Identification of Antimutagenic Substances (Ames Test) from *Boesenbergia pandurata* Schl. (Fingerroot) and *Languas galanga* (Galanga)

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Abstract

Methanolic extracts of *Boesenbergia pandurata* Schl. (fingerroot) and *Languas galanga* (galanga) that are spices commonly consumed in Thailand, showed a potent inhibitory activity on the mutagenesis induced by 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1) in *Salmonella typhimurium* TA98. Then the isolation and characterization of the antimutagenic compounds from both plants was carried out using reversed phase column chromatography. Six active compounds (FR1, FR2, FR3, FR4, FR5 and FR6) from fingerroot and two active compounds (G1 and G2) from galanga were isolated. The physico-chemical properties of these compounds were determined by liquid chromatography-mass spectrometry and UV-absorption spectrometry. FR1 and FR3 were identified as chalcone derivatives, and, FR2 and FR4 were identified as flavanone derivatives. FR5 and FR6 could not be identified due to insufficient information. G1 and G2 were estimated to be phenylpropanoid derivatives. The six isolated compounds from fingerroot exhibited a more pronounced antimutagenic effect than two compounds from galanga, at the concentration of 25 μg/plate. The antimutagenic activity of all the isolated compounds was fairly stable upon heat treatment and persisted after heating at 105℃ for 15 min.

Additional key words: antimutagenicity, *Boesenbergia pandurata* Schl., *Languas galanga*, Thai spices, Ames test

Abbreviations: HPLC, high performance liquid chromatography; UV, ultra violet; LC-MS, liquid chromatography-mass spectrometry

Food is a basic requirement for human health. Although, foods contain mutagens and/or carcinogens occurring either naturally or induced during preparations[2, 29], some plants contain a number of antimutagens and/or anticarcinogens[31]. Epidemiological studies have suggested that a large consumption of fruits and vegetables may contribute cancer prevention[3, 4, 30], due to the presence of substances such as carotenes, fibers and polyphenols, that display antimutagenic or anticarcinogenic properties[5, 30, 31]. In Asian countries, many edible plants originate from various plant families and/or genera, that are different from those of the plants consumed in Western countries. Therefore, the possibility of detecting antimutagens in Asian edible plants is being investigated.

In Thailand, there are hundreds of edible plant species[16]. However, research on the cancer preventing properties of edible Thai plants is limited. In recent years, Murakami *et al.* studied the anti-tumor promoting effect of various edible Thai plants[18, 23, 24]. The studies revealed that edible Thai plants consisted of a larger number of active species than edible plants in Japan[24]. As carcinogenesis involves at least two steps; initiation and promotion[11, 30], mutagenesis plays a role in the initiation stage followed by tumor promotion in the second stage. Hence, additional information on the antimutagenicity of edible Thai plants may be able to elucidate some aspects of the inhibition of the carcinogenesis process.

Commonly consumed plants from the family of Zingiberaceae were selected for the study. In Thailand, ginger, turmeric, fingerroot and galanga which are members in Zingiberaceae are commonly used as spices. Ginger and turmeric are consumed in many countries and a large number of studies were conducted to determine the beneficial properties of the existing substances. However, information on the beneficial effect of fingerroot and galanga on health is limited. Therefore, only fingerroot and galanga were used in this study.

Fingerroot, known as 'krachai' in Thailand, has a characteristic appearance with several slender, long tubers sprouting in the same direction from the central part of the rhizome. Fingerroot is used as folk medicine in Southeast Asia[14, 15, 25, 33]. Interestingly, fingerroot is an ingredient in foods consumed daily in Thailand. Studies on the medicinal properties of fingerroot may be able to elucidate its role as a functional food. One study on the anti-tumor promoting effect of a substance extracted from fingerroot was published[22]. The current investigation deals with the antimutagenic properties of the plant.

Galanga, known as 'khaa' in Thailand, has an appearance similar to that of ginger, but its aroma is completely different. Galanga is also used in Malaysia to prepare meat dishes[28]. Many countries in Southeast Asia as well as in China and Saudi Arabia consider galanga as a medicinal plant[1, 20, 26]. Compared with fingerroot, a larger number of studies were conducted. Many substances from galanga essential oil were isolated and examined for their antimicrobial and anticarcinogenic activities[27, 37]. Some substances extracted from seeds and rhizomes of galanga were reported[9, 18, 20, 22]. To enhance the value of galanga as a functional food, additional research on the antimutagenicity of existing constituents is required.

Materials and Methods

Plant materials

Fingerroot, *Boesenbergia pandurata* Schl. (syn. *Kaempferia pandurata* Roxb.), and galanga, *Languas galanga* (syn. *Alpinia galanga*) were purchased from a local market in Bangkok, Thailand.

Extraction of antimutagenic compounds

Fresh rhizomes (approximately 1 kg) of fingerroot or galanga were homogenized and extracted twice with a total of 3 l of methanol. Plant extracts obtained were concentrated with a vacuum rotary evaporator. Chloroform (250 ml) was added into the concentrated extracts to remove highly hydrophobic compounds. The remaining solid was then soaked and washed with 500 ml of methanol to obtain a methanol-soluble fraction. The solid obtained after washing with methanol was used as water-soluble fraction. The water-soluble fractions were freeze-dried before the antimutagenicity assay was carried out.

Antimutagenicity assay (Ames test)

The assay was performed using *Salmonella typhimurium* TA98 (frameshift mutant) as a tester strain and 3-amino-1,4 dimethyl-*5H*-pyrido-[*4,3-b*]indole (Trp-P-1, an indirect-acting mutagen) as mutagen precursor. The assay mixture for the antimutagenicity test contained 100 μl of 0.1M phosphate buffer (pH 7.0), 50 μl of Trp-P-1 (1 μg/ml in dimethyl sulfoxide), 100 μl of S-9 mix (from rat liver, Kikkoman Co. Ltd., Noda, Japan), 50 μl of plant substances in dimethyl sulfoxide and 100 μl of an overnight culture of TA 98 in Difco nutrient broth. The assay mixture was incubated at 37℃ for 20 min in a shaking water bath. Then, molten top agar (3 ml) was added to the assay mixture before pouring onto a Vogel-Bonner agar plate. All the plates were incubated at 37℃ for 48 h. Number of *his*$^+$ revertant colonies were counted and expressed as percentage of antimutagenicity, after subtracting of spontaneous revertants. In this screening test, amounts of applied plant substances were 0.15, 0.3, 0.6 and 1.2 mg/plate. The concentrations of 0.6 and 1.2 mg/plate were selected in order to ensure that the fraction which contained a small amount of antimutagen(s) would not be overlooked[35].

Isolation of antimutagenic compounds

Concentrated methanol-soluble fractions from fingerroot (335 mg) and galanga (500mg), which were found to exhibit an antimutagenic effect, were applied to reversed phase column chromatography. Methanol-soluble fraction of fingerroot was loaded on the column (Wakogel, LP-40 C18, 3 x 25 cm) equilibrated with water containing 0.5% formic acid, and eluted by stepwise gradient of 35% (100 ml), 50% (80 ml), 60% (100 ml), 70% (100 ml), 80% (100 ml) and 100% (100 ml) acetonitrile (ACN). Methanol-soluble fraction of galanga was applied to the same column and eluted by a stepwise gradient of 10% methanol (50 ml), 50% methanol (50 ml), 35% ACN (100 ml) and 50% ACN (100 ml). Eluted substances were monitored by measuring the absorbance at 280 nm using a spectrophotometer (Shimadzu UV-1200, Kyoto, Japan). Fractions obtained as major peaks were combined respectively and examined for their antimutagenic effect. Active fractions were then subjected to preparative HPLC for final purification.

HPLC condition

Substances in the antimutagenic active fractions were analyzed using a Tosoh (Tokyo, Japan) automated liquid chromatography unit. The system consisted of a PX-8020 system controller, a CCPM-II pump, a PD-8020 photodiode array detector, a AS-8020 auto injector and a CO-8020 column oven. Analytical HPLC system was equipped with a TSKgel super ODS column (4.6x100 mm, Tosoh) and the analysis was carried out at 40℃. For the preparative HPLC system, a Tsk gel ODS-80Ts column (20 x 250 mm, Tosoh) was used and the chromatography was carried out at ambient temperature. The mobile phase system consisted of a linear gradient from water containing 0.5% formic acid to acetonitrile.

Heating effect on plant substances

Active plant substances as antimutagens were examined for the effect of heating by autoclaving at 105℃ for 15 min. Heat treatment was performed before the addition of the remaining mixture solution for the antimutagenicity test as mentioned above.

Structural characterization of antimutagenic compounds

UV absorption spectra of the active compounds were obtained from the HPLC unit. Liquid chromatograph mass spectrometer (M-1200AP, Hitachi, Tokyo, Japan) was used to obtain mass spectra.

Results and Discussion

Antimutagenicity of crude extracts from fingerroot and galanga

Water-soluble and methanol-soluble fractions from fingerroot and galanga were examined for their antimutagenic effect by Ames-test before proceeding to further isolation. Methanol-soluble fractions of fingerroot and galanga showed a highly potent inhibitory effect on mutagenesis (97 - 98%) at a concentration of 0.3 mg/plate (Fig. 1). These results indicate that fingerroot and galanga display a high potential antimutagenic effect on Trp-P-1 as shown when compared to extract from lemon grass[34], another common Thai spice. The amount of fingerroot and galanga extracts applied could be less than 1.5% of lemon grass extract in order to obtain 84% antimutagenicity[34].

Water-soluble fraction from galanga also acted as a potent antimutagen. However, the fraction which exhibited a high antimutagenic activity, contained major compounds as in the case of methanol-soluble fraction, as revealed by HPLC. Hence, the water-soluble fraction of galanga was omitted. Water-soluble fraction of fingerroot was not used for the isolation process due to the low antimutagenicity of 40% at a concentration of 1.2 mg/plate (Fig. 1). Only methanol-soluble fractions of both plants were used for the isolation of antimutagenic substances.

Antimutagenicity of isolated compounds from fingerroot

Figure 2A shows a chromatogram of the methanol-soluble fraction of fingerroot obtained by reversed phase column chromatography. Seven fractions, designated as KC1, KC2, KC3, KC4, KC5, KC6 and KC7, were pooled and prepared for the antimutagenicity assay. All the fractions, except for KC1, showed a marked inhibitory effect in the

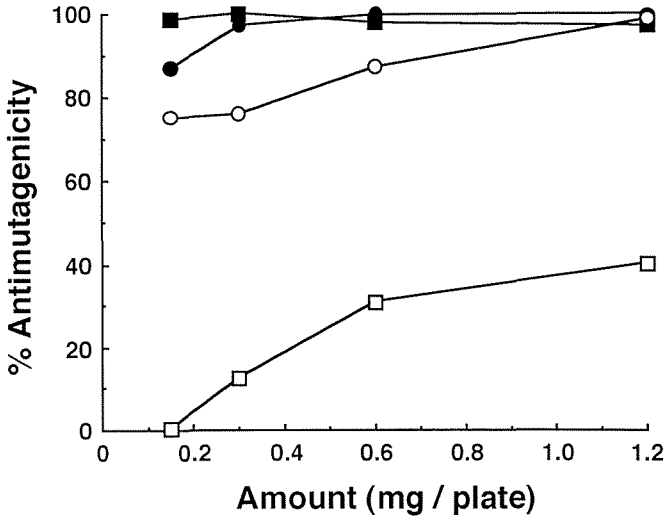

Fig. 1. Antimutagenicity of water-soluble and methanol-soluble fractions from fingerroot and galanga at various concentrations. □, water-soluble fraction from fingerroot; ■, methanol-soluble fraction from fingerroot; ○, water-soluble fraction from galanga; ●, methanol-soluble fraction from galanga.

range of 85 to 100% (Fig. 2A). Fraction KC1 inhibited the mutation by only 49%. Therefore, KC1 was not included for further isolation of the compounds. The fractions that showed high antimutagenic properties were eluted with acetonitrile in aqueous solution in a proportion of more than 50%. Although the fraction KC2 exhibited 85% antimutagenicity, the major compound in KC2 was not included in this study due to the too small amount obtained. The other active fractions contained compounds designated as FR1, FR2, FR3, FR4, FR5 and FR6 by HPLC (Fig. 3A). Compounds FR1 and FR2 were detected in fractions KC3, FR3 in fraction KC4, FR4 in

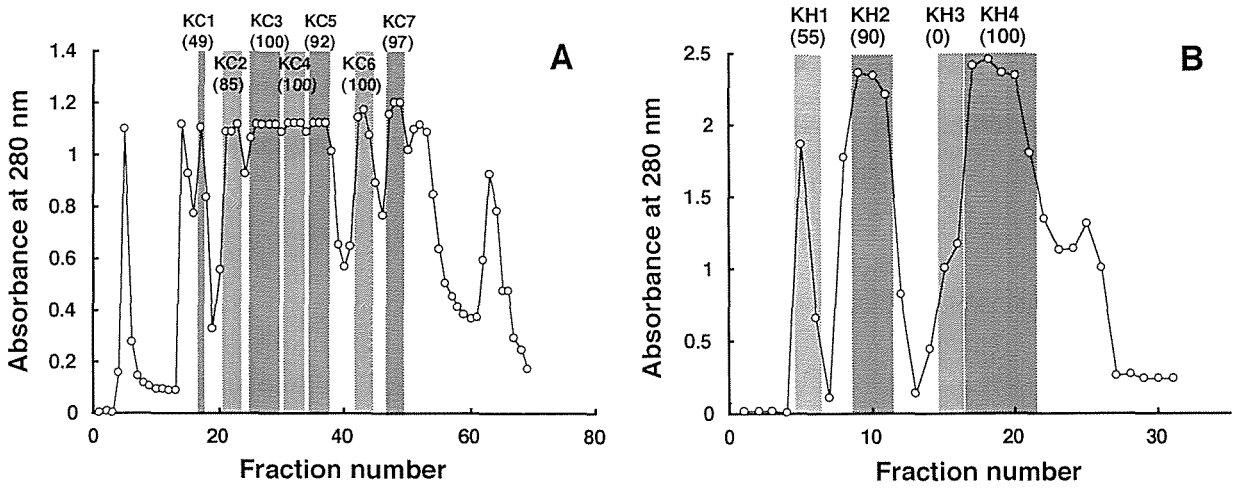

Fig. 2. Chromatograms of methanol-soluble fraction of fingerroot, 2A and galanga, 2B, obtained by reversed phase column chromatography. Fractions were pooled and designated as fractions KC1, KC2, KC3, KC4, KC5, KC6 and KC7 for fingerroot and KH1, KH2, KH3 and KH4 for galanga, indicated by shaded columns. Each concentrated fraction was dissolved in 1.0 ml DMSO, and was used to determine the antimutagenicity (approximately 0.1 mg/plate). Values in parenthesis indicated the percentage of antimutagenicity.

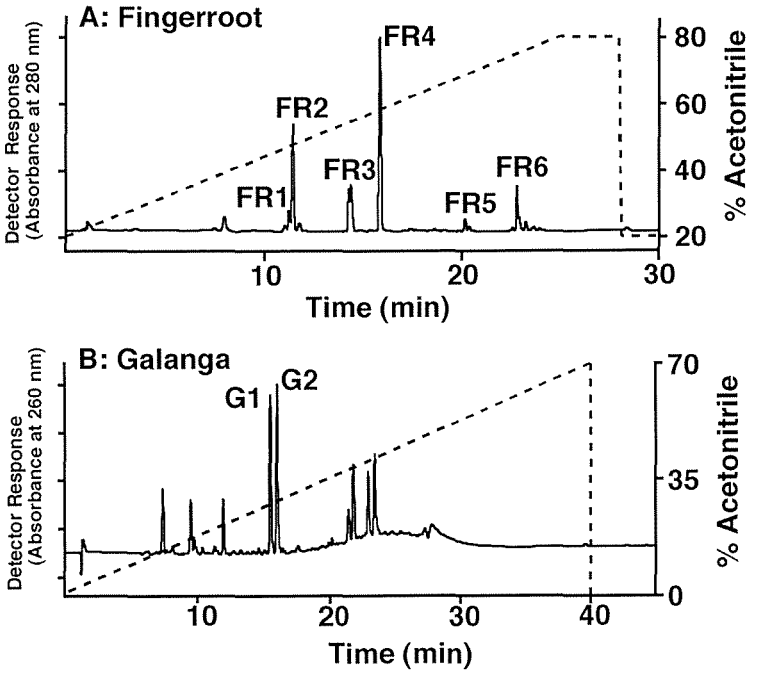

Fig. 3. HPLC analysis of fingerroot and galanga, from methanol-soluble fraction

fraction KC5, FR5 in fraction KC6, and FR6 in fraction KC7. Thereafter, the compounds were purified by preparative HPLC and examined for their antimutagenic properties.

All six isolated compounds inhibited the mutation induced by Trp-P-1 in *S. typhimurium* TA98. At the concentration of 25 μ g/plate, compounds FR1, FR2, FR3, FR4 and FR5 showed a substantially high mutagenic inhibition in the range of 89 to 94% (Fig. 4). Only compound FR6 was a weak antimutagen. However, FR6 also displayed a high antimutagenic effect of 97% at a concentration of 150 μ g/plate (data not shown).

In fingerroot, major antimutagenic substances that were reported are flavonoids[14, 19, 25]. Antimutagenic effects of flavonoids regarding 2-amino-3-methylimidazo [4,5-*f*] quinoline (IQ), one of the indirect-acting mutagens, in *S. typhimurium*, were analyzed by Edenharder *et al.*[6]. The results obtained by Edenharder *et al.*[6] might be applicable to this present work, since Trp-P-1 and IQ are activated by the same molecular species of cytochrome P450 enzymes[9]. Edenharder *et al.*[6] demonstrated that flavonoids inhibited the mutagenicity of IQ depending on the chemical structure. Assuming that compounds FRs 1 to 6 were flavonoids, based on the spectra obtained, the antimutagenicity results may agree with the findings of Edenharder *et al.*[6]. Based on the antimutagenic properties, it is liked that these six isolated compounds contain a keto group at carbon atom 4 of the flavane nucleus, or a double bond between carbon 2 and 3, or no substitution of hydroxyl group at carbon atoms 6 or 2'. Pinostrobin, one of major compound in fingerroot [14, 21, 25], was examined by Edenharder *et al.*[6] and was found to be an effective antimutagen. Regarding the antimutagenic potential of other major flavonoids reported in fingerroot, pinocembrin and two chalcone derivatives (Fig. 5), it is suggested that these three compounds may act as active antimutagens due to maintenance of functional structures as indicated by Edenharder *et al.*[6].

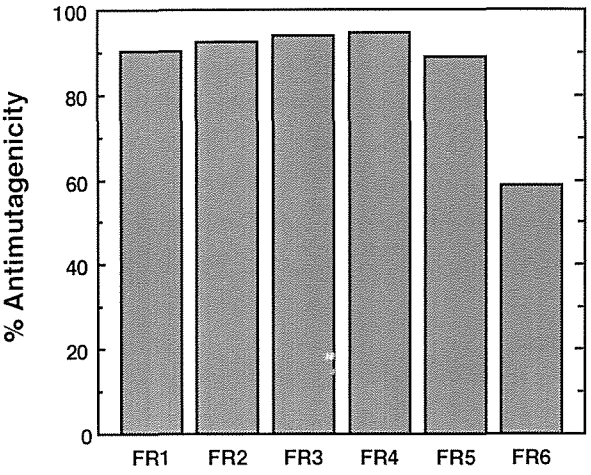

Fig. 4. Antimutagenicity of compounds from fingerroot, at a concentration of 25 μ g/plate

Characterization of isolated compounds from fingerroot

The data obtained from molecular weight determination and UV absorption spectrometry are summarized in Table 1. Based on these data, FR2 and FR4 were estimated to be two flavanone derivatives, pinocembrin and pinostrobin, respectively (Fig. 5). It had already been reported that these compounds occur in Thai yellow fingerroot[14, 19, 21]. Isolated compounds from Thai fingerroot included pinostrobin, alpinetin, pinocembrin, 2', 6'-dihydroxy-4'-methoxychalcone, cardamonin, boesenbergin A and boesenbergin B. Rhizomes of fingerroot from Indonesia also contained pinostrobin, alpinetin, pinocembrin and cardamonin[25]. FR3 and FR1 were assumed to be chalcone derivatives. FR3 could be either 2',6'-dihydroxy-4'-methoxychalcone or cardamonin, and FR1 could be a chalcone derivative as 2',6'-dihydroxy-4'-methoxychalcone or cardamonin, but with a hydroxyl group instead of a methoxy group at carbon 4' or 6'. Therefore, FR5 and FR6 could not be identified.

In this study, FRs 1 to 4 were the major compounds based on the chromatogram (HPLC) recorded at 280 and 330 nm. Based on the report of Jaipetch *et al.*[14], the major compounds isolated from fresh Thai fingerroot were 2', 6'-dihydroxy-4'-methoxychalcone, pinocembrin, pinostrobin, and cardamonin. Similarly, pinocembrin and

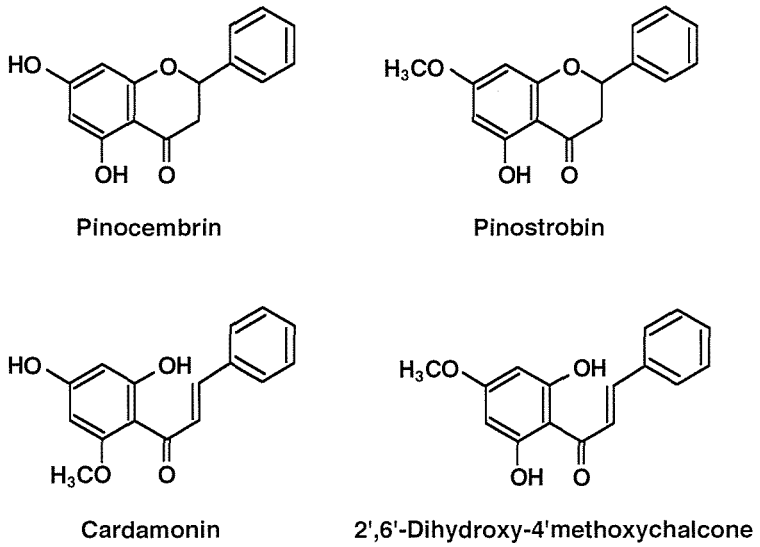

Fig. 5. Compounds reported to occur in fingerroot

Table 1. Physico-chemical properties of isolated antimutagenic compounds from fingerroot and galanga

Compound	Molecular weight	λ_{max} in UV absorption spectrum (nm)
FR1	256	340 (broad)
FR2	256	289,330 (shoulder)
FR3	270	340 (broad)
FR4	270	289,330 (shoulder)
FR5	392	290
FR6	406	290
G1	192	252
G2	164	261

pinostrobin opposed to be FR2 and FR4, respectively, two out of the four major compounds identified in this study.

Antimutagenicity of isolated compounds from galanga

From the methanol-soluble fraction of galanga, 4 fractions, designated as KH1, KH2, KH3, and KH4 were collected (Fig. 2B). Two fractions, KH2 and KH4, displayed a high antimutagenic effect. However, after the analysis of the compounds present in KH2 and KH4 by analytical HPLC, KH2 was not included in further isolation process, since KH2 is composed of the first 3 minor compounds, which were eluted before G1 as illustrated in Fig. 3B. The fraction KH4 was eluted with 50% acetronitrile in the reversed phase column chromatography. The fraction KH4 contained G1 and G2, which are major compounds in methanol-soluble fraction, as shown by HPLC (Fig. 3B).

Compounds G1 and G2 were less active as antimutagens than all the compounds obtained from fingerroot at a concentration of 25 μ g/plate (Fig. 6). However, when the concentration increased to 150 μ g/plate, G1 and G2 exhibited a high antimutagenic effect above 90%. Wall *et al.*[35] investigated inhibitory effect of 22 phenolic compounds on mutagenesis by five mutagens. At the same application level of 150 μ g/plate, only 8% of the experimental phenolic compounds showed an antimutagenicity greater than 90%. Based on this findings, G1 and G2 were considered to be

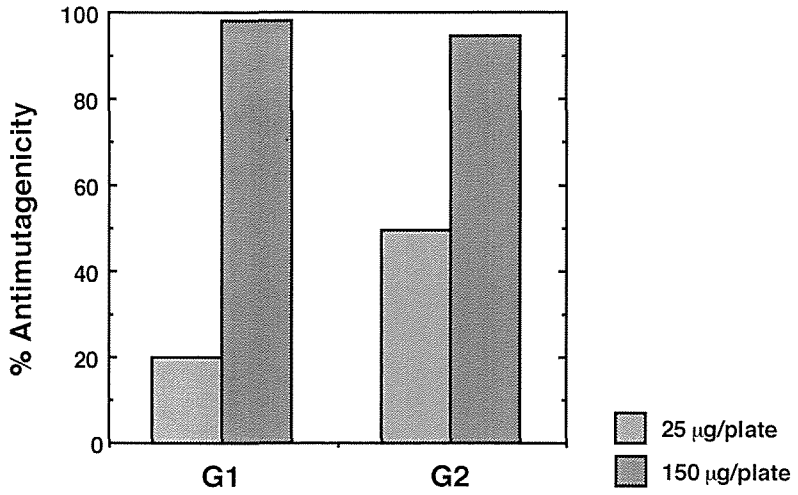

Fig. 6. Antimutagenicity of compounds from galanga

secondary plant metabolites which have a great potential as antimutagens. The results from this study revealed another property of the compounds present in galanga in addition to the antitumour, anti-ulcer, antifungal, inhibitor of phagocytosis of macrophages and xanthine oxidase inhibition properties already reported[10, 12, 13, 20, 24, 27, 32, 36, 37].

Characterization of isolated compounds from galanga

From the data shown in Table 1, compounds G1 and G2 were considered to be simple phenylpropanoids, with a basic structure consisting of a three-carbon side chain attached to an aromatic ring. One compound that was often isolated from rhizomes and seeds of galanga was 1'-acetoxychavicol acetate[12, 18, 20]. Since the molecular weight of 1'-acetoxychavicol acetate is 234, neither G1 nor G2 corresponded to 1'-acetoxychavicol acetate. Galanal A, galanal B, (E)-8, 17-epoxy-labd-12-ene-15, 16-dial, 1'-acetoxy-eugenol acetate, *trans*-3, 4-dimethoxycinnamyl alcohol, *trans*-4-methoxycinnamyl alcohol and trans-4-hydroxy cinnamaldehyde are other compounds found in methanolic extracts from seeds and rhizomes of galanga[10, 12, 20, 22]. Molecular weights of galanal A, galanal B, (E)-8, 17-epoxy-labd-12-ene-15, 16-dial and 1'-acetoxyeugenol acetate are 318, 318, 302 and 264, respectively. Among these compounds, those with a molecular weight close to that of G1 and G2 are *trans*-3, 4-dimethoxycinnamyl alcohol, *trans*-4-methoxycinnamyl alcohol and trans-4-hydroxy cinnamaldehyde with molecular weights of 194, 164 and 148, respectively.

Effect of heating on isolated compounds

When the rhizomes of fingerroot and galanga are utilized as ingredients in the Thai diet, heat is involved in the preparation process. Since the antimutagen(s) extracted from vegetables were reported to be either heat-stable or heat-sensitive[8, 17], an experiment on the antimutagenicity of heat-treated compounds from fingerroot and galanga should be carried out. In this study, it was shown that heating at 105℃ for 15 min did not reduce the antimutagenic activity of the compounds from fingerroot and galanga (Table 2). The four compounds from fingerroot, FRs 1 to 4, that were characterized as flavonoids, were heat stable[7]. The activity of FR6 increased by 28% after heat treatment. It is possible that part of FR6 was converted to another compound that displayed a higher activity by heat treatment. However, further analyses were not performed due to the small amount obtained. G1 and G2 from galanga, that were assumed to be phenylpropanoids, were fairly stable to heat treatment.

Table 2. Effect of heat on antimutagenicity of compounds from fingerroot; FR1, FR2, FR3, FR4, FR5 and FR6, at 25 μ g/plate and from galanga; G1 and G2, at 150 μ g/plate

Compound	% Inhibition after heating[a]
FR1	100.0
FR2	102.2
FR3	99.2
FR4	101.3
FR5	105.4
FR6	128.0
G1	99.2
G2	100.8

[a] Mutation-inhibitory relationship of heat-treated compounds to unheated compounds when % antimutagenicity of unheated treatment is taken as 100%

Conclusion

In this investigation, it was demonstrated that the methanol-soluble fractions from fingerroot and galanga exhibited a higher antimutagenic activity than the water-soluble fractions. The antimutagenic activity of both plant species was attributed to several components, with six active compounds from fingerroot and two active compounds from galanga. Physico-chemical information from the isolated compounds suggested that compounds from fingerroot were chalcone derivatives and flavanone derivatives, and, compounds from galanga were phenylpropanoid derivatives. However, two compounds from fingerroot could not be identified. Compounds isolated from fingerroot exhibited a greater antimutagenic potency than those from galanga at the same concentration. And all the isolated compounds remained active as antimutagens after heat treatment, which is considered to be important for food ingredients. It has been suggested that fingerroot and galanga which are important edible plant spices may play a role in cancer chemoprevention, and, may have a potential as effective functional foods.

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抗変異原性（エームステスト）を有するフィンガールート（*Boesenbergia pandurata* Schl.）及びガランガ（*Languas galanga*）成分について

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摘　要

フィンガールート（タイ名クラチャイ，*Boesenbergia pandurata* Schl.)及びガランガ（タイ名カー，*Languas galanga*）はショウガ科に属する食用植物であり，タイにおいては一般的なスパイスとして用いられている。ショウガ科植物の多くは強い抗変異源性を示すことが知られており，ガンの化学的予防において重要であるが，フィンガールート及びガランガについては詳細な研究がなされていなかった。今回，変異原性物質 Trp-P-1 によりサルモネラ菌 *Salmonella typhimurium* TA98株において誘発される復帰突然変異を抑制する強度を測定する系を用い，両植物のメタノール抽出液について検討したところ，強い抗変異原活性が見られた。次に，逆相カラムクロマトグラフィーにより有効成分の分離・精製を行った。フィンガールートからは6種類（FR1～6），ガランガからは2種類（G1及びG2）の活性物質がそれぞれ単離された。LC-MS分析の結果及び紫外吸収スペクトルの特徴から，それぞれの物質について構造推定が行われた。FR1及びFR3はカルコン誘導体，FR2及びFR4はフラバノン誘導体であることが推定された。FR5及びFR6については，構造推定のために十分な情報が得られなかった。G1及びG2はフェニルプロパノイド誘導体であると予想された。フィンガールートから単離された6種類の物質は，25 μ g/plateの濃度において，ガランガから単離された2種類の物質よりも高い抗変異原性を示した。FR1～5は特に強い抗変異原性を示し，同濃度において約90%の突然変異を抑制した。また，今回単離されたすべての物質は，高い熱安定性を示し，105℃，15分間の加熱後でも失活は見られなかった。従って，通常の加熱調理においてこれらスパイスにおける抗変異原性は失われることはないものと考えられた。

キーワード：抗変異原性，*Boesenbergia pandurata* Schl, *Languas galanga*, タイ産ハーブ，エームス試験