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Original Research Article

Comparative Chemical Composition and *In-Vitro* Cytotoxic Activity of *Syzygium aromaticum* against Selected Cancer Cell Lines

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ABSTRACT

The dried flower buds of clove (*Syzygium aromaticum*) are among the most commonly used spices. It is famous as a source of volatile oils and eugenol and possesses various pharmacological activities. However, there is little scientific information on the medicinal and biological importance of ethanol extract of the plant. In this study, 50% ethanol extracts of *S. aromaticum* young leaves, leaves, young flower buds, and flower buds were analyzed for their chemical composition using LC-MS. The cytotoxic activity of the extracts against A549, HT-29, Hela, and MDA-MB-231 cell lines was evaluated using MTT assay. Three major phenolic compounds, namely; 2, 4, 6-trihydroxyacetophenone-3-C-*β*-D-glucoside, biflorin and isobiflorin, were detected in the extracts, with 2,4,6- trihydroxyacetophenone-3-C-*β*-D-glucoside being the most abundant in all the plant parts, except in the leaves. All extracts displayed low cytotoxic activity against A549 and HT-29. Both flower buds and young flower buds containing high amounts of 2,4,6-trihydroxyacetophenone-3-C-*β*-D-glucoside exhibited moderate cytotoxic activity against Hela and MDA-MB-231 cell lines with IC_{50} values of 40.88 and 48.52 µg/mL; 35.69 and 39.56 µg/mL for flower buds and young flower buds, respectively. Extracts of flower buds and young flower buds of *S. aromaticum* can be sources of novel bioactive compounds that might be useful as anticancer agents.

Keywords *Syzygium aromaticum*, Clove extracts, 2,4,6-trihydroxyacetophenone-3-C-*β*-D-glucoside, Cytotoxic activity

Introduction

Syzygium aromaticum (L.) Merr. & L.M. Perry, is commonly known as clove tree in the Myrtaceae family. Clove flower bud has been used for a long time in the management of toothaches. The most abundant compound in the clove is eugenol.[1] The flower, leaf, and stem of clove tree are used as materials for clove oil production. Clove oil is used extensively in foods, cosmetics, and in dentistry. Indonesia and Madagascar are the leading clove exporters.[1] In Indonesia, clove production is high, but its cigarette industry uses up almost all the leaves material.[1] However, the Indonesian government aims to reduce the population of smokers by increasing tariffs on cigarettes, which may decrease the domestic demand for clove.[2] There are projections for cloves production to increase annually.[1] To meet these targets, there is a need to reduce the cigarette industry's dependency, and Indonesian clove farmers must export most of their products. The demand for clove can also increase if other uses are explored beyond its traditional benefits, significantly increasing the clove market share value. One of the aims of this study is to observe whether clove can be used for other purposes, especially in medicine. Cancer is a significant health problem globally; about 1 in 6 deaths is due to cancer. The most common deaths result from lung, colorectal, stomach, liver, and breast cancers.[3] Medicinal plants have played a significant part as sources of anticancer compounds. Examples of anticancer agents derived from plants include the vinca alkaloids from Madagascar periwinkle, isoquinoline alkaloids from the Opium poppy, camptothecin derivatives from *camptotheca spp*, taxane derivatives from Pacific yew tree, podophyllotoxin derivatives from Mayapple, and cannabinoids from cannabis.[4] The investigation of plants continue to hold a promise for the treatment of cancer. In this study, extracts of flower buds, young flower buds, leaves and young leaves of *S. aromaticum* were evaluated for their cytotoxic activity against A549 (lung carcinoma), HT-29 (colorectal adenocarcinoma), Hela (cervical adenocarcinoma), and MDA-MB-231 (breast adenocarcinoma) cell lines. The study also intends to validate the plant extracts as an alternative source of anticancer agents. Furthermore, a comparative analysis of phenolic compounds in the plant extracts was carried out.

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Materials and Methods

Plant material and chemicals

S. aromaticum sample was collected from Chanthaburi province, Thailand in September 2020, and identified by Apiradee Korpphaiboon, a Botanist in the Department of Agriculture, Chanthaburi Horticultural Research Center. A Voucher specimen number #10102 was assigned. The plant material was washed to remove contaminants and dried at 50°C in a hot air oven for 24 h. The plant samples were separately pulverized in a blender and stored at room temperature, away from light.

Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), glutamine, penicillin, and streptomycin were purchased from Invitrogen (USA). MTT reagent (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)) was purchased from Life

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Technologies (USA). HPLC grade acetonitrile and water, and analytical grade ethanol were all purchased from B and J (South Korea). Analytical grade formic acid and DMSO were purchased from Merck (Germany). Distilled water was purchased from Puris, Expe-CB Ele10 Water System (South Korea).

Extraction of plant materials

Each pulverized sample (young leaves, leaves (expanded-1 and mature stage), young flower buds, and flower buds (budding-2 stage)) of *S. aromaticum* (500 mg) was separately extracted with 50% ethanol (40 mL) by sonication for 30 min. Each resulting mixture was filtered and evaporated to dryness using a rotatory evaporator to afford extracts labelled as young leaves (YL), leaves (L), young flower buds (YF) and flowers buds (F) of *S. aromaticum*.

Chemical composition of each extract

Each extract 1 mg was reconstituted with 1 mL of 50% ethanol and filtered through a 0.45 μm nylon filter before analysis. The chemical composition of each extract was determined using LC-MS (a Dionex Ultimate TM 3000 HPLC coupling with Bruker Amazon SL mass spectrometer). The column was Acclaim® 120-C18 column (3 µm, 2.1 mm x 150 mm). The analysis was performed in a gradient mode using 0.1% formic acid-acetonitrile ratio from 100:0 to 85:15 (0-5 min), then isocratically at a ratio of 85:15 (5-25 min), the gradient mode at 85:15 to 45:55 (25-50 min), the isocratic mode at a ratio of 45:55 (50-60 min), then isocratically at a ratio of 0:100 (60-70 min), and re-equilibrated at 100:0 for five minutes. The mobile phase flow rate was 0.12 mL/min, and the column temperature was 25 ^{o}C. The mass spectrometer was equipped with an ESI ion source and a quadrupole-ion trap. The analyses were performed in negative mode, recorded on a mass range of m/z 100-1,000. The desolvation gas flow rate was 7.0 L/min at 200 ^{o}C. The capillary voltage was 4,500 V, and nebulizer pressure was set at 2 bars. The samples were injected in triplicate. The MS data was compared to the in-house database of the Sino-Thai Traditional Medicine Research Center, College of Pharmacy, Rangsit University.

Cell viability assay

The A549, HT-29, Hela, and MDA-MB-231 cell lines were cultured in DMEM medium supplemented with 10% FBS, 5% penicillin, 5% streptomycin, and 2mM L-glutamine. Cells were cultured at 37 ^{o}C and 5% CO_2 in a humidified incubator.

Cell viability was determined using an MTT assay as described in a previous report with slight modification.[5] Cells were seeded at 1.4 x 10^4 cells/mL on a 96-well plate. They were treated for 72 h with seven different concentrations (6.25, 12.5, 25, 50, 100, 200, and 400 µg/mL) of the extracts dissolved in DMSO in triplicate wells. For the negative control, the protocol was repeated but without the extracts. After 24h, 10 µL of MTT reagent was added into each well and incubated for 4hr at 37 ^{o}C and 5% CO_2. Cell viability's absorbance at every concentration was measured using Biorad Microplate Reader and calculated using the equation below:

$$\text{Percentage cell viability} = \frac{\text{OD570 sample}}{\text{OD570 control}} X\ 100$$

The percentage of cell viability was plotted against concentration, and the IC_{50} values were determined from the graph for each extract.

Results and Discussion

The compounds present in the ethanol extract of the leaves, young leaves, flower buds, and young flower buds of *S. aromaticum* were identified based on a database comparing the retention times and molecular ion. Different phenolic compounds, including acetophenone glycoside (2,4,6-trihydroxyacetophenone-3-C-β-D-glucoside), chromone glycosides (biflorin, isobiflorin), flavonoids (quercetin), and phenolic acids (quinic and gallic acids) were detected in each plant extract (Table 1). For each extract, i.e. YL, L, YF, and F, the presence of these compounds are as shown in the chromatogram below (Figure 1). In all, a total of 6 compounds were identified. 2,4,6-trihydroxyacetophenone-3-C-β-D-glucoside (*m/z* 329.1) was the main compound found in high amounts in YF and F of clove extract, but low in YL and L. The estimated amounts using peak area from the chromatogram are in the following order F>YF>YL>L. This compound was first reported in clove leaves and later isolated from the flower buds.[6-7] The mass spectrum of 2,4,6 trihydroxyacetophenone-3-C-β-D-glucoside was shown in figure 2 and the chemical structure was shown in figure 3. Biflorin (*m/z* 353.1) and isobiflorin (*m/z* 353.1), chromone glycosides, was found in all extracts. These compounds were also previously isolated from clove flower buds.[7] This study revealed that these compounds were copiously expressed in the flowers more than in leaves (F>YF>L>YL). Other reports show that "biflorin" was referred as a naphthoquinone from *Caparia biflora* L.[8] The chromatogram revealed the presence of quinic (*m/z* 191.1) and gallic acids (*m/z* 169.1) which are precursors in the synthesis of secondary plant metabolites, especially in the shikimate pathway. The study results also show that quinic acid amounts were more in the leaves while gallic acid was low in all the four extracts. The report from another study corroborates these findings.[9] On the other hand, the amount of quercetin (*m/z* 302.0), a bioactive flavonoid, was low in all the samples. This compound was previously reported in clove flower buds by Ryu *et al*.[10] The biosynthesis of metabolites in clove occurs mostly via the shikimate pathway that leads to the synthesis of eugenol. However, it was suggested that the acetate pathway also plays a crucial role in leaves and flower buds since higher amounts of 2,4,6-trihydroxyacetophenone-3-C-β-D-glucoside, biflorin, and isobiflorin were present in the plant.

S. aromaticum extracts' cytotoxic activity against the different cell lines was examined in a concentration-response experiment at a concentration of 6.25 – 400 µg/mL in the culture medium incubated for 72 h. Samples F and YF inhibited the viability of all cell lines in a concentration-dependent manner (Figure 4), while samples YL and L did not show activities against A549 and HT-29 (IC_{50} > 100 µg/mL). The IC_{50} values of all samples in table 2 revealed that only flower samples showed moderate cytotoxic activity against Hela and MDA-MB-231 (IC_{50} 30-50 µg/mL). This result correlated with that of a previous study.[11] However, the authors did not indicate the metabolite responsible for the aqueous and ethanol extract's cytotoxicity. In another study, oleanolic acid, a compound isolated from the ethyl acetate extract of *S. aromaticum* was reported to be responsible for the plant's cytotoxic activity against HT-29[12]. In this current study, oleanolic acid was absent in the 50% ethanol extracts. Its cytotoxic activity against HT-29 was low at IC_{50} value of 50-100 µg/mL. It could be inferred that the YF and F extract's cytotoxicity must be due to other compounds. The criteria for cytotoxic activity was implied in the literature.[13-15]

2,4,6-trihydroxyacetophenone-3-C-β-D-glucoside, biflorin, and isobiflorin detected in the four extracts of *S. aromaticum* were earlier

Table 1: List of compounds identified in *S. aromaticum* extract as determined by comparing retention time and molecular ion and localization with estimated content of these compounds in the plant

Compounds	Rt (min)	Molecular ion (ion (*m/z*)	Parts/content
Quinic acid	3.4	191.1	YL,L/high YF,F/low
Gallic acid	11.5	169.1	YL,L/low YF,F/low
2,4,6-trihydroxyacetophenone-3-C-β-D-glucoside	15.2	329.1	YL,L/low YF,F/high
Isobiflorin	15.5	353.1	YL/low L,YF,F/high
Biflorin	16.7	353.1	YL/low L,YF,F/high
Quercetin	37.1	301.0	YL,L/low YF,F/low

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F = flower buds, YF = young flower buds, L = leaves, YL = young leaves reported to possess no cytotoxicity against human ovarian cancer cells (A2780).[7] Biflorin and isobiflorin may likely be responsible or may that has a similar structure with biflorin displayed activity against Hep-G2. However, its activity against HT-29 and MDA-MB-231 cells was low.[16] Quercetin seems to be a promising compound that represents cytotoxic activity in this study. It was reported to possess high cytotoxicity against all test cell lines with specific mechanisms.[17-20] However, all samples showed moderate cytotoxic activity against some of the cell lines from our results. This observation may be related to trace amounts of quercetin in all extract samples. Of particular interest is the fact that from the IC_{50} values of the test samples against Hela and MDA-MB-231 cell lines, only YF and F samples which possessed high content of 2,4,6-trihydroxyacetophenone-3-C-*β*-D-glucoside showed moderate cytotoxic activity against these cell lines. Also, literature reports showed that acetophenone derivative possessed cytotoxic activity against Hela and MDA-MB-231.[21-22] Cytotoxic activity and the contents of the compounds are in this order F>YF>YL>L. The cytotoxic activity exhibited by the four extracts of the plant may be due to the presence of trihydroxyacetophenone glycoside or possibly due to the synergistic action of some constituents in the plant. Further studies would be centered on isolation and characterization of the glycoside and its cytotoxic investigation against different cancer cells. The cytotoxicity of isolated compounds can be further compared with the standard drug such as doxorubicin which possessed high anticancer activity (IC50 0.47-8.03 µM).[23]

Table 2: IC_{50} values of clove extract samples against A549, HT-29, Hela, and MDA-MB-231

Extract	IC_{50} (µg/mL)			
	A549	**HT-29**	**Hela**	**MDA-MB-231**
F	53.61	60.04	40.88	35.69
YF	76.23	> 100	48.52	39.56
L	> 100	> 100	93.36	79.41
YL	> 100	70.7	68.08	58.31

F = flower buds, YF = young flower buds, L = leaves, YL = young leaves

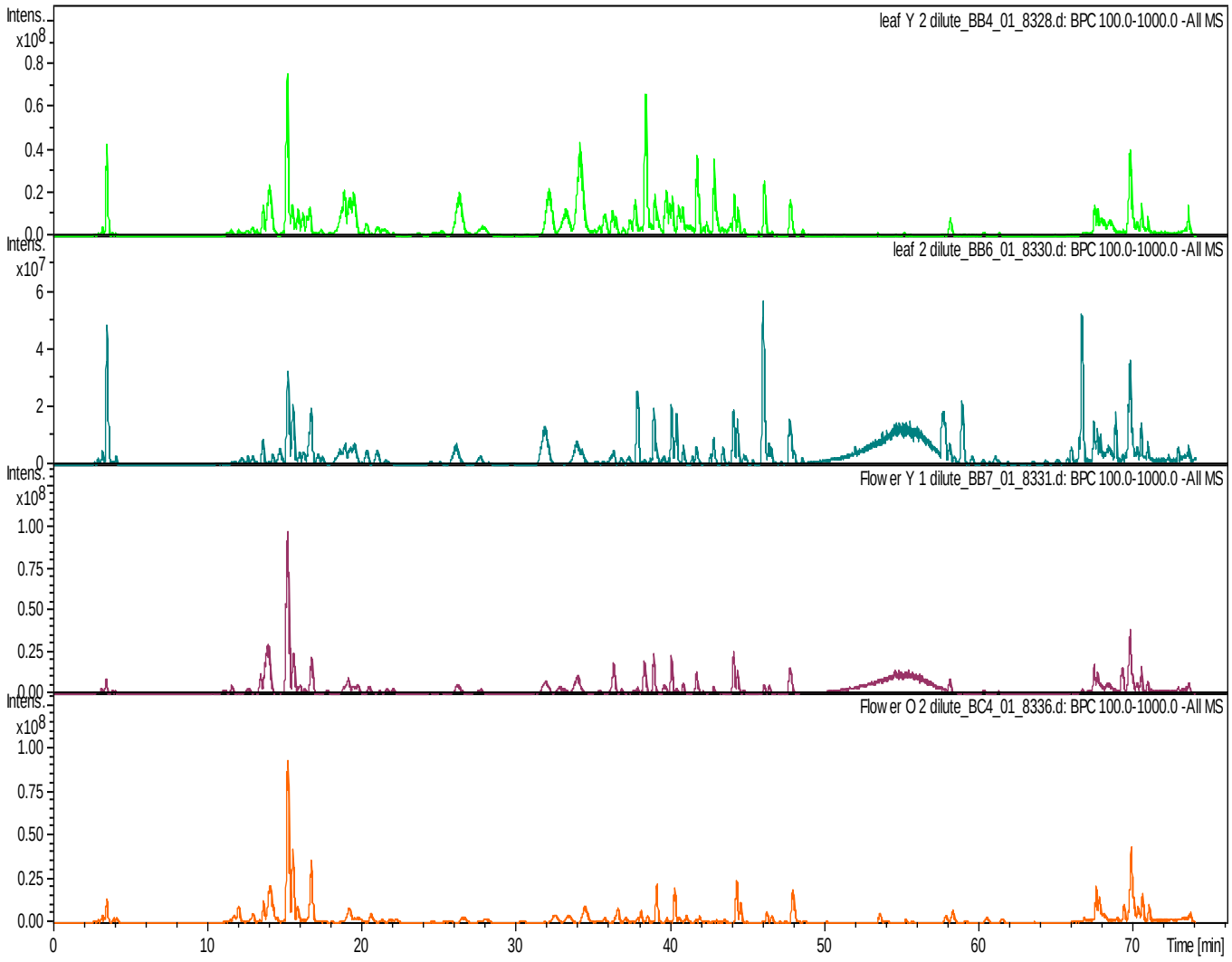

Figure 1: LC-MS chromatogram in ESI negative mode of *S. aromaticum* extract (YL, L, YF, and F; from upper to lower) (At Rt 3.4 min is quinic acid; 11.5 min is gallic acid, 15.2 min is 2,4,6-trihydroxyacetophenone-3-C-*β*-D-glucoside, 15.5 min is isobiflorin, 16.7 min is biflorin, and 37.1 min is quercetin)

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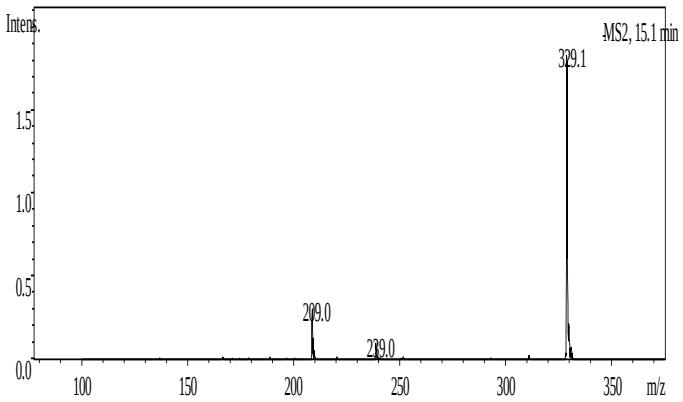

Figure 2: The mass spectrum of 2,4,6 trihydroxyacetophenone-3-C-β-D-glucoside.

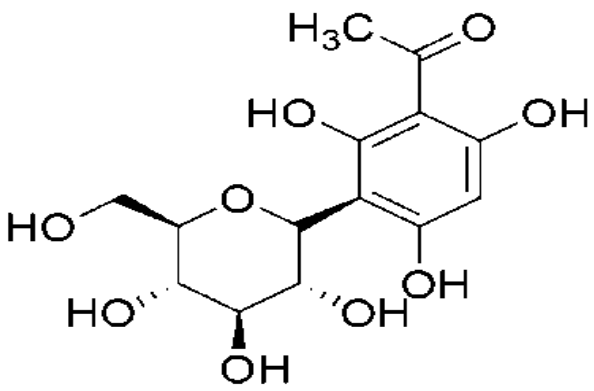

Figure 3: The chemical structure of 2,4,6-trihydroxyacetophenone-3-C-β-D-glucoside

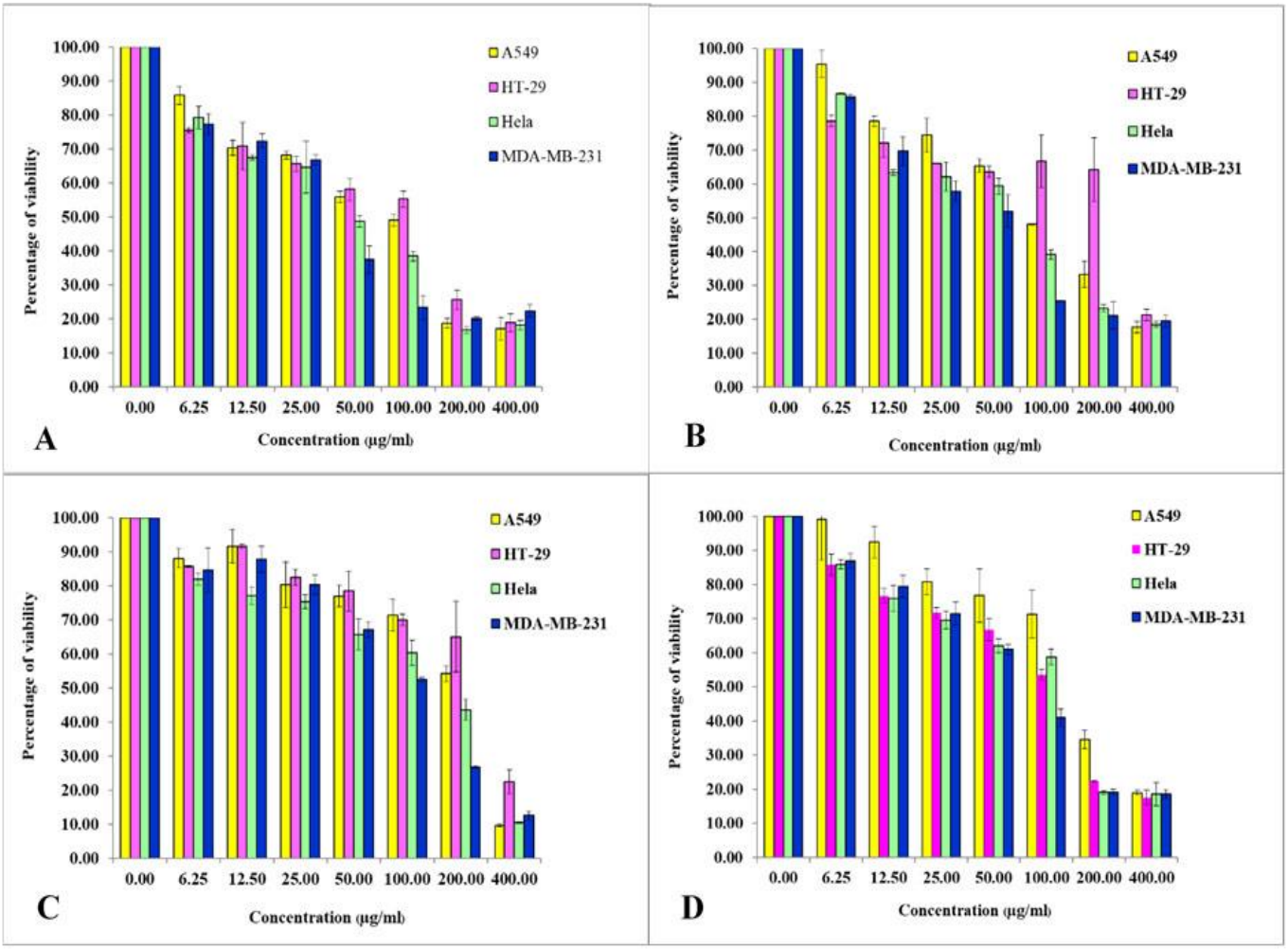

Figure 4: Concentration-dependent manner of 50% ethanol *S. aromaticum* extract on A549, HT-29, Hela, and MDA-MB-231 cell lines. A was flower buds, B was young flower buds, C was leaves, and D was young leaves. Experiments were performed in triplicate

Conclusion

Ethanol extracts of *S. aromaticum* flower buds exhibited moderate cytotoxic activity against cervical and breast adenocarcinoma (Hela and MDA-MB-231). The presence of 2,4,6-trihydroxyacetophenone-3-C-*β*-D-glucoside acting individually or synergistically with other constituents could have contributed to the cytotoxic activity of the extract. This indicates that flower buds of *S. aromaticum* might be a potential source of lead compounds in the treatment of cervical and breast cancer.

Conflict of interest

The authors declare no conflicts of interest.

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Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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