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Binding effects of *Alpinia galanga* oil and its nanoemulsion to GABA_A receptors in rat cortical membranes

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Abstract

Anesthetic activity of *Alpinia galanga* oil (AGO) has been reported however the mechanism of action in mammals has not been clear. In the present study, the binding effects of AGO and its three active components to gamma-aminobutyric acid type A (GABA_A) receptor in cortical membranes of Sprague-Dawley rats were firstly investigated using a [³H]muscimol binding assay. Dimethyl sulfoxide (DMSO) was used to deliver these test samples. The results showed that only AGO and methyl eugenol displayed positive modulation at the highest concentration whereas 1,8-cineole and 4-allylphenyl acetate were inactive. An oil-in-water nanoemulsion containing 20%w/w AGO (NE-AGO) was formulated to deliver AGO instead of DMSO. This NE-AGO significantly enhanced a specific [³H]muscimol binding to 179% of the control with EC₅₀ of 391 µg/mL. The result correlates well to the amount of methyl eugenol in AGO. This result confirms that the anesthetic activity of AGO and methyl eugenol is associated with GABA_A receptor modulation, while that of 1,8-cineole and 4-allylphenyl acetate is not and may instead be related to other mechanisms. AGO showed well-tolerated by human cells. Therefore, the formulated NE-AGO might be a promising alternative anesthetic product for humans.

Introduction

Alpinia galanga is an edible plant of the Zingiberaceae family which is widely cultivated in Southeast Asian countries ^{1,2}. *A. galanga* is well-known in Asian folk medicine and has been used for centuries as a food additive, an antimicrobial agent, a local anesthetic, an analgesic, and an antipruritic ³⁻⁶. *A. galanga* oil (AGO) is rich in 1,8-cineole and 4-allylphenyl acetate ⁷. AGO was recently shown to act as an anesthetic in fish ⁸. Further, 1,8-cineole has been shown to reduce locomotor activity in rodents when administered orally or intraperitoneally ^{9,10}. To the best of our knowledge, the mechanism of anesthetic action of AGO

has not yet been elucidated. Moreover, 4-allylphenyl acetate has not been reported for anesthetic effect in rodents, whereas methyl eugenol, a minor compound of AGO has been reported as a surgical anesthetic in rodents ¹¹.

Generally, anesthetics have been used to relieve pain and suffering during surgery and post-surgery by inhibiting or depressing propagation of the signal along the nerves ¹². The mechanism of action of these anesthetics are a blockade of the N-methyl-D-aspartate receptor receptors, inhibition of dopaminergic receptors, or enhancement of the function of γ -aminobutyric acid type A (GABA_A) receptors ¹³. In addition, GABA_A receptors are the principal ionotropic receptors for fast inhibitory neurotransmission in the mammalian central nervous system ¹⁴. GABA_A receptors are clinically employed targets for a range of structurally diverse positive allosteric modulators such as isoflurane, etomidate, propofol, barbiturates, and benzodiazepines ^{15–17}. Given the structural resemblance of the main AGO constituents with myristicin, another previously reported GABA_A receptor positive allosteric modulators ¹⁸ (Fig. 1), we hypothesized that the anesthetic mechanism of action of AGO involves central GABA_A receptors.

Figure 1. Chemical structures of 1,8-cineole (a), 4-allylphenyl acetate (b), methyl eugenol (c), and myristicin (d).

The poor water-solubility of active compounds in essential oils remains a challenge in bioassay studies employing aqueous buffers. To overcome this problem, many organic solvents have been used for dissolving or delivery the active compounds before activity testing, however, potentially compromising the biological evaluation ¹⁹. AGO is also water-insoluble, therefore it needs a potential delivery system for solubility enhancement. Nanoemulsion is one of the promising nano delivery system that can improve aqueous solubility of many hydrophobic active compounds ^{20–22}. The nanoemulsions of several water-insoluble compounds have been developed for using in various bioassay studies ^{23–26}.

In the present study, the chemical components of AGO extracted from the fresh rhizomes of *A. galanga* were analyzed. AGO and its three selected chemical components, 1,8-cineole, 4-allylphenyl acetate, and methyl eugenol were investigated for the possible mechanism of action on anesthetic activity in mammals using rat brain GABA_A receptors. Moreover, a nanoemulsion containing AGO (NE-AGO) was formulated to reduce the amount of organic solvent used for dissolving AGO and to investigate for its potential on AGO delivery. The toxicity of AGO on normal human cells was also investigated for the further studies of NE-AGO in humans.

Results and Discussion

Extraction and chemical analysis of AGO. AGO appeared as a clear pale yellowish liquid. The chromatograms of AGO obtained from gas chromatography-mass spectrometry (GC-MS) demonstrated 14 identifiable chemical components that represented 97.93% of the total chemical components of AGO (Table

1). The main chemical components of AGO were 1,8-cineole ($41.94 \pm 0.13\%$), 4-allylphenyl acetate ($35.70 \pm 0.14\%$), and methyl eugenol ($3.23 \pm 0.02\%$), respectively. The identified chemical components of AGO are similar to what has been found in a previous study⁸. However, the percent content of these components was slightly different. Changes in composition of essential oils from the same plants are sometimes found due to harvest period or post-harvest air drying periods of the plants^{27,28}.

No.	Components	Retention time (min)	Amount (%)
1	β -Pinene	5.08	0.72 ± 0.07
2	1,8-Cineole	6.62	41.94 ± 0.13
3	α -Terpineol	11.49	2.64 ± 0.06
4	Terpinen-4-ol	11.89	3.07 ± 0.04
5	Chavicol	15.85	1.33 ± 0.08
6	4-Allylphenyl acetate	19.02	35.70 ± 0.14
7	Geranyl acetate	20.40	0.55 ± 0.01
8	Methyl eugenol	21.39	3.23 ± 0.02
9	α -Farnesene	22.32	0.58 ± 0.05
10	β -Bisaboloene	25.29	0.78 ± 0.01
11	β -Sesquiphellandrene	25.87	0.53 ± 0.01
12	Eugenyl acetate	26.22	1.19 ± 0.02
13	9-Octadecenoic acid	44.86	2.34 ± 0.12
14	9-Octadecenamide	46.53	3.39 ± 0.07
Total			97.99 ± 0.34

Table 1. Chemical components in AGO.

Preparation and characterization of NE-AGO. As AGO is immiscible with water, DMSO is always used to dissolve AGO and enhance water miscibility. However, DMSO shows several disadvantages due to its toxic effects to animals and humans and binding interferences. Nanoemulsions can be prepared without the use of organic solvents²⁹. Incorporation of water-insoluble drugs into the o/w nanoemulsions can improve their water miscibility³⁰. In the present study, NE-AGO was formulated without the use of organic solvent to improve the aqueous miscibility of AGO and to avoid any binding interference of DMSO in the employed binding assay. It was found that the formulated NE-AGO containing 20%w/w AGO appeared as a translucent mixture with white-bluish color and showed no phase separation. It was found that after adding the NE-AGO with water to make a 100-fold dilution, a fast-dispersing time of the formulation with less than 5 min was observed. Moreover, the small droplet size of 49 ± 2 nm of AGO was obtained with a size distribution expressed as polydispersity index (PDI) of only 0.24 ± 0.01 . The small PDI score indicated that this formulation presented a narrow droplet size distribution. The size distribution curve showed a single peak, and the peak intensity of this formulation was 99.8% (Fig. 2). Zeta potential of the diluted NE-AGO was negative with a value of -14.4 ± 0.6 mV. Adsorption of hydroxyl ions in the aqueous system onto the surface of the droplets may leads to slightly negative zeta potential value, and may be the explanation of the negative zeta-potential of this formulation^{31,32}.

[³H]Muscimol binding studies. [³H]Muscimol was used as a high-affinity radioligand to label GABA_A receptors in a synaptosomally enriched cortical homogenate preparation from Sprague-Dawley rats as previously reported³³. AGO at a concentration of 1 mg/mL (in DMSO) showed specific modulation of [³H]muscimol at 130% similar to diazepam (positive control) at a concentration of 100 μ M (Fig. 3a), in

accordance with other reports^{34,35}. These binding results indicate that AGO has constituents able to modulate [³H]muscimol binding, which is prompting further studies. These involved testing of the three main constituents; 1,8-cineole, 4-allylphenyl acetate, and methyl eugenol, from AGO, and indicated that only methyl eugenol was a modulator. A similar specific modulate [³H]muscimol binding of AGO and methyl eugenol was according to the different concentrations used in the test. The concentration of methyl eugenol used (i.e. 100 µM) is about 55-folds higher than that of methyl eugenol actually found in AGO (3.23%). Moreover, the concentration of 1,8-cineole (42%) and 4-allylphenyl acetate (36%) used, are also 3.6 and 4.9 folds higher than the actual amount found in AGO. Thus, the result showed that the modulate [³H]muscimol binding of these compounds was similar to that of AGO at 10 µg/mL.

Figure 2. Size distribution of NE-AGO.

In this study, DMSO was used for dissolving AGO and its components, therefore, DMSO was also tested separately. It was shown that DMSO significantly decreased the specific binding of [³H]muscimol at concentrations above 3%w/w, limiting the accuracy of the compound testing above 300 µM. Thus, an accurate assessment of EC₅₀ values could not be obtained from the oil or methyl eugenol containing DMSO. Moreover, DMSO at a concentration above 1%w/w dissolved the membrane while the nanoemulsion did not. To improve aqueous solubility and precluding the use of DMSO, the NE-AGO was instead used for testing. The binding effect of blank NE-AGO (the nanoemulsion without AGO) as a negative control at the highest tested concentration was not significantly different from 100% binding, indicated that the excipient itself did not significantly modulate binding levels (Fig. 3b). Instead, the EC₅₀ value, potent agonist value (pEC₅₀ ± S.E.M.), and maximum binding levels (mean ± S.E.M.) of AGO was obtained in the NE-AGO formulation described and found to be 391 µg/mL (3.41 ± 0.02) and 179 ± 10 % of the control.

Cytotoxicity study of AGO on human normal cells. Cytotoxicity studies often use peripheral blood mononuclear cells (PBMCs) as model cells for assessing the inhibitory effects of chemicals on cell proliferation of human normal cells^{36,37}. Thus, PBMCs were used to evaluate cytotoxicity in this study. The dose-response curves showed high viability of PBMCs (> 80%) after exposure to AGO in DMSO at all contact times and concentrations (Fig. 4). Normally, PBMC viability values higher than 80% are regarded as safe for human use³⁸. In addition, the IC₅₀ values of AGO in DMSO at all contact times were higher than 500 µg/mL. There was no significant difference in the concentration and contact time of AGO in DMSO on the viability of PBMCs among the groups exposed to AGO in DMSO for 1, 3, 6, 12, and 24 h ($p < 0.05$). However, the toxicity profile of PBMCs exposed to AGO in DMSO for 48 h showed concentration-dependency ($p < 0.01$). These results indicated that AGO had no cytotoxic activity on PBMCs. The cell viability was higher than 90% when the blank NE-AGO at a final concentration of 500 µg/mL was used, indicating the non-toxicity of the blank NE-AGO.

Figure 3. Modulation of [^3H]muscimol binding to rat brain cortical homogenate. The 100% binding (dashed red line) denotes the total specific binding by 30 nM [^3H]muscimol (a). Data are presented as mean percentage of specific binding $\pm$ S.E.M. (b). Red point indicates the blank NE-AGO vehicle at the highest concentration. Asterisk (*) indicates significant differences between groups with 100% binding ($p < 0.05$).

GABA_A receptors are the most common targets for general anesthetics. However, general anesthesia can be induced via other pathways such as enhancing inhibitory signals or blocking excitatory signals. Generally, anesthetics act as GABA_A receptor agonists, N-methyl-D-aspartate receptor antagonists, $\alpha 2$ -Adrenoceptor agonists, or dopaminergic receptor antagonists. In the present study, GABA_A receptors were emphasized due to their being the most common target. The anesthetic activity of AGO could potentially be due to methyl eugenol via the involvement of GABA_A receptors³⁹, while the activity of 1,8-cineole and 4-allylphenyl acetate might be related to other mechanisms. There is no correlation between the constituent content and the mechanism. However, The EC₅₀ and maximal binding levels of the NE-AGO formulation are correlates well to the measured content of methyl eugenol present in this essential oil (180 μM). The results confirmed that the anesthetic activity of AGO could potentially be due to methyl eugenol via the involvement of GABA_A receptors.

Figure 4. Dose-response curves of viability of PBMCs exposed to AGO in DMSO for 1 h (a), 3 h (b), 6 h (c), 12 h (d), 24 h (e), and 48 h (f). Data are presented as means $\pm$ S.E.M. and were analysed based on a one-way ANOVA followed by Tukey's post-hoc test ($p < 0.05$). Asterisk (*) indicates significant differences between concentrations of AGO.

In addition, 1,8-cineole has been reported to directly affects Na^+ channels of the superior cervical ganglion neurons that are likely to be the major cause of excitability blockade⁴⁰. Furthermore, 1,8-cineole-induced blockade of excitability is like that of classic local anesthetics, such as lidocaine and procaine. However, further studies are required to determine whether the pharmacokinetic characteristics of the AGO and the three main constituents; 1,8-cineole, 4-allylphenyl acetate, and methyl eugenol, will influence its acceptability using this essential oil to be a new local anesthetic in human.

Conclusions

Among AGO and its three main compounds, methyl eugenol, 1,8-cineole, and 4-allylphenyl acetate, it can be concluded that the possible anesthetic mechanism of action of AGO and methyl eugenol is associated with GABA_A receptor modulation. The activity of 1,8-cineole and 4-allylphenyl acetate is not due to GABA_A receptor modulation and might instead be related to other mechanisms. AGO is well-tolerated by human cells. Nanoemulsion is a good delivery system for AGO. NE-AGO might be useful as an alternative anesthetic nanoformulation for further studies in humans.

Materials and Methods

Materials. Fresh rhizomes of *A. galanga* were collected from the medicinal plant garden of Chiang Mai University, Chiang Mai, Thailand in February 2018 according to WHO Guidelines on Good Agricultural and Collection Practices (GACP) for Medicinal Plants. The plant was identified by Wannaree Charoensup (a botanist, Department of Pharmaceutical Sciences, Faculty of Pharmacy, Chiang Mai University, Thailand), and the voucher specimen (no. 009245) was deposited at the Herbarium of the Northern Research Center for Medicinal Plants, Faculty of Pharmacy, Chiang Mai University, Thailand.

Fetal bovine serum (FBS), penicillin, streptomycin, L-glutamine, and RPMI 1640 were purchased from GIBCO InvitrogenTM (Waltham, MA, USA). Lymphoprep and 4-allylphenyl acetate were purchased from Axis-Shield PoC AS (Oslo, Norway) and ABCR GmbH (Karlsruhe, Germany). Methyl eugenol, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and DMSO were from Sigma-Aldrich (St Louis, MO, USA). 1,8-cineole, polyoxyethylene sorbitan monooleate (Tween 80), phosphate buffer solution (PBS), and dichloromethane were of analytical grade and supplied by Merck Millipore (Darmstadt, Germany). Diazepam and [³H]muscimol (36.6 Ci/mmol) were obtained from Nycomed Danmark A/S (Hobro, Denmark) and Perkin Elmer (Boston, MA, USA).

Extraction and chemical analysis of AGO. Fresh rhizomes of *A. galanga* were washed with clean water and cut into small pieces before subjecting to hydro-distillation for 3 h. The obtained AGO was analyzed for their chemical compositions by GC-MS on an Agilent 6890 gas chromatograph coupled to electron impact (EI, 70 eV) using a Hewlett Packard (HP) mass selective detector (MSD), model HP 5973-MSD (Agilent Technologies Inc, USA). The HP5-MSI column; 30.0 m × 0.25 mm i.d. × 0.25 μm film thickness (Agilent Technologies Inc, USA) was used as a capillary column. The analytical conditions were modified from a previous study^{41,42}. Briefly, AGO was diluted with dichloromethane to 1:100 (v/v) and 1 μL of this mixture was injected into GC-MC. The injection and detector temperatures were 250°C and 280°C, respectively. The oven temperature was 70°C. The sample was held isothermally for 3 min and the temperature was then increased at 3°C/min to 188°C, and then at 20°C/min to 280°C, followed holding for 3 min. Helium was used as carrier gas at a flow rate of 1 mL/min. The experiments were performed in triplicate with at least three independent experiments.

Preparation and characterization of NE-AGO. NE-AGO composed of 20%w/w AGO, 10%w/w Tween 80, and 70% w/w water was prepared according to a method previously described as it was reported that NE-AGO formulated by this method was chemically stable when stored at various temperatures (4°C, 20°C, and 40°C) for several weeks (0, 4, 8, 12 weeks)⁷. Briefly, the aqueous phase composed of Tween 80 and water was mixed using a vortex mixer for 5 min. The oil phase was composed of AGO. The water phase was added to the oil phase under stirring at 50°C for 5 min before subjecting to a high-speed stirring of 16,000 rpm using an Ultra-Turrax T25 (Janke and Kunkel GmbH, Germany) for 5 min and passed through a high-pressure homogenizer (Avestin Inc., Canada) under a pressure of 10,000 psi for 7 cycles at room temperature.

The droplet size, size distribution, and zeta potential of the obtained NE-AGO were determined using dynamic light scattering by photon correlation spectroscopy (Zetasizer Nano ZS, Malvern Instruments Ltd., UK). The PDI value indicates the width of the size distribution. The size measurements were obtained by averaging at least ten measurements at a fixed angle of 173° at 25°C. NE-AGO was diluted (1:100 v/v) with purified water to have a suitable scattering intensity before measurement. The experiments were carried out in triplicate with at least three independent experiments.

[³H]muscimol binding assay. Rat brain cortical synaptosomes from adult male Sprague-Dawley rats were prepared according to the method previously described⁴³. All animal experiments were carried out in accordance with the European Communities Council Directive (2010/63/EU) and the ARRIVE guidelines for the care and use of laboratory animals and the Danish legislation regulating animal experiments. Modulation of [³H]muscimol binding to rat brain cortical homogenate of AGO and its main compounds (dissolved in DMSO) were compared. DMSO was used as a vehicle control. On the day of the assay, the membranes were quickly thawed in binding buffer (50 mM Tris-HCl buffer; pH 7.4), then homogenized and washed three times by centrifugation (48,000 × g at 4°C). The [³H]muscimol binding assay was performed in a 96-well format essentially as previously described^{43,44}. For this purpose, aliquots of membrane preparation (75-100 μg protein/aliquot) were incubated with a test substance and radioligand

[³H]muscimol (5 nM) in a total volume of 250 µL at 0°C for 60 min. Nonspecific binding was determined in the presence of 1 mM GABA whereas 100 µM diazepam was used as a control for positive modulation. After incubation for 1 h at 0-4°C, the binding reaction was terminated by rapid filtration through GF/C unifilters (PerkinElmer) using a 96-well Packard FilterMate cell harvester, followed by three successive washes with ice-cold binding buffer, the addition of Microscint scintillation fluid (PerkinElmer) and quantification of the filter-bound radioactivity in a Packard TopCount microplate scintillation counter. The experiments were performed in triplicate and repeated in at least three independent experiments. Data analysis was performed using GraphPad Prism 7.0b (GraphPad Software Inc, La Jolla, CA, USA). Counts per min values were converted to specific binding by subtracting non-specific binding. For modulation curves, data were fitted by non-linear regression analysis using the equation for sigmoidal concentration-response with variable slope as follows (1):

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\log \text{IC}_{50} - X) \times \text{Hill-Slope}))}, \quad (1)$$

where Y is the response, X is the logarithm of the concentration, Top and Bottom are the plateaus in same units as Y, and log IC₅₀ is the concentration giving a response halfway between Bottom and Top. The Hill-Slope is the steepness of the curve. All data were determined in triplicate and repeated in at least three independent experiments (as denoted in the figure legends).

Cytotoxicity study on PBMCs. The potential toxicity of AGO and blank NE-AGO on normal cells was assessed by a cytotoxicity test against human PBMCs. An MTT assay was used according to previous report with some modifications^{36,45}. This study was approved by the Ethics Committee, Faculty of Associated Medical Sciences, Chiang Mai University (No. AMSEC-64EM-002. Informed consent was waived by the Ethics Committee of this Faculty of Associated Medical Sciences because all samples were anonymous. The research presents no more than minimal risk of harm to subjects and involves no procedures. Briefly, blood samples were collected by venipuncture from healthy volunteers and transferred into 15 mL of heparin-coated test tubes. Blood was diluted at a 1:1 ratio (v/v) with 0.1 M PBS and layered onto Lymphoprep at a volume ratio of 3:1. PBMCs were collected after centrifugation at 1000 × g for 30 min and then washed 3 times with PBS. The PBMCs were resuspended in complete RPMI 1640 culture medium supplemented with 10% FBS, 100 unit/mL of penicillin, 100 µg/mL of streptomycin, and 1 mM of L-glutamine. The PBMCs were seeded in a 96-well tissue culture plate (1×10⁵ cells/well) and incubated at 37°C, 5% CO₂ atmosphere, and 95% relative humidity for 24 h. After the incubation, various concentrations of AGO or blank NE-AGO in DMSO (15-500 µg/mL) in a complete RPMI 1640 culture medium (100 µL) were added into each well and incubated for another 1, 3, 6, 12, 24, and 48 h for AGO and 48 h for blank NE-AGO. A mixture composed of 0.5% v/v DMSO in a complete RPMI 1640 culture medium was used as a vehicle control. MTT stock dye solution (5 mg/mL MTT dye in PBS) was added to each well (15 µL) after removal of 100 µL of the medium, and the plate was incubated at 37°C in a 5% CO₂ atmosphere. After 4 h, the supernatant was removed, followed by the addition of DMSO (200 µL) to each well and mixed thoroughly to dissolve the dye crystals. Absorbance was measured using an AccuReaderTM M965/965+ microplate reader (Metertech Inc., Taiwan) at 570 nm with a reference wavelength of 630 nm. All experiments were performed in triplicate and at least three independent experiments confirming the data. The percent of cell viability was calculated using the following equation (2):

$$\% \text{ Cell viability} = (\text{MA}_{\text{test}} / \text{MA}_{\text{control}}) \times 100 \quad (2)$$

where MA_{test} and MA_{control} are mean absorbance in test wells and mean absorbance in vehicle control wells.

Statistical analysis. The data are presented as mean $\pm$ S.E.M. Statistical evaluation of cytotoxicity study was performed by a one-way ANOVA followed by Tukey's posthoc or Dunnett's test where $p < 0.05$ was considered to indicate significant differences.

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Conceptualization, A.M., T.R., and S.O.; methodology, N.K., S.A., and P.W.; investigation, N.K. and P.W.; data curation, N.K. and P.W.; data analysis and interpretation, N.K., P.W., S.A., S.O., A.M., T.R., and K.S; writing—original draft, N.K.; writing—review and editing: P.W., A.M., T.R., S.O. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Faculty of Associated Medical Sciences, Chiang Mai University (protocol code AMSEC-64EM-002).

Competing interests

The authors declare no competing interests.

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