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Research Article

Comparison of the potential of extracts from the flower, fruit pulp, and seed of *Cassia fistula* L. on MCF-7 breast cancer cell growth and cell migration

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Abstract

This study aimed to determine the anticancer effects of extracts prepared from various parts of *Cassia fistula* L. (CF), i.e., flower extract (FE), fruit pulp extract (FPE) and seed extract (SE), on MCF-7 breast cancer cells. The anticancer effects of the extracts were assessed for cell toxicity, cell proliferation, cell migration, cell apoptosis, and production of reactive oxygen species (ROS). Effective cancer treatments have focused on inhibiting epidermal growth factor receptor (EGFR) signalling. Thus, the expression of EGFR protein after extract-treated cells was also determined. Following a 72 incubation, high potential cytotoxicity on MCF-7 cells was observed after SE treatment, followed by FE and FPE treatment. FE, FPE, and SE significantly inhibited cell growth at concentrations of 500, 1,000, and 250 µg/mL, respectively. Also, FE, FPE, and SE markedly suppressed migration of MCF-7 cells at concentrations of 500, 500, and 100 µg/mL, respectively. These results can be concluded that SE had the highest potential anticancer effect on MCF-7 cells when compared with FE and FPE. Thus, SE might be a potential source of preventative and therapeutic agents against breast cancer. Since most anticancer drugs cause ROS production in cancer cells and it is known that ROS induce cell death; therefore, cell apoptosis and ROS formation induced by SE were further studied. The results showed SE induced MCF-7 cell apoptosis in a concentration-dependent way. SE caused a significant increase in ROS formation when compared with the control group. Western blot analysis showed low levels of EGFR protein expression after SE-treated cells at 1,000 mg/mL. Therefore, besides ROS formation, it may be concluded that the downregulation of EGFR protein expression is potentially one of the fundamental mechanisms driving the anticancer effects of SE.

Keywords: apoptosis; cell toxicity; golden shower tree; protein expression; western blotting data

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Introduction

Cassia fistula L. (CF) (common name: the golden shower tree) belongs to the family Fabaceae. CF is the national plant of Thailand, which is cultivated in numerous regions (Limtrakul *et al.*, 2016). In addition, this plant is a popular herb in Ayurveda, which is an alternative medicine system that is alleged to be based on the science of good health and well-being. Ayurvedic CF formulations have been used to treat heart illness, hematemesis, pruritus, leucoderma, abdominal lumps and metabolic disorders (Bahorun *et al.*, 2005; Kaur *et al*., 2020). In women, breast cancer is the second largest cause of cancer associated mortality (Siegel *et al.*, 2016). Alternative cancer treatments derived from medicinal plants are currently being investigated in light of the resistance that develops towards other forms of medication and the side effects associated with chemotherapies (Mitra and Dash, 2018). Anticancer activities have been identified from various parts of CF, as follows. Gupta *et al.* (2000) reported that methanolic extract derived from the seeds of CF [seeds extract (SE)] was able to both reduce the size and quantity of Ehrlich ascites carcinoma breast cancer cells and prolong the age of the treated rats. Duraipandiyan *et al.* (2012) reported that rhein, a substance isolated from an extract of the CF flowers [i.e., flower extract (FE)], exerted cytotoxic effects on colon cancer cells (specifically, COLO 320DM cells) in both a concentration and incubation time dependent manner. Irshad *et al*. (2014) reported that extract derived from the CF fruit pulp [i.e., fruit pulp extract (FPE)] and seeds prepared using ethyl acetate or n-butanol as a solvent possessed anticancer activities on MCF-7 breast and SiHa cervical cancer cells. Kaur *et al*. (2020) reported that extract of the CF pods prepared using hexane as a solvent both exerted cytotoxic effects on HeLa cervical cancer cells and inhibited their growth. On the basis of these findings, it was possible to conclude that extracts derived from various parts of CF may be an effective source of anti-breast cancer compounds; however, in terms of further experiments, it would be interesting, for example, to investigate how extracts derived from this medicinal plant may restrict the cell migration of MCF-7 cells in connection with breast cancer cell metastasis. To the best of the authors' knowledge, there are only a limited number of published studies that have evaluated anticancer activities of the CF extract on MCF-7 cell migration (Irshad *et al.*, 2014). One aim of the present study was therefore to investigate the effects of the CF extract on MCF-7 cell migration. In addition, we are not aware of the prior publication of any comparative studies on the effects of extract derived from various parts of CF on cell growth, cell apoptosis and reactive oxygen species (ROS) production in MCF-7 breast cancer cells. Therefore, the present study aimed to compare the anticancer activities of extracts prepared from the flower (FE), fruit pulps (FPE) and seeds (SE) of the CF on MCF-7 breast cancer cells. Furthermore, given that excess ROS generation has been associated with cell cycle arrest or apoptosis (Shehat and Tigno-Aranjuez, 2020), and also that the majority of chemotherapeutic drugs produce ROS in cancer cells, the underlying mechanism of action of the CF extracts was also investigated, including the possible involvement of ROS generation and cell death. A tyrosine kinase called the epidermal growth factor receptor (EGFR) is involved in the growth, division, and mitosis of cells as well as the development of cancer (Mitra and Dash, 2018; Kong *et al*., 2021). It also has a significant impact on the emergence of medication resistance (Uribe *et al.*, 2021). Inhibiting EGFR signalling has become a key component of modern cancer therapies. Thus, the expression of the EGFR protein in cells treated with extract was also identified.

Materials and Methods

Materials

The Gibco® cell culture medium ingredients were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA), whereas all chemicals and solvents were obtained from Sigma Aldrich (Merck KGaA, Darmstadt, Germany).

Preparation of FE, FPE and SE

CF plants were harvested between February and April 2021 from Phayao, Thailand. The CF specimen (voucher no. MSUT_7659) was kept in the Faculty of Science, Mahasarakham University. The flowers, fruit pulp and seeds were cleaned, dried in an oven at 60 °C (Memmert GmbH & Co.KG, Schwabach, Germany) and cut into small pieces. Subsequently, the small pieces of the plants were extracted with 95% ethanol using a maceration method. The mixture was then filtered, and the filtrate was subsequently concentrated using a rotary evaporator at a controlled temperature of 50 °C (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany). The percentage yields of FE, FPE and SE were 16.18, 17.52 and 4.58% (w/w), respectively. The appearance of all extracts was as a viscous, yellowish paste as shown in Figure 1.

(A) (B) (C)

Figure 1. The appearance of extracts prepared from (A) flower, (B) fruit pulp and (C) seed of *Cassia fistula* L.

Cell culture

MCF-7 cells were grown in Gibco® Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin G and 100 µg/mL streptomycin. The cells were incubated at 37 °C in an atmosphere of 5% CO_2. Every 3 days, the cells were trypsinized with 0.25% trypsin/EDTA, after which the cells were subcultured in fresh DMEM.

Cell cytotoxic assay

The sulforhodamine B (SRB) is the method used to measure cellular protein content which indicates the cell density. Therefore, it was used to investigate the cytoxicity of the FE, FPE, and SE on MCF-7 cells. In a brief, MCF-7 cells ($1x10^4$ cells) were cultured in a 96-well plate for 24–72 h with the extract solution at final concentrations of 0-250 µg/mL. SRB dye was then added to the wells. The protein-bound dye was dissolved in 200 µL of a Tris base solution (pH 7.4). Thereafter, the absorbances of the cellular protein-bound dye were measured at 540 nm. The extract's cytotoxicity on MCF-7 cells was then assessed by comparing it to untreated extract cells (control group) and calculating the 50% inhibitory concentration (IC_{50}) values from the dose-response curve.

Clonogenic assay

Clonogenic assay (or colony formation assay) was used to examine the influence of FE, FPE and SE on the ability of a single cell to grow and form a colony. The cells were plated in a 6- well plate at a density of 500 cells/well and cultured in DMEM for 24 h at 37 °C. The extract solution (100 µL/well) was subsequently added at final doses of 0-1,000 µg/mL, and the cells were incubated for a further 24 h. The cells were then washed, new culture medium was added, and the cells were subsequently grown for a further 10 days. Every 2 days, the medium was replaced with fresh medium. Finally, the cultured cells were stained with crystal violet (0.5% in methanol), and a direct counting method was used to determine the number of colonies.

Cell migration assay

The effects of FE, FPE and SE on the inhibition on cell migration was assessed using scratch wound-healing assay. The cells were plated in a 24-well plate at a density of $2.5x10^5$ cells/well, and incubated for 24 h at 37 °C. Subsequently, a sterile pipette tip was used to scratch the surface of the cells, making a straight wound. The extract solution (100 µL/well) was added at a concentration of 0-1,000 µg/mL, and the cells were incubated in DMEM for a further 48 h. The area of the uncovered part of the wound was determined, and an inverted optical microscope (TS100; Nikon Corporation, Japan) was subsequently used to capture the images. From the area data, the percentage of relative closure of the straight wound was computed, and where the closure of the straight wound was relatively restricted, this suggested that the extract had suppressed migration of the cells.

Cell apoptosis assay

The cells were seeded in a 6-well plate at 2.5×10^5 cells/well and then treated with 100 µL of the extracts at concentration of 50-250 µg/mL. After a 24-h incubation period, the cells were harvested and washed twice in PBS and then collected in 0.25% trypsin-EDTA. After that, the cells were added with 100 µL binding buffer, 5 µL Annexin V-FITC, and 1.5 µL propidium iodide solution (Cat No. 558547, BD Biosciences, CA, USA). An additional 15-minute incubation at room temperature in the dark was done. Finally, a flow cytometer was used to quantify viable, early apoptotic, late apoptotic, and necrotic cells. BD Accuri C6 Plus software (BD Biosciences, CA, USA) was used to evaluate the data.

ROS production assay

ROS production induced by the extracts was evaluated using the 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) fluorescent probe. The cells ($2.5x10^5$ cells/well) were cultured in a 6-well plate in the dark for 30 min with extract in final concentrations ranging from 50 to 250 µg/mL plus 25 mM DCF-DA. Subsequently, the treated cells were washed with PBS. Flow cytometer was then used to measure ROS levels. The fluorescence of DCF-DA was monitored for the ROS generation assay, and increased ROS levels suggested a shift to the right (Buranrat *et al.*, 2020).

Western blotting analysis

The SE was applied to the cells at a concentration of 1,000 g/mL and left on them for 24 h at 37 °C. The cells were then collected, washed, and then lysed in RIPA lysis buffer on ice for 30 min. The resulting lysates were then centrifuged at 12,000 x g for 30 min at 4°C. After collecting the supernatant, the protein content in the supernatant was measured using Bradford technique. The samples containing a total of protein (20 µg) were electrophoresed with 12% SDS-PAGE and shifted to an Immobilon® polyvinylidene fluoride membrane. Tris-buffered saline with 0.1% Tween-20 (TBST) was added to the blotting membranes for 2 h at room temperature. Primary antibodies including EGFR and β-actin (ACTB) as the loading control protein (dilution, 1:1,000) were added to the blotting membranes overnight at 4°C, followed by the secondary antibody a (horseradish peroxidase-conjugated (dilution, 1:2,500)) at room temperature for 2 h for 60 min, and the membranes were then detected with the ECL substrate (Bio-Rad Laboratories, Inc.). Immunoreactive bands were visualized and analyzed using ChemiDocTM MP imaging equipment with Image Lab software (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Statistical analysis

The data was reported as means with standard deviations. Sigma Stat software version 3.5 was used to do a one-way analysis of variance (ANOVA) with a post-hoc least significant difference (LSD) test (Systat Software Inc., San Jose, CA, USA). A statistically significant difference was defined as one with a p-value of less than 0.05.

Results and Discussion

SE has the highest cytotoxic effect on MCF-7 cells

The results from the experiments that investigated the cytotoxicity of the extracts SE, FPE and FE on MCF-7 cells revealed that FE exerted significant cytotoxicity on MCF-7 cells at a concentration of 1,000 µg/mL after incubation of the cells for 24, 48 and 72 h (Figure 2A), whereas FPE showed significant cytotoxicity on MCF-7 cells at a treated dose of 1,000 µg/mL for 24 and 48 h, and at a treated dose of 250 µg/mL for 72 h (Figure 2B). SE exerted significant cytotoxicity on MCF-7 cells at treated doses of 500, 250 and 32.25 µg/mL after 24, 48 and 72 h of incubation, respectively (Figure 2C). At 72 h, the IC_{50} values of FE (Figure 2A), FPE (Figure 2B) and SE (Figure 2C) were 1,393.8±180.2, 1,617.8±189.9 and 160.0±36.7 µg/mL, respectively. When comparing SE with FE and FPE, these data revealed that SE had the highest level of cytotoxicity on MCF-7 cells. These results differed from those published by Irshad *et al.* (2014), who reported that CF FPE with ethyl acetate and n-butanol (IC_{50} = 422.2 and 564.5 µg/mL, respectively) exerted more significant cytotoxic effects on MCF-7 cells compared with CF SE (IC_{50} = 451.4 and 575.6 µg/mL, respectively). This discrepancy might have arisen since the cytotoxic agents in CF FPE dissolved more readily in ethyl acetate or n-butanol than they did in ethanol as a solvent, whereas the cytotoxic agents from CF SE dissolved in ethanol more readily than in ethyl acetate or n-butanol.

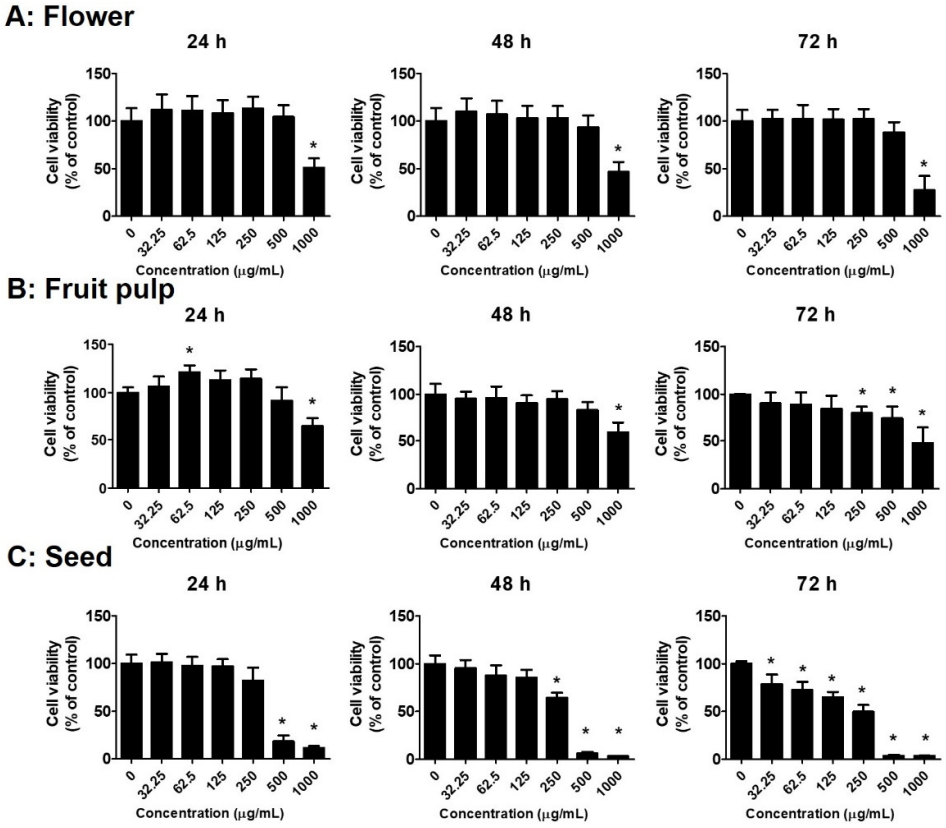

Figure 2. Cytotoxic effects of extract from (A) flower, (B) fruit pulp and (C) seed of *Cassia fistula* L. on MCF-7 cells. The cells were mixed with the extracts at final concentrations of 0–1,000 µg/mL for 24, 48 and 72 h. *p<0.05 when compared with the control

SE is the most effective extract at inhibiting the growth of the MCF-7 cells

The extracts from CF reduced MCF-7 cell growth in a dose-dependent way. The cell growth was significantly inhibited when the cells were treated with FE (Figure 3A), FPE (Figure 3B) and SE (Figure 3C) at concentrations of 500, 1,000 and 250 µg/mL, respectively. The findings from the present study revealed that CF SE could inhibit MCF-7 cell growth more than CF FPE. These results are disagreement with those published by Irshad *et al.* (2014), who reported that the CF FPE could inhibit MCF-7 cell growth more effectively than the CF SE.

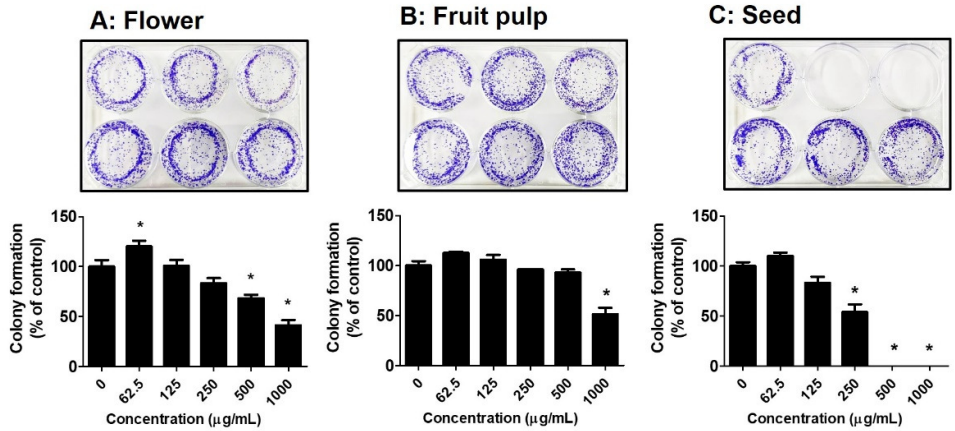

Figure 3. Effects of extract from (A) flower, (B) fruit pulp and (C) seed of *Cassia fistula* L.on MCF-7 cell growth. Cells were mixed with the extracts at final concentrations of 0–1,000 µg/mL. *p<0.05 when compared with the control

SE is the most effective extract at inhibiting the migration of the MCF -7 cells

The potential of cancer cells to undergo metastasis is illustrated by the scratch wound-healing assay experiments. which were performed to assess the MCF-7 cell migration (Amith *et al.*, 2016). As shown in Figures 4A-C, MCF-7 cell migration was significantly suppressed by FE, FPE, and SE at concentrations of 500, 500 and 100 µg/mL, respectively. SE was shown to have the greatest potential in terms of restricting MCF-7 cell migration. These findings suggested that SE could suppress the migration of MCF-7 breast cancer cells, thereby preventing them from spreading.

Cell apoptosis and ROS production

Based on the previous results, SE was selected as the extract of choice for further investigations on cell apoptosis and ROS formation. Figure 5A depicts the impact of SE on the induction of apoptosis in MCF-7 cells. The percentages of viable, early apoptotic, late apoptotic and non-viable cells are represented in the lower left, lower right, upper right and upper left quadrants, respectively. SE triggered apoptosis in MCF-7 cells in a concentration-dependent way, and the percentages of late apoptotic MCF-7 cells treated with 0, 50, 100 and 250 µg/mL of the extract were found to be 6.3, 7.6, 9.5 and 12.0%, respectively. Following the calculation of the percentages of ROS production, it was found that, at concentrations of 0, 50, 100 and 250 µg/mL, the percentages of ROS formation induced with SE were 0.7±0.2%, 1.5±0.4%, 2.3±0.4% and 9.1±1.0%, respectively (Figure 5B). Therefore, compared with the untreated cells (control group), SE significantly increased ROS generation at a concentration of 250 µg/mL. Rhein has been found to induce cell apoptosis and ROS formation (Wang *et al.*, 2020; Kong *et al.*, 2021). Using the high-performance liquid chromatography technique, the rhein contents in SE were 0.34 ± 0.02 mg/g. Thus, SE may have an impact on cell apoptosis and ROS formation due to the presence of rhein.

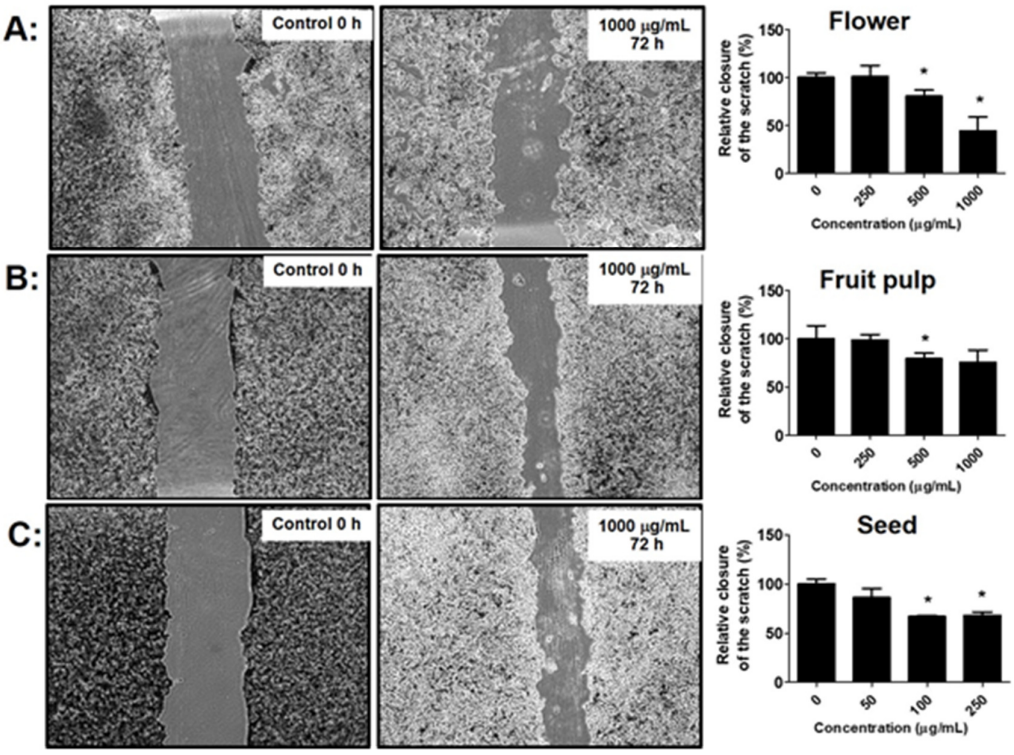

Figure 4. Effects of extract from (A) flower, (B) fruit pulp and (C) seed of *Cassia fistula* L. on MCF-7 cell migration. Cells were treated with the extracts at concentrations of 0–1,000 µg/mL for 72 h. *$p<0.05$ when compared with the control

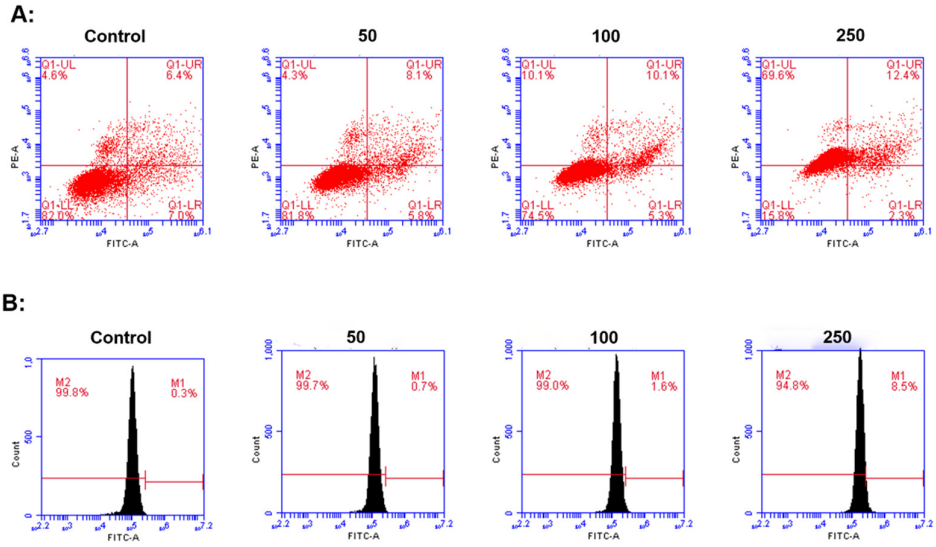

Figure 5. Flow cytometry histograms of cell apoptosis (A) and ROS formation (B) of MCF-7 cells treated with the seed extract of *Cassia fistula* L. Cells were treated with the extracts at concentrations of 0-250 µg/mL

Western blot analysis

The expression of EGFR protein in SE-treated MCF-7 cells was illustrated in Figure 6A. Compared with the untreated cells, SE suppressed the expression of EGFR protein in the cells. After calculation of the amount of the EGFR relative to the β-actin, SE significantly suppressed the expression of EGFR protein at 0.65±0.18 when compared with the β-actin (control protein) at the SE concentration of 1,000 µg/mL (Figure 6B). The result obtained indicated that SE induce apoptosis through EGEF protein inhibition. Compared with a previous study (Kanwal *et al*., 2022), this study revealed that EGFR protein expression is one potentially target for the anticancer effects of *Cassia fistula*.

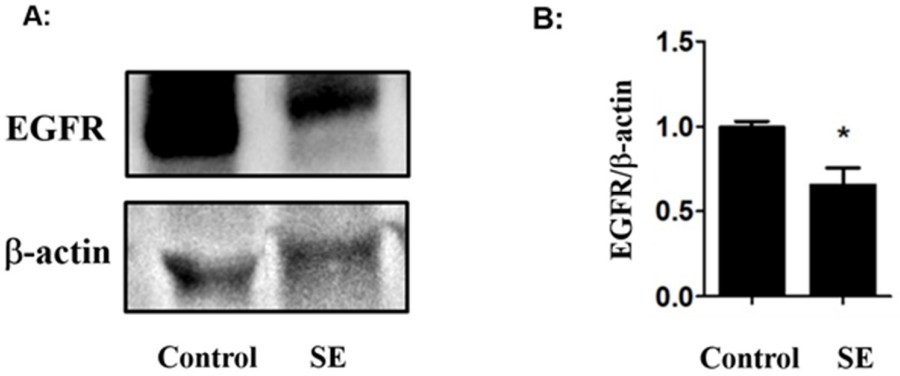

Figure 6. Effect of *Cassia fistula* L seed extract (SE) on the expression levels of epidermal growth factor receptor (EGFR) in MCF-7 cells. (A) Western blotting data of the EGFR levels after treated with the SE at concentration of 1,000 µg/mL for 24 h; (B) The quantification of the EGFR relative to the β-actin (control protein); *$p < 0.05$ when compared with the control group.

This is the first report to determine the anticancer effect of *Cassia fistula* seed extract via EGFR protein expression suppression levels. Compared with our previous report (Boontha *et al*., 2023), the seed extract induced MCF-7 cells apoptosis pattern similar to pod shell extract and leaf extract via induced apoptosis through EGFR protein suppression. However, seed extract inhibited EGFR expression less effective than pod shell extract and leaf extract. This might be due to seed extract containing rhein contents (0.38 mg/g), an active anticancer agent in *Cassia fistula*, less than pod shell extract (1.55 mg/g) and leaf extract (0.76 mg/g).

Conclusions

Based on the results obtained in this study, the following conclusions may be drawn. First, all the extracts from the three parts of CF were shown to inhibit the cell viability, cell growth and cell migration of human breast cancer (MCF-7) cells. CF SE exhibited the most potent anticancer activity towards MCF-7 cells when compared with CF FE and CF FPE. CF SE was also shown to induce cell apoptosis, which was accompanied by an increase in ROS production. Also, SE induce apoptosis through EGEF protein inhibition. Thus, downregulation of EGFR protein expression is potentially one of the fundamental mechanisms driving the anticancer effects of SE. Taken together, these findings have provided a theoretical foundation for CF SE as a putative target for chemotherapeutic medicines and chemoprevention agents against breast cancer; however, further studies are required in order to elucidate the underlying mechanism of action.

Authors' Contributions

S.B. designed the study and the experiments, conducted the project, prepared the extract, analyzed data, and wrote the manuscript. B.B. was responsible for MCF-7 cell study. K.S. characterized the extract. T.P. assisted in experimental work and appraised the manuscript.

All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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