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ORIGINAL ARTICLE

T47D Cell-inhibiting Indonesian Medicinal Plants and Active Constituents of *Alpinia galanga* Rhizome

Muhammad Da'i, Arini Fadhilah, Juwita Rahmawati, Alfian Mahardika Forentin, Tepy Usia[1], Maryati Maryati, Azis Saifudin

Department of Pharmaceutical Chemistry, Universitas Muhammadiyah Surakarta, Jawa Tengah 57102, [1]Research Center of National Drug and Food Control, Jakarta 10560, Indonesia

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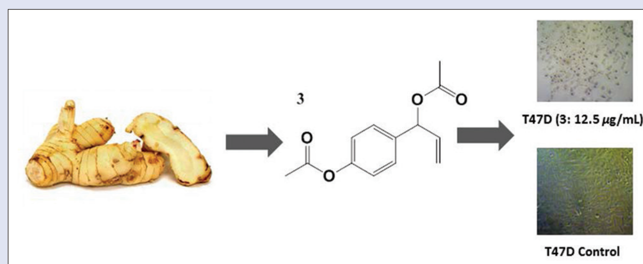
ABSTRACT

Background: The screening of Indonesian medicinal plant extracts against T47D cell line and identifying active chemical constituents of *Alpinia galanga* rhizome were conducted. **Materials and Methods:** Thirty methanol extracts of Indonesian medicinal plants were screened for possible anti-breast cancer. The T47D cell line was used as a target model. Chromatography techniques were used to purify the chemical constituents of *A. galanga* rhizome. Structural elucidation of isolated compounds was conducted by means of 1D and 2 D NMR (nuclear magnetic resonance) spectrometry. Their activities were also examined on MCF7, WiDr, and HeLa cell lines. **Results:** Five samples (*A. galanga*, *Clonorchis sinensis*, *Piper cubeba*, *Santalum album*, and *Vitex trifolia*) showed a strong activity with 96.4%, 91.9%, 87.6%, 82.6%, and 88.7% inhibition, respectively. Since *A. galanga* exhibited the most potent activity, its IC_{50} value was determined with a dose-dependent effect with the IC_{50} value of 21.2 μg/mL. Its methanol extract was also separated based on chromatography techniques producing the following four compounds: *trans*-p-coumaryl alcohol (1); *p*-coumaryl acetate (2); [1'S]-1'-acetoxy chavicol (3); and *trans*-p-coumaryl diacetate (4). Compounds 3 and 4 showed significant activity with the IC_{50} values of 17.3 and 25.4 μg/mL, respectively. On the other hand, compounds 1 and 2 showed weak activity. All compounds exhibited a similar feature against MCF7, WiDr, and HeLa cell lines. **Conclusions:** On the structure-activity relationship observation, acetoxy group at a para position could be a key contributor to the effect. Thus, an acetoxy phenylpropanoid could be a good model for future anti-breast cancer lead compound development. Furthermore, extract plants should have demonstrated their potential to inhibit cancer cells.

Key words: Acetoxy phenylpropanoid, *Alpinia galangal*, breast cancer, cytotoxic, herbal medicine, T47D

SUMMARY

- The screening of thirty Indonesian medicinal plant extracts for anti-breast cancer cell line was conducted
- The methanol extract of *Alpinia galanga* extract showed the most active sample
- Active chemical constituents of *A. galanga* were isolated and studied their activity-structure relationship.



Abbreviations used: TLC: Thin layer chromatography, RPMI: Roswell Park Memorial Institute, NMR: Nuclear magnetic resonance, HMBC: Heteronuclear multiple bond correlations.

Correspondence:

Dr. Azis Saifudin,
Faculty of Pharmacy, Universitas Muhammadiyah Surakarta, Pabelan, Kts Solo, Jawa Tengah 57102, Indonesia.
E-mail: azis.saifudin@ums.ac.id
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INTRODUCTION

Breast cancer remains a major cause of death worldwide. During 2012, there were 1.67 million cases reported.[1] It is estimated that it was between 2.1% and 2.6% population in medium index countries, and high index suspected with breast cancer[2] and the rate of incidence of 2% per annum. Globally, it is predicted that there will be 22.2 million incidences reported by 2030. In the United States alone, it is projected that there will be >3.1 million women living with a history of invasive breast cancer, and an additional almost 250,000 women were diagnosed in 2014.[3] To date, an efficient pharmacotherapy method has not sufficiently eradicated cancer. Yet, there are also tremendous side effects on the treated patients, who have experienced hair loss, sexual disorders, and/or depression.[4-7] From a health economic viewpoint, many patients in third world countries often deter from affording such an expensive medication process.[8] Therefore, there is an urgent effort to discover an alternative lead compound for anti-breast cancer. Since small molecules, especially secondary metabolites, have played a significant role in anti-cancer discoveries, it is promising to search for a leading compound from natural resources.[9,10] We considered that the safety of herbal medicine and daily condiments have been acknowledged for centuries. They have been involved on a daily basis for preventing any harmful impacts of internal biochemical reactions, microbial attacks, as well as the attacks of other strange substances, which could include some types of cancer cells. Therefore, the presence of cancer cells-inhibiting chemical constituents in the daily used plants may become a reality.

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Thus, to leverage natural resources from indigenous medicinal plants, we have screened 30 plant species including some spices that are commonly used with cuisine in Indonesia. To explore the possibility of those spices to inhibit cancer, a T47D cell line has been employed as a breast cancer model. The most active plant species was then investigated for its active constituents through column chromatography techniques. To extend the anticancer potential inhibiting effect, the isolated compounds were examined related to their activity to other cell lines: MCF7 (breast), WiDR (colon), and HeLa (cervix cancer).

MATERIALS AND METHODS

Plant materials

The plants were purchased from Widodo herbal trading Solo City and authenticated by one of the authors (AS). Their voucher specimens were deposited at the Laboratory of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Muhammadiyah Surakarta.

Screening

A total of 50 g of each plant material were extracted using methanol 100 mL twice. Since methanol had the highest extractability toward the secondary metabolites rather any other solvents,[11-13] each extract was evaporated to remove methanol. The concentration of the stock solution for the assay was 1 mg/mL in 10% dimethyl sulfoxide (DMSO), whereas the concentration of each well was 25 μg/mL. The maximum DMSO concentration in the well was 0.1%.

Inhibitory activity test

The cytotoxic test was conducted based on colorimetry through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in accordance with Khanavi *et al*. and Turak *et al*.[14,15]

Cell growing

The cell cultures were maintained in the Laboratory of Cell Culture at Faculty of Pharmacy, Universitas Muhammadiyah Surakarta. T47D, MCF7, WiDr, and HeLa cells were cryopreserved in nitrogen in a tube before the seedling process. To cultivate, T47D was grown in sterile conical and suspended in 5–10 mL of RPMI media. They were centrifuged within 10 min to result in a pellet. It was then added with 1 mL of each medium with FBS 20% and re-suspended and divided into four flask cultures. They were then placed in a CO_2 incubator for 24 h. Each medium was replaced with the fresh one until being confluent (around 80%). The same procedures were conducted for the other cells, except MCF7 that was grown in DMEM medium.

Cell harvesting

To collect the cells, phosphate-buffered saline was sprayed on the flask wall and added with 50 μL trypsin. The flasks were then placed in the CO_2 incubator for around 5–10 min with the addition of 3–5 mL, subsequently. The flask was then placed under an inverted microscope confirming its condition and contaminant. To count the cell number, 10 μL of it was placed in a hemocytometer.

3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay

Each cell solution was pipetted in a 96-well microplate with the density of 5 × 103/well and further incubated for 24–48 h until reaching an appropriate condition for the inhibitory test. The plant extract was added to the final concentration of 25 μg/mL of each well. The treated cells together with positive controls epirubicin and doxorubicin (Kalbe Farma Ltd, Indonesia) and DMSO control were re-incubated in CO_2 for 24 h. The medium was discarded and then 100 μL of MTT (5 mg/mL in medium) was pipetted. The plate was then incubated for another 24 h. To dissolve the produced formazan, 50 μL sodium dodecyl sulfate 10% was pipetted in each well. The plate was wrapped in an aluminum foil and placed at room temperature overnight. Finally, the absorbance was measured through an Elx800 Biotek microplate reader (λmax = 540 nm). The test was made in a Duplo and conducted in three independent experiments. The remaining living cells were counted based on the following formula:

$$\text{Cell viability} = \frac{\text{Treated cell absorbance-}\ \text{Media control absorbance}}{\text{Cell control absorbance-}\ \text{Media control absorbance}} \times 100\%$$

Extracts, which were capable of inhibiting >80% of the cell, were further determined for their IC_{50} values through the following series of extract concentration: 2, 4, 8, 10, and 20 μg/mL. The most potent sample was determined for their IC_{50} in three independent experiments. Since *Alpinia galanga* extract was capable of inhibiting more than 97% cell population and showed the most potent sample with an IC_{50} value of 21.2 μg/mL, it was further investigated for its chemical constituents.

Extraction and purification of *Alpinia galanga* extract

Spectroscopical measurement

NMR spectra of isolated compounds were measured using a JEOL ECS400-spectrometer with tetramethylsilane (TMS) as an internal standard. Chemical shifts were given on the δ scale from the TMS standard and coupling constants (*J*) in Hz. The structure of obtained compounds was identified by means of one dimension and two dimensions ^1H and ^{13}C nuclear magnetic resonance (1 and 2 D NMR).

Purification

A total of 1.0 kg of *A. galanga* rhizome was macerated in methanol 10 L within 24 h. The extraction was repeated twice. The obtained extract was concentrated under a reduced pressure into vacuo to obtain 55 g of extract. A portion of 15.0 g extract was separated on a silica chromatography column (30 cm × 3.5 cm) with chloroform-hexane (9:1) and continued with methanol in a chloroform (0; 0.5; 1.0; 1.5; 2.0; 2.5%, respectively) solvent system. The volume of each solvent was 600 mL and the collecting fraction was 100 mL. On the basis of thin layer chromatography profile analyses, this separation work resulted in six fractions: (a) 0.6 g, (b) 2.6 g, (c) 1.7 g, (d) 4.6 g, (e) 3.2 g, and (f) 1.6 g. From the inhibitory test, fractions D and E were found capable of inhibiting >90% T47D cells with the IC_{50} values of 35 and 26 μg/mL, respectively. In contrast, the rest of the fractions showed weak effects. Fraction E was further separated on a silica column (15 cm × 2.5 cm) with hexane in an ethyl acetate (100%, 80%, 60%, 50%, 30%, and 10%) solvent system, which resulted in five fractions: E1 (120 mg), E2 (345 mg), E3 (980 mg), E4 (458), and E5 (269 mg). Fractions E3 and E4 exhibited a similar activity with >90% inhibition. Since fraction E4 possessed a significant quantity, it was purified by preparative TLC with a hexane-EtOAc (3:1) solvent system to result in two mixtures according to 1H NMR spectra: E4-1 (98 mg) and E4-2 (130 mg). Each mixture was separated on TLC with $CHCl_3$-MeOH (9:1). The former resulted in 1 (43 mg), and 2 (10.8 mg), whereas the latter was furnished with 3 (54 mg) and 4 (8.2 mg), respectively. The purities of 1–4 were 95%, 95%, 92%, and 90% according to TLC and 1D NMR spectra analyses.

The inhibitory capacity of all of the obtained compounds was re-assayed against T47D, MCF7, HeLa, and WiDr cell lines with the same procedure as shown above.

RESULTS

Inhibitory activity test

Of the examined samples, *A. galanga, Clonorchis sinensis, Piper cubeba, Santalum album,* and *Vitex trifolia* exhibited 96.4%, 91.9%, 87.6%, 82.6%, and 88.7% inhibition, respectively. On the other hand, *frutescens*, *Litsea cubeba*, and *M. aromaticum* had an effect with a moderate inhibition mode, ranging from 54% to 72%; whereas, the rest of the samples showed a weak activity with <40% inhibition [Table 1].

Elucidation structure of isolated compounds

The ^{1}H NMR spectra of all compounds showed a similar pattern of appearance with two doubled signals of aromatic protons between δ_H 6.2-7.44 ppm. Each represents two protons based on the integration ratio. They had the coupling constant (*J*) of 8.8 Hz, which indicated those protons at onto position. Meanwhile, ^{13}C NMR showed the signals of six aromatic carbons between δ_C 117–150 ppm, which confirmed a typical symmetric benzyl. Three aliphatic carbons were apparently present to all compounds based on COSY connectivity analyses. HMBC correlation analyses showed that 1–4 possessed a phenylpropanoid skeleton. For 1, the location of the oxygenated methyl at δ_H 3.37 ppm was deduced based on HMBC correlated to the aliphatic carbon at δ_H 73.5 ppm. The acetyl group of 2 was deduced at the aliphatic side chain based on its proton methyl (δ_H 2.28 ppm) correlation with carbonyl carbon at δ_C 169.7 ppm. The methyl at δ_H 2.08 ppm of 3 was deduced as part of acetoxy at C-6 on the basis of its correlation and oxygenated methylene (δ_H 2.28 ppm) correlation to carbonyl carbon at δ_C 170.8 ppm. COSY correlations confirmed the presence of 3-propenyl moiety; it was located adjacent to the latter acetoxy based on the HMBC correlations of proton δ_H 4.71 ppm with that carbonyl carbon. Finally, 1D NMR spectrum of compound 4 was similar to that of 2, except with the addition of a methyl peak. Based on the HMBC correlation, the methyl was located as a para position to acetoxy-1-propenyl ether. On accurate observation, the spectra have good agreements with *trans-p*-coumaryl alcohol (1), *p*-coumaryl acetate (2),[16] [1'S]-1'-acetoxy chavicol (3), *trans-p*-coumaryl diacetate (4),[17] respectively [Figure 1]. Their NMR spectra are available by referring to the corresponding authors.

Inhibitory activity test of isolated compounds (1–4)

Of the isolated compounds, 3 and 4 showed the significant activities with more than 80% inhibitory at a concentration of 25 µg/mL. They had the IC_{50} values of 17.3 and 25.4 µg/mL, respectively. Meanwhile, 1 and 2 exhibited a number of mild activities with <40% and 30% inhibition, respectively. In further examination, it showed that both compounds had a similar fashion on MCF7, HeLa, and WiDr cell lines. On MCF7, 3 and 4 had the IC_{50} values of 14.2 and 23.0 µg/mL, on HeLa cells they had the IC_{50} values of 26.9 and 37.6 µg/mL, and against WiDr with IC_{50} values of 11.2 and 18.2 µg/mL, respectively.

Compounds 3 and 4 exhibited significant activities on T47D with IC_{50} values of 17.3 and 25.4 µg/mL. They showed cytotoxic activity even at low concentrations [Figure 2]. Further, they can significantly inhibit against MCF7, HeLa, and WiDr. Interestingly, both compounds also are able to inhibit MCF7 with significant activities in small concentrations, i.e., 6.25 and 12.5 µg/mL [Figure 3]. MCF7 is a type of breast cancer cell which is notably resistant toward some drug of choices for nonestrogenic receptor-independent (such as doxorubicin). However, it can be inhibited by both isolated compounds as well. Their inhibitory activities are shown by a lack of proliferation with large unoccupied zones on the bottom wells. Some T47D and MCF7 cells experienced blackened

Table 1: Plant name, local name, family, used plant and cell viability treated with each extract (25 µg/mL)

No	Scientific name	Local name	Familia	Used part	Cell viability
1	*Abelmoschus moschatus*	Waron	Malvaceae	Seed	98.8±0.04
2	*Alpinia galanga*	Laos	Zingiberaceae	Rhizome	3.16±0.02
3	*Amomum cardamomum*	Kapulaga	Zingiberaceae	Fruit and seed	82.8±0.03
4	*Annona muricata* L.	Daun Sirsat	Annonaceae	Leaf	72.4±0.02
5	*Baeckea frutescens* L.	Jongrahap	Myrtaceae	Leaf	45.3±0.02
6	*Cassia sinensis*	Ketepeng	Fabaceae	Leaf	8.1±0.01
7	*Clinacanthus nutans*	Dandang Gula	Acanthaceae	Leaf	82.9±0.01
8	*Equisetum debile* Roxb	Greges Otot	Equisetaceae	Aerial part	66.2±0.04
9	*Foeniculum vulgare*	Adas	Apiaceae	Aerial part	68.9±0.01
10	*Graptophyllum pictum* (L.) Griff.	Daun Ungu	Acanthaceae	Leaf	78.2±0.01
11	*Guazuma ulmufolia*	Jati Belanda	Sterculiaceae	Leaf	66.7±0.03
12	*Gynura segetum* (Lour.) Merr.	Daun Dewa	Asteraceae	Leaf	98.4±0.05
13	*Hemigraphis alternata* (Burm.f.) T. Anders	Remet Getih	Acanthaceae	Aerial part	93.6±0.01
14	*Imperata cylindrica* Raeusch.	Alang-alang	Poaceae	Aerial part	88.0±0.04
15	*Justicia gendarussa* Burm. f.	Gondoruso	Acanthaceae	Aerial part	77.4±0.01
16	*Litsea cububa pers*	Kerangean	Lauraceae	Fruit	27.9±0.00
17	*Massoia aromaticum* Becc.	Masoyi	Lauraceae	Bark	33.1±0.01
18	*Myristica fragrans*	Pala	Myristaceae	Seed	85.9±0.04
19	*Oroxylum indicum* Vent.	Kayu Lanang	Bignoniaceae	Bark	60.2±0.01
20	*Parameria laevigata (Juss.) Moldenke*	Kayu Rapet	Apocynaceae	Bark	92.3±0.03
21	*Parkia roxburghii* G.Don	Dawung	Mimosaceae	Seed	67.1±0.01
22	*Piper retrofractum* Vahl	Cabe	Piperaceae	Fruit	54.9±0.02
23	Pipper cubeba	Kemukus	Piperaceae	Fruit	12.4±0.00
24	*Plantago major* L.	Sindokan	Plantaginaceae	Aerial part	77.4±0.04
25	*Psophocarpus tetragonolobus*	Botor	Fabaceae	Seed	87.9±0.03
26	*Santalum album* L.	Cendana	Santalaceae	Bark	17.4±0.02
27	*Saricocalix crispus*	Keji Beling	Acanthaceae	Leaf	76.8±0.03
28	*Sterculia javanica* R. Br.	Pronojiwo	Sterculiaceae	Seed	91.6±0.03
29	*Strychnos ligustrina*	Widoro Laut	Loganiaceae	Wood	79.3±0.01
30	*Vitex trifolia*	Legundi	Verbenaceae	Leaf	11.3±0.01

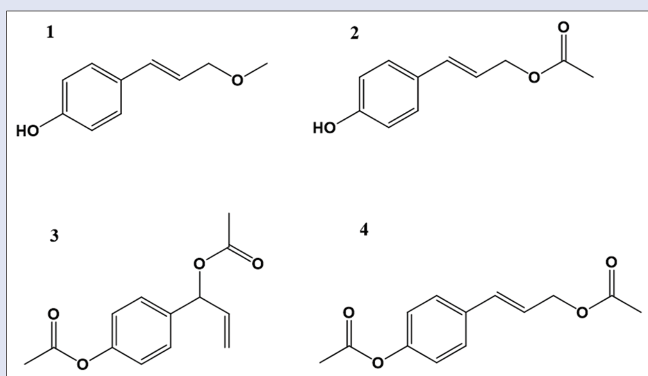

Figure 1: Isolated compounds: *trans-p*-coumaryl alcohol (1), p-coumaryl, diacetate (2), [1'S]-1'-acetoxy chavicol (3), *trans-p*-coumaryl diacetate (4)

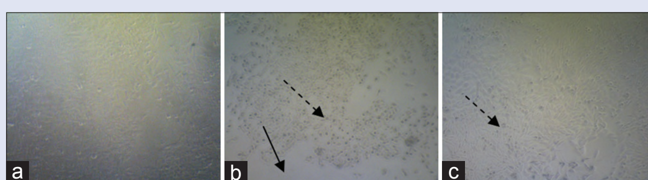

Figure 2: (a) T47D cell control, cells under treatment with compounds 3 (b) and 4 (c) at a concentration of 6.25 μg/mL (40x magnification). Both treated cells show blackened nuclear due to cytolysis (dashed arrow). The empty area shows a lack of proliferation of treated cells (solid arrow)

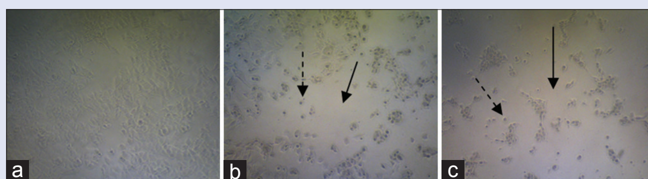

Figure 3: MCF7 cell control (a), cells under the treatment with compounds 3 (b) and 4 (c) at a concentration of 6.25 μg/mL. Both treated cells experienced cytolysis by showing blackened nucleus (dashed arrow) and exhibit some extensively inhibited zones (solid arrow)

cytoplasms [Figures 2 and 3b-c], which could be due to the cytotoxic activities of compounds 3 and 4.

DISCUSSION

Endowed with a tropical climate, Indonesia is one of the richest countries in biodiversity in the world with the potential to provide leading compounds for drug discovery program. In continuing the effort to search bioactive molecules from Indonesian natural resources, various extract plants have been screened against T47D cell lines. Among the potent samples, here is the first report on the significant effect of *C. sinensis*, *P. cubeba*, *S. album*, and *V. trifolia* against this breast cancer cell line. Moreover, there is no conventional usage for a cancer-related activity of these plant materials. Thus, they can be involved in future studies related to cancer treatments. Since *A. galanga* showed the strongest activity, its activity was further observed through a series of activity concentration inhibitions; it had a dose-dependent inhibition with an apparent IC_{50} value of 21.2 μg/mL. Interestingly, *A. galanga* rhizome has been used as a spice in daily cooking in Indonesia in relatively low quantities. In traditional medicine, it has been used as a remedy for numerous ailments such as itchy skin, digestion, colic, dysentery, enlarged spleen, respiratory disorders, mouth and stomach cancer, systemic infections, cholera aphrodisiac, and removing mucus.[18] It is also sometimes used to treat muscle pains.[19] Therefore, this plant has been used for these purposes for centuries. For these reasons, its active chemical constituents were further investigated through flash chromatography techniques, which resulted in four compounds (1–4).

In the concentration of 25 μg/mL, compounds 1 and 2 are only able to inhibit <25% cells while 3 and 4 can inhibit >90% cell population. Although all the isolated compounds possess a phenylpropanoid skeleton, from the structure-activity relationship, the acetoxy group at position C-6 might be the main contributor to the effect, clearly shown by 3 and 4 activities. The treated cells showed to change the morphology of the nucleus tend to hyperploid, and the empty area of bottom well-demonstrated some inhibited zones [Figures 2 and 3]. Thus, this could be a firm evidence for this functional group effect. On the other hand, rather than a linear side chain group,[4] 3-propenyl provided more potent activities.[3] Of T47D, the treated cells exhibited cytotoxic due to a necrotic mechanism after a period because the early stage of growing both compounds did not affect cell proliferation. Thus, on observation after 48 h posttreatment, the cell densities were relatively similar between control and treated cell samples with 3 and 4. On the other hand, on MCF7 both compounds have a cytotoxic effect as well as an inhibit proliferation since the early stage of cell growth, thus showing by vast empty regions of the well bottoms as well as cytolysis processes. To the best of our knowledge, this is the first report of breast cancer inhibitory activity relationship of para-acetoxy phenylpropanoids.

Compound 3, ([1'S]-1'-acetoxy chavicol), also known as ACA, was reported to significantly inhibit the growth of human head and neck squamous cell carcinoma cell line HN4 and induced cell apoptosis through downregulation of miR-23a, which repressed PTEN as a tumor suppressor.[20] Another study concluded that ACA and or cisplatin altered hsa-miR-138, hsa-miR-210, and hsa-miR-744 expression, which is involved in signaling pathways of apoptosis and cell cycle progression.[21] According to Awang *et al.*,[22] this compound possesses cytotoxic effect by inducing apoptosis and cell cycle arrest mechanism on MCF7. It should be noted that since both compounds can show significant activity on the other cell types, they could have potential to be active in other cancer types. Thus, by this study, [1'S]-1'-acetoxy chavicol (3) and trans-p-coumaryl diacetate (4) may be potential leads for further model development of anti-breast, cervical, and lung cancer drug discovery.

Hence, *A. galanga* rhizome extract and its active isolated compounds could be important materials to be developed as a remedy or preventive agent for cancer. It should also be worth noting here that since *A. galanga*, *C. sinensis*, *P. cubeba*, *S. album*, and *V. trifolia* also performed potent inhibition activity, they can be an interest for further isolation works as well as for further studied materials for anti-cancer research.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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