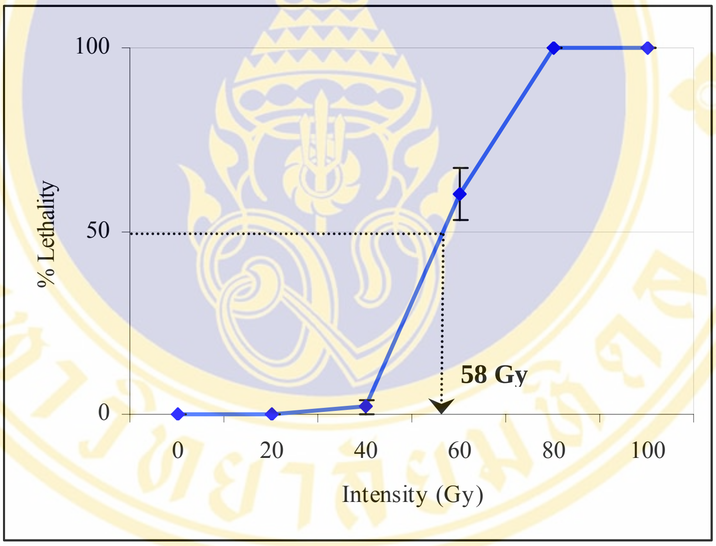


Figure 7 Observation of LD_{50} intensity of *Curcuma longa* at 60 days after acute irradiation with gamma rays. (All data are shown in appendix, table 21.)

Induced mutation with gamma rays

Curcuma longa was irradiated with gamma rays at 5 intensities, 0 (control), 40, 50, 60, and 70 Gy. After irradiation, the treated plants were shoots induced by incubating in shoot induction medium for 4 weeks, and another 4 weeks in MS medium later on.

The frequency of shoots responding to shoot formation and the regeneration rate are shown in figure 8. The frequency of responding shoots and the regeneration rates decreased when the treated intensity of gamma rays increased. Numbers of regenerated shoots per explant of 40, 50, 60, and 70 Gy treatments were 1.96±0.15, 1.55±0.14, 0.91±0.26, and 0.83±0.57 respectively while the control was 3.71±0.37.

Figure 9 demonstrates growth of the gamma rays-treated plants after 8 weeks of culture.

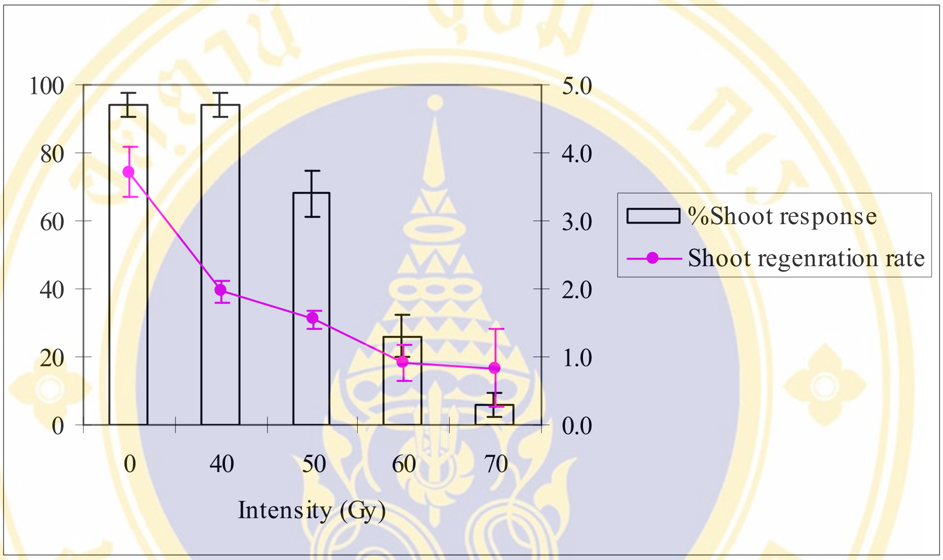


Figure 8 Frequency of shoot response and shoot regeneration rate of gamma rays-treated *Curcuma longa* after incubating in shoot induction medium for 4 weeks and in MS medium for 4 weeks. Vertical line indicate S.E. (All data are shown in appendix, table 22.)

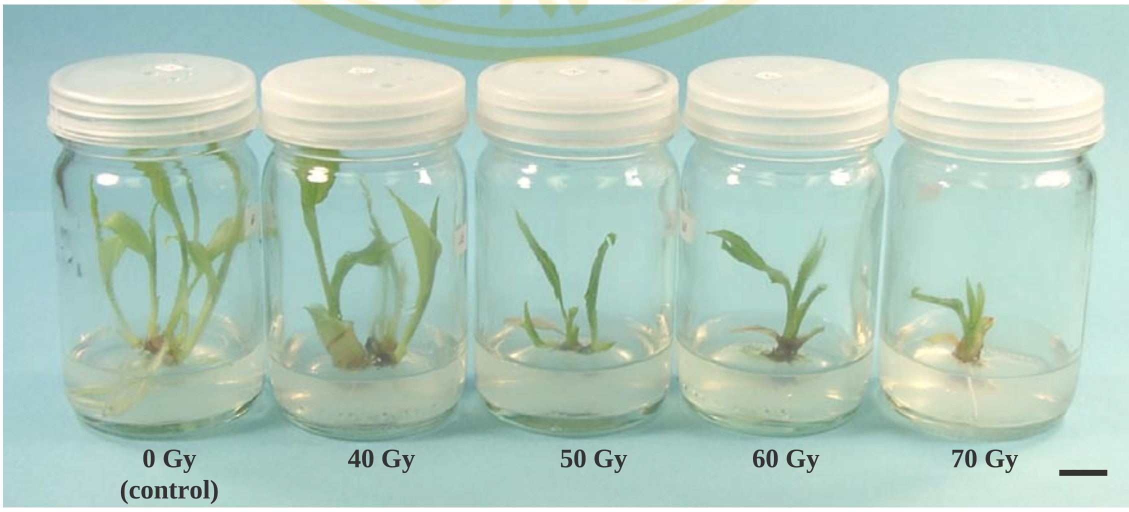


Figure 9 Gamma rays-treated *Curcuma longa* after incubating in shoot induction medium for 4 weeks and in MS medium for 4 weeks. Scale bar = 1 cm.

The frequency of lethality after exposing to the mutagens was observed in order to assess the sensitivity of *Curcuma longa* over the test dosage and treated-duration. Increasing of dosage and duration of colchicine and gamma ray treatment correlates to increasing of lethality frequency. Ratanapan (2001) studied mutational induction of *Curcuma longa* L. and *Curcuma zedoaria* Rosc. by incubating their shoot tips in 0.25% colchicine solution for 1-4 days. Lethality of both plants increased proportionally to the length of the treatment (56). As same as the effect of colchicine, strength of gamma rays affects lethality frequency which also found in the study of *Digitalis obscura* (6), lotus (50), and banana (51).

After mutational induction, the treated plants were shoots induced in shoot induction medium containing 0.1 TDZ for 4 weeks then transferred to MS medium for 4-8 weeks until gaining of regenerated shoots (M2). The regeneration cycle was repeated using M2 as initial plants to get M3 regenerated plants.

3. Cultivation and harvest

The regenerated plants (M3) from mutational induction experiments were acclimatized in the plastic trays for 2 weeks before potting (figure 10).

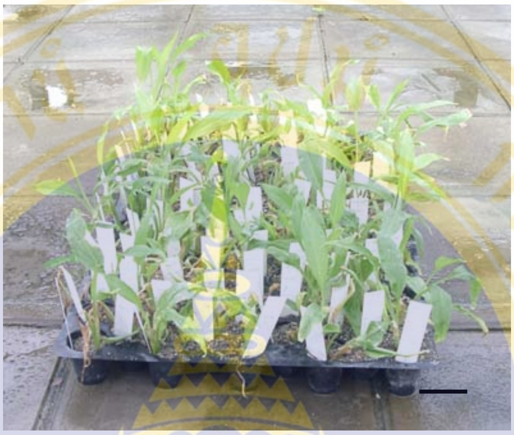


Figure 10 Two-week old acclimatized *Curcuma longa* before potting. Scale bar = 5 cm.

This stage, 2 selection criteria were submitted to qualify the potential clones for medicinal purposes.

Selection criterion 1: Before bringing the acclimatized M3 plants to grow in greenhouse conditions, the plants were selected according to survival ability (completely rooting and healthy appearance). Thus, 141 plants from colchicine experiment and 67 plants from gamma rays experiment were chosen to cultivate in greenhouse.

During cultivation stage, abnormal characters of the plants apparently appeared in leaves and rhizomes. Figure 11 and 12 show the abnormal characters of leaves and rhizomes.

Growth of 4-months old cultivated plants shows in figure 13. Height of the survival treated plants was compared with average height of the control plants (figure 14 and 15).

After 8 months, their rhizomes were harvested. Data of corm size, finger size and number of fingers, and dry weight show in figure 16-21.

Figure 11 Abnormal character found in leaves of the treated *Curcuma longa* growing in greenhouse. (A) Growth of overall cultivated plants; (B) Leaf of C31-1 plant (0.75% colchicine, 1 day) treatment plant represents crinkled area (arrow); (C) Abnormal plant and rolled edge leaf of C11-25 plant (0.25% colchicine, 1 day); (D) Crinkled leaf of G50-21 plant (50 Gy gamma rays); (E) Longitudinal convex on leaf of G40-7 plant (40 Gy gamma rays); (F) leaf with split end of G40-7 plant. Scale bars: A = 30 cm, B-F = 2.5 cm.

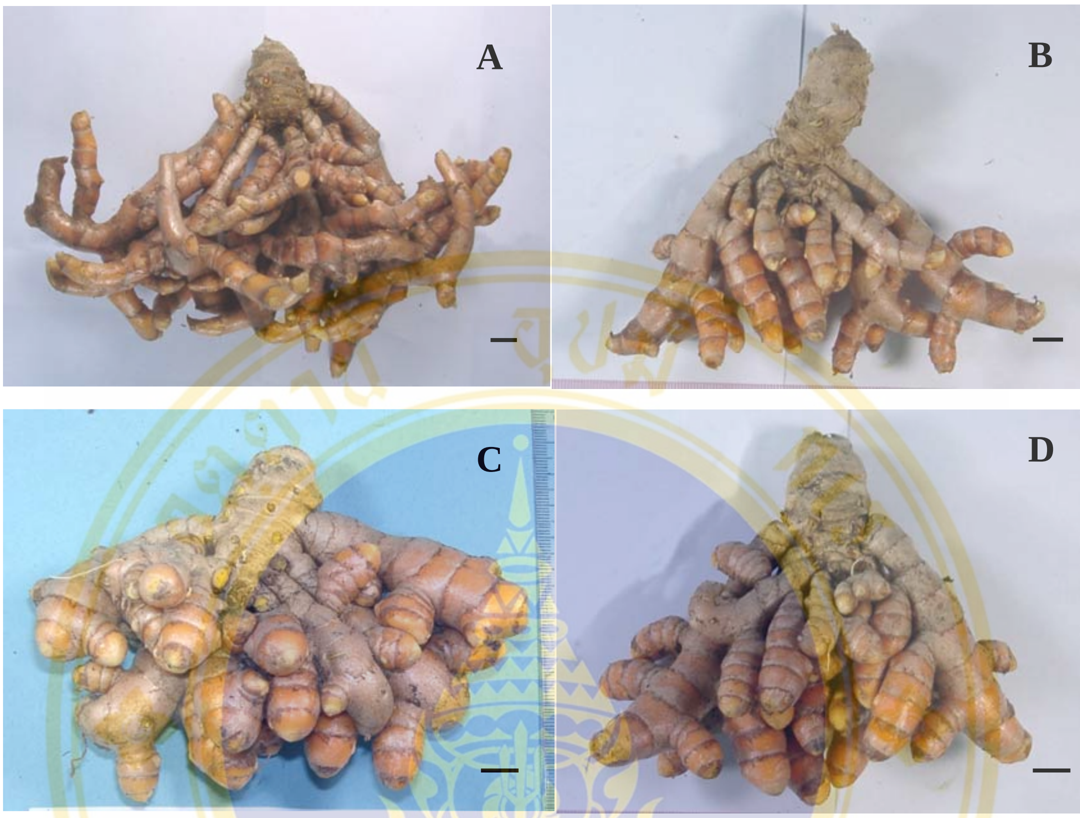


Figure 12 Abnormal character found in rhizome of the treated *Curcuma longa* after harvesting. (A) Control plant; (B) C33-5 plant (0.75% colchicine for 3 days); (C) G50-14 plant (50 Gy gamma rays treatment) ; (D) 60-2 Gy (60 Gy gamma rays treatment). Scale bars = 1 cm.

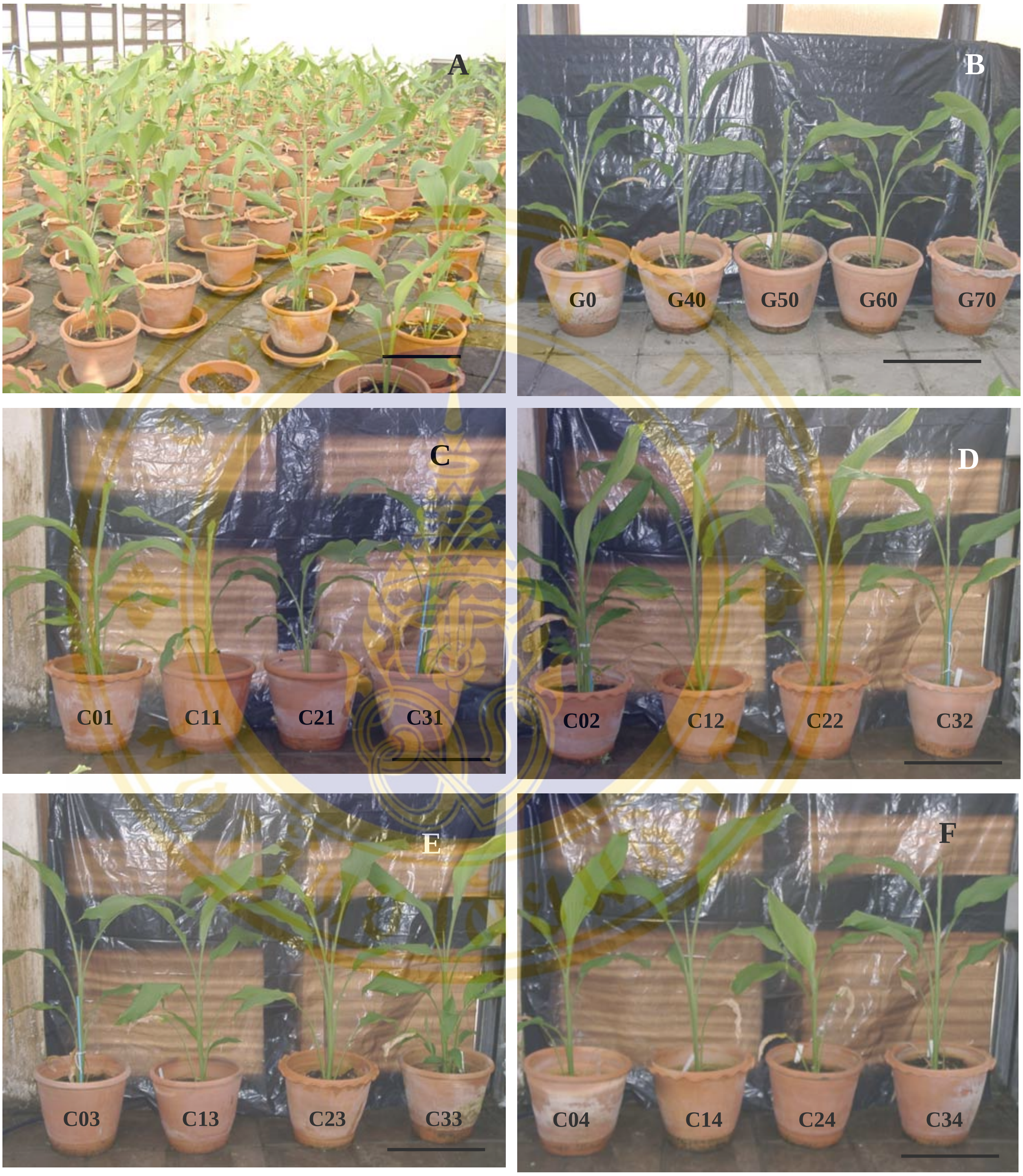


Figure 13 Four-month old *Curcuma longa* in greenhouse. (A) Growth of overall *Curcuma longa*; (B) Gamma rays treatments; (C) 1-day treatments; (D) 2-days treatments; (E) 3-days treatments; (F) 4-days treatments. Scale bars = 30cm.

(Plant code: G0, 40, 50, 60, 70 = 0 , 40, 50, 60, 70 Gy gamma rays respectively; C01, C11, C21, C31 = 0, 0.25, 0.50, 0.75% colchicine for 1 day; C02, C12, C22, C32 = 0, 0.25, 0.50, 0.75% colchicine for 2 days; C03, C13, C23, C33 = 0, 0.25, 0.50, 0.75% colchicine for 3 days; C04, C14, C24, C34 = 0, 0.25, 0.50, 0.75% colchicine for 4 days.)

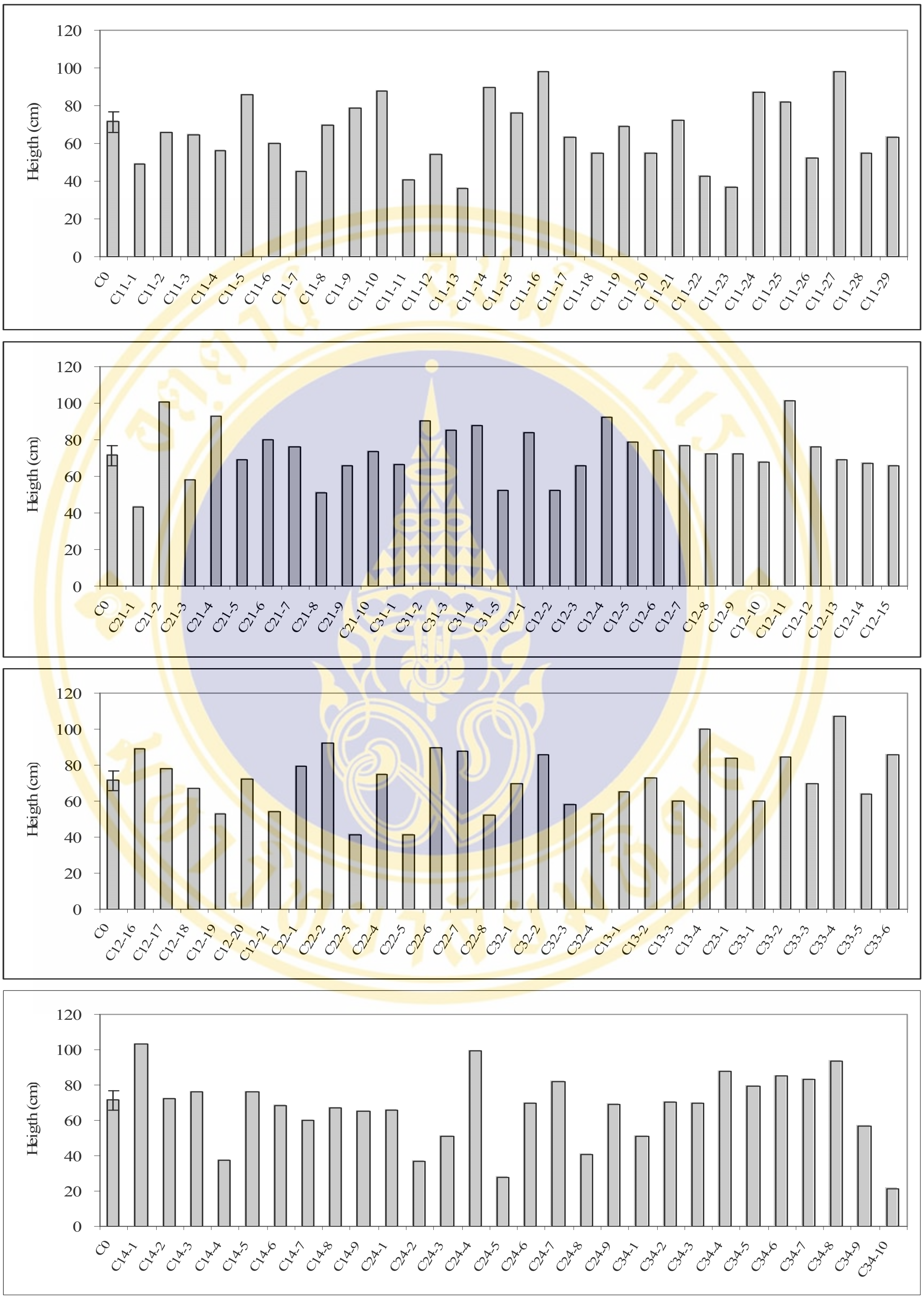


Figure 14 Plant height of colchicine-treated *Curcuma longa* at 4 months of cultivation in greenhouse conditions. C0 represents as average height of 12 control plants with S.E. (All data are shown in appendix, table 23.)

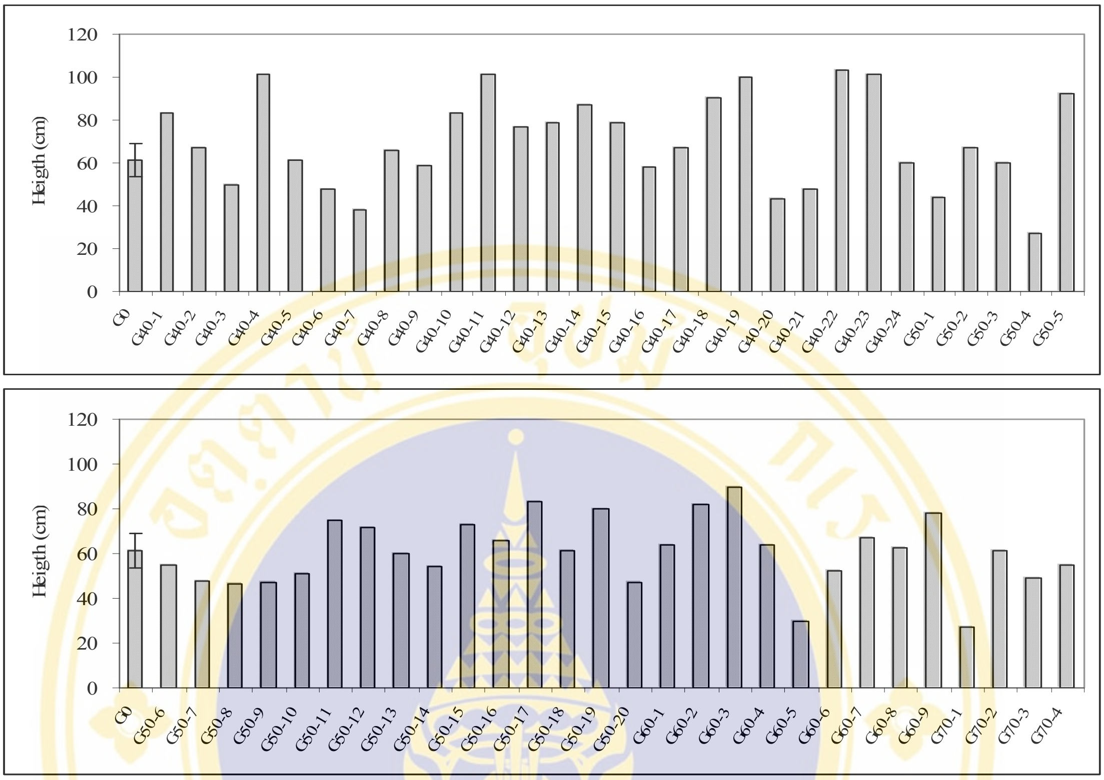


Figure 15 Plant height of gamma rays-treated *Curcuma longa* at 4 months of cultivation in greenhouse conditions. G0 represents as average height of 10 control plants with S.E. (All data are shown in appendix, table 24.)

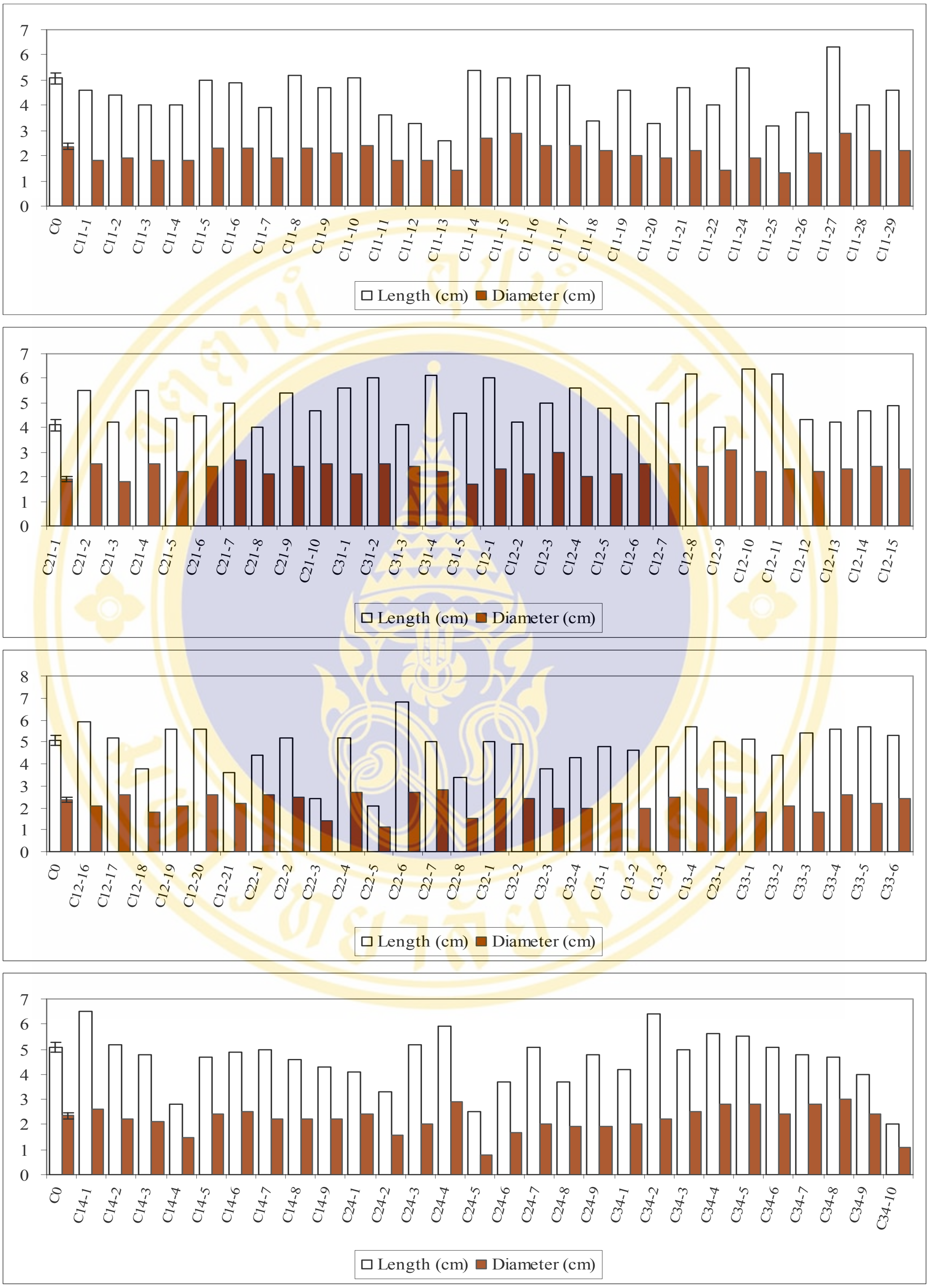


Figure 16 Corm size of M3 colchicine-treated plants after cultivation for 8 months in greenhouse conditions. C0 represents as average length and diameter of 12 control plants with S.E. (All data are shown in appendix, table 23.)

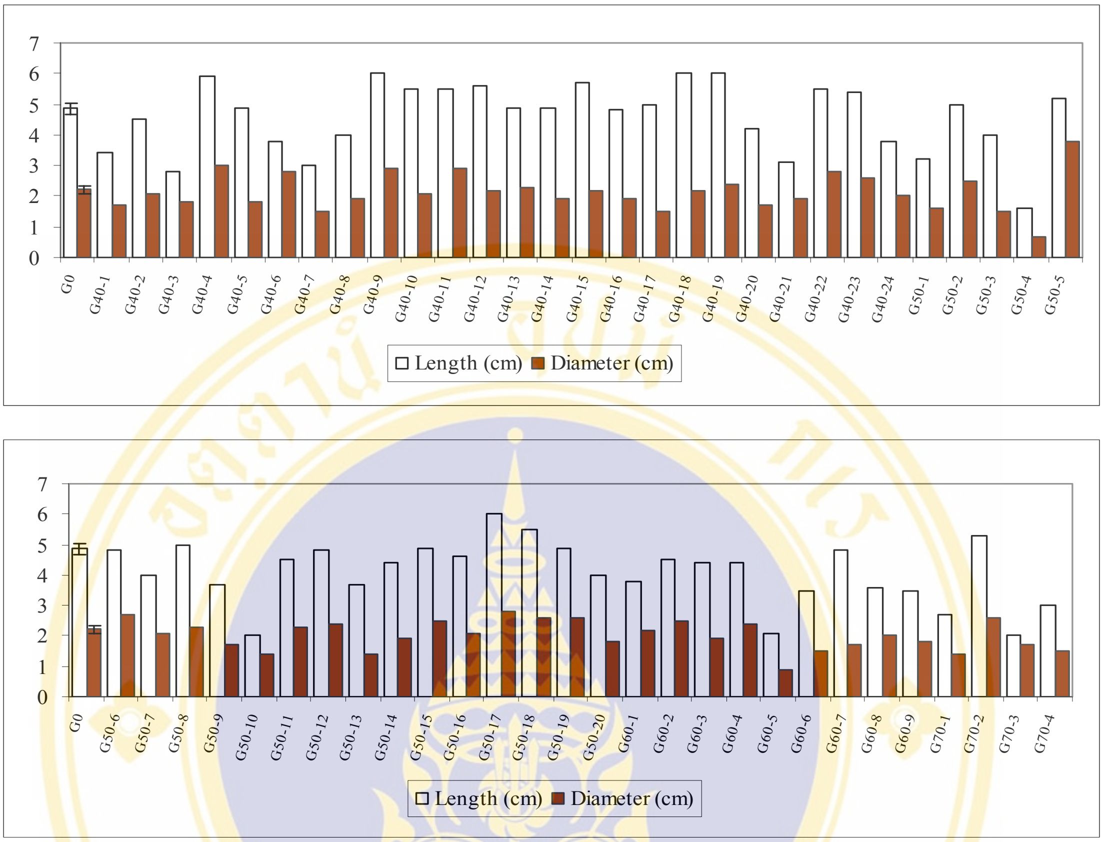


Figure 17 Corm size of M3 gamma rays-treated plants after cultivation for 8 months in greenhouse conditions. G0 represents as average length and diameter of 10 control plants with S.E. (All data are shown in appendix, table 24.)

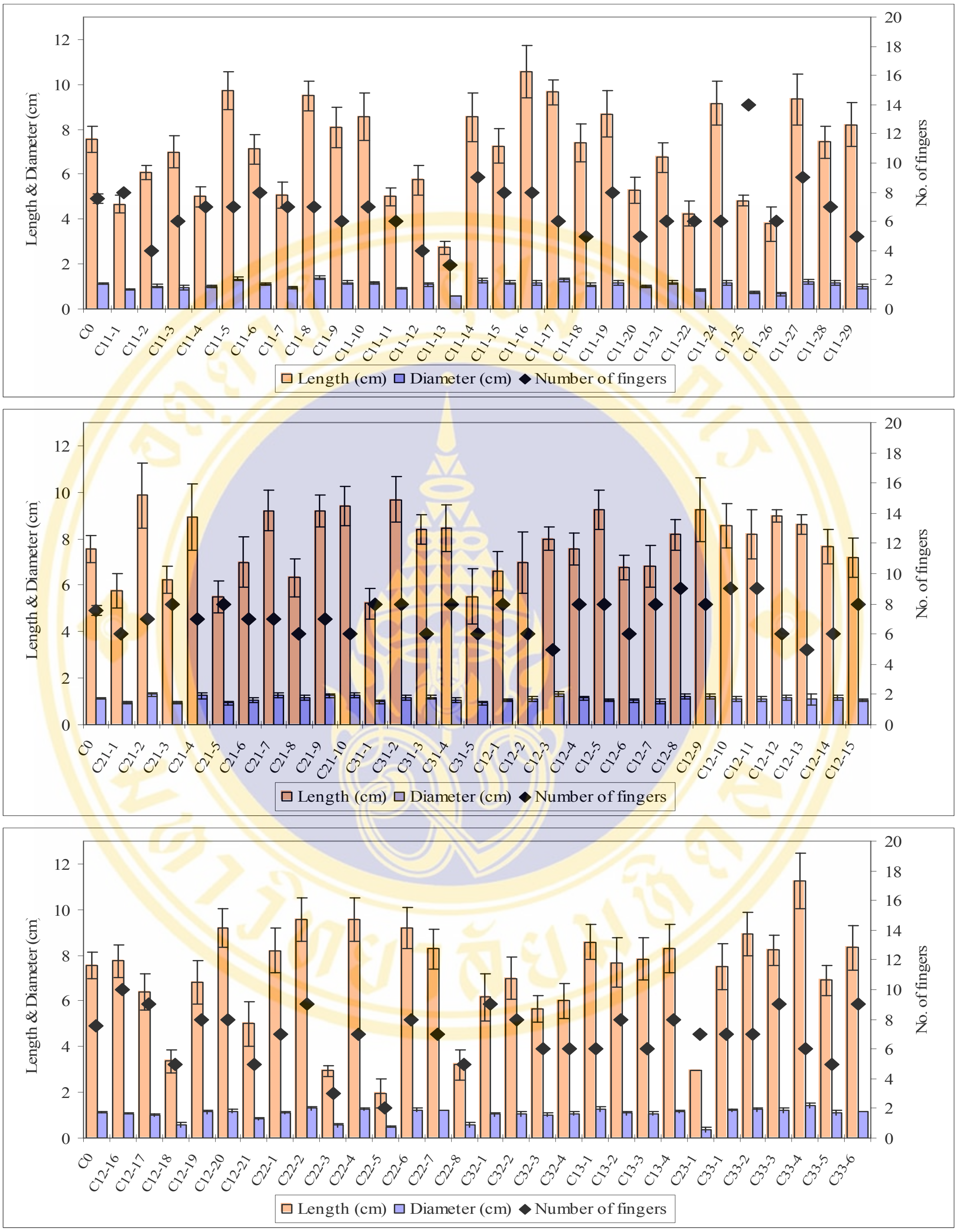


Figure 18 Finger size and number of fingers of M3 colchicine-treated plants after cultivation for 8 months in greenhouse conditions. C0 represents as average finger size and number of fingers of 12 control plants. Vertical line indicates S.E. (All data are shown in appendix, table 23.)

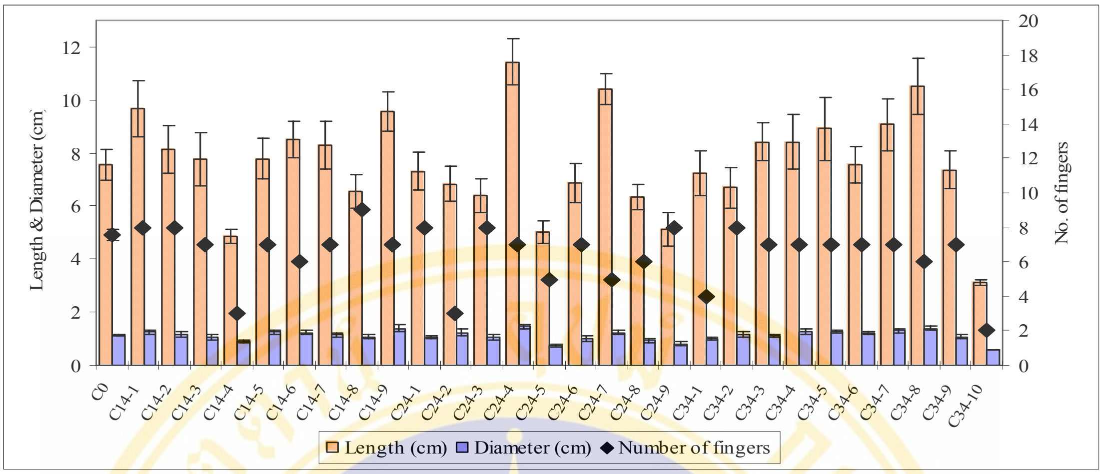
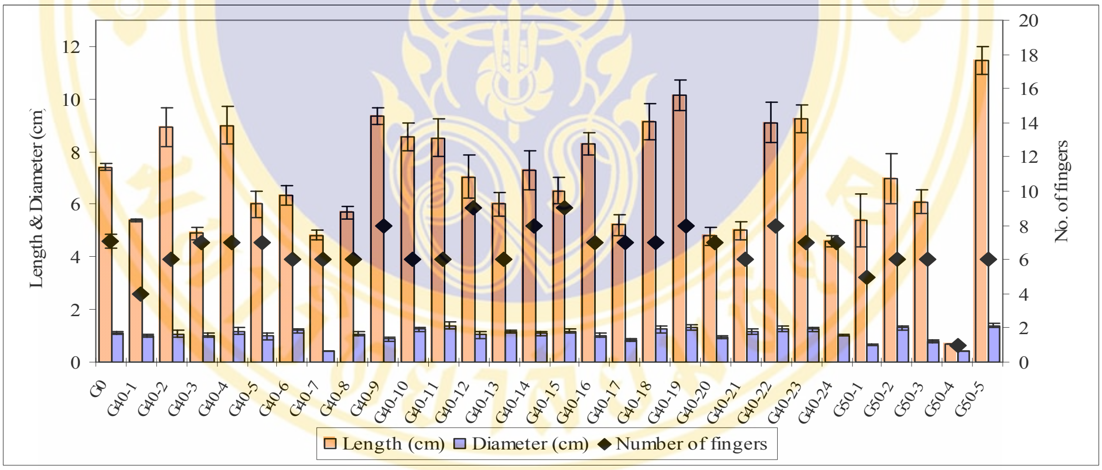


Figure 18 Finger size and number of fingers M3 colchicine-treated plants after cultivation for 8 months in greenhouse conditions. C0 represents as average finger size and number of fingers of 12 control plants (continued). (All data are shown in appendix, table 23.)



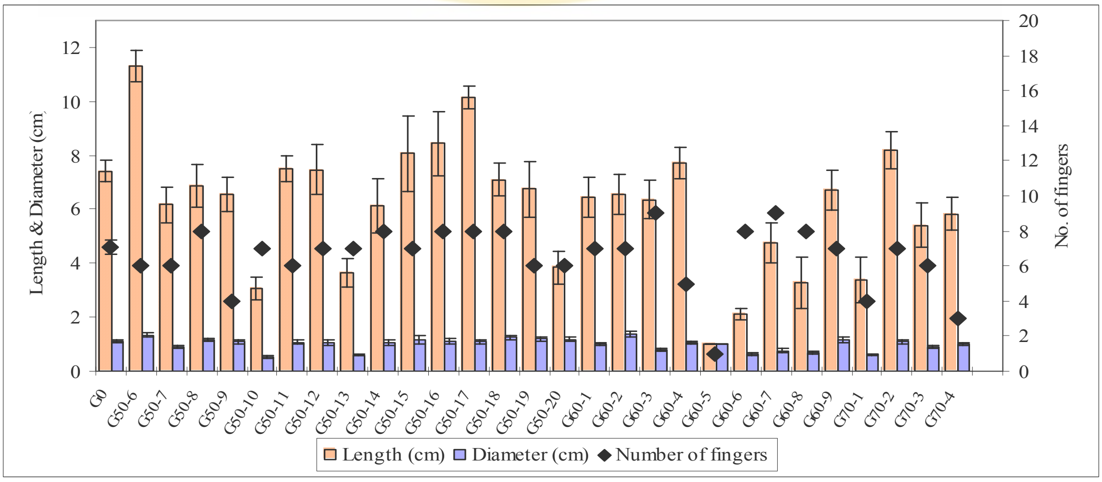


Figure 19 Finger size and number of fingers of M3 gamma rays-treated plants after cultivation for 8 months in greenhouse conditions. G0 represents as average finger size and number of fingers of 10 control plants with S.E. (All data are shown in appendix, table 24.)

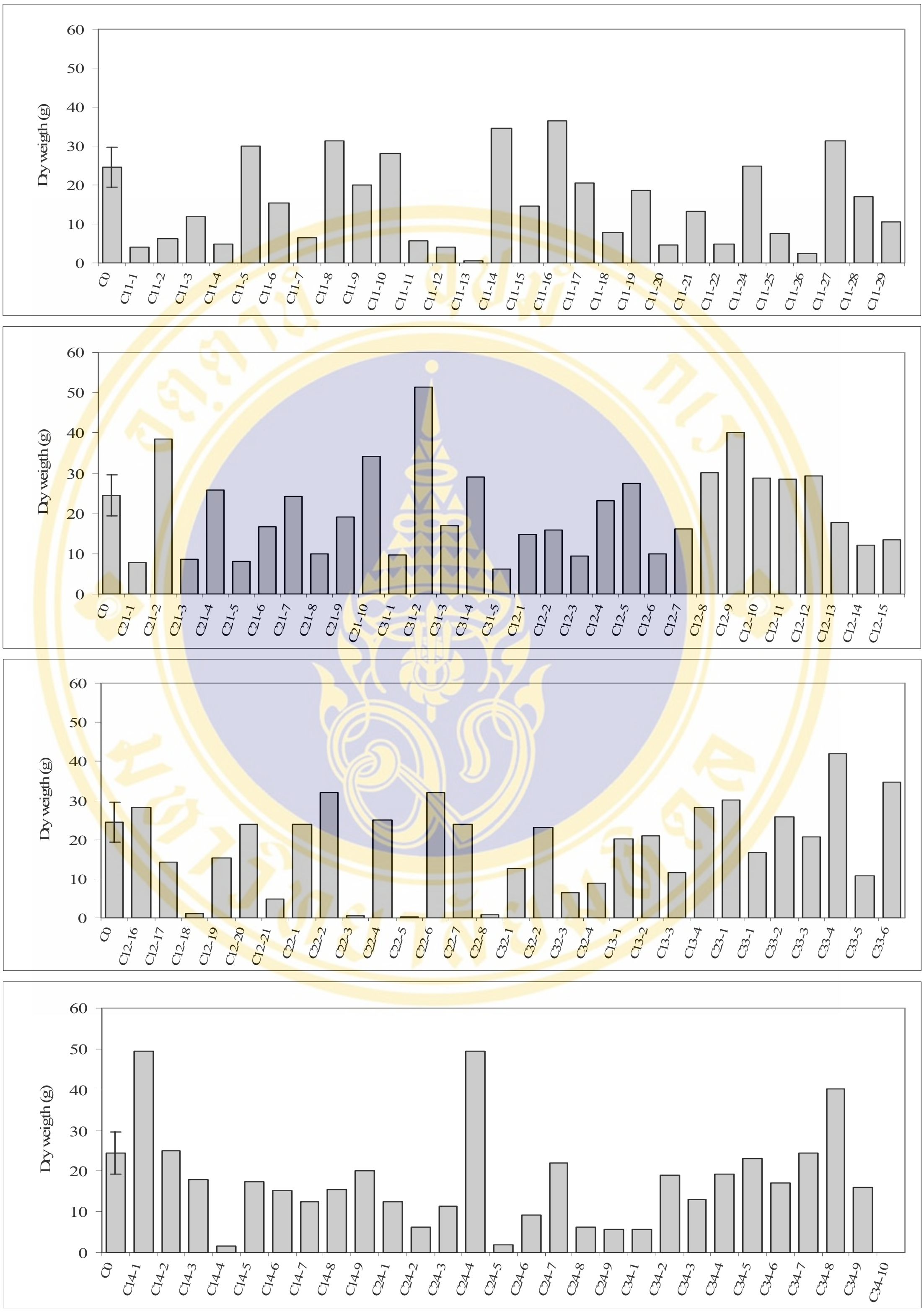


Figure 20 Dry weight (g) of fingers obtained from colchicine-treated *Curcuma longa*. C0 represents as average weight of 12 control plants with S.E. (All data are shown in appendix, table 23.)

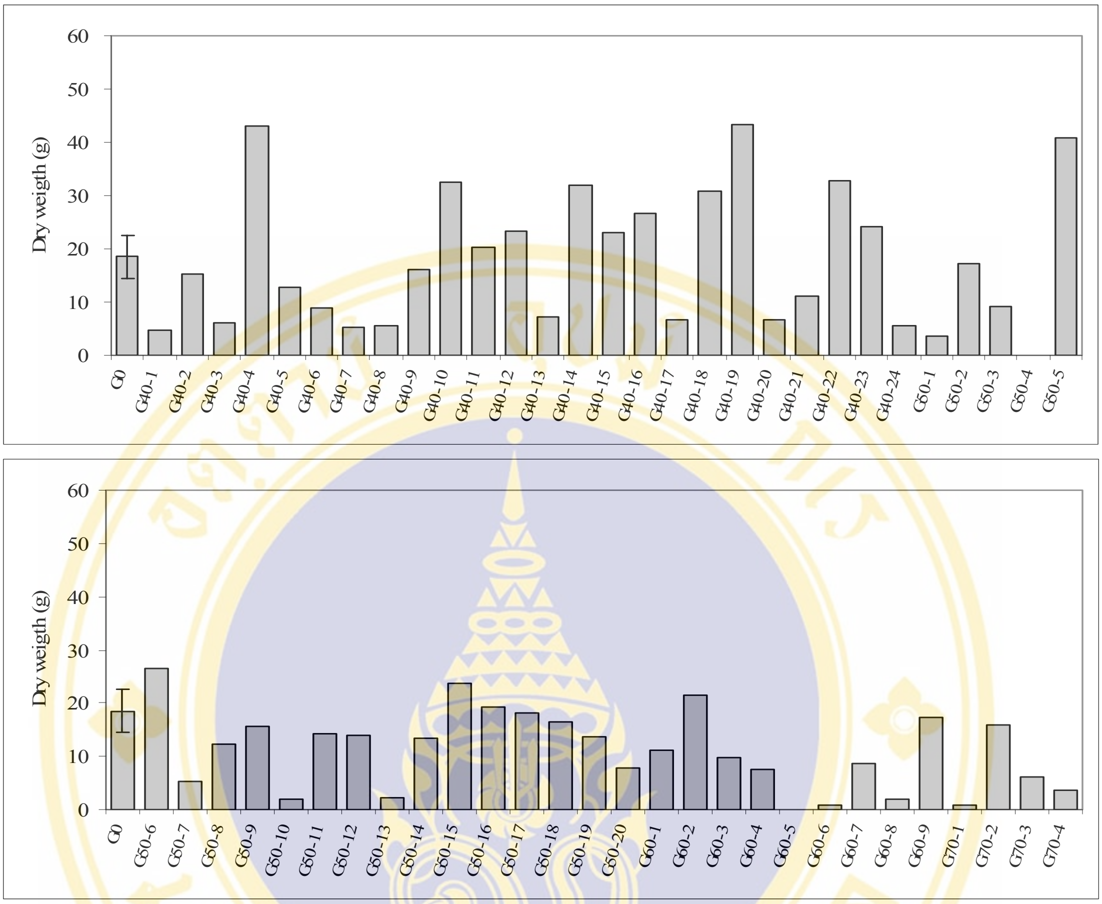


Figure 21 Dry weight (g) of fingers obtained from gamma rays-treated *Curcuma longa*. G0 represents as average weight of 10 control plants with S.E. (All data are shown in appendix, table 24.)

Selection criterion 2: after the plants were harvested, dry weight of fingers of each plants was assessed as phytomass production. By comparison to average value of dry weight of all control plants in each mutational induction experiment, the plants presenting superior dry weight were chosen. This step, a number of 37 (excluded 5 control plants) and 16 (excluded 5 control plants) plants from colchicine experiment and gamma rays experiment respectively were qualified, and then they were investigated the chemical contents and cytological character.

Table 7 summarizes number of the qualified plants from the selection criteria 1 and 2.

Table 7 Number of the qualified *Curcuma longa* through selection criteria 1 and 2.

Treatment*	Total number of M3 plant	Number of plants from Selection Criterion 1	Number of plants from Selection Criterion 2
C11	67	32	6 (8.96%)**
C21	21	13	4 (19.05%)
C31	13	6	2 (15.38%)
C12	42	24	9 (21.43%)
C22	20	12	5 (25.00%)
C32	24	5	1 (4.17%)
C13	16	4	1 (6.25%)
C23	14	3	1 (7.14%)
C33	21	6	3 (14.29%)
C14	20	11	2 (10.00%)
C24	20	13	1 (5.00%)
C34	37	12	2 (5.41%)
Total	315	141	37 (11.75%)
G40	212	29	11 (5.19%)
G50	79	22	4 (5.06%)
G60	48	12	1 (2.08%)
G70	10	4	0 (0.00%)
Total	349	67	16 (4.58%)

* C11, C21, C31 = 0.25, 0.50, 0.75% colchicine for 1 day; C12, C22, C32 = 0.25, 0.50, 0.75% colchicine for 2 days; C13, C23, C33 = 0.25, 0.50, 0.75% colchicine for 3 day; C14, C24, C34 = 0.25, 0.50, 0.75% colchicine for 4 days; G40, G50, G60, G70 = 40, 50, 60, 70 Gy gamma rays respectively.

** The percentage of qualified plants selected from the total number of mutation-induced plant (M3).

4. Chemical analysis

4.1 Quantitative determination of curcumin, demethoxycurcumin, and bisdemethoxycurcumin

4.1.1 Study of extraction methods

The amount of total curcuminoids from all test extracts was determined by using UV spectrophotometer as a detector, and calculation carried out by extrapolation from regression equation of curcumin calibration curve (figure 22).

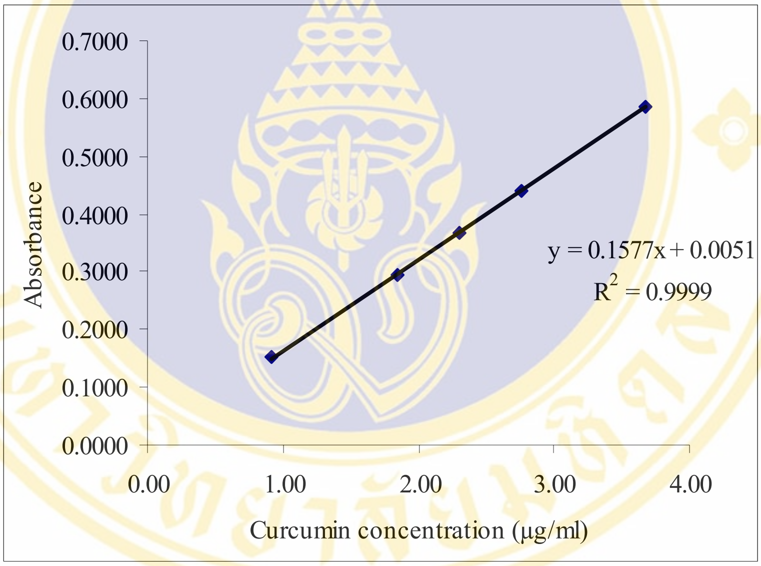


Figure 22 Calibration curve of standard curcumin when detected by UV spectrophotometer at wavelength of 420 nm.

In experiment A, effect of number of extraction was examined. Number of extraction affected amount of total curcuminoids (p<0.001) in the extract as presented in table 8. Increasing of number of extraction, 0.5 h each, significant increased the total curcuminoids content. The extraction for 4 times provided the highest amount of total curcuminoids. However, there was no significant difference between 3 and 4 times extraction. Thus, extraction for 3 times was considered to be the optimum number of extraction.

The effect of duration and number of extraction was examined according to experiment B. Two difference durations, 0.5 h and 1 h, were assessed together with three difference numbers of extraction, 1-3 times. Table 9 demonstrates that both duration and number of extraction had significant effects on the amount of curcuminoids (p=0.044, p<0.001 respectively). However, there were no interaction effect between duration and number of extraction (p=0.078 by 2-ANOVA test).

In experiment C, the appropriate volume of methanol for the extraction was studied (table 10). The methanol volumes of 10, 15, 20, and 30 ml affected to the amount of total curcuminoids in the extracts. Increasing of methanol volume exhibited significant decrease (p=0.007) in total curcuminoids contents, and the highest amount presented in the extract of 10 ml methanol.

Thus, an appropriate extraction method for *Curcuma longa* in this study was to extract with 10 ml of methanol for 0.5 h, 3 times.

Table 8 Effect of number of extraction (experiment A).

Duration of extraction (h)	Number of extraction (time)	Total curcuminoids content (%)
0.5	1	$5.54 \pm 0.105^{a*}$
	2	6.48 ± 0.209^{b}
	3	$6.74 \pm 0.123^{c,d}$
	4	6.76 ± 0.122^{d}
	p-value	<0.001

[*] Means with different letters are significant different according to Duncan's multiple range test at 5% level.

Table 9 Effect of duration and number of extraction (experiment B).

Duration of extraction (hour)	Total curcuminoids content (%)		
	1 time	2 times	3 times
0.5	5.54±0.105	6.48±0.210	6.74±0.123
1	5.34±0.093	6.55±0.082	6.46±0.105
p-value*		0.078	

* According to interaction factor of duration and number of extraction Means with different letters are significant different according to Duncan's multiple range test at 5% level.

Table 10 Effect of volume of methanol (experiment C).

Volume of MeOH (ml)	Total curcuminoids Content (%)
10	6.74±0.123[a*]
15	6.47±0.100[b]
20	6.35±0.051[b]
30	5.89±0.075[c]
p-value	0.007

* Means with different letters are significant different according to Duncan's multiple range test at 5% level.

4.1.2 HPLC method

Method validation

Performance of this method validation obeyed requirement of USP, and assay category I was fit to the analyses due to this HPLC method was to determine major components or active ingredient in medicinal plant (57).

System suitability

The system suitability was tested to verify the suitability of the chromatographic system before all analyses. The relative standard deviation (RSD) of retention times (Rt) and relative peak areas of five consecutive injections of curcumin, demethoxycurcumin, and bisdemethoxycurcumin were not more than 2% which can be accepted according to USP25/NF20 (57). Details are shown in table 11.

Table 11 System suitability test of standard curcumin, demethoxycurcumin, and bisdemethoxycurcumin.

N	Rt (min)			Peak area		
	Curcumin	Demethoxy-curcumin	Bisdemethoxy-Curcumin	Curcumin	Demethoxy-curcumin	Bisdemethoxy-curcumin
1	12.97	11.89	10.89	1519398	424268	121907
2	13.01	11.93	10.92	1523696	417728	122687
3	12.83	11.70	10.68	1524742	432613	120494
4	12.94	11.83	10.80	1547445	431571	124510
5	13.03	11.94	10.93	1555526	434401	121157
RSD	0.61	0.83	0.97	1.06	1.63	1.27

Figure 23 shows peak character of curcumin, demethoxycurcumin, and bis-demethoxycurcumin when they were individually injected into the HPLC system, and when they were mixed together.

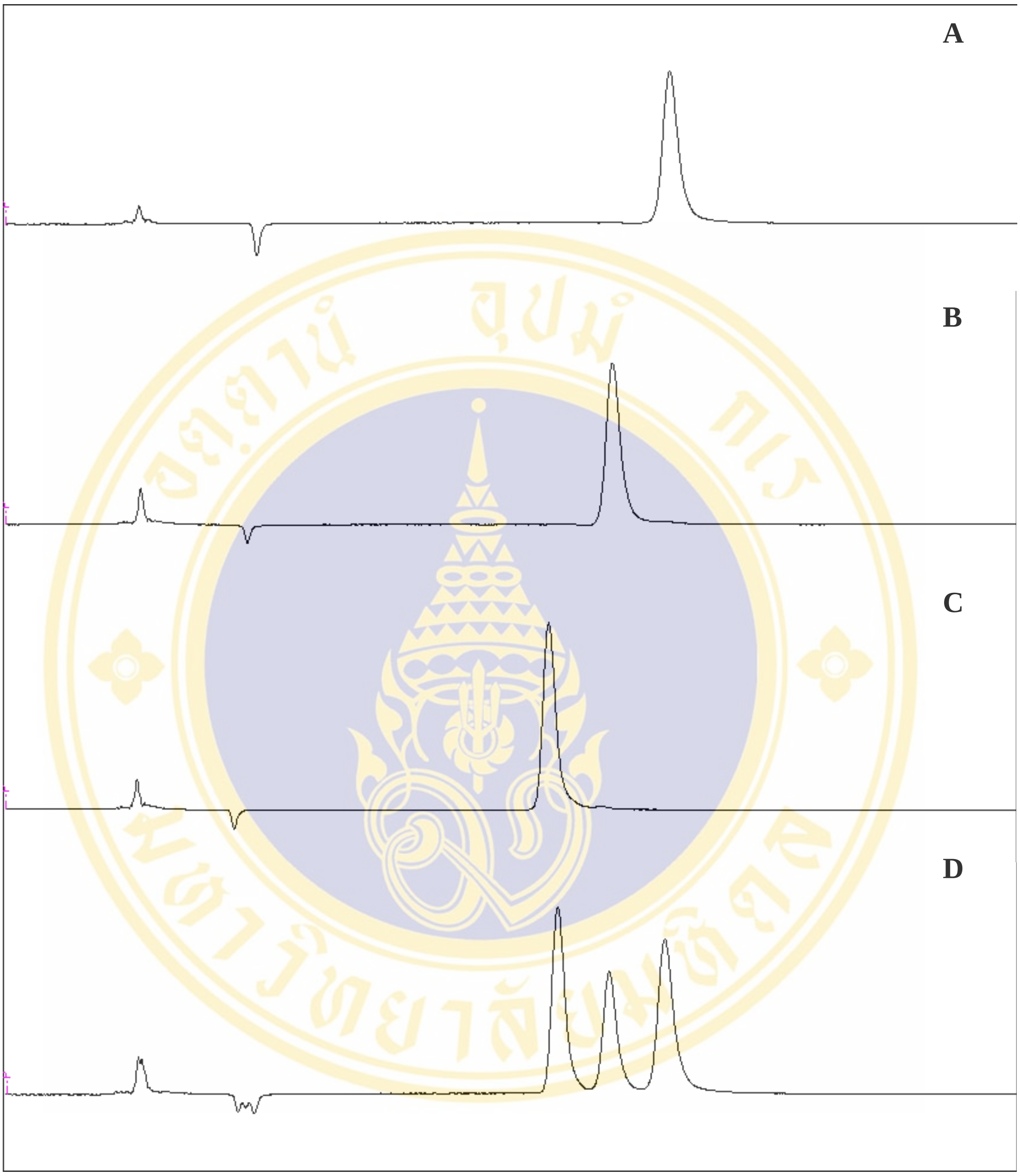


Figure 23 HPLC chromatograms of three standard curcuminoids.

(A) Curcumin peak presented at Rt 13.03 min.

(B) Demethoxycurcumin peak presented at Rt 11.94 min.

(C) Bisdemethoxycurcumin peak presented at Rt 10.93 min.

(D) Mixture of those three curcuminoids.

Linearity and range

Linear relationships between peak areas versus concentrations of curcumin (5-70 µg/ml), demethoxycurcumin (1.4-70 µg/ml), and bisdemethoxycurcumin (1.5-50 µg/ml) were observed with the values of correlation coefficient, y-intercept, and slope, presented in figure 24-26.

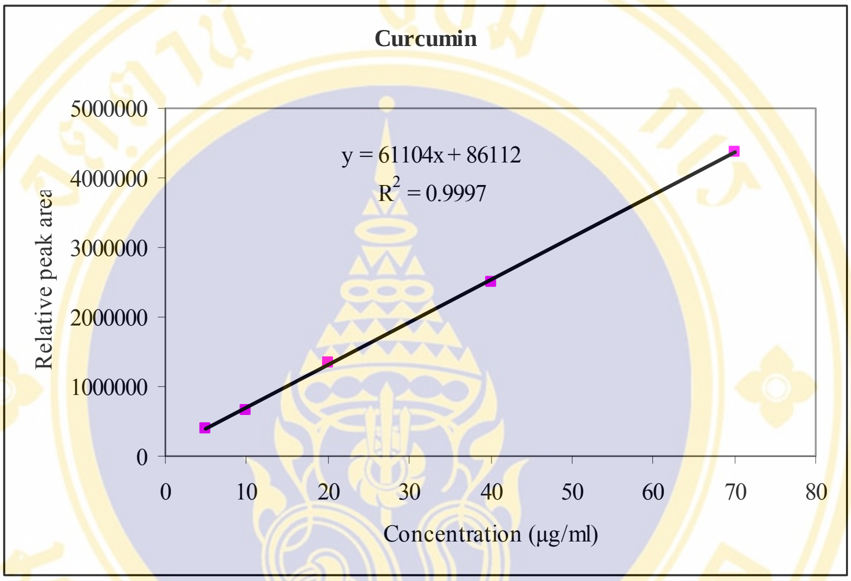


Figure 24 Calibration curve of curcumin performing by HPLC.

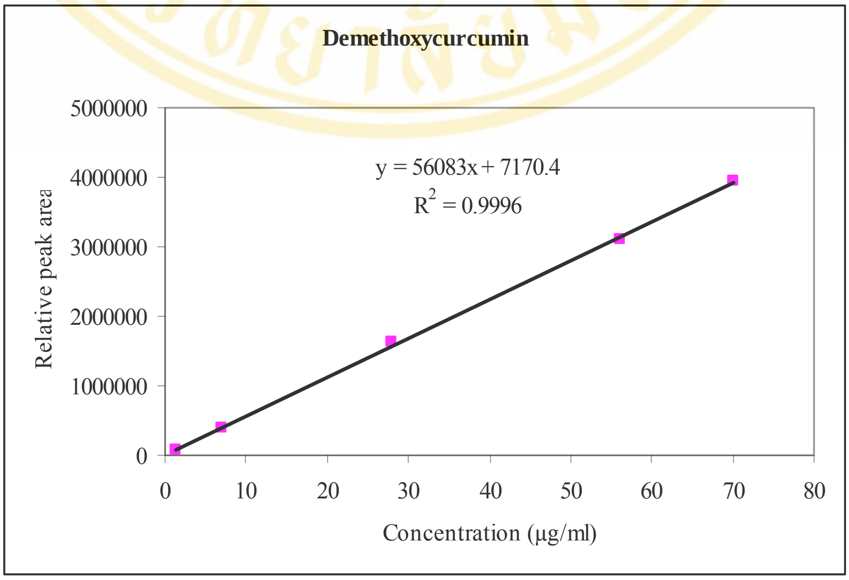


Figure 25 Calibration curve of demethoxycurcumin performing by HPLC.

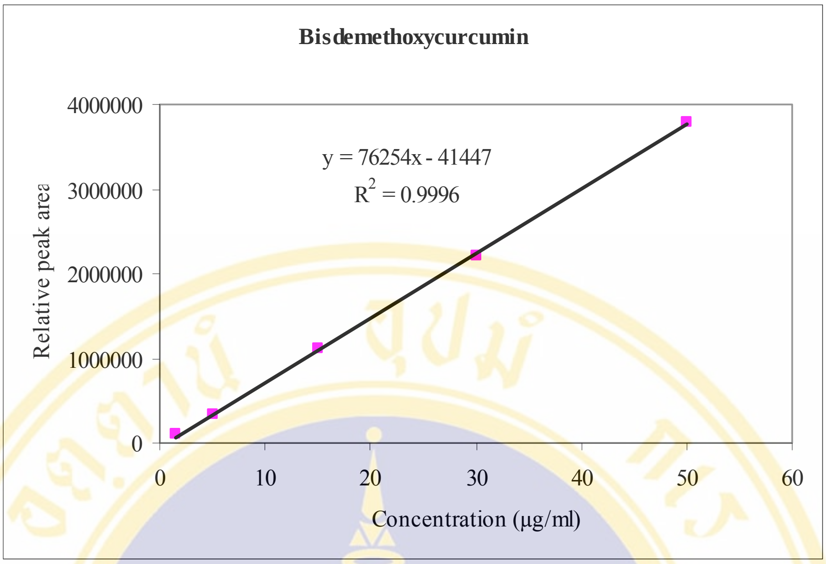


Figure 26 Calibration curve of bisdemethoxycurcumin performing by HPLC.

Accuracy and precision

The percentage recoveries over the spike values and relative peak area of 3 concentrations (3 replications each) of curcumin (20-70 µg), demethoxycurcumin (28-70 µg), and bisdemethoxycurcumin (20-70 µg) with 3 concentrations are demonstrated in table 12.

Accuracy measurements of curcumin, demethoxycurcumin, and bisdemethoxycurcumin showed % recovery of 98.71-104.18, 95.76-106.08, and 97.37-105.22 respectively.

Precision was measured by RSD values, and curcumin, demethoxy-curcumin, and bisdemethoxycurcumin showed that of 2.57, 3.47, and 1.69 respectively.

The AOAC has provided the acceptance criteria according to amount of the analyte. Measurement of 100 µg of analyte, % recovery could be obtained between 90-107%, and RSD should not larger than 5.3 (58).

Table 12 Test of accuracy and precision in method validation for determination of curcumin, demethoxycurcumin, and bisdemethoxycurcumin by HPLC.

Amount added (µg)	% Recovery			Mean	SD	RSD
	1	2	3			
Curcumin						
20	104.18	100.50	100.37			
40	101.32	101.77	102.45	100.23	2.58	2.57
70	98.71	100.00	99.12			
Demethoxycurcumin						
28	105.66	101.78	104.06			
56	106.08	98.11	99.08	101.25	3.51	3.47
70	101.23	99.52	95.76			
Bisdemethoxycurcumin						
20	99.62	99.37	101.28			
40	105.22	97.92	99.08	100.94	1.71	1.69
70	103.31	98.89	97.37			

4.1.4 Quantitative determination of curcumin, demethoxycurcumin, and bisdemethoxycurcumin in samples

Pulverized fingers of 42 samples (included 5 samples of the control) from colchicine experiment and 21 samples (included 5 samples of the control) from gamma ray experiment were extracted with methanol following method A3 in extraction experiment. Each sample extract was triplicate injected into HPLC system. Percentages by weight of curcumin, demethoxycurcumin, and bisdemethoxycurcumin are shown in figure 27 and 28.

Among the colchicine-treated *Curcuma longa*, the amounts of curcumin, demethoxycurcumin and bisdemethoxycurcumin were 1.24-4.28, 0.42-1.65, and 0.38-1.52% (w/w) respectively while average amounts in the control plants were 3.45±0.21, 1.19±0.07, and 1.03±0.06% (w/w) respectively. In the gamma rays-treated plants, the amounts of curcumin, bisdemethoxycurcumin, and demethoxycurcumin were 1.50-4.62, 0.56-1.74, and 0.24-1.79% (w/w) respectively, average amounts in the control were 3.31±0.49, 1.29±0.12, and 0.98±0.17% (w/w) respectively.

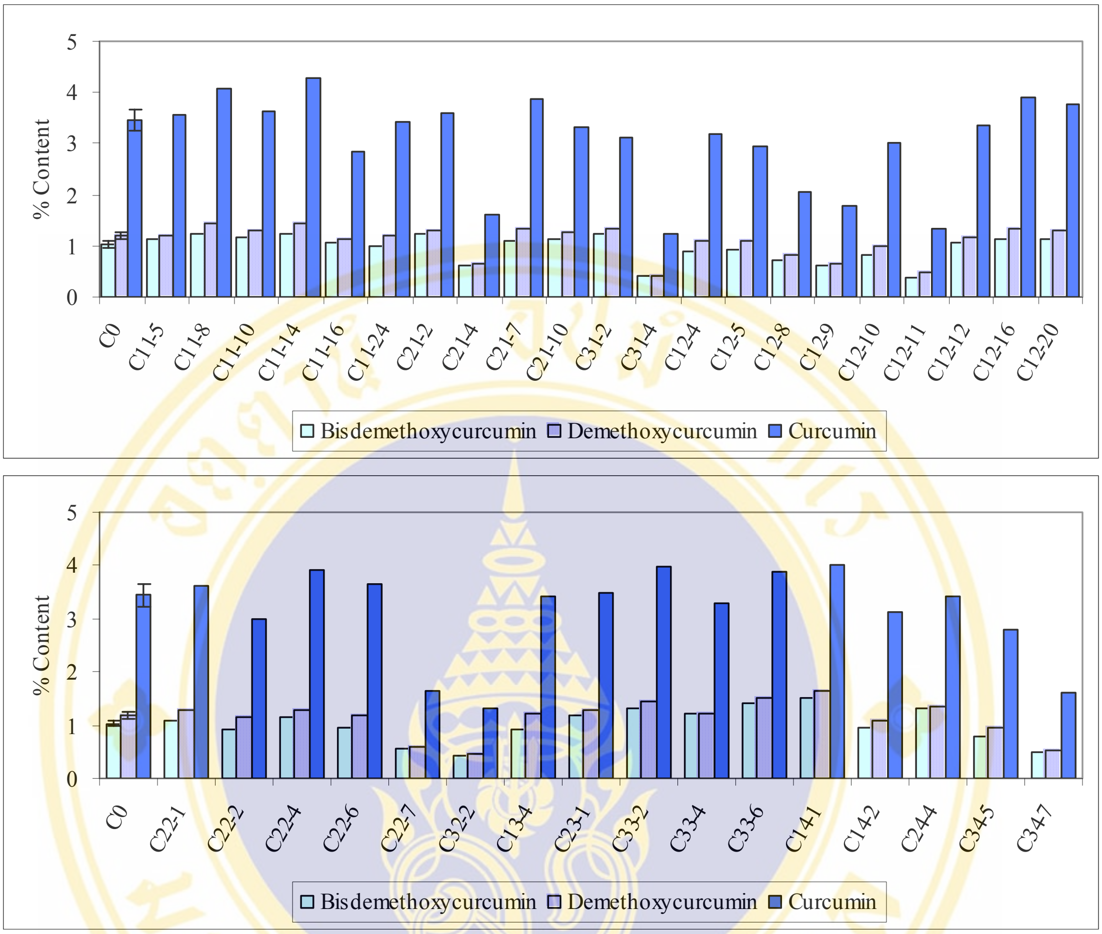


Figure 27 Percentage by weight of bisdemethoxycurcumin, demethoxycurcumin and curcumin contents in colchicine-treated *Curcuma longa*. C0 indicated control plant with average value from 5 plants with S.E. (All data are shown in appendix, table 25.)

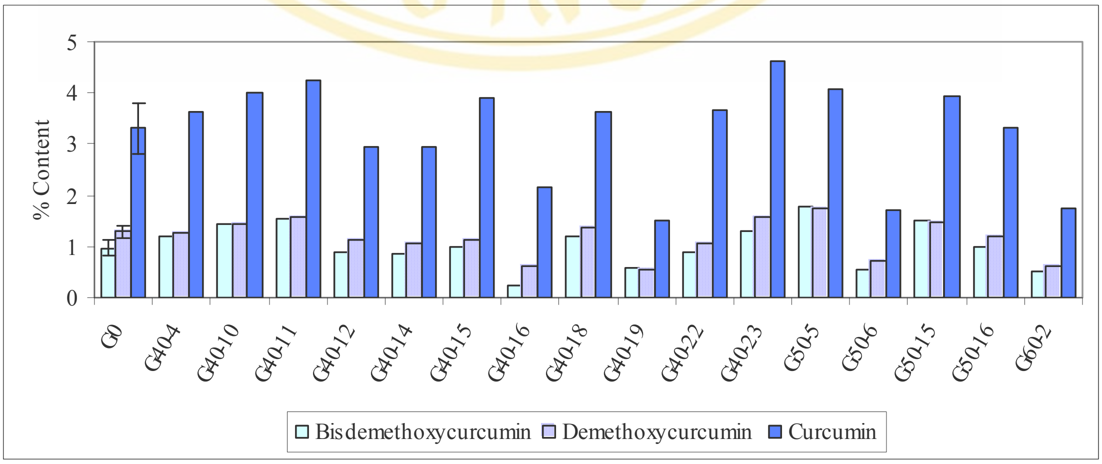


Figure 28 Percentage by weight of bisdemethoxycurcumin, demethoxycurcumin and curcumin contents in gamma rays-treated *Curcuma longa*. G0 indicated control plant with average value from 5 plants with S.E. (All data are shown in appendix, table 26.)

Volatile oil content in the selected samples from colchicine-treated plants and gamma rays-treated plants were determined following the method of Thai herbal pharmacopoeia 1995 (54). The volume was measured, and percentage of oil volume by weight was calculated.

Pulverised fingers of colchicine-treated plants contained 2.00-6.20% (v/w) volatile oil while the control plants contained 4.75$\pm$0.34% (figure 29). The gamma rays-treated plants contained 3.00-6.00% (v/w) volatile oil while the control plants contained 4.78$\pm$0.38% (figure 30).

Qualitative analysis by GC-MS represented 3 major constituents in volatile oil of all selected plants that were *ar*-turmerone, turmerone, and curlone, and the others can be detected including β-sesquiphellandrene, (-)-zingiberene, and α-curcumene. Table 13 and 14 show % relative peak area of the constituents of volatile oil found in selected colchicine-treated and gamma rays-treated plants determined by GC-MS. The GC chromatogram and its corresponding MS spectra of *Curcuma longa* oil is shown in appendix.

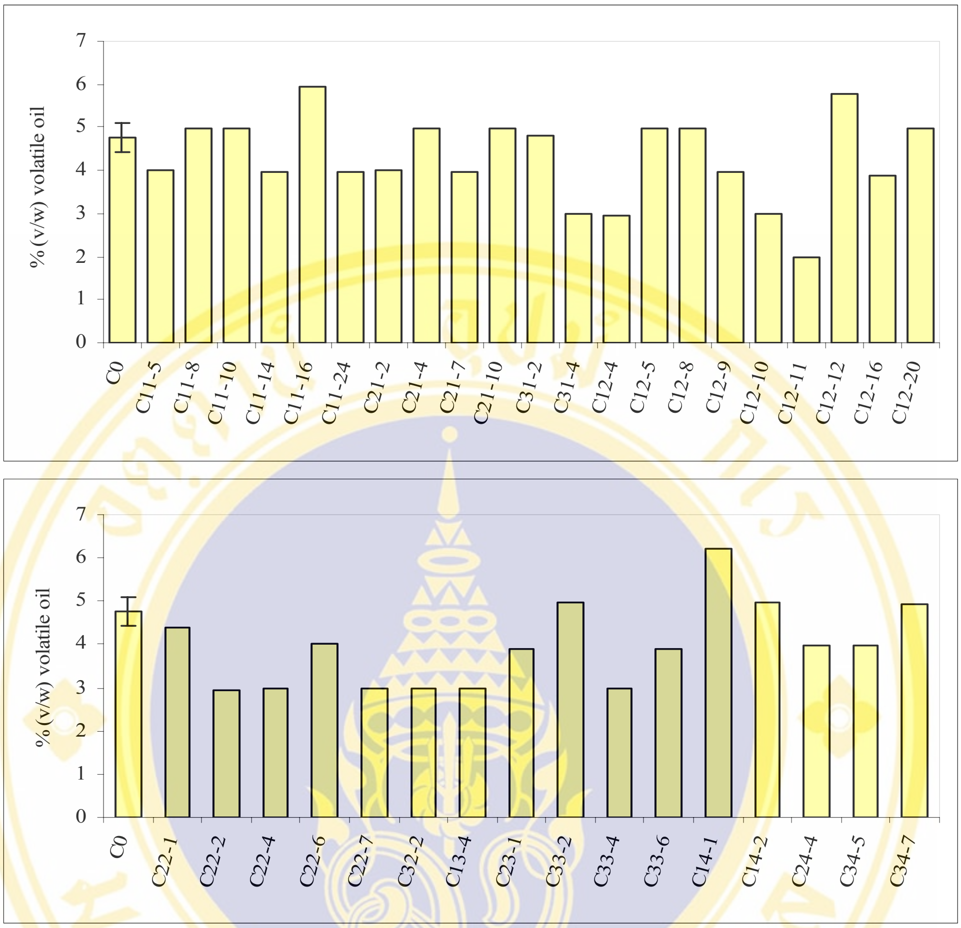


Figure 29 Volatile oil content of colchicine-treated *Curcuma longa*. C0 indicated control plant with average value from 5 plants with S.E. (All data are shown in appendix, table 27.)

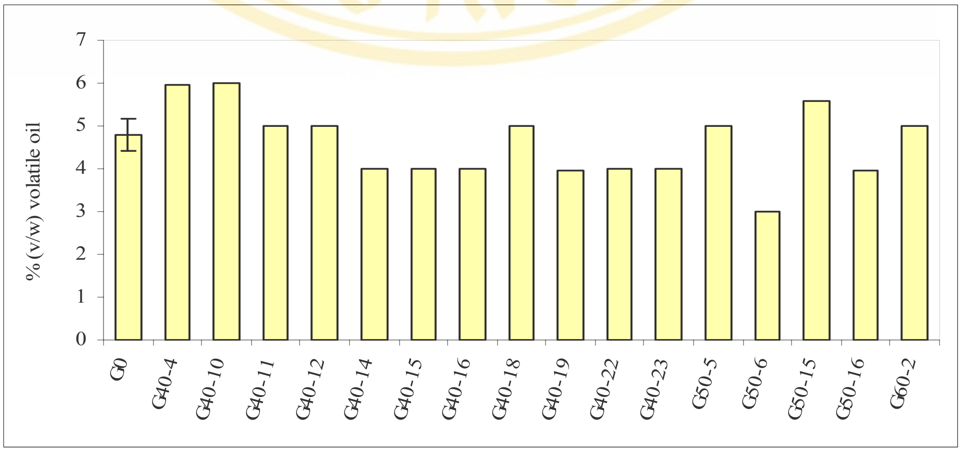


Figure 30 Volatile oil content of gamma rays-treated *Curcuma longa*. G0 indicated control plant with average value from 5 plants with S.E. (All data are shown in appendix, table 28.)

Table 13 Percentage relative peak area of constituents of volatile oil found in selected colchicine-treated *Curcuma longa* determining by GC-MS.

	% Relative peak area					
Plant code*	α-Curcumene	(-)-Zingiberene	*β*-Sesquiphellan-drene	*ar*-Turmerone	Turmerone	Curlone
C0-1	2.25	4.54	5.91	24.95	37.90	18.03
C0-2	1.25	3.33	4.50	22.87	42.26	20.09
C0-3	2.44	3.21	5.21	30.80	31.48	19.19
C0-4	2.82	2.89	5.64	32.06	29.32	19.64
C0-5	2.08	2.83	4.69	30.35	32.47	20.27
C11-5	1.43	1.08	2.79	34.05	31.52	20.77
C11-8	1.85	3.82	4.76	25.38	38.10	18.97
C11-10	1.86	2.91	4.31	28.26	36.56	19.56
C11-14	1.27	1.42	2.79	33.19	32.41	20.40
C11-16	1.40	4.54	5.26	19.33	44.05	19.15
C11-24	2.40	3.77	5.37	30.50	33.11	18.48
C21-2	1.32	1.56	3.09	30.35	35.28	20.95
C21-4	2.08	4.25	5.54	23.44	39.34	18.79
C21-7	2.58	2.84	5.40	32.77	28.82	20.46
C21-10	1.60	2.69	4.18	28.37	36.07	20.36
C31-2	2.02	5.23	6.28	20.25	41.20	18.00
C31-4	2.05	3.56	5.10	28.03	34.72	19.61
C12-4	1.64	1.94	3.81	33.29	31.28	20.47
C12-5	1.75	3.78	5.13	22.77	39.36	19.98
C12-8	1.45	5.01	5.56	19.99	42.88	18.62
C12-9	1.15	1.90	3.05	28.76	36.16	21.71
C12-10	2.38	3.19	5.38	30.23	32.44	19.49
C12-11	trace	0.53	0.90	33.62	30.14	22.24
C12-12	1.73	3.58	4.71	24.52	39.02	19.42
C12-16	1.70	2.49	3.97	30.78	33.80	19.90
C12-20	1.59	3.66	4.79	23.57	40.04	19.44
C22-1	2.28	2.90	4.89	30.91	32.49	19.85
C22-2	2.37	3.66	5.56	29.08	32.81	19.14
C22-4	2.56	2.32	4.93	35.17	26.59	20.36
C22-6	1.94	2.41	4.18	32.04	31.22	19.95
C22-7	2.46	4.33	6.27	25.66	32.60	18.71
C32-2	1.47	3.20	4.21	25.73	38.29	19.73
C13-4	2.37	4.05	5.75	27.43	34.10	18.52
C23-1	1.51	3.27	4.35	23.71	41.37	19.67
C33-2	2.55	2.85	5.40	31.94	30.51	19.98
C33-4	2.33	3.98	5.68	26.60	36.62	18.35
C33-6	3.31	5.21	7.79	26.04	34.17	16.82

* Plant code explanation: C0 = control plant of colchicine-treated experiment; C11, C21, C31 = 0.25, 0.50, 0.75% colchicine for 1 day; C12, C22, C32 = 0.25, 0.50, 0.75% colchicine for 2 days; C13, C23, C33 = 0.25, 0.50, 0.75% colchicine for 3 day; the number behind dash indicates identification number of the plants.

Table 13 Percentage relative peak area of constituents of volatile oil found in selected colchicine-treated *Curcuma longa* determining by GC-MS (continued).

	% Relative peak area					
Plant code*	α-Curcumene	(-)-Zingiberene	*β*-Sesquiphellan-drene	*ar*-Turmerone	Turmerone	Curlone
C14-1	1.68	5.03	5.69	19.77	42.31	18.54
C14-2	2.49	3.11	5.36	31.44	31.43	19.52
C24-4	2.34	2.07	4.49	34.53	27.41	20.93
C34-5	2.27	2.66	5.09	31.46	30.26	20.93
C34-7	2.05	3.65	5.12	27.04	35.61	19.37

* Plant code explanation: C14, C24, C34 = 0.25, 0.50, 0.75% colchicine for 4 days; the number behind dash indicates identification number of the plants.

Table 14 Percentage relative peak area of constituents of volatile oil found in selected gamma rays-treated *Curcuma longa* determining by GC-MS.

	% Relative peak area					
Plant code*	α-Curcumene	(-)-Zingiberene	*β*-Sesquiphellan-drene	*ar*-Turmerone	Turmerone	Curlone
G0-1	2.28	4.06	5.66	26.31	36.84	18.26
G0-2	1.98	4.17	5.45	24.96	36.42	19.33
G0-3	2.24	3.94	5.45	27.40	34.36	19.41
G0-4	1.52	2.86	4.27	25.86	38.37	20.64
G0-5	2.18	1.85	4.21	34.87	28.17	20.69
G40-4	2.30	2.66	4.83	31.67	30.27	20.77
G40-10	2.87	2.61	5.54	33.08	28.36	20.61
G40-11	2.06	4.19	5.58	24.63	38.67	18.84
G40-12	2.11	4.71	5.90	23.86	37.88	18.29
G40-14	3.75	2.85	6.94	35.28	25.31	18.85
G40-15	3.00	2.30	4.83	38.16	24.11	19.55
G40-16	1.74	3.56	4.99	27.69	34.54	19.46
G40-18	1.99	4.72	5.91	22.22	39.01	18.25
G40-19	2.50	4.11	6.19	26.41	35.89	18.27
G40-22	1.92	2.98	4.50	29.24	33.63	19.96
G40-23	1.85	3.94	5.28	24.54	37.75	18.90
G50-5	1.90	3.25	4.54	27.91	35.14	19.75
G50-6	2.05	2.41	4.23	33.32	29.27	20.63
G50-15	1.89	3.94	5.06	24.22	39.85	18.79
G50-16	1.53	3.98	4.96	21.43	41.85	19.62
G60-2	1.46	3.56	4.57	22.84	40.74	19.76

* Plant code explanation: G0 = control plant of gamma rays-treated experiment; G40, G50, G60 = 40 Gy, 50 Gy, and 60 Gy gamma rays treatment respectively; the number behind dash indicates identification number of the plants.

5. Cytological observation

Chromosome number in young bud and/or root tip cells of *Curcuma longa* was observed. It was found that untreated plants have 2n = 63 (figure 31). This result is similar to somatic number of *Curcuma longa* collecting from India which also was 2n = 63 (59).

Due to limitation plant materials, only 10 colchicine-treated plants and 10 gamma ray-treated plants were studied. Figure 32 and 33 show the chromosome morphology of colchicine-treated and gamma rays-treated plants respectively. The result reveals that most of the observed plants have the same chromosome number, 2n = 63 (table 15 and 16).

Even cytological character by mean of chromosome number in 20 treated plants from totally 53 qualified plants through selection criteria of phytomass production showed unchanged, the morphological variation apparently appeared in leaves and rhizomes at stage of cultivation. These may reveal genetically changed happening in the treated plants, especially in gamma rays-treated plants, which can be caused by point mutation or structural change, and such changes can not identify by chromosome morphology. A number of chromosomal aberrations in some cases have been found that there was no change in the quantity of genetic material; however, it caused a new phenotypic effect (60).

This investigation found the difficulty in determination of chromosome number because *Curcuma longa* possess very small chromosome of about 0.6-1.7 µm in length (59), and also rich in number (61). Furthermore, all the regenerated shoots withered during studying (October-December 2005), and even, to avoid such obstacle, some regenerated shoots were brought into *in vitro* culture to obtain regenerated roots, such process can not performed in all treated plants because number of regenerated shoots also limited.

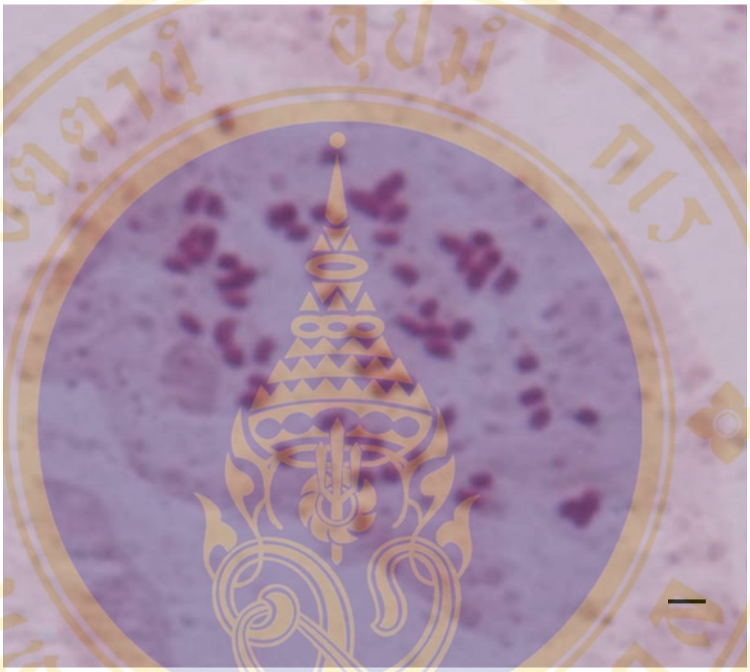


Figure 31 Metaphase chromosome of untreated *Curcuma longa*, 2n = 63. Scale bar = 1 µm.

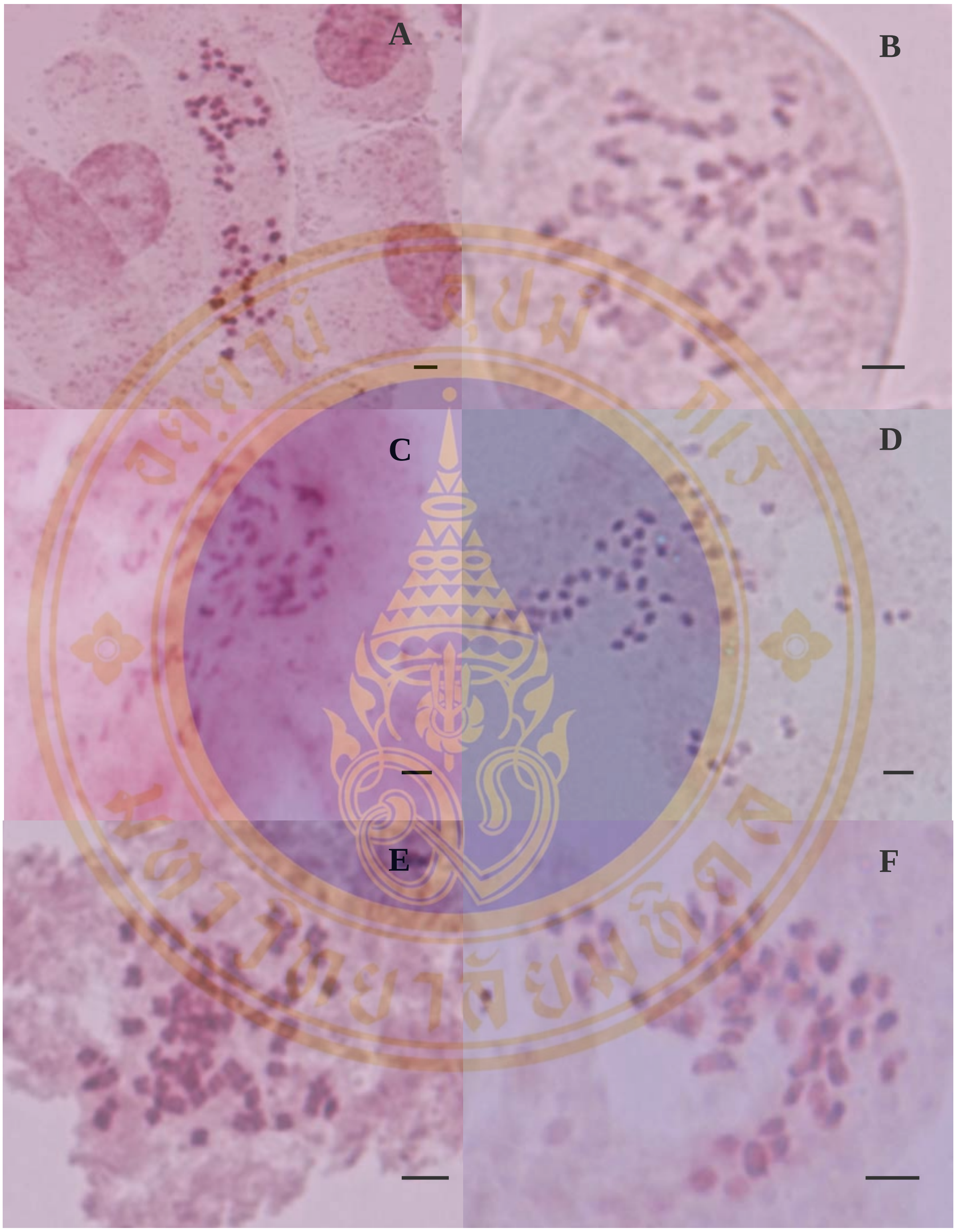


Figure 32 Metaphase chromosome in cells of colchicine-treated *Curcuma longa*, 2n=63. (A) C11-16 plant (0.25% colchicine for 1 day); (B) C12-5 plant (0.25% colchicine for 2 days); (C) C22-7 plant (0.25% colchicine for 3 days); (D) C23-1 plant (0.50% colchicine for 3 days); (E) C33-6 plant (0.75% colchicine for 3 days); (F) C34-7 plant (0.75% colchicine for 4 days). Scale bars = 2 µm.

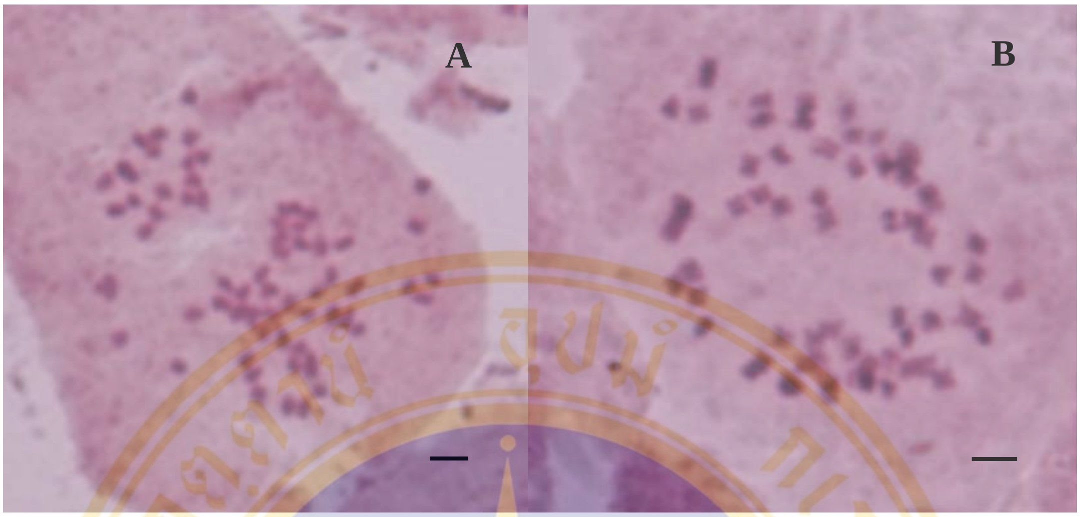


Figure 33 Metaphase chromosome in cells of gamma rays-treated *Curcuma longa*, 2n=63. (A) G40-11 plant (40 Gy gamma rays); (B) G50-5 plant (50 Gy gamma rays). Scale bars = 2 µm.

Table 15 Chromosome number determination of the colchicine-treated *Curcuma longa*.

Plant code*	Treatment: Concentration of colchicine	Treatment: Treatment duration (day)	Chromosome number (2n)
C11-16	0.25%	1	63
C12-5	0.25%	2	63
C12-16	0.25%	2	63
C22-4	0.50%	2	63
C22-7	0.50%	2	63
C23-1	0.50%	3	63
C33-4	0.75%	3	63
C33-6	0.75%	3	63
C24-4	0.50%	4	63
C34-7	0.75%	4	63

* Plant code represents code of colchicine treatment and identification number of the plants. Letter C = colchicine, the first and the second number behind letter C (in front of dash) indicate concentration of colchicine and treated-duration respectively, and the number behind dash indicate identification number of plants from the treatment.

Table 16 Chromosome number determination of the gamma rays-treated *Curcuma longa*.

Plant code*	Dose of gamma ray (Gy)	Chromosome number (2n)
G40-11	40	63
G40-14	40	63
G40-18	40	63
G40-19	40	63
G40-22	40	63
G40-23	40	63
G50-5	50	63
G50-15	50	63
G50-16	50	63
G60-2	60	63

* Plant code represents code of gamma ray treatment and identification number of the plants. Letter G = gamma rays, the number behind letter G (in front of dash) indicates dose of gamma rays, and the number behind dash indicate identification number of plants from the treatment.

6. Evaluation of the mutants from mutational induction experiments

The investigation was proposed to induce the valuable *Curcuma longa* for medicinal purposes. Curcuminoids and volatile oil contents were used to justify the value of derived mutants, and they were assessed as yield production per plant. The variants of both colchicine and gamma rays experiments show variability in both curcuminoids and volatile oil yield production (table 17 and 18). C14-1 contained the highest yield in both curcuminoids (3.56 g/plant) and volatile oil (3.07 ml/plant) while G50-5 contained the highest yield of only curcuminoids (3.10 g/plant) and, however, also high yield of volatile oil (2.04 ml/plant).

Concerning superior chemical characteristics (yield production of total curcuminoids and volatile oils) comparing to the control group, a number of 18 plants consisting of C11-5, C11-8, C11-10, C11-14, C11-16, C21-2, C21-10, C31-2, C12-5, C12-12, C12-20, C22-6, C23-1, C33-2, C33-4, C33-6, C14-1 and C24-4, and a number of 4 plants consisting of G40-4, G40-10, G40-18, and G50-5 were qualified. Details are shown in table 17 and 18.

Gavidia (1999) induced variants *Digitalis obscura* by gamma rays irradiation at intensity range of 20-100 Gy. The variations corresponded to aneuploid changes, and the plants showed high variability in their cardenolide production (6).

Additionally, it is remarkable that those qualified plants also shows variation in growth ability according to data of plant height, corm size, finger number, and finger size comparing to the control group and all other cultivated plants. Such evidence can be promoted by genetic effect and/or environmental effect. Since it would be considered that the plants were brought from the same clone and all plants were cultivated under the same controlled conditions, thus, genetic effect could be the most reason in variation of their morphology. Hence, even chromosome checking can not be performed in all treated plants, variation in morphology and chemical contents imply the cytological effect conducting by the mutagens in both mutational induction treatments.

Table 17 Summarized data of plant morphology, chemical contents, and yield chemical productions of the selected colchicine-treated *Curcuma longa*.

		Corm			Finger			% (w/w)					Yield production of	
Plant code	Height (cm)	Length (cm)	Diameter (cm)	No. of Finger	Length (cm)	Diameter (cm)	Dry Weight (g)	Curcumin	Dem.	Bisdem.	Total curcumi-noids	% (v/w) Volatile oil	Total curcumi-noids (g)/plant	Volatile oil (ml)/plant
C0	71.32	5.08	2.36	7.58	7.94	1.13	23.74	3.45*	1.19*	1.03*	5.67*	4.75*	1.31*	1.12*
(n=12)	*(5.74)*[a]	*(0.22)*	*(0.12)*	*(0.34)*	*(0.50)*	*(0.03)*	*(3.74)*	*(0.21)*	*(0.07)*	*(0.06)*	*(0.34)*	*(0.34)*	*(0.09)*	*(0.19)*
C11-5**	86.0	5.0	2.30	7	9.71	1.34	30.12	3.56	1.21	1.12	5.90	3.99	1.78	1.20
C11-8**	70.0	5.2	2.30	7	9.50	1.39	31.43	4.08	1.43	1.22	6.74	4.98	2.12	1.57
C11-10**	88.0	5.1	2.40	7	8.57	1.16	28.20	3.65	1.31	1.16	6.12	4.97	1.73	1.40
C11-14**	90.0	5.4	2.70	9	8.56	1.26	34.59	4.28	1.43	1.24	6.95	3.98	2.40	1.38
C11-16**	98.0	5.2	2.40	8	10.56	1.16	36.38	2.85	1.14	1.06	5.05	5.94	1.84	2.16
C11-24	86.8	5.5	1.90	6	9.17	1.17	24.85	3.41	1.20	1.00	5.61	3.98	1.39	0.99
C21-2**	100.5	5.5	2.50	7	9.86	1.30	38.54	3.61	1.30	1.23	6.15	3.99	2.37	1.54
C21-4	93.0	5.5	2.50	7	8.93	1.24	25.76	1.61	0.64	0.61	2.86	4.98	0.74	1.28
C21-7	76.0	5.0	2.70	7	9.21	1.27	24.09	3.88	1.33	1.09	6.29	3.97	1.52	0.96
C21-10**	73.5	4.7	2.50	6	9.42	1.27	34.25	3.34	1.27	1.14	5.75	4.98	1.97	1.71
C31-2**	90.5	6.0	2.50	8	9.69	1.18	51.44	3.13	1.35	1.22	5.70	4.83	2.93	2.48
C31-4	88.0	6.1	2.20	8	8.44	1.06	28.97	1.24	0.42	0.41	2.07	2.99	0.60	0.87
C12-4	92.0	5.6	2.00	8	7.56	1.14	23.10	3.19	1.09	0.88	5.16	2.96	1.19	0.68
C12-5**	78.5	4.8	2.10	8	9.25	1.05	27.34	2.96	1.11	0.91	4.98	4.99	1.36	1.36
C12-8	72.0	6.2	2.40	9	8.17	1.23	30.08	2.05	0.83	0.71	3.59	4.97	1.08	1.50
C12-9	72.0	4.0	3.10	8	9.25	1.21	40.03	1.79	0.66	0.63	3.08	3.94	1.23	1.58
C12-10	68.0	6.4	2.20	9	8.56	1.12	28.71	3.01	0.99	0.82	4.82	2.98	1.38	0.85
C12-11	101.0	6.2	2.30	9	8.17	1.11	28.47	1.34	0.48	0.38	2.20	2.00	0.63	0.57
C12-12**	76.0	4.3	2.20	6	9.00	1.17	29.21	3.34	1.15	1.06	5.55	5.78	1.62	1.69
C12-16	89.0	5.9	2.10	10	7.75	1.08	28.33	3.90	1.34	1.13	6.37	3.86	1.80	1.09
C12-20**	72.5	5.6	2.60	8	9.19	1.19	23.99	3.78	1.31	1.12	6.21	4.96	1.49	1.19

* The presented data were averaged from 5 control plants.
** The plants that represent superior characters on total curcuminoids and volatile oil yield production comparing to the control.
[a] The standard error (S.E.) value.

Table 17 Summarized data of plant morphology, chemical contents, and yield chemical productions of the selected colchicine-treated *Curcuma longa* (continued).

		Corm			Finger			% (w/w)					Yield production of	
Plant code	Height (cm)	Length (cm)	Diameter (cm)	No. of Finger	Length (cm)	Diameter (cm)	Dry Weight (g)	Curcumin	Dem.	Bisdem.	Total curcumi-noids	% (v/w) Volatile oil	Total curcumi-noids (g)/plant	Volatile oil (ml)/plant
C22-1	79.3	4.4	2.60	7	8.21	1.11	24.01	3.62	1.29	1.09	6.00	4.37	1.44	1.05
C22-2	92.5	5.2	2.50	9	9.56	1.30	32.06	2.99	1.14	0.92	5.06	2.94	1.62	0.94
C22-4	75.0	5.2	2.70	7	9.57	1.27	25.02	3.92	1.29	1.15	6.36	2.99	1.59	0.75
C22-6**	90.0	6.8	2.70	8	9.19	1.24	32.01	3.66	1.18	0.97	5.81	4.00	1.86	1.28
C22-7	88.0	5.0	2.80	7	8.29	1.20	23.95	1.65	0.59	0.55	2.80	2.99	0.67	0.72
C32-2	86.0	4.9	2.40	8	7.00	1.06	23.18	1.33	0.46	0.43	2.22	2.98	0.51	0.69
C13-4	100.0	5.7	2.90	8	8.31	1.16	28.13	3.43	1.21	0.92	5.56	2.99	1.56	0.84
C23-1**	84.0	5.0	2.50	7	8.47	1.27	30.11	3.48	1.27	1.19	5.94	3.91	1.79	1.18
C33-2**	84.5	4.4	2.10	7	8.93	1.24	25.90	3.99	1.44	1.33	6.76	4.98	1.75	1.29
C33-4**	107.0	5.6	2.60	6	11.25	1.42	42.01	3.28	1.21	1.22	5.71	2.99	2.40	1.26
C33-6**	85.5	5.3	2.40	9	8.33	1.19	34.79	3.88	1.50	1.41	6.79	3.91	2.36	1.36
C14-1**	103.5	6.5	2.60	8	9.69	1.26	49.44	4.03	1.65	1.52	7.20	6.20	3.56	3.07
C14-2	72.0	5.2	2.20	8	8.13	1.16	24.87	3.13	1.09	0.96	5.19	4.97	1.29	1.24
C24-4**	99.5	5.9	2.90	7	11.43	1.46	49.47	3.41	1.36	1.31	6.08	3.96	3.01	1.96
C34-5	79.5	5.5	2.80	7	8.93	1.27	23.05	2.79	0.96	0.79	4.54	3.98	1.05	0.92
C34-7	83.5	4.8	2.80	7	9.07	1.31	24.45	1.60	0.54	0.50	2.64	4.92	0.64	1.20

* The presented data were averaged from 5 control plants.
** The plants that represent superior characters on total curcuminoids and volatile oil yield production comparing to the control.
[a] The standard error (S.E.) value.

Table 18 Summarized data of plant morphology, chemical contents, and yield chemical productions of the selected gamma rays-treated *Curcuma longa*.

Plant code	Height (cm)	Corm: Length (cm)	Corm: Diameter (cm)	No. of Finger	Finger: Length (cm)	Finger: Diameter (cm)	Finger: Dry Weight (g)	% (w/w): Curcumin	% (w/w): Dem.	% (w/w): Bisdem.	% (w/w): Total curcumi-noids	% (v/w) Volatile oil	Yield production of: Total curcumi-noids (g)/plant	Yield production of: Volatile oil (ml)/plant
G0	61.25	4.85	2.22	7.10	7.42	1.12	23.96	3.31*	1.29*	0.98*	5.58*	4.78*	1.61*	1.34*
(n=10)	*(7.75)* [a]	*(0.20)*	*(0.14)*	*(0.41)*	*(0.52)*	*(0.05)*	*(11.34)*	*(0.49)*	*(0.12)*	*(0.17)*	*(0.78)*	*(0.38)*	*(0.48)*	*(0.34)*
G40-4**	101.0	5.9	3.00	7	9.00	1.19	42.97	3.64	1.28	1.18	6.11	5.96	2.63	2.56
G40-10**	83.0	5.5	2.10	6	8.55	1.25	32.37	4.00	1.43	1.42	6.86	6.00	2.22	1.94
G40-11	101.5	5.5	2.90	6	8.53	1.40	20.36	4.24	1.59	1.53	7.36	5.00	1.50	1.02
G40-12	77.0	5.6	2.20	9	7.04	1.03	23.45	2.94	1.13	0.89	4.96	5.00	1.16	1.17
G40-14	87.0	4.9	1.90	8	7.30	1.09	31.83	2.94	1.06	0.85	4.85	4.00	1.55	1.27
G40-15	78.5	5.7	2.20	9	6.52	1.18	23.15	3.91	1.12	0.99	6.02	3.98	1.39	0.92
G40-16	58.0	4.8	1.90	7	8.30	1.03	26.55	2.16	0.62	0.24	3.03	4.00	0.80	1.06
G40-18**	90.5	6.0	2.20	7	9.14	1.26	30.94	3.63	1.36	1.18	6.17	4.98	1.91	1.54
G40-19	100.0	6.0	2.40	8	10.15	1.33	43.39	1.50	0.56	0.58	2.63	3.98	1.14	1.73
G40-22	103.0	5.5	2.80	8	9.10	1.26	32.87	3.65	1.06	0.89	5.60	4.00	1.84	1.31
G40-23	101.0	5.4	2.60	7	9.26	1.24	24.04	4.62	1.57	1.30	7.50	3.98	1.80	0.96
G50-5**	92.0	5.2	3.80	6	11.48	1.38	40.76	4.08	1.74	1.79	7.61	5.00	3.10	2.04
G50-6	55.0	4.8	2.70	6	11.30	1.33	26.38	1.72	0.73	0.53	2.99	3.00	0.79	0.79
G50-15	73.0	4.9	2.50	7	8.07	1.17	23.82	3.93	1.48	1.52	6.93	5.60	1.65	1.33
G50-16	66.0	4.6	2.10	8	8.44	1.13	19.22	3.31	1.21	1.00	5.52	3.97	1.06	0.76
G60-2	82.0	4.5	2.50	7	6.57	1.39	21.56	1.75	0.61	0.53	2.89	4.99	0.62	1.08

* The presented data were averaged from 5 control plants.
** The plants that represent superior characters on total curcuminoids and volatile oil yield production comparing to the control.
[a] The standard error of mean (S.E.) value.

CHAPTER V
CONCLUSIONS

1. Mutational induction of valuable *Curcuma longa* using colchicine and gamma rays

Curcuma longa was sensitive to dose and duration when treated with colchicine at 0.25, 0.50, and 0.75% (w/w) for 1-4 days and gamma rays at intensities of 40, 50, 60, and 70 Gy. Strength of both mutagens and together with length of treatment affects frequency of lethality and shoot regeneration rate. Lethality increased and the percentage of shoot formation decreased when strength and length of the mutagens increased.

After proceeding of induction experiments and inducing of M3 plants, the plants were selected following the selection criterion 1 which considered to survival ability. A number of 141 (44.76%) colchicine treated-plants and 67 (19.20%) gamma rays-treated plants were cultivated in greenhouse conditions for 8 months. During cultivation, both colchicine and gamma rays-treated plants showed apparently morphological variation in leaves and rhizomes. The variation was also demonstrated by data of plant height, corm size, number of fingers, finger size, and finger dry weight.

After harvesting, fingers dry weight was assessed as phytomass production of plants. The selection criterion 2 was performed considering to phytomass production. This step, 37 (11.75%) of colchicine treated-plants and 16 (4.58%) gamma rays-treated plants were chosen to further chemically and cytological study.

Evaluation of the chemical contents in the treated plants was done by comparing to average amount according to percentage by weight and yield production found in the control plants. In colchicine treated plants, contents of curcumin, bisdemethoxycurcumin, and demethoxycurcumin were 1.24-4.28, 0.42-1.65, and 0.38-

1.52% (w/w) respectively while the average amount of the control plants were 3.45±0.21, 1.19±0.07, and 1.03±0.06% (w/w) respectively. In gamma rays treated plants, contents of curcumin, bisdemethoxycurcumin, and demethoxycurcumin were 1.50-4.62, 0.56-1.74, and 0.24-1.79% (w/w) respectively while the amounts of the control plants were 3.31±0.49, 1.29±0.12, and 0.98±0.17% (w/w) respectively. The selected plants of colchicine experiment contained 2.00-6.20% (v/w) volatile oil while that of 4.75±0.34% presented in the control plants. The selected plants of gamma rays experiment contained 3.00-6.00% (v/w) volatile oil while the control plants showed that of 4.78±0.38%. Their volatile oil mainly contained *ar*-turmerone, turmerone, and curlone.

A number of 18 (5.71%) colchicine-treated plants which derived from treatments of 0.25, 0.50, and 0.75% colchicine for 1 day, 0.25, 0.50, and 0.75% colchicine for 2 days, 0.75% colchicine for 3 days, and 0.25 and 0.75% colchicine for 4 days, and a number of 4 (1.15%) gamma rays-treated plants which derived from treatments of 40 Gy and 50 Gy gamma rays exhibited higher amounts of curcuminoids and volatile oil than those of the control plants.

Cytological observation was proceeded by using Feulgen squash method. Choromosome number of the control plants was 2n = 63. The observation found unchanged in chromosome number of 20 selected plants.

2. The establishment of HPLC analysis of *Curcuma longa*

An simply isocratic HPLC system was developed over Hypersil BDS-C18 column (4.6 mm x 250 mm) to determine curcumin, demethoxycurcumin, and bisdemethoxycurcumin content by using proportional 2% acetic acid and acetronitrile (55:45) as mobile phase as mobile phase, flow rate 1 ml/min, running time of 20 min, detected at 425 nm and without temperature controller. Method validation which consisting of suitability, linearity and range, and accuracy and precision test fitted to acceptance criteria of USP25 and AOAC. Sensitivity of the analysis recommended in the range of 5-70 µg/ml, 1.4-70 µg/ml, and 1.5-50 µg/ml for curcumin, demethoxycurcumin, and bisdemethoxycurcumin respectively.

In this investigation, the suitable extraction method was studied by determine the total curcuminoids contents using UV spectrophotometer at 420 nm-wavelength. Plant extraction by sonication 300 mg amount of pulverized fingers with 10 ml methanol for 0.5 h, 3 times because it provided exhausted extracts.

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APPENDIX

Table 19 Frequency of lethality of colchicine-treated *Curcuma longa* after cultured in shoot induction medium for 4 weeks, and in MS medium for 4 weeks (figure 4).

Treatment	N	% Lethality
0.00% Colchicine, 1 day	30	13.33±6.31
0.25% Colchicine, 1 day	33	53.13±8.83
0.50% Colchicine, 1 day	32	81.25±7.01
0.75% Colchicine, 1 day	32	90.63±5.24
0.00% Colchicine, 2 days	31	3.23±3.23
0.25% Colchicine, 2 days	32	68.75±8.32
0.50% Colchicine, 2 days	32	68.75±8.32
0.75% Colchicine, 2 days	30	83.33±6.92
0.00% Colchicine, 3 days	30	16.67±6.92
0.25% Colchicine, 3 days	30	83.33±6.92
0.50% Colchicine, 3 days	30	96.67±3.33
0.75% Colchicine, 3 days	31	80.65±7.21
0.00% Colchicine, 4 days	30	23.33±7.85
0.25% Colchicine, 4 days	30	90.00±5.57
0.50% Colchicine, 4 days	30	96.67±3.33
0.75% Colchicine, 4 days	31	87.10±6.12

Table 20 Frequency of shoot response and shoot regeneration rate of colchicine-treated *Curcuma longa* after cultured in shoot induction medium for 4 weeks, and in MS medium for 4 weeks (figure 5).

Treatment	N	% Shoot response	Shoot regeneration rate
0.00% Colchicine, 1 day	30	93.33±4.63	2.61±0.19
0.25% Colchicine, 1 day	33	78.79±7.23	2.85±0.28
0.50% Colchicine, 1 day	32	43.75±8.91	2.36±0.36
0.75% Colchicine, 1 day	32	31.25±8.32	1.70±0.26
0.00% Colchicine, 2 days	31	90.32±5.40	2.57±0.24
0.25% Colchicine, 2 days	32	71.88±8.08	2.35±0.17
0.50% Colchicine, 2 days	32	46.88±8.96	2.07±0.18
0.75% Colchicine, 2 days	30	56.67±9.20	2.18±0.37
0.00% Colchicine, 3 days	30	96.67±3.33	2.38±0.17
0.25% Colchicine, 3 days	30	43.33±9.20	2.00±0.23
0.50% Colchicine, 3 days	30	33.33±8.75	1.80±0.29
0.75% Colchicine, 3 days	31	38.71±8.89	2.00±0.21
0.00% Colchicine, 4 days	30	83.33±6.92	2.58±0.28
0.25% Colchicine, 4 days	30	16.67±6.92	2.20±0.49
0.50% Colchicine, 4 days	30	23.33±7.85	2.71±0.36
0.75% Colchicine, 4 days	31	25.81±7.99	2.75±0.31

Table 21 Observation of LD_{50} intensity of *Curcuma longa* at 60 days after acute irradiation with gamma rays (figure 7).

Intensity (Gy)	N	% Lethality
0	50	0.00±0.00
20	50	0.00±0.00
40	50	2.00±2.00
60	50	60.00±7.00
80	50	100.00±0.00
100	50	100.00±0.00

Table 22 Frequency of shoot response and shoot regeneration rate of gamma rays-treated *Curcuma longa* after cultured in shoot induction medium for 4 weeks, and in MS medium for 4 weeks (figure 8).

Intensity (Gy)	N	%Shoot response	Shoot regeneration rate
0	50	94.00±3.39	3.71±0.37
40	50	94.00±3.39	1.96±0.15
50	50	68.00±6.66	1.55±0.14
60	50	26.00±6.27	0.91±0.26
70	50	6.00±3.39	0.83±0.57

Table 23 Summarize data of plant height at 4-moths old cultivation, and corm size, finger number, finger size, and dry weight of finger after harvest of colchicine-treated *Curcuma longa* (figure 14, 16, 18, and 20).

Plant code*	Plant height (cm)	Corm		Finger			
		Length (cm)	Diameter (cm)	Number	Length±S.E. (cm)	Diameter±S.E. (cm)	Dry weight (g)
C0 (n=12)	71.32±5.74	5.08±0.22	2.36±0.12	7.58±0.34	7.94±0.50	1.13±0.03	24.51±5.14
C11-1	49.0	4.6	1.80	8	4.66±0.39	0.86±0.02	4.09
C11-2	66.0	4.4	1.90	4	6.08±0.32	1.03±0.06	6.13
C11-3	64.5	4.0	1.80	6	7.00±0.70	0.95±0.11	11.94
C11-4	56.0	4.0	1.80	7	5.00±0.45	1.01±0.06	4.84
C11-5	86.0	5.0	2.30	7	9.71±0.83	1.34±0.07	30.12
C11-6	60.0	4.9	2.30	8	7.13±0.65	1.10±0.05	15.46
C11-7	45.0	3.9	1.90	7	5.07±0.57	0.94±0.06	6.55
C11-8	70.0	5.2	2.30	7	9.50±0.66	1.39±0.09	31.43
C11-9	78.5	4.7	2.10	6	8.08±0.91	1.18±0.08	20.01
C11-10	88.0	5.1	2.40	7	8.57±1.04	1.16±0.07	28.20
C11-11	40.8	3.6	1.80	6	5.00±0.41	0.90±0.03	5.78
C11-12	54.0	3.3	1.80	4	5.75±0.66	1.10±0.08	4.15
C11-13	36.0	2.6	1.40	3	2.73±0.29	0.60±0.00	0.44
C11-14	90.0	5.4	2.70	9	8.56±1.08	1.26±0.10	34.59
C11-15	76.0	5.1	2.90	8	7.25±0.77	1.19±0.08	14.66
C11-16	98.0	5.2	2.40	8	10.56±1.14	1.16±0.09	36.38
C11-17	63.0	4.8	2.40	6	9.67±0.56	1.30±0.07	20.53
C11-18	55.0	3.4	2.20	5	7.40±0.83	1.08±0.08	7.84
C11-19	69.0	4.6	2.00	8	8.69±1.03	1.16±0.10	18.68
C11-20	55.0	3.3	1.90	5	5.30±0.58	1.00±0.06	4.55
C11-21	72.0	4.7	2.20	6	6.75±0.66	1.17±0.08	13.16
C11-22	42.5	4.0	1.40	6	4.25±0.56	0.87±0.08	4.95
C11-23	37.0	2.8	1.20	4	1.85±0.39	0.55±0.05	0.00
C11-24	86.8	5.5	1.90	6	9.17±0.99	1.17±0.11	24.85
C11-25	82.0	3.2	1.30	14	4.82±0.23	0.75±0.05	7.48
C11-26	52.5	3.7	2.10	6	3.78±0.79	0.67±0.06	2.34
C11-27	98.0	6.3	2.90	9	9.33±1.13	1.22±0.12	31.40
C11-28	55.0	4.0	2.20	7	7.43±0.73	1.16±0.09	17.14
C11-29	63.5	4.6	2.20	5	8.20±0.98	1.00±0.09	10.41
C21-1	43.0	4.1	1.90	6	5.75±0.75	0.95±0.07	7.71
C21-2	100.5	5.5	2.50	7	9.86±1.38	1.30±0.10	38.54
C21-3	58.0	4.2	1.80	8	6.25±0.58	0.96±0.06	8.64

* Plant code explanation: C0 = control plant of colchicine-treated experiment, all data represent with S.E.; C11 = 0.25% colchicine for 1 day; C21 = 0.50% colchicine for 1 days; C31 = 0.75% colchicine for 1 days; C12 = 0.25% colchicine for 2 day; C22 = 0.50% colchicine for 2 days; C32 = 0.75% colchicine for 2 days; C13 = 0.25% colchicine for 3 day; C21 = 0.50% colchicine for 3 days; C31 = 0.75% colchicine for 3 days; C14 = 0.25% colchicine for 4 day; C21 = 0.50% colchicine for 4 days; C31 = 0.75% colchicine for 4 days; the number after dash indicates identification number of the plants.

Table 23 Summarize data of plant height at 4-moths old cultivation, and corm size, finger number, finger size, and dry weight of finger after harvest of colchicine-treated *Curcuma longa* (figure 14, 16, 18, and 20) (continued).

Plant code*	Plant height (cm)	Corm		Finger			
		Length (cm)	Diameter (cm)	Number	Length ±S.E. (cm)	Diameter±S.E. (cm)	Dry weight (g)
C21-4	93.0	5.5	2.50	7	8.93±1.45	1.24±0.12	25.76
C21-5	69.0	4.4	2.20	8	5.50±0.67	0.93±0.06	8.07
C21-6	80.0	4.5	2.40	7	7.00±1.07	1.04±0.10	16.59
C21-7	76.0	5.0	2.70	7	9.21±0.86	1.27±0.08	24.09
C21-8	51.0	4.0	2.10	6	6.32±0.84	1.17±0.09	9.86
C21-9	65.5	5.4	2.40	7	9.21±0.69	1.24±0.06	19.02
C21-10	73.5	4.7	2.50	6	9.42±0.83	1.27±0.12	34.25
C31-1	66.5	5.6	2.10	8	5.21±0.66	1.00±0.08	9.82
C31-2	90.5	6.0	2.50	8	9.69±0.97	1.18±0.09	51.44
C31-3	85.0	4.1	2.40	6	8.42±0.64	1.18±0.06	16.97
C31-4	88.0	6.1	2.20	8	8.44±1.01	1.06±0.09	28.97
C31-5	52.0	4.6	1.70	6	5.50±1.19	0.93±0.08	6.24
C12-1	84.0	6.0	2.30	8	6.63±0.84	1.05±0.07	14.72
C12-2	52.0	4.2	2.10	6	7.00±1.32	1.10±0.12	15.95
C12-3	66.0	5.0	3.00	5	8.00±0.50	1.32±0.10	9.31
C12-4	92.0	5.6	2.00	8	7.56±0.70	1.14±0.07	23.10
C12-5	78.5	4.8	2.10	8	9.25±0.86	1.05±0.06	27.34
C12-6	74.0	4.5	2.50	6	6.75±0.53	1.03±0.06	9.86
C12-7	77.0	5.0	2.50	8	6.81±0.89	1.00±0.08	16.22
C12-8	72.0	6.2	2.40	9	8.17±0.65	1.23±0.11	30.08
C12-9	72.0	4.0	3.10	8	9.25±1.39	1.21±0.12	40.03
C12-10	68.0	6.4	2.20	9	8.56±0.95	1.12±0.10	28.71
C12-11	101.0	6.2	2.30	9	8.17±1.06	1.11±0.12	28.47
C12-12	76.0	4.3	2.20	6	9.00±0.26	1.17±0.09	29.21
C12-13	69.0	4.2	2.30	5	8.60±0.43	1.10±0.25	17.84
C12-14	67.0	4.7	2.40	6	7.67±0.75	1.17±0.09	12.16
C12-15	66.0	4.9	2.30	8	7.19±0.77	1.06±0.07	13.32
C12-16	89.0	5.9	2.10	10	7.75±0.86	1.08±0.07	28.33
C12-17	78.0	5.2	2.60	9	6.39±0.72	1.00±0.07	14.38
C12-18	67.0	3.8	1.80	5	3.36±0.51	0.58±0.06	1.20
C12-19	53.2	5.6	2.10	8	6.81±0.94	1.14±0.08	15.37
C12-20	72.5	5.6	2.60	8	9.19±0.98	1.19±0.10	23.99
C12-21	54.5	3.6	2.20	5	5.00±0.84	0.86±0.09	4.78

* Plant code explanation: C0 = control plant of colchicine-treated experiment, all data represent with S.E.; C11 = 0.25% colchicine for 1 day; C21 = 0.50% colchicine for 1 days; C31 = 0.75% colchicine for 1 days; C12 = 0.25% colchicine for 2 day; C22 = 0.50% colchicine for 2 days; C32 = 0.75% colchicine for 2 days; C13 = 0.25% colchicine for 3 day; C21 = 0.50% colchicine for 3 days; C31 = 0.75% colchicine for 3 days; C14 = 0.25% colchicine for 4 day; C21 = 0.50% colchicine for 4 days; C31 = 0.75% colchicine for 4 days; the number after dash indicates identification number of the plants.

Table 23 Summarize data of plant height at 4-moths old cultivation, and corm size, finger number, finger size, and dry weight of finger after harvest of colchicine-treated *Curcuma longa* (figure 14, 16, 18, and 20) (continued).

Plant code*	Plant height (cm)	Corm		Finger			
		Length (cm)	Diameter (cm)	Number	Length (cm)	Diameter (cm)	Dry weight (g)
C22-1	79.3	4.4	2.60	7	8.21±0.99	1.11±0.07	24.01
C22-2	92.5	5.2	2.50	9	9.56±0.94	1.30±0.09	32.06
C22-3	41.0	2.4	1.40	3	2.93±0.23	0.57±0.07	0.66
C22-4	75.0	5.2	2.70	7	9.57±0.95	1.27±0.09	25.02
C22-5	41.0	2.1	1.10	2	1.95±0.65	0.50±0.00	0.22
C22-6	90.0	6.8	2.70	8	9.19±0.91	1.24±0.10	32.01
C22-7	88.0	5.0	2.80	7	8.29±0.87	1.20±0.07	23.95
C22-8	52.5	3.4	1.50	5	3.20±0.68	0.60±0.03	0.92
C32-1	70.0	5.0	2.40	9	6.17±1.02	1.03±0.10	12.63
C32-2	86.0	4.9	2.40	8	7.00±0.93	1.06±0.13	23.18
C32-3	58.0	3.8	2.00	6	5.63±0.58	1.02±0.07	6.56
C32-4	53.0	4.3	2.00	6	6.00±0.76	1.08±0.05	8.77
C13-1	65.0	4.8	2.20	6	8.58±0.76	1.27±0.10	20.12
C13-2	73.0	4.6	2.00	8	7.69±1.06	1.09±0.12	21.08
C13-3	60.2	4.8	2.50	6	7.83±0.92	1.07±0.07	11.62
C13-4	100.0	5.7	2.90	8	8.31±1.06	1.16±0.11	28.13
C23-1	84.0	5.0	2.50	7	8.47±1.13	1.27±0.13	30.11
C33-1	60.0	5.1	1.80	7	7.50±1.02	1.20±0.08	16.77
C33-2	84.5	4.4	2.10	7	8.93±0.95	1.24±0.11	25.90
C33-3	70.0	5.4	1.80	9	8.22±0.66	1.22±0.08	20.79
C33-4	107.0	5.6	2.60	6	11.25±1.20	1.42±0.09	42.01
C33-5	64.0	5.7	2.20	5	6.90±0.66	1.10±0.08	10.67
C33-6	85.5	5.3	2.40	9	8.33±0.97	1.19±0.10	34.79
C14-1	103.5	6.5	2.60	8	9.69±1.06	1.26±0.08	49.44
C14-2	72.0	5.2	2.20	8	8.13±0.91	1.16±0.08	24.87
C14-3	76.0	4.8	2.10	7	7.79±1.00	1.06±0.10	17.90
C14-4	37.5	2.8	1.50	3	4.87±0.27	0.90±0.06	1.61
C14-5	76.0	4.7	2.40	7	7.79±0.91	1.26±0.08	17.49
C14-6	68.5	4.9	2.50	6	8.50±0.75	1.22±0.09	15.28
C14-7	60.0	5.0	2.20	7	8.29±0.70	1.16±0.08	12.58
C14-8	67.0	4.6	2.20	9	6.56±0.61	1.07±0.07	15.60
C14-9	65.0	4.3	2.20	7	9.57±0.74	1.40±0.12	20.01
C24-1	66.0	4.1	2.40	8	7.31±0.69	1.08±0.06	12.39

* Plant code explanation: C0 = control plant of colchicine-treated experiment, all data represent with S.E.; C11 = 0.25% colchicine for 1 day; C21 = 0.50% colchicine for 1 days; C31 = 0.75% colchicine for 1 days; C12 = 0.25% colchicine for 2 day; C22 = 0.50% colchicine for 2 days; C32 = 0.75% colchicine for 2 days; C13 = 0.25% colchicine for 3 day; C21 = 0.50% colchicine for 3 days; C31 = 0.75% colchicine for 3 days; C14 = 0.25% colchicine for 4 day; C21 = 0.50% colchicine for 4 days; C31 = 0.75% colchicine for 4 days; the number after dash indicates identification number of the plants.

Table 23 Summarize data of plant height at 4-moths old cultivation, and corm size, finger number, finger size, and dry weight of finger after harvest of colchicine-treated *Curcuma longa* (figure 14, 16, 18, and 20) (continued).

Plant code*	Plant height (cm)	Corm		Finger			
		Length (cm)	Diameter (cm)	Number	Length (cm)	Diameter (cm)	Dry weight (g)
C24-2	37.0	3.3	1.60	3	6.83±0.67	1.23±0.12	6.33
C24-3	51.0	5.2	2.00	8	6.40±0.65	1.05±0.10	11.53
C24-4	99.5	5.9	2.90	7	11.43±0.88	1.46±0.09	49.47
C24-5	28.0	2.5	0.80	5	5.00±0.42	0.76±0.05	1.85
C24-6	70.0	3.7	1.70	7	6.86±0.75	1.01±0.10	9.17
C24-7	82.0	5.1	2.00	5	10.40±0.58	1.22±0.08	22.04
C24-8	40.5	3.7	1.90	6	6.33±0.48	0.93±0.08	6.19
C24-9	69.0	4.8	1.90	8	5.13±0.62	0.80±0.08	5.62
C34-1	51.0	4.2	2.00	4	7.25±0.85	1.00±0.04	5.65
C34-2	70.2	6.4	2.20	8	6.69±0.77	1.15±0.11	19.04
C34-3	70.0	5.0	2.50	7	8.43±0.69	1.11±0.05	13.05
C34-4	87.8	5.6	2.80	7	8.43±1.03	1.26±0.11	19.23
C34-5	79.5	5.5	2.80	7	8.93±1.19	1.27±0.06	23.05
C34-6	85.0	5.1	2.40	7	7.57±0.69	1.20±0.06	16.97
C34-7	83.5	4.8	2.80	7	9.07±0.98	1.31±0.09	24.45
C34-8	93.5	4.7	3.00	6	10.50±1.05	1.38±0.07	40.20
C34-9	56.5	4.0	2.40	7	7.36±0.70	1.07±0.07	16.09
C34-10	21.0	2.0	1.10	2	3.10±0.10	0.60±0.00	0.00

* Plant code explanation: C0 = control plant of colchicine-treated experiment, all data represent with S.E.; C11 = 0.25% colchicine for 1 day; C21 = 0.50% colchicine for 1 days; C31 = 0.75% colchicine for 1 days; C12 = 0.25% colchicine for 2 day; C22 = 0.50% colchicine for 2 days; C32 = 0.75% colchicine for 2 days; C13 = 0.25% colchicine for 3 day; C21 = 0.50% colchicine for 3 days; C31 = 0.75% colchicine for 3 days; C14 = 0.25% colchicine for 4 day; C21 = 0.50% colchicine for 4 days; C31 = 0.75% colchicine for 4 days; the number after dash indicates identification number of the plants.

Table 24 Summarize data of plant height at 4-moths old cultivation, and corm size, finger number, finger size, and dry weight of finger after harvest of gamma rays-treated *Curcuma longa* (figure 15, 17, 19, and 21).

Plant code*	Plant height (cm)	Corm		Finger			
		Length (cm)	Diameter (cm)	Number	Length (cm)	Diameter (cm)	Dry weight (g)
G0 (n=10)	61.25±7.75	4.85±0.20	2.22±0.14	7.1±0.41	7.42±0.52	1.12±0.05	18.48±4.08
G40-1	83.0	3.4	1.70	4	5.38±0.13	1.00±0.07	4.73
G40-2	67.0	4.5	2.10	6	8.93±1.47	1.08±0.11	15.29
G40-3	50.0	2.8	1.80	7	4.90±0.43	1.03±0.07	6.10
G40-4	101.0	5.9	3.00	7	9.00±1.30	1.19±0.13	42.97
G40-5	61.5	4.9	1.80	7	6.00±0.95	0.99±0.12	12.66
G40-6	48.0	3.8	2.80	6	6.34±0.72	1.20±0.07	8.94
G40-7	38.0	3.0	1.50	6	4.83±0.34	0.40±0.11	5.19
G40-8	66.0	4.0	1.90	6	5.68±0.47	1.08±0.08	5.68
G40-9	58.5	6.0	2.90	8	9.35±0.58	0.88±0.07	16.03
G40-10	83.0	5.5	2.10	6	8.55±1.06	1.25±0.08	32.37
G40-11	101.5	5.5	2.90	6	8.53±1.43	1.40±0.12	20.36
G40-12	77.0	5.6	2.20	9	7.04±1.32	1.03±0.13	23.45
G40-13	79.0	4.9	2.30	6	6.02±0.91	1.15±0.06	7.28
G40-14	87.0	4.9	1.90	8	7.30±1.29	1.09±0.09	31.83
G40-15	78.5	5.7	2.20	9	6.52±0.84	1.18±0.09	23.15
G40-16	58.0	4.8	1.90	7	8.30±0.81	1.03±0.09	26.55
G40-17	67.0	5.0	1.50	7	5.21±0.75	0.86±0.06	6.72
G40-18	90.5	6.0	2.20	7	9.14±1.28	1.26±0.13	30.94
G40-19	100.0	6.0	2.40	8	10.15±1.03	1.33±0.13	43.39
G40-20	43.0	4.2	1.70	7	4.79±0.66	0.94±0.06	6.64
G40-21	48.0	3.1	1.90	6	5.00±0.67	1.17±0.10	11.17
G40-22	103.0	5.5	2.80	8	9.10±1.33	1.26±0.11	32.87
G40-23	101.0	5.4	2.60	7	9.26±0.96	1.24±0.08	24.04
G40-24	60.0	3.8	2.00	7	4.59±0.38	1.03±0.04	5.54
G50-1	44.0	3.2	1.60	5	5.40±0.99	0.66±0.05	3.51
G50-2	67.0	5.0	2.50	6	6.95±0.95	1.30±0.07	17.27
G50-3	60.0	4.0	1.50	6	6.08±0.45	0.82±0.05	9.07
G50-4	27.0	1.6	0.70	1	0.70	0.40	0.00
G50-5	92.0	5.2	3.80	6	11.48±0.53	1.38±0.07	40.76
G50-6	55.0	4.8	2.70	6	11.30±0.57	1.33±0.08	26.38
G50-7	48.0	4.0	2.10	6	6.17±0.65	0.90±0.07	5.41
G50-8	46.5	5.0	2.30	8	6.88±0.80	1.15±0.06	12.22
G50-9	47.0	3.7	1.70	4	6.55±0.63	1.10±0.08	15.56
G50-10	51.0	2.0	1.40	7	3.07±0.44	0.54±0.06	1.94

* Plant code explanation: G0 = control plant of gamma rays-treated experiment, all data represent with S.E.; G40 = 40 Gy treatment; G50 = 50 Gy treatment; G60 = 60 Gy treatment; G70 = 70 Gy treatment; the number after dash indicates identification number of the plants.

Table 24 Summarize data of plant height at 4-moths old cultivation, and corm size, finger number, finger size, and dry weight of finger after harvest of gamma rays-treated *Curcuma longa* (figure 15, 17, 19, and 21) (continued).

Plant code*	Plant height (cm)	Corm		Finger			
		Length (cm)	Diameter (cm)	Number	Length (cm)	Diameter (cm)	Dry weight (g)
G50-11	75.0	4.5	2.30	6	7.50±0.48	1.08±0.07	14.20
G50-12	71.5	4.8	2.40	7	7.47±0.92	1.04±0.11	13.96
G50-13	60.0	3.7	1.40	7	3.64±0.52	0.60±0.04	2.25
G50-14	54.0	4.4	1.90	8	6.13±1.02	1.06±0.11	13.53
G50-15	73.0	4.9	2.50	7	8.07±1.41	1.17±0.15	23.82
G50-16	66.0	4.6	2.10	8	8.44±1.17	1.13±0.11	19.22
G50-17	83.0	6.0	2.80	8	10.13±0.42	1.09±0.07	18.17
G50-18	61.0	5.5	2.60	8	7.10±0.59	1.25±0.08	16.48
G50-19	80.0	4.9	2.60	6	6.75±1.02	1.20±0.07	13.75
G50-20	47.0	4.0	1.80	6	3.83±0.63	1.18±0.08	7.93
G60-1	64.0	3.8	2.20	7	6.43±0.74	1.00±0.07	11.24
G60-2	82.0	4.5	2.50	7	6.57±0.74	1.39±0.09	21.56
G60-3	90.0	4.4	1.90	9	6.37±0.69	0.78±0.05	9.63
G60-4	64.0	4.4	2.40	5	7.70±0.58	1.04±0.06	7.59
G60-5	30.0	2.1	0.90	1	1.00	0.50	0.09
G60-6	52.5	3.5	1.50	8	2.13±0.23	0.64±0.03	0.89
G60-7	67.0	4.8	1.70	9	4.76±0.77	0.74±0.08	8.63
G60-8	62.5	3.6	2.00	8	3.28+0.96	0.68±0.06	2.07
G60-9	78.0	3.5	1.80	7	6.71±0.75	1.16±0.11	17.26
G70-1	27.0	2.7	1.40	4	3.40±0.84	0.63±0.02	0.88
G70-2	61.5	5.3	2.60	7	8.19±0.69	1.10±0.07	16.03
G70-3	49.0	2.0	1.70	6	5.42±0.81	0.90±0.07	6.11
G70-4	55.0	3.0	1.50	3	5.83±0.60	1.00±0.06	3.52

* Plant code explanation: G0 = control plant of gamma rays-treated experiment, all data represent with S.E.; G40 = 40 Gy treatment; G50 = 50 Gy treatment; G60 = 60 Gy treatment; G70 = 70 Gy treatment; the number after dash indicates identification number of the plants.

Table 25 Percentage of curcumin, demethoxycurcumin, and bisdemethoxycurcumin of colchicine-treated *Curcuma longa* after cultivation for 8 months in greenhouse (figure 27).

Plant code*	% (w/w)		
	Curcumin	Demethoxycurcumin	Bisdemethoxycurcumin
C0 (n=5)	3.45±0.21	1.19±0.07	1.03±0.06
C11-5	3.56	1.21	1.12
C11-8	4.08	1.43	1.22
C11-10	3.65	1.31	1.16
C11-14	4.28	1.43	1.24
C11-16	2.85	1.14	1.06
C11-24	3.41	1.20	1.00
C21-2	3.61	1.30	1.23
C21-4	1.61	0.64	0.61
C21-7	3.88	1.33	1.09
C21-10	3.34	1.27	1.14
C31-2	3.13	1.35	1.22
C31-4	1.24	0.42	0.41
C12-4	3.19	1.09	0.88
C12-5	2.96	1.11	0.91
C12-8	2.05	0.83	0.71
C12-9	1.79	0.66	0.63
C12-10	3.01	0.99	0.82
C12-11	1.34	0.48	0.38
C12-12	3.34	1.15	1.06
C12-16	3.90	1.34	1.13
C12-20	3.78	1.31	1.12
C22-1	3.62	1.29	1.09
C22-2	2.99	1.14	0.92
C22-4	3.92	1.29	1.15
C22-6	3.66	1.18	0.97
C22-7	1.65	0.59	0.55
C32-2	1.33	0.46	0.43
C13-4	3.43	1.21	0.92
C23-1	3.48	1.27	1.19
C33-2	3.99	1.44	1.33
C33-4	3.28	1.21	1.22
C33-6	3.88	1.50	1.41
C14-1	4.03	1.65	1.52
C14-2	3.13	1.09	0.96
C24-4	3.41	1.36	1.31
C34-5	2.79	0.96	0.79
C34-7	1.60	0.54	0.50

* Plant code explanation: C0 = control plant of colchicine-treated experiment, all data represent with S.E.; C11 = 0.25% colchicine for 1 day; C21 = 0.50% colchicine for 1 days; C31 = 0.75% colchicine for 1 days; C12 = 0.25% colchicine for 2 day; C22 = 0.50% colchicine for 2 days; C32 = 0.75% colchicine for 2 days; C13 = 0.25% colchicine for 3 day; C21 = 0.50% colchicine for 3 days; C31 = 0.75% colchicine for 3 days; C14 = 0.25% colchicine for 4 day; C21 = 0.50% colchicine for 4 days; C31 = 0.75% colchicine for 4 days; the number after dash indicates identification number of the plants.

Table 26 Percentage of curcumin, demethoxycurcumin, and bisdemethoxycurcumin of gamma rays-treated *Curcuma longa* after cultivation for 8 months in greenhouse (figure 27).

Plant code*	% (w/w)		
	Curcumin	Demethoxycurcumin	Bisdemethoxycurcumin
G0 (n=5)	3.31±0.49	1.29±0.12	0.98±0.17
G40-4	3.64	1.28	1.18
G40-10	4.00	1.43	1.42
G40-11	4.24	1.59	1.53
G40-12	2.94	1.13	0.89
G40-14	2.94	1.06	0.85
G40-15	3.91	1.12	0.99
G40-16	2.16	0.62	0.24
G40-18	3.63	1.36	1.18
G40-19	1.50	0.56	0.58
G40-22	3.65	1.06	0.89
G40-23	4.62	1.57	1.30
G50-5	4.08	1.74	1.79
G50-6	1.72	0.73	0.53
G50-15	3.93	1.48	1.52
G50-16	3.31	1.21	1.00
G60-2	1.75	0.61	0.53

* Plant code explanation: G0 = control plant of gamma rays-treated experiment, all data represent with S.E.; G40 = 40 Gy treatment; G50 = 50 Gy treatment; G60 = 60 Gy treatment; G70 = 70 Gy treatment; the number after dash indicates identification number of the plants.

Table 27 Volatile oil content of colchicine-treated *Curcuma longa* (figure 28).

Plant code*	%(v/w) Volatile oil
C0 (n=5)	4.75±0.34
C11-5	3.99
C11-8	4.98
C11-10	4.97
C11-14	3.98
C11-16	5.94
C11-24	3.98
C21-2	3.99
C21-4	4.98
C21-7	3.97
C21-10	4.98
C31-2	4.83
C31-4	2.99
C12-4	2.96
C12-5	4.99
C12-8	4.97
C12-9	3.94
C12-10	2.98
C12-11	2.00
C12-12	5.78
C12-16	3.86
C12-20	4.96
C22-1	4.37
C22-2	2.94
C22-4	2.99
C22-6	4.00
C22-7	2.99
C32-2	2.98
C13-4	2.99
C23-1	3.91
C33-2	4.98
C33-4	2.99
C33-6	3.91
C14-1	6.20
C14-2	4.97
C24-4	3.96
C34-5	3.98
C34-7	4.92

* Plant code explanation: C0 = control plant of colchicine-treated experiment, the data represents with S.E.; C11 = 0.25% colchicine for 1 day; C21 = 0.50% colchicine for 1 days; C31 = 0.75% colchicine for 1 days; C12 = 0.25% colchicine for 2 day; C22 = 0.50% colchicine for 2 days; C32 = 0.75% colchicine for 2 days; C13 = 0.25% colchicine for 3 day; C21 = 0.50% colchicine for 3 days; C31 = 0.75% colchicine for 3 days; C14 = 0.25% colchicine for 4 day; C21 = 0.50% colchicine for 4 days; C31 = 0.75% colchicine for 4 days; the number after dash indicates identification number of the plants.

Table 28 Volatile oil content of gamma rays-treated *Curcuma longa* (figure 28).

Plant code*	%(v/w) Volatile oil
G0 (n=5)	4.78±0.38
G40-4	5.96
G40-10	6.00
G40-11	5.00
G40-12	5.00
G40-14	4.00
G40-15	3.98
G40-16	4.00
G40-18	4.98
G40-19	3.98
G40-22	4.00
G40-23	3.98
G50-5	5.00
G50-6	3.00
G50-15	5.60
G50-16	3.97
G60-2	4.99

* Plant code explanation: G0 = control plant of gamma rays-treated experiment, the data represents with S.E.; G40 = 40 Gy treatment; G50 = 50 Gy treatment; G60 = 60 Gy treatment; G70 = 70 Gy treatment; the number after dash indicates identification number of the plants.

GC chromatogram of *Curcuma longa* oil with corresponding MS spectra

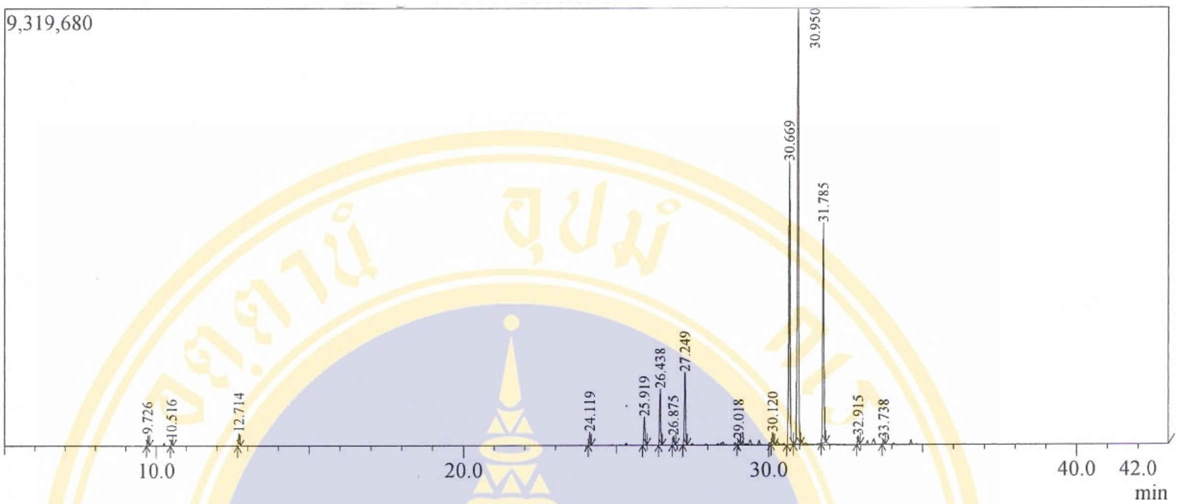
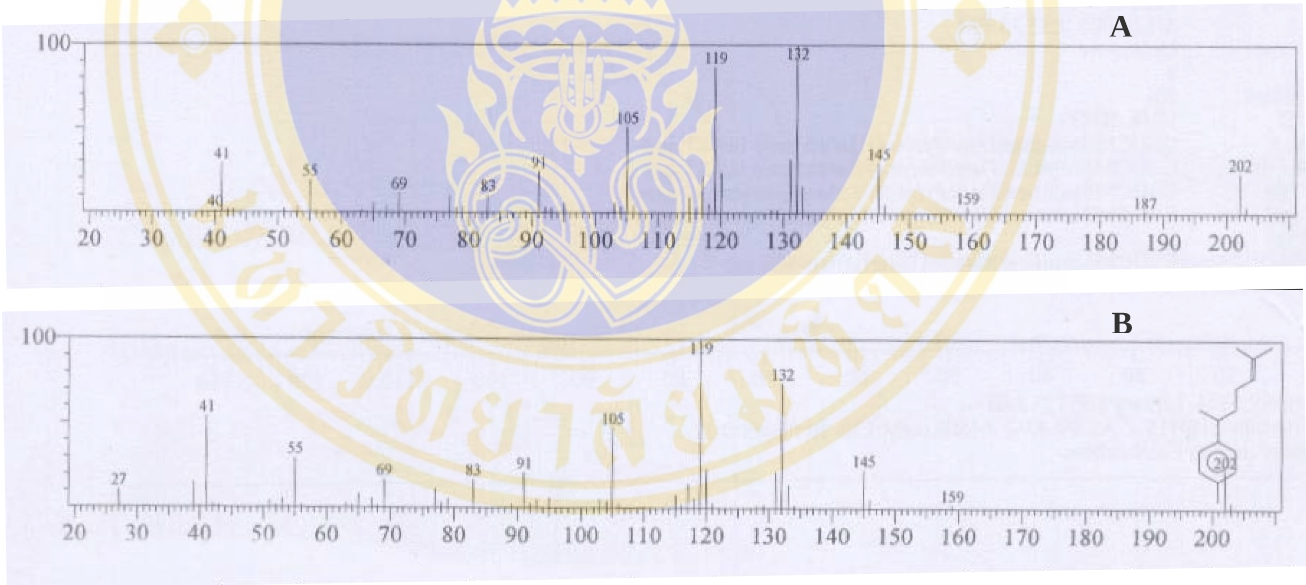


Figure 34 GC chromatogram of *Curcuma longa* oil.



Finger 35 Corresponding MS spectrum of the GC peak at Rt 25.919 min (A) which is identified as α-curcumene, MW=202 (B).

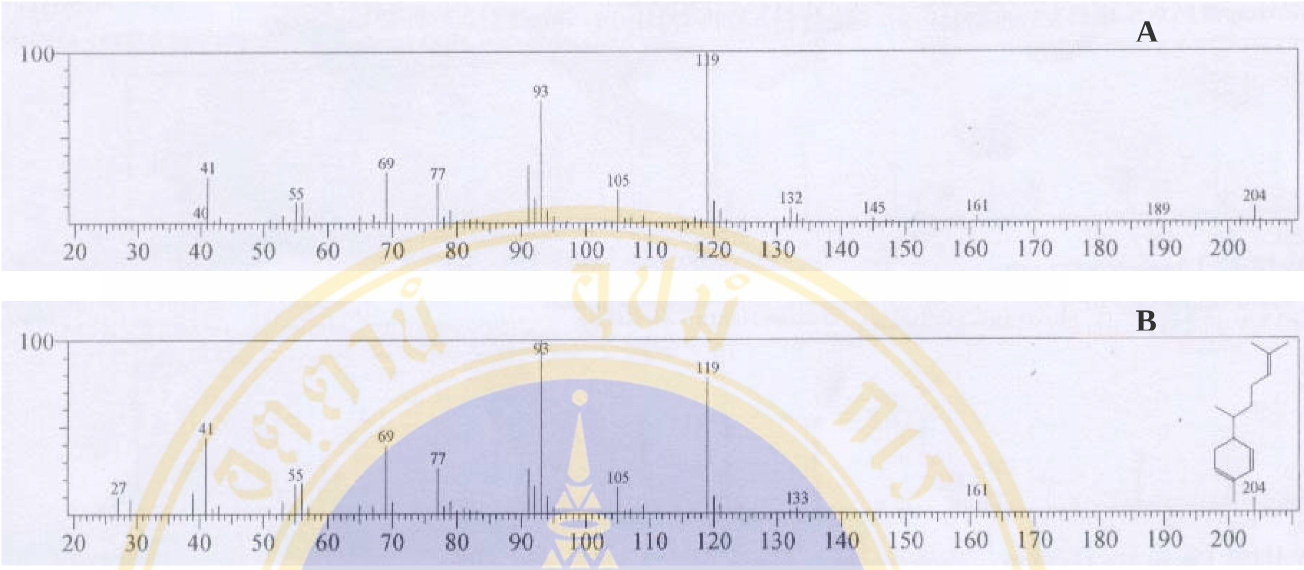


Figure 36 Corresponding MS spectrum of the GC peak at Rt 26.438 min (A) which is identified as (-)-zingiberene, MW=204 (B).

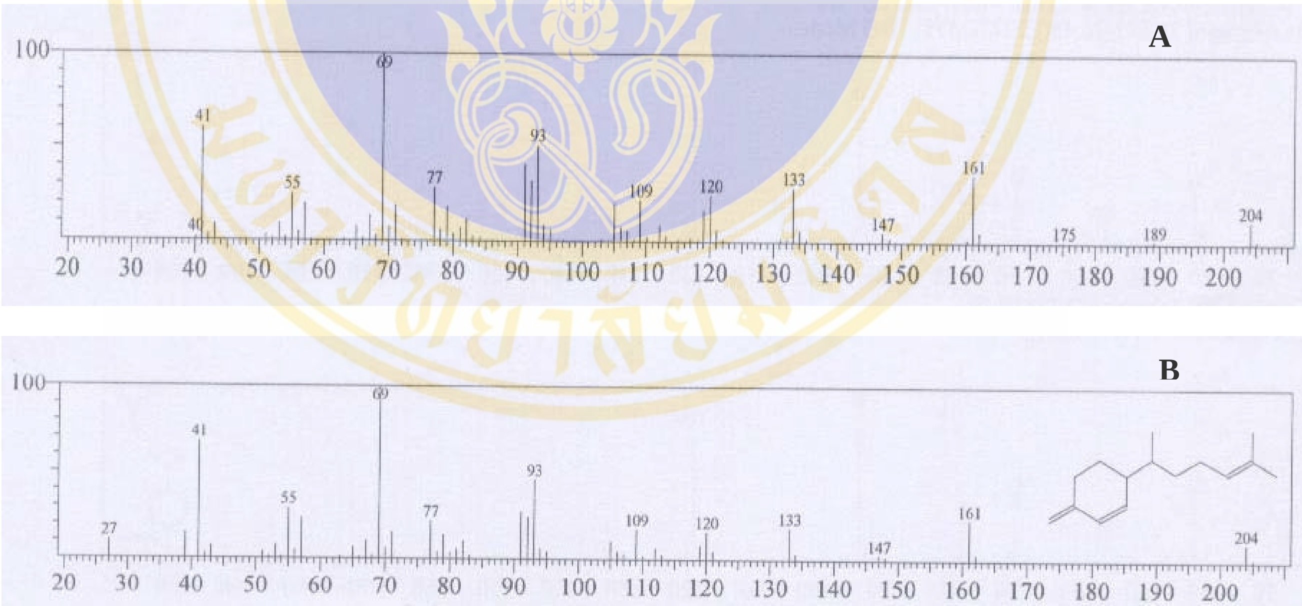


Figure 37 Corresponding MS spectrum of the GC peak at Rt 27.249 min (A) which is identified as *β*-sesquiphellandrene, MW=204 (B).

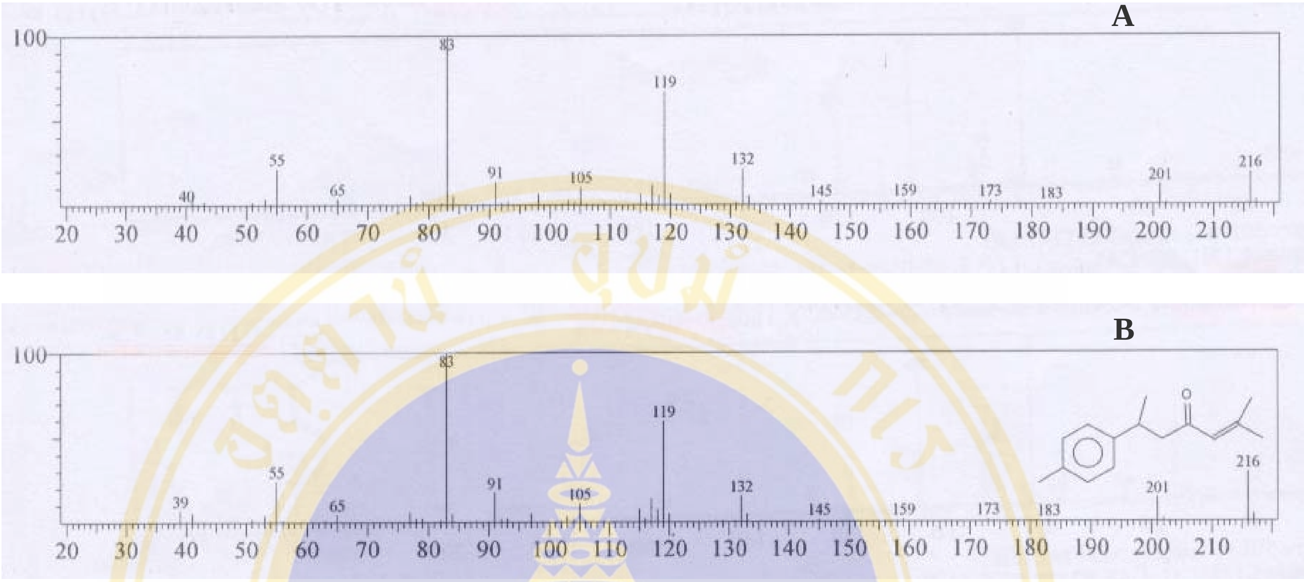


Figure 38 Corresponding MS spectrum of the GC peak at Rt 30.669 min (A) which is identified as *ar*-turmerone, MW= 216 (B).

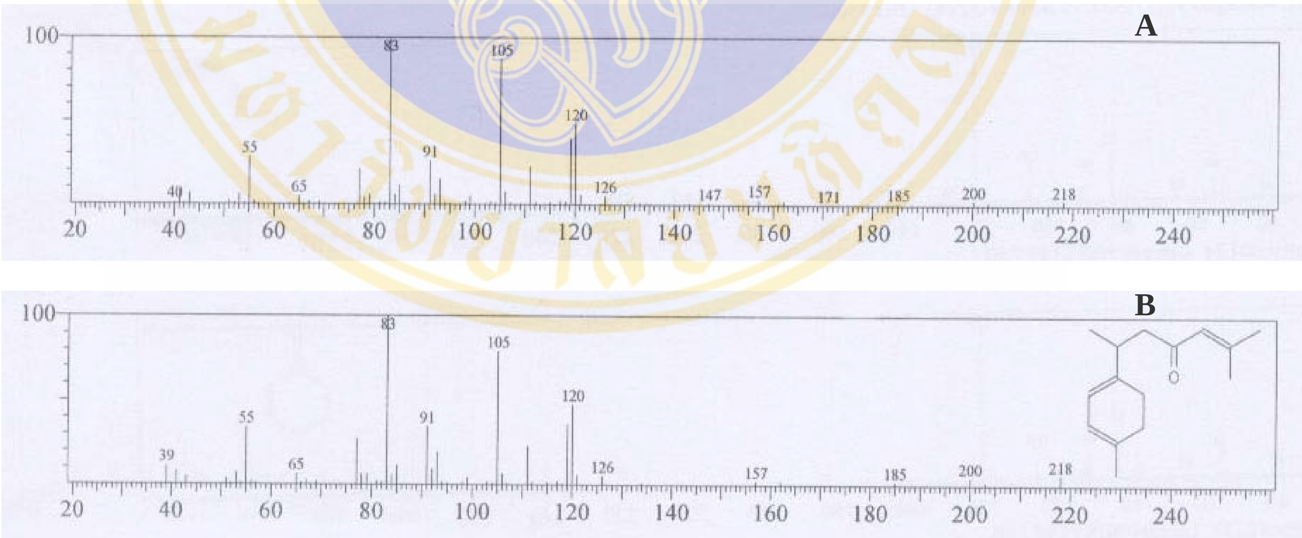


Figure 39 Corresponding MS spectrum of the GC peak at Rt 30.950 min (A) which is identified as turmerone, MW=218 (B).

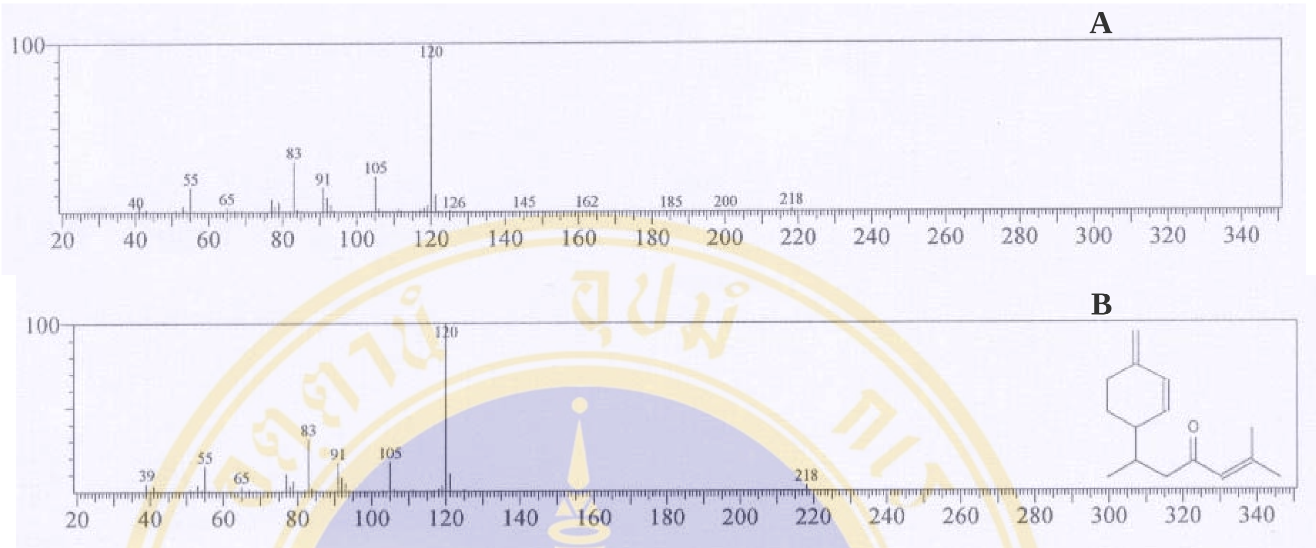


Figure 40 Corresponding MS spectrum of the GC peak at Rt 31.785 min (A) which is identified as curlone, MW=218 (B).

Preparation of reagents in cytological study

1. *p*-Dichlorobenzene solution

A 1.6 g amount of *p*-dichlorobenzene was dissolved with 100 ml distilled water in a glass stopcock bottle. The solution was saturated by incubating in hot air oven at 60^{o}C overnight.

2. Schiff's reagent

Boiling of 200 ml distilled water, and 1 g basic fuchsin was added over and stirred. The solution was cooled down to 50^{o}C, and then it was filtered through filter paper (Whatman No.1). The filtrate was added 30 ml 1N HCl. The mixture was further cooled down, then, 1 g sodium metabisulphite was added. The mixture was kept in the dark overnight. A few amount of activated charcoal was added into the mixture, and kept in refrigerator overnight, then, filtered through filter paper (Whatman No.1).

3. Aceto-orcein

Boiling 55 ml glacial acetic acid, and 2 g amount of orcein was added and stirred well. The mixture was cooled down, and 45 ml distilled water was added and filtered through filter paper (Whatman No.42).

BIOGRAPHY

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