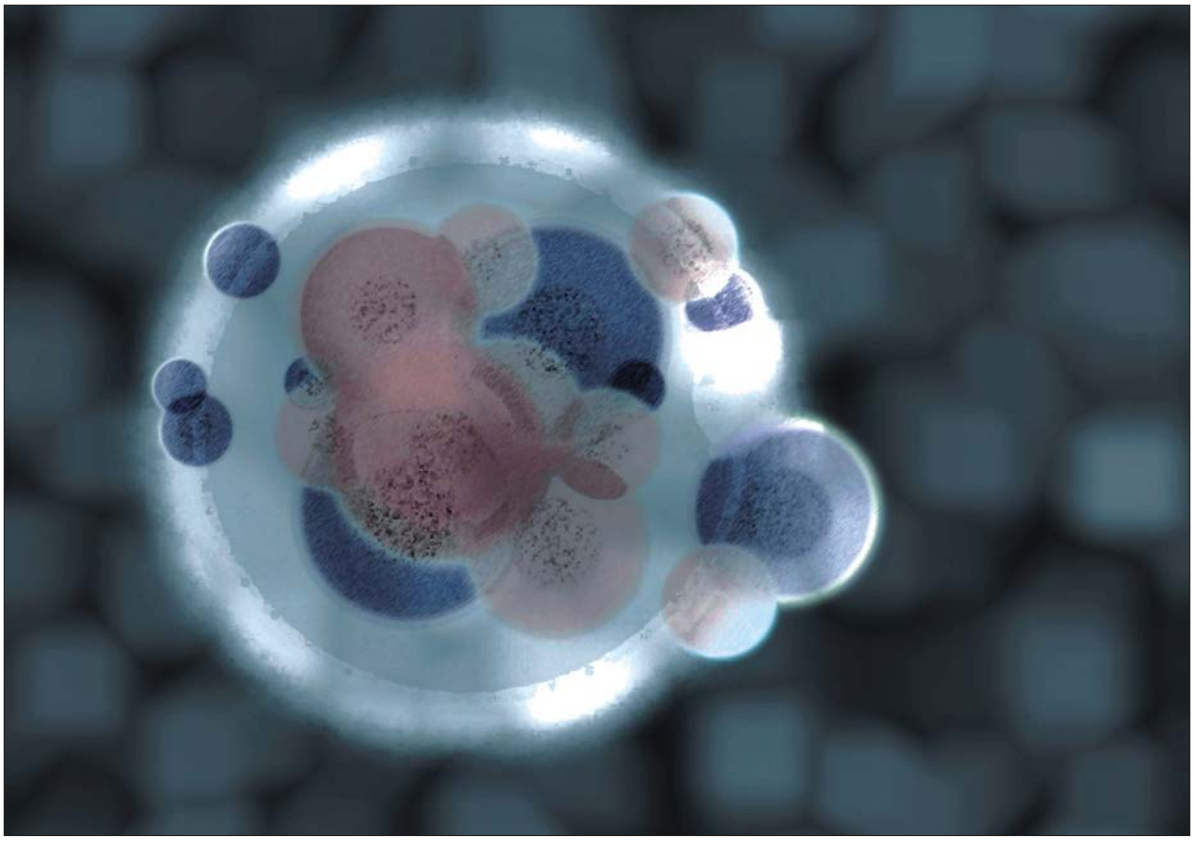

Journal of Biological Sciences

ISSN 1727-3048

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Journal of Biological Sciences 12 (6): 355-360, 2012
ISSN 1727-3048 / DOI: 10.3923/jbs.2012.355.360

Antibacterial Activity, Antimutagenic Activity and Cytotoxic Effect of an Essential Oil Obtained from *Amomum uliginosum* K.D. Koenig

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Abstract: The essential oil from the fruits of *Amomum uliginosum* K.D. Koenig has been commonly used in Thai herbal formula for the treatment of gastrointestinal diseases. However, the biological activity of the essential oil obtained from *A. uliginosum* fruits has never been reported. This study was aimed to investigate the pharmacological activities and cytotoxic effect of the essential oil derived from the fruit of *A. uliginosum*. The essential oil derived from the authentic fruits and the commercial fruits were prepared by distillation with the yield of 2.90±0.00 and 2.90±0.30% v/w, respectively. The chemical constituents of the essential oil were analyzed by GC-MS. Twenty one and 13 chemical compounds were found in the essential oil derived from the authentic and commercial fruits, respectively. The major chemical compound presented in the essential oil from both sources was bornyl acetate. The essential oil exhibited an antibacterial activity against gastrointestinal disorder-related bacteria with the lowest MIC of 75 μL mL^{-1} against *E. coli* O157:H7 DMST 12743 and *S. aureus* DMST 8013. The essential oil exerted the cytotoxic effect on 3T3 fibroblasts with the IC_{50} of 0.0725±0.0045 μL mL^{-1}. The strong antimutagenic effect in *Salmonella typhimurium* TA 98 was found at the concentration of 3.75 mL $plate^{-1}$. These results scientifically confirmed the use of the essential oil derived from *A. uliginosum* for the medicinal purposes. Additionally, its potential application for the use as cancer chemoprevention is also indicated from this study.

Key words: *Amomum uliginosum*, antibacterial activity, antimutagenic activity, cytotoxicity test

INTRODUCTION

Bastard cardamom or Tavoy cardamom, which is commonly known as "Reo" in Thailand, belongs to the genus of *Amomum*, family Zingiberaceae. The plants and crude drugs derived from them have been used in many countries for culinary and medicinal purposes, especially for the treatment of gastrointestinal disorders. Various species of the genus of *Amomum*, known as cardamoms, are widely distributed among several countries in South Asia and Southeast Asia. *Amomum kravanh* Pierre (Round Siam cardamom) is mostly found in Thailand and Cambodia. *A. aromaticum* Roxb. and *A. subulatum* Roxb. (Bengal cardamom or Nepal cardamom) are commonly cultivated in India. *A. xanthioides* Wall. (Bastard cardamom) can be found in many countries such as India, Laos, Cambodia, Vietnam, southern China and Thailand (Bhatnagar, 1948).

In China, the fruits of bastard cardamom (*Amomum villosum* Lour.) are used medicinally to treat various gastrointestinal-related ailments such as indigestion, flatulence, gastralgia, nausea, vomiting and dysentery with cold symptoms (Dharmananda, 1997; PHC, 2002). In addition, they can also be used as antiseptics, astringents, analgesics and febrifuges (De Padua *et al*., 1999; Tsumura, 1991).

In Thailand, "Reo" belongs to several species from the genus of *Amomum* sp. Two kinds of Reo, which are called "Reo-Noi" and "Reo-Yai", have been used widely in Thai traditional medicines. *A. uliginosum* K. D. Koenig is known as "Reo-Noi" in Thai. The essential oil from the fruits of *A. uliginosum* has been commonly used in Thai herbal formula for the treatment of gastrointestinal diseases. The morphology and botanical characteristics of *A. uliginosum* K. D. Koenig were described previously (De Padua *et al*., 1999). It has been demonstrated that the crude extract of its fruits possesses antibacterial activity against *Helicobacter pylori*, which are peptic ulcer-causing bacteria (Lee *et al*., 2007). Although, the essential oil derived from the fruits of *A. uliginosum* has been thought to play an important role for its medicinal properties. However, the biological activity of the essential oil obtained from *A. uliginosum* fruits has never been reported. The objectives of this study were thus

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aimed to investigate the antibacterial activity against pathogenic bacteria causing diarrhea, antimutagenic activity and cytotoxic effect of the essential oil derived from the fruits of *A. uliginosum*.

MATERIALS AND METHODS

Plant materials: The authentic specimens of *Amomum uliginosum* K.D. Koenig were collected from Khao Soi Dao, Chanthaburi Province, Thailand (No.MSU.PH-ZIN-A1) in May 2008.

The authentic samples were dried by hot-air oven at the temperature of 50°C for 2 days. The voucher specimens were deposited in the Herbarium of Pharmaceutical Chemistry and Natural Product Research Unit, the Faculty of Pharmacy, Mahasarakham University, Thailand. They were compared with the authentic specimens at the Forest Herbarium Department of National Parks, Wildlife and Plant Conservation. The dried fruits of commercially available Reo-Noi were purchased from the herbal drugstore in Bangkok, Thailand.

Chemicals: Muller Hilton agar and Muller Hilton broth were provided by Himedia (India). Ethanol and dimethyl sulfoxide (DMSO) was obtained from Carlo Erba (France). Hexane was purchased from Lab Scan (Ireland). Norfloxacin was obtained from Sigma-Aldrich (USA). Gentamicin was obtained from Fluka (USA). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum, L-glutamine, penicillin and streptomycin were purchased from Sigma-Aldrich (USA).

Cultures and growth conditions: *Salmonella typhi* DMST 22842, *Staphylococcus aureus* DMST 8013, *Escherichia coli* DMST 15537, *Shigella sonnei* DMST 561 and *E. coli* O157:H7 DMST 12743 were obtained from the Department of Medical Science, Ministry of Public Health, Thailand. Cultures were maintained and grown in Muller Hilton broth at 37°C.

Distillation of *A. uliginosum* essential oil: The authentic and commercials dried fruits of *A. uliginosum* were milled and sieved using sieve number 18. Two hundred mL of distilled water was added to 15 g of *A. uliginosum* powder. The powder was distilled for 2.5 h with the constant rate (2-3 mL min^{-1}). Due to a limited quantity of authentic *A. uliginosum* fruits, they were distilled in triplicate. However, the commercial *A. uliginosum* fruits were sufficient for distillation for five times.

Determination of the *A. uliginosum* essential oil constituents by gas chromatography - mass spectrometry (GC-MS): The chemical constituents of *A. uliginosum* essential oil obtained from both authentic and commercial fruits (10 μL mL^{-1} in hexane) were identified by Gas Chromatograph Mass Spectrometer (GCMS-QP 2010, Shimadzu®, Japan) equipped with autoinjector (AOC-5000, Shimadzu®, Japan). Capillary column used was Rtx®-5 MS (30 m×0.25 mm id.×0.25 μm film thickness). Carrier gas used was helium at a flow rate 1.55 mL min^{-1}. Oven temperature was started at 80°C and hold for 2 min, then the temperature was increased to 280 at 10°C min^{-1} and hold for 1 min. Injection system comprised; injection temperature 250°C, injection volume 1 μL, split mode 1:100. MS conditions consisted of positive ion detection, interface temperature: 230°C, ion source: 0 kV, scan mode: positive ion, full scan m/z over 35-550 amu at 1,111 amu sec^{-1}. The compounds were identified by comparing their mass spectra with the NIST mass spectral libraries.

Determination of the MIC and MBC values of *A. uliginosum* essential oil against the tested bacteria: A broth microdilution method as described by Tarawneh *et al.* (2010) was used to determine the Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentrations (MBCs) of the essential oil with slight modification. Briefly, Muller Hilton broth was supplemented with 10% DMSO to dissolve the essential oils. Inoculums' size of approximately 10^7 colony forming unit mL^{-1} was used in this study. The MIC value was determined as the lowest concentration of the essential oil in the broth medium that inhibited the visible growth of the test bacteria. To determine the MBC, 10 μL of broth was spotted onto Muller Hilton agar. After incubation for 24 h, the number of surviving organism was counted. The lowest concentration where less than 0.1% of the initial inoculum survived was defined as MBC. The antibacterial activities of norfloxacin and gentamicin, the standard antibiotics used, were performed in the same manners. Each experiment was performed in triplicate.

Cytotoxicity test of *A. uliginosum* essential oil: The MTT assay was performed according to the protocol described by Plumb *et al.* (1989) with slight modification. Mouse embryonic fibroblasts (3T3; ATCC Cat. No. CRL-1658) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 unit mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. The cells were seeded in a 96-well plate at a density of 3,000 cells/well for 48 h at 37°C in a fully humidified, 5% CO_2. The essential oil of *A. uliginosum* at various concentrations were added to the cells and incubated for 24 h. After 24 h, the culture medium was removed and the cells were incubated with fresh medium for a further 24 h. The optical density of the Formazan blue color, formed in the viable cells after the

MTT assay, was read on a microplate reader (Molecular Devices, USA) at a wavelength of 570 nm. The data were analyzed with the SoftMax Program (Molecular Devices, USA) to determine the IC_{50}.

Antimutagenic activity assay of the essential oil of *A. uliginosum*: Antimutagenic activity of the essential oil of *A. uliginosum* was studied using the Ames test according to the method described by Yahagi *et al.* (1975). Briefly, 0.1 mL DMSO containing 0.47, 0.94, 1.87 or 3.75 mL of sample was mixed with 0.5 mL of sodium phosphate buffer (0.2 M, pH 7.4) and 0.1 mL of the overnight activated culture of *S. typhimurium* TA 98. One hundred ng of the mutagen (nitrite treated 1-aminopyrene) was added to the mixture as a positive control. The mixture was pre-incubated at 37°C for 20 min before adding 2.0 mL of molten top agar containing L-histidine (0.025 mM), biotin (0.025 mM). The mixture was then poured onto a minimal glucose agar plate. The histidine revertant colonies were counted after 48 h incubation at 37°C. Each sample was performed in triplicate. The antimutagenic activity was expressed as a percentage of mutagenic inhibition.

RESULTS

Distillation of *A. uliginosum* essential oil: The percent yield of the essential oil obtained from the authentic fruits and the commercial fruits were 2.90±0.00 % v/w (n = 3) and 2.90±0.30% v/w (n = 5), respectively (Table 1).

Determination of the *A. uliginosum* essential oil constituents by gas chromatography - mass spectrometry (GC-MS): Twenty one chemical compounds were identified in the essential oil obtained from the authentic fruits, while only 13 chemical compounds were found in the essential oil derived from the commercial fruits. The total amounts of the identified chemical compounds in the essential oil obtained from the authentic fruits and the commercial fruits were 88.09 and 83.98%, respectively (Table 2).

Determination of the MIC and MBC values of *A. uliginosum* essential oil against the tested bacteria: Since the quantity of the essential oil derived from the authentic fruits of *A. uliginosum* was not sufficient for the antibacterial, antimutagenic and cytotoxicity tests, thus the essential oil obtained from the commercial fruits of *A. uliginosum* were used in the following experiments.

The antibacterial activity of *A. uliginosum* essential oil in comparison to the standard antibacterial agents, norfloxacin and gentamicin, is shown in Table 3. The MICs of the essential oil were in the range of 75-150 μL mL^{-1}, with the lowest MIC of 75 μL mL^{-1} against *E. coli* O157:H7 DMST 12743 and *S. aureus* DMST 8013. The lowest MBCs of the essential oil were 150 μL mL^{-1} against every tested strain of bacteria except *S. typhi* DMST 22842. The MBC of the essential oil against *S. typhi* DMST 22842 was more than 150 μL mL^{-1}.

Table 1: Percent yield of the essential oil obtained from *A. uliginosum* fruit

	Authentic fruit (n = 3)	Commercial fruit (n = 5)
Percent yield (% v/w)	2.90±0.00	2.90±0.30

Table 2: The chemical constituents of the essential oil obtained from the fruits of authentic and commercial *A. uliginosum*

	Composition (%)	
Compound	Authentic	Commercial
Linalool	-	0.37
Camphor	25.50	25.35
Borueol	2.64	5.51
Geraniol	0.24	-
Bornyl acetate	34.96	31.25
Linalyl acetate	-	0.30
δ-Elemene	0.17	-
Geranyl acetate	1.22	-
α-Bergamotene	0.53	-
β-Farnesene	0.64	-
Germacrene-D	2.64	0.72
Naphthalene	-	0.42
Bicyclogermacrene	-	2.66
Cadinene	1.25	-
β-Bisabolene	1.36	-
α-Longipinene	0.38	-
β-Sesquiphellandrene	2.32	-
Sesquisabinene hydrate	0.67	-
Nerolidol	0.74	0.63
Germacrene-B	0.37	0.13
Pentalene	-	1.18
Selina-6-en-4-ol	0.67	-
α-Bisabolol	0.40	-
Farnesol	7.03	12.92
Farnesal	3.22	2.54
Farnesyl acetate	1.14	-
Total	88.09	83.98

Cytotoxicity test of *A. uliginosum* essential oil: The lowest % of cell viability was achieved at the concentrations of 0.75, 0.375 and 0.1875 μL mL^{-1} of *A. uliginosum* essential oil (Table 4). The essential oil at the concentrations of 1.5 and 0.0938 μL mL^{-1} also had a cytotoxic effect with the % of cell viability lower than 50% (4.0±0.5 and 14.0±2.0%, respectively). The % of cell viability drastically increased when the concentrations of *A. uliginosum* essential oil were decreased to 0.0469 and 0.0234 μL mL^{-1}. The essential oil at the lowest concentration tested (0.0234 μL mL^{-1}) appeared to have no cytotoxic effect on 3T3 fibroblasts with the % of cell viability of 99.0±3.0%. The results of MTT assay indicated that the essential oil of *A. uliginosum* had a cytotoxic effect on 3T3 fibroblasts with the IC_{50} of 0.0725 ±0.0045 μL mL^{-1}.

Table 3: MICs and MBCs of the essential oil of *A. uliginosum* against the tested bacteria in comparison to norfloxacin and gentamicin (n = 3)

	A. uliginosum essential oil		Norfloxacin		Gentamicin	
Bacteria	MIC (μL mL^{-1})	MBC (μL mL^{-1})	MIC (μL mL^{-1})	MBC (μL mL^{-1})	MIC (μL mL^{-1})	BC (μL mL^{-1})
Escherichia coli DMST 15537	150	150	0.78	6.25	0.78	1.56
Escherichia coli O157:H7 DMST 12743	75	150	0.78	1.56	0.78	0.78
Staphylococcus aureus DMST 8013	75	150	6.25	12.50	0.39	0.78
Shigella sonnei DMST 561	150	150	0.78	3.12	1.56	1.56
Salmonella typhi DMST 22842	150	More than 150	0.78	3.12	0.20	0.20

MICs: Minimum inhibitory concentrations, MBCs: Minimum bactericidal concentrations

Table 4: Evaluation of *A. uliginosum* essential oil cytotoxicity by MTT test in 3T3 fibroblasts

Conc. (μL mL^{-1})	Cell viability (%)	IC_{50} (μL mL^{-1})
1.5	4.0±0.5	
0.75	3.0±0.1	
0.3750	3.0±0.1	
0.1875	3.0±0.2	0.0725±0.0045
0.0938	14.0±2.0	
0.0469	89.0±1.0	
0.0234	99.0±3.0	

Values are Mean±SD, n = 3

Table 5: Antimutagenic activity in *S. typhimurium* TA 98 of *A. uliginosum* essential oil

Conc. (mL/plate)	Inhibition (%)
0.47	3.75±0.75
0.94	36.41±13.04
1.87	44.84±8.98
3.75	69.13±2.94

Values are Mean±SD, n = 3

Antimutagenic activity assay of the essential oil of *A. uliginosum*: The antimutagenic activity of the essential oil of *A. uliginosum* is summarized in Table 5. The essential oil at the concentration of 3.75 mL plate^{-1} exhibited strong antimutagenic activity with the percentage inhibition of 69.13±2.94. Moderate antimutagenic activity was found when the lower concentrations of the essential oil were tested (0.94 and 1.87 mL plate^{-1}). Low antimutagenic activity was observed at the concentration of 0.47 mL plate^{-1}.

DISCUSSION

Although, the fruits of *A. uliginosum* have been used widely in Thai traditional medicine, especially for the treatment of gastrointestinal related ailment, the present study is the first report of the pharmacological activities and cytotoxic effect of the essential oil derived from *A. uliginosum* fruit. The yield of essential oil distilled from the authentic fruits of *A. uliginosum* was similar to that of the commercial fruits (2.90 ±0.00 and 2.90±0.30% (v/w), respectively). The chemical constituents of both authentic and commercial fruits of *A. uliginosum* were determined by using GC-MS. The chemical constituents derived from the authentic fruits and the commercial fruits were different, with the number of the chemical compounds of 21 and 13 in the authentic fruits and the commercial fruits, respectively (Table 2). However, the major compound found in both samples was bornyl acetate, with the relative quantity of 34.96 and 31.25% in the authentic fruits and the commercial fruits, respectively. Camphor is the second mostly found chemicals in both samples of the essential oil tested. However, the relative quantities of farnesol and borneol in the commercial fruits derived essential oil was higher than that in the authentic essential oil. The chemical compounds which were not presented in the commercial fruits derived essential oil were geraniol, δ-elememe, α-bergamotene, β-farnesene, cadinene, β-bisabolene, α-longipinene, β-sesquiphellandrene, sesquisabinene hydrate, selina-6-en-4-ol, α-bisabolol and farnesyl acetate. However, the relative quantities of these chemicals were rather low (approximately not more than 2%), thus the lack of these chemicals in the essential oils are considered as not significant. Three chemical compounds (linalool, naphthalene , bicyclogermacrene) were found only in the commercial fruit derived essential oil. However, the amounts of these chemical compounds were again relatively low. The difference in the chemical constituents between the authentic sample and the commercial fruits derived sample may be due to various planting factors such as climate conditions, geography, collection time, as well as stage of plant development (Runyoro *et al*., 2010). Since the amount of the essential oil derived from the authentic fruits of *A. uliginosum* was not sufficient for the test of its biological activities, thus the essential oil obtained from the commercial fruits of *A. uliginosum* were used in the following experiments.

The antibacterial activities of the essential oil derived from the fruits of *A. uliginosum* were tested against 5 strains of bacteria, which are the common cause of diarrhea. The MICs and MBCs of the essential oil were in the range of 75-150 μL mL^{-1}. This indicates that the essential oil derived from the fruits of *A. uliginosum* possesses significant antibacterial activity against the tested bacteria. The lowest MIC (75 μL mL^{-1}) was found against *E. coli* O157:H7 DMST 12743 and *S. aureus* 8013 which usually cause gastrointestinal infection with severe acute hemorrhagic diarrhea (Kuntz and Kuntz, 1999).

Bornyl acetate, the major chemical found in the essential oil derived from the fruits of *A. uliginosum*, is a common chemical compound presenting in various essential oils (Yang *et al*., 2009). Camphor, borneol and farnesol are also the main constituents found in the essential oil of *A. uliginosum*. According to the previous reports, bornyl acetate, camphor and borneol possessed the antibacterial activity against *S. aureus* and *E. coli*. It was demonstrated that the MICs of bornyl acetate, camphor and borneol against *S. aureus* were 1.95, 2.70 and 1.25 mg mL^{-1}, respectively, while the MICs of these compounds against *E. coli* were 4.88, 1.33 and 4.50 mg mL^{-1}, respectively (Runyoro *et al*., 2010). The antibacterial activity of the essential oil found in this study is similar to that of other types of essential oil such as the essential oil derived from *Chrysanthemum indicum*, in which bornyl acetate, camphor and borneol are the major chemical constituents (Shunying *et al*., 2005). Additionally, the essential oil obtained from *Salvia eremophila* Boiss which contains high amount of bornyl acetate and borneol also showed a prominent antibacterial activity (Ebrahimabadi *et al*., 2010). Inoue *et al*. (2004) reported that farnesol could disrupt the cell membrane of *S. aureus* and this might be one of its modes of antibacterial action. Collectively, these findings strongly indicate that bornyl acetate, camphor, borneol and farnesol are the key components which are responsible for the antibacterial activity of the essential oil. However, the antibacterial activity of the essential oil may only be a part of its role in the treatment of gastrointestinal diseases. Other biological activities of the essential oil which might be related to its actions in the treatment of gastrointestinal disorders, such as antimotility and anti-flatulence, should be investigated further.

The essential oil derived from *A. uliginosum* appeared to have a cytotoxic effect on 3T3 fibroblasts with the IC_{50} of 0.0725±0.0045 μL mL^{-1}. The concentrations of the essential oil possessing the cytotoxic effect were relatively low. The vehicle at the concentration used in this study did not perform any cytotoxic activity (data not shown). This may be the disadvantage of using this essential oil for the treatment of gastrointestinal disorders. However, the cell used in this study was 3T3 fibroblast cell line which is biologically different from the human cell. Further studies are thus needed to investigate the cytotoxic activity of the essential oil derived from *A. uliginosum* in other cell types, especially the human gastrointestinal epithelial cells, which are likely to have a direct contact to the essential oil at the high concentrations after the oral ingestion. Nevertheless, the cytotoxic effect of the essential oil may indicate other promising medicinal applications, such as cancer chemotherapy. Additional studies on its anticancer activity are thus needed to prove this speculation. In addition, the essential oil derived from *A. uliginosum* has been used traditionally as a combination with other active herbal compounds. Generally, the effective concentration of the essential oil used in the combination is at very low concentration which may not reach the cytotoxic level. Moreover, there is no toxicity report with the use of the essential oil in the traditional remedy. The effectiveness of the traditional remedy in the treatment of gastrointestinal diseases may be caused by the synergistic action of the various active compounds in the formulation. Further experiment thus should be done to prove this speculation.

The antimutagenic activity of the essential oil of *A. uliginosum* was investigated using the Ames test. The essential oil exerted its antimutagenic activity in a concentration-dependent manner. The potency of the mutagenic inhibition was classified according to the protocol of Calomme *et al*. (1996). Strong antimutagenic activity with the percentage inhibition of 69.13±2.94 was found when the essential oil at the concentration of 3.75 mL $plate^{-1}$ was tested. The essential oil at the concentrations of 0.94 and 1.87 mL $plate^{-1}$ exhibited moderate antimutagenic activity. The lowest concentration of the essential oil tested (0.47 mL $plate^{-1}$) possessed low antimutagenic activity with the percentage of inhibition of 3.75±0.75. Antimutagenic activity of the other type of essential oil, such as lavender oil, tested in *S. typhimurium* TA 98 was reported previously (Evandri *et al*., 2005). To our knowledge, this is the first report of the antimutagenic activity of the essential oil of *A. uliginosum*. It has been established that antimutagens play an important role in the prevention of cancer (Vaidya *et al*., 2008). The cytotoxic activity of the essential oil of *A. uliginosum* was also demonstrated in this study. Thus, the essential oil of *A. uliginosum* potentially serves as a good candidate for cancer chemoprevention. Therefore it is of interest to investigate the anticancer activity of this essential oil in the near future.

In conclusion, the antibacterial activity of *Amomum uliginosum* fruit-derived essential oil against some gastrointestinal disorder-related bacteria was demonstrated in this study. This is the first scientific evidence supporting its use for the medicinal purposes. Additionally, the essential oil also exerted the cytotoxic and antimutagenic effects. Thus, its potential application for the use as cancer chemoprevention is also indicated from this study.

ACKNOWLEDGMENT

The study was financially supported by Mahasarakham University research grant (Fiscal year 2011).

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