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Cassia fistula L. pod shell and leaf extracts induce cell cytotoxicity and suppress cell migration by downregulating EGFR in MCF-7 cells

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ABSTRACT

Introduction: *Cassia fistula* Linn. (CF) is a well-known Thai medicinal plant and a source of many bioactive compounds. The present work designed to examine the anticancer effects of pod shell and leaf of CF extracts on a human breast cancer cell line via the suppression of tumor development and metastasis. The expression of an epidermal growth factor receptor (EGFR) expression was interested to explore.

Methods: The *in vitro* anticancer activities of the CF pod shell and leaf extracts, including cell cytotoxicity, cell growth, cell migration, cell death, and reactive oxygen species (ROS) formation were evaluated using sulforhodamine B (SRB), colony forming, wound healing, and flow cytometric analysis, respectively. The EGFR protein expression was determined by western blot analysis, which is related to increased cancer cell growth and resistance to apoptosis.

Results: CF pod shell and leaf extracts showed significant toxicities against MCF-7 cells and inhibited the cancer cells proliferation and migratory ability of breast cancer MCF-7 cells in concentration- and time-dependent manners. Both extracts induced late cell apoptosis and significantly generated ROS formation at a dose of 250 mg/mL. Western blotting data exhibited low levels of EGFR protein expression after treating with the extracts at a dose of 1000 mg/mL.

Conclusion: CF pod shell and leaf extracts are able to reduce breast cancer cell proliferation, increase cell apoptosis, and suppress cell migration through the downregulation of EGFR expression indicating anticancer activities.

Implication for health policy/practice/research/medical education:
Cassia fistula pod shell and leaf extracts exhibited anticancer activities on human breast cancer MCF-7 cells. These extracts might be potential sources of chemotherapeutic agents for breast cancer treatment.

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Introduction

The breast cancer is a prominent cause of death in women cancer in the world (1,2). The primary cause of breast cancer death is metastasis, which occurs when initial cancer spreads to other organs such as the bones, brain, liver, lymph nodes, and lungs. The ability of cancer cells to invade or migrate is significantly linked to the progression and spread of cancer (3). Therefore, suppression of cancer cell migration is a major role in breast cancer treatment. Tumor development and metastasis are dependent on angiogenesis (3,4). Epidermal growth factor receptor (EGFR) is an important factor in angiogenesis resulting in the growth and spread of malignancies. EGFR is a transmembrane receptor tyrosine kinase (an HER

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family member) that controls cell adhesion, motility, and apoptosis (5,6). Hence, inhibition of EGFR expression is considered to be a role success in the treatment of breast cancer. The resistance and toxicities in chemotherapy are also important factors in breast cancer failure (7). To overcome these issues, medicinal plant-based cancer therapies are currently being studied.

Cassia fistula Linn. (CF) or golden shower tree is a well-known Thai medicinal plant and is a source of many bioactive compounds (8). It has been used as a popular herb in the alternative medicine system i.e., Ayurveda and Unani medicines. CF has been suggested for the treatment of haematemesis, pruritus, leucoderma, and diabetes, as well as skin ailments, liver problems, and tuberculous glands in Ayurveda and Unani medicines (9,10). Various parts of CF have been found to have anticancer activities (11-14). Rhein is one of the main active components, which have been found in various parts of CF (12,15-20). For anticancer activity, rhein, an active compound isolated from the CF flower extract, showed cytotoxicity on colon cancer cells (COLO 320DM) in concentration- and time-dependent manners (12). Also, rhein has been shown to inhibit cell proliferation in the human breast, colon, central nervous system, and lung cancer cells and to activate apoptosis in many human cancer cells (18,21). Thus, rhein might be an anticancer agent of the CF extracts. Among the extracts prepared from various parts of CF, the extracts prepared from pod shells and leaves are promising sources for chemotherapeutic agents. However, there are limited published data that have investigated the anticancer activities of the extract of CF on the suppression of MCF-7 cell migratory ability and induction of ROS production in the cells. The inhibition levels of EGFR protein expression of the CF pod shell and leaf extract-treated cells have not been investigated. The fundamental purpose of chemotherapy medications is cytotoxicity. Also, cancer cell growth inhibition is necessary for the inhibition of tumor development. Hence, this work needs to examine the *in vitro* anticancer activities of the CF pod shell and leaf extracts, including cell cytotoxicity, cell growth, cell migration, cell death, and reactive oxygen species (ROS) formation on breast cancer cells, MCF-7. The underlying mechanism of CF extracts by downregulation of EGFR expression was also investigated.

Materials and Methods

Chemicals and reagents

Chemicals and solvents were purchased from TTK science (Bangkok, Thailand). The solvents used for the extraction of CF and for the determination of total flavonoids were analytical grades, while the solvents used for the determination of rhein contents were high-performance liquid chromatography (HPLC) grades. The cell culture mediums used were acquired from Gibco BRL Life Technologies (Grand Island, NY, USA), while chemicals used for studying anticancer effects on cancer cells were acquired from Sigma Aldrich (Merck KGaA, Darmstadt, Germany).

The CF extraction

The ripe pods and leaves of the CF were collected in April 2021 from Phayao province, Thailand. The herbarium of CF was identified and kept by staff at the Faculty of Science, Mahasarakham University (voucher no. MSUT_7659). The pod shells were separated from the CF ripe pods before being used for extraction. The CF pod shell and leaf extracts were prepared as follows. The pod shell and leaves were washed and dried at 60˚C for 24 hours in an oven. Then, dried plants were cut into small pieces and extracted using 95% ethanol. The obtained mixture was filtered and concentrated by a rotary evaporator at a controlled temperature of 50˚C.

Total flavonoid and rhein content determination

Flavonoids have been shown to suppress invasion, apoptosis, metastasis, angiogenesis, and cell growth in various cancer cells (22). Thus, the flavonoid contents of the prepared extracts were determined by using standardized colorimetric assays with rutin (23). The data were presented as rutin equivalent (RE) per gram of crude extract.

Rhein contents of the prepared extracts were determined using HPLC (LC-20AD, Shimadzu, Japan) equipped with an ultraviolet-visible detector (SPD-20A, Shimadzu, Japan). The chromatography was performed on Hypersil BDS C18 (150 × 4.6 mm, 5 µm particle size). The mobile phase solution was consisted of methanol and 0.01% (v/v) aqueous phosphoric acid (85:15), run with the flow rate at 1.0 mL/min, and the peak was detected at 254 nm. The sample volume was injected for 20 µL per of each sample. The rhein content was calculated from the peak area of the HPLC chromatogram and compared with the rhein standard and sample. The sample solution was prepared by dissolving the extracts (5 mg for the CF pod shell extract and 10 mg for the CF leaf extract) in 10 mL of methanol. After the extracts were completely dissolved by sonication, they were filtrated through 0.45-µm nylon membrane filters and then analyzed for rhein content using HPLC.

Cell culture

Human breast cancer MCF-7 cell was cultured at 37˚C in an atmosphere of 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic (penicillin G and streptomycin). After cells were confluent about 70-80%, the cells were trypsinized with 0.25% trypsin-EDTA and then subcultured in a fresh medium every 3 days.

Sulforhodamine B (SRB) method

To evaluate the cytotoxicity of the CF pod shell and leaf

extracts on the MCF-7 cells, SRB was used. The cells were seeded into 96-well plates (1×10^4 cells/well) for overnight. Next day, 100 µL of the medium, containing the CF pod shell and leaf extracts, was mixed to each well with a final dose of 0-1000 µg/mL and further incubated for 24-72 hours. Cells were fixed, 0.4% SRB dying solution were added and incubated at 37˚C for 30 minutes. Protein-bound dye was then solubilized by adding 200 µL Tris base buffer (pH 7.4). The optical density was measured at 540 nm using a spectrophotometer (Opsys MR™). The cytotoxicity of CF pod shell and leaf extracts cancer cells inhibition were determined by comparing them with the untreated cells (control group) and using the dose-response curve to get the 50% inhibitory concentration (IC_{50}).

Colony forming ability method

The effects of CF pod shell and leaf extracts on the capability of a single cell to form a colony were evaluated using a colony forming ability assay. Cancer cells were plated in a 6-well culture plate (500 cells/well), for overnight and then the CF pod shell and leaf extract solutions were incubated to wells to obtain a final dose of 0-1000 µg/mL for 24 hours. At the end of incubation period, cancer cells were washed with PBS buffer, added with complete DMEM medium, and the cells were further incubated for 10 days. The cells were added with 0.5% crystal violet solution, air dried, captured, and the cancer colonies were counted manually. The colony formation was calculated using the following equation:

$$\text{Colony formation (\%)} = \frac{\text{(number of colony formation in the extract-treated cells)}}{\text{(number of colony formation in the untreated cells)}} \times 100$$

Wound-healing method

The effects of CF pod shells and leaf extracts on the suppression of cancer cell migration were performed using a wound-healing method (24). The cells were plated in a 24-well culture plate (2.5×10^5 cells/well) for overnight and 200 µL sterile pipette tips were created to make a artificial wounds. Thereafter, 100 µL/well of the CF pod shell and leaf extract solutions at various concentrations (0-1000 µg/mL) were incubated for 48 hours. Images of the scratch wound were taken with an inverted microscope (TS100; Nikon Corporation, Tokyo, Japan) following 48 hours of treatment. The cell migration rates were measured as a percentage of the wound's area reduction, which increases as cells move over time.

Cell apoptosis analysis

The effects of CF pod shell and leaf extracts on cancer cell apoptosis were carried out using flow cytometry. Cancer cells were plated in a 6-well culture plate (2.5×10^5 cells/well), and 100 µL of the extract solutions at various concentrations (0-1000 µg/mL) were incubated for 24 hours. Cancer cells were added with PBS buffer to wash before being collected in trypsin-EDTA solution (0.25%). Next, 100 µL binding assay buffer was added to each tube with further adding 5 µL Annexin V-FITC and then incubated for 15 minutes in the dark at room temperature. After that 1.5 µL propidium iodide solution were added and measured the cancer cells apoptosis using BD Accuri C6 Plus software (BD Biosciences, CA, USA).

Reactive oxygen species (ROS) formation analysis

The effects of CF pod shell and leaf extracts on ROS formation were carried out using flow cytometry. Cancer cells were seeded in a 6-well culture plate (2.5×10^5 cells/well) for overnight. They were then treated with the extract solutions (in final concentrations of 0 to 1000 µg/mL) for 24 hours and the cells were harvested. Cancer cells were incubated with 25 µM 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) for 30 minutes in the dark and measured the ROS levels using BD Accuri C6 Plus software (BD Biosciences, CA, USA).

Western blot analysis

Cancer cells were seeded into 6-well culture plate (2.5×10^5 cells/well) for overnight and exposed to CF pod shell and leaf extracts at various doses of 0-1000 µg/mL for 24 hours. After that, the cells were then harvested and solubilized in ice-cold RIPA buffer for 30 minutes and the lysate solution was centrifuged, the supernatant was collected, and the protein concentration was determined using Bradford's reagent. The sample proteins (20 µg) were analyzed with 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membrane. The blotting membranes were blocked with TBST buffer for 1 hour at room temperature. The membranes were then added with primary antibodies for EGFR and β-actin (ACTB) as the loading control (dilution, 1:1000) at 4˚C for overnight. Consequently, membranes were added with secondary antibody (dilution, 1:2500) for 2 hours at room temperature. The ECL substrate (Bio-Rad Laboratories, Inc.) was used to detect immune-active bands. A ChemiDoc™ MP imaging equipment with Image Lab software (Bio-Rad Laboratories Inc., Hercules, CA, USA) was used to collect and analyze images of specific protein bands.

Statistical analysis

Quantified data were determined by one-way analysis of variance, followed by the least significant difference post hoc test. Sigma Stat software version 3.5 (Systat Software Inc., San Jose, CA, USA) was used to examine the results. *P* value less than 0.05 was considered to indicate a statistically significant difference.

Results

The percentage yields following the extraction of the CF pod shell and leaf extracts were 6.59% and 12.88% (w/w),

respectively. The CF pod shell crude extract contained a flavonoid content of 30.6 ± 1.4 mg RE/g whereas the CF leaf crude extract contained a flavonoid content of 904.1 ± 89.2 mg RE/g. The rhein chromatogram peaks were well resolved using HPLC analysis under isocratic elution with retention durations of 3.2 minutes, and the rhein contents in the CF pod shell and leaf extracts were 1.55 ± 0.02 and 0.76 ± 0.02 mg/g, respectively.

The CF pod shell and CF leaf extracts induced cytotoxicity in MCF-7 cancer cells

As depicted in Figure 1, CF pod shell and leaf extracts at doses ranging from 32.25 to 1000 µg/mL had dose- and time-dependent toxicities on MCF-7 cells following incubation at 24-72 hours. The CF pod shell extract exhibited significant toxicity against MCF-7 cells at 1000, 1000, and 62.5 µg/mL after incubation periods at 24, 48, and 72 hours, respectively, as compared with the untreated cells (Figure 1A). On the other hand, the CF leaf extract showed significant toxicity against MCF-7 cells at 500, 500, and 62.5 µg/mL after treated with the extract at 24, 48, and 72 hours, respectively (Figure 1B). At 72 hours, the IC_{50} of CF pod shell and leaf extracts on MCF-7 cells were 180.87 ± 14.35 and 331.97 ± 64.17 µg/mL, respectively. This data indicated that CF pod shell extract was more toxic to MCF-7 cells than the CF leaf extract.

The CF pod shell and leaf extracts inhibited MCF-7 cancer cell growth

The CF pod shell and leaf extracts inhibited cancer cell growth in a concentration-dependent manner (Figure 2). The CF pod shell extract strongly suppressed cancer cell replication at 500 µg/mL (Figure 2A), whereas the CF leaf extract exhibited a significant inhibition of cancer cell growth at 62.5 µg/mL (Figure 2B). The IC_{50} of CF pod shell and leaf extracts on MCF-7 cells were 697.03 ± 117.35 and 34.53 ± 10.57 µg/mL, respectively. The data demonstrated that CF leaf extract inhibited the MCF-7 cell growth more effective than the CF pod shell extract.

The CF pod shell and leaf extracts decreased MCF-7 cancer cell migration

Similar results were observed in the reduction of the MCF-7 cancer cell growth assay. The CF pod shell and leaf extracts decreased cancer cell migration in a concentration-dependent manner. Compared with the untreated cells, the results revealed that the wound size was reduced to ~20 and 10% after being exposed to 1000 µg/mL of CF pod shell (Figure 3A) and leaf extracts (Figure 3B). The IC_{50} of the CF pod shell and leaf extracts in MCF-7 cells were 399.40 ± 44.70 and 280.96 ± 16.22 µg/mL, respectively. The data demonstrated that the CF leaf extract suppressed MCF-7 cell migration more effectively than the CF pod shell extract.

The CF pod shell and leaf extracts induced MCF-7 cancer cell apoptosis

The impacts of the CF pod shell and CF leaf extracts on the activation of MCF-7 cell apoptosis are presented in Figures 4A and 4B. The percentages of viable (lower left), early apoptotic (lower right), late apoptotic (upper right), and necrotic cells (upper left) were shown and the data reported that both extracts triggered cancer cell apoptosis in a concentration-dependent manner. Pod shell extract increased late apoptotic cells of MCF-7 cells after treating with 0, 100, 250, and 1000 µg/mL and the data were ~4.6,

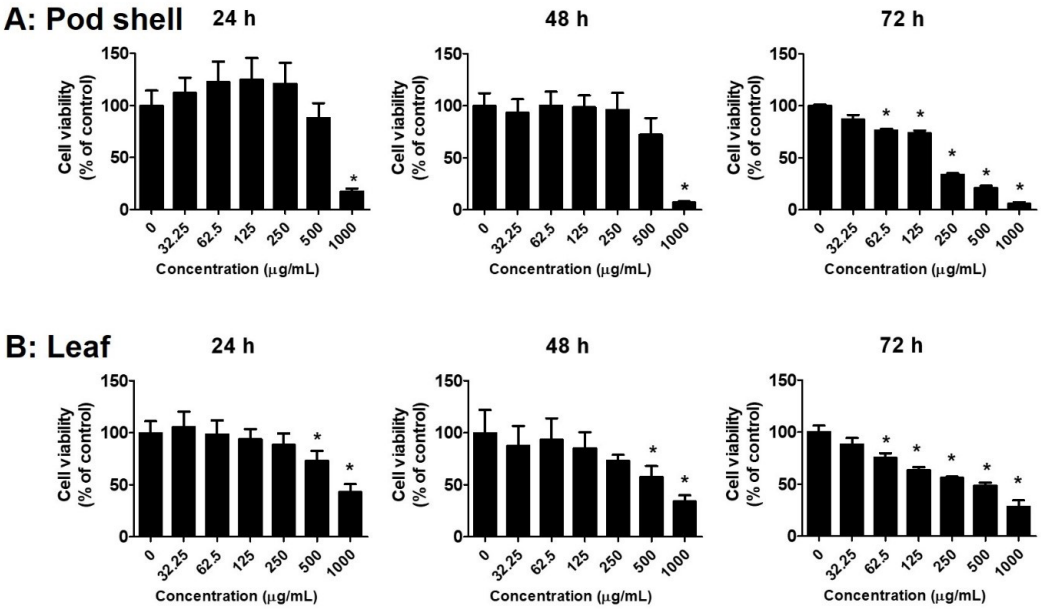


Figure 1. Cytotoxicity effects of *Cassia fistula* (A) pod shell and (B) leaf extracts on MCF-7 cancer cells. **P*<0.05 when compared with the untreated control group.

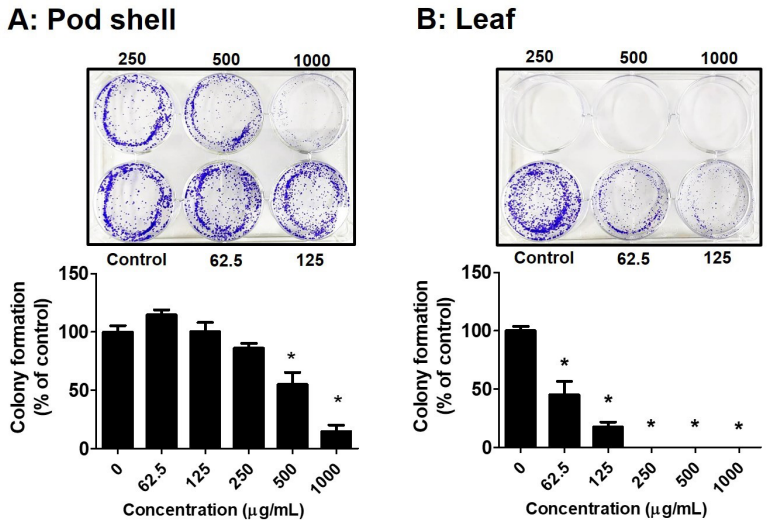


Figure 2. Effects of *Cassia fistula* (A) pod shell and (B) leaf extracts on MCF-7 cell growth. **P*<0.05 when compared with the untreated control group.

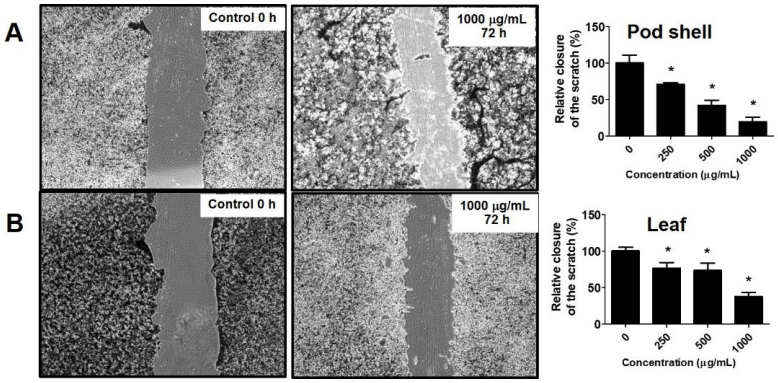


Figure 3. Effects of *Cassia fistula* (A) pod shell and (B) leaf extracts on MCF-7 cell migration. **P*<0.05 when compared with the untreated control group.

11.0, 12.3, and 11.9%, respectively (Figure 4A), correlated with leaf extract and the data were ~5.5, 10.4, 10.7, and 16.0%, respectively (Figure 4B). These data demonstrated that CF leaf extract induced MCF-7 cancer cell apoptosis more effectively than the CF pod shell extract.

The CF pod shell and leaf extracts induced ROS formation in MCF-7 cancer cells

ROS generation is required for the triggering of cancer cells apoptosis and depolarization of mitochondrial membranes. Phytochemicals have been shown to increase the oxidative stress level in cancer cells by inducing ROS production (25). Thus, the ROS production induced with the CF pod shell and leaf extracts was evaluated. For the formation of ROS experiment, the DCF-DA fluorescence-probe was used, and higher ROS levels indicated a shift to the right (26). At concentrations of 0, 250, 500, and 1000 µg/mL, ROS production in the CF pod shell extract-treated MCF-7 cells were 3.3 ± 0.8, 26.5 ± 8.5, 12.9 ± 3.5, and 1.2 ± 0.1%, respectively (Figure 5A), whereas ROS production in the CF leaf extract-treated MCF-7 cells was 1.0 ± 0.1, 7.4 ± 0.3, 11.9 ± 0.9, and 16.0 ± 0.9%, respectively (Figure 5B). The results showed that both extracts significantly increased ROS production at a dose of 250 µg/mL when compared with the untreated control group. Next, CF leaf extract induced ROS formation in a concentration-dependent manner at doses of 0-1000 µg/mL (Figure 5B). However, the CF pod shell extract at a low concentration (250 µg/mL) induced the highest ROS level, and at high concentrations (500-1000 µg/mL) decreased ROS levels by increasing the dose (Figure 5A).

The CF pod shell and leaf extracts reduced EGFR expression in MCF-7 cancer cell

EGFR protein expression in the CF pod shell and leaf extract in MCF-7 cancer cells is depicted in Figure 6A. The CF pod shell and leaf extracts inhibited the expression of EGFR in MCF-7 cells. Following the determination of the amount of the EGFR relative to the ACTB, the 1000 µg/mL of CF pod shell and leaf extracts significantly inhibited the expression of EGFR protein at 0.17 ± 0.10 and 0.41 ± 0.05, respectively, compared with the untreated control group

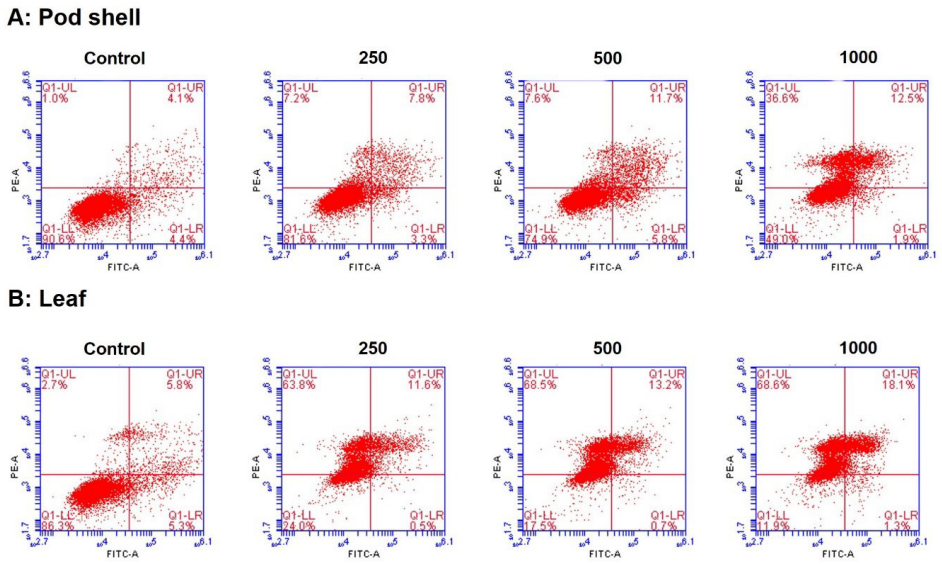
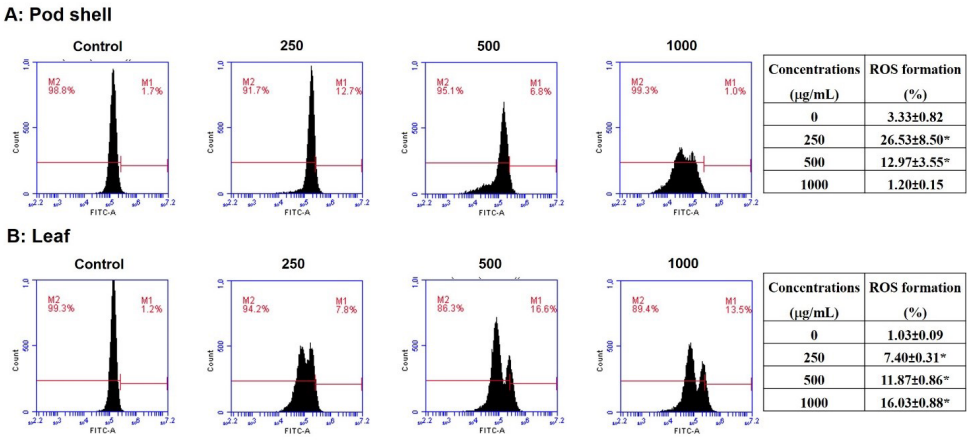


Figure 4. Flow cytometry histograms of the apoptosis of MCF-7 cells treated with *Cassia fistula* (A) pod shell and (B) leaf extracts at concentrations of 0-1000 µg/mL.



Concentrations (µg/mL)	ROS formation (%)
0	3.33±0.82
250	26.53±8.50*
500	12.97±3.55*
1000	1.20±0.15

Concentrations (µg/mL)	ROS formation (%)
0	1.03±0.09
250	7.40±0.31*
500	11.87±0.86*
1000	16.03±0.88*

Figure 5. Flow cytometry histograms of ROS production in MCF-7 cancer cells incubated with *Cassia fistula* (A) pod shell and (B) leaf extracts at concentrations of 0-1000 µg/mL. *P<0.05 when compared with the untreated control group. ROS: Reactive oxygen species.

(Figure 6B). Data demonstrated that the CF pod shell and leaf extracts induced apoptosis through EGEF protein suppression and the CF pod shell extract inhibited EGFR expression more effectively than the CF leaf extract.

Discussion

Breast cancer is the most frequent cancer among women and continues to be the second greatest cause of cancer mortality. One reason for this high mortality rate is the cancer cells' invasive tendency, which leads to breast cancer metastasis (3). As a result, if cancer development and aggressive behavior is suppressed, breast cancer metastasis will be inhibited, and the patients' survival rate will be increased. EGFR plays a key role in angiogenesis, which contributes to cancer cell proliferation and dissemination (5,6). In addition, EGFR levels are associated with increased proliferation and resistance to cancer cell apoptosis. Therefore, the downregulation of EGFR has been proposed for breast cancer treatment. An herbal plant is a promising source for breast cancer treatment based on drug-resistant and side effects from chemotherapy. Phytochemicals are secondary plant metabolites, which can prevent cancer by suppressing the ROS-scavenging pathway, activating oxidative stress-related cell death, stimulating apoptosis-correlated signals, and decreasing pro-survival pathway that promote cancer cell proliferation (22). The flavonoids in the aerial parts of the CF have already been confirmed (10). Flavonoids are a class of polyphenolic chemicals with anti-oxidant properties. They have shown to suppress invasion, metastasis, angiogenesis, apoptotic processes, and cell cycle arrest in cancer cells (22). Flavonoids operate antioxidants activity under normal circumstances; however, they are strong pro-oxidants in several pathways in cancer cells inhibiting pro-inflammatory pathways

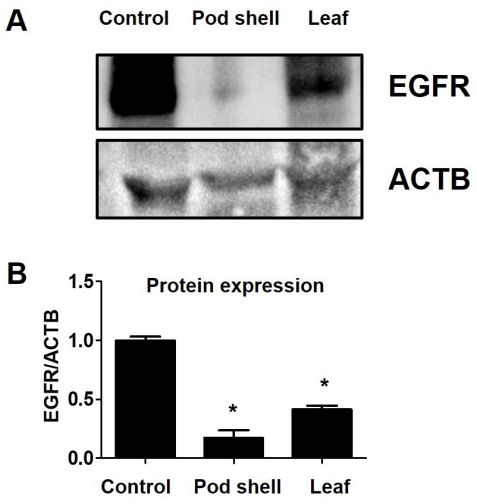


Figure 6. Flow cytometry histograms of ROS production in MCF-7 cancer cells incubated with *Cassia fistula* (A) pod shell and (B) leaf extracts at concentrations of 0-1,000 µg/mL. *P<0.05 when compared with the untreated control group. ROS: Reactive oxygen species.

and activating apoptotic pathways. This gives them a dual effect regarding ROS homeostasis (22). Thus, plants with antioxidant activity could increase the ROS levels in cancer cells.

CF is a well-known Thai medicinal plant as a source of many bioactive compounds i.e., anthraquinone derivatives, flavonoids, sterols, and terpenoids. Anticancer activities of the extract from different parts of the CF have been reported (11-14). In this work, the potential effects of CF pod shell and leaf extracts on human breast MCF-7 cancer cells were explored. Data showed that flavonoid contents were 30.6 ± 14.0 and 904.1 ± 89.2 mg RE/g for the CF pod shell and leaf crude extracts, respectively. The rhein contents in the CF pod shell and leaf extracts were 1.55 ± 0.02 and 0.76 ± 0.02 mg/g, respectively. Flavonoid contents were greater level in the CF leaf extract than in the CF pod shell extract. Also, the rhein content was higher in the CF pod shell extract than in the CF leaf extract. These results were inconsistent with those of a previous study showing that the content of flavonoid was higher in the CF pod extract than in the CF leaf extract (10). This might be due to the effects of geographic location, plant developmental stage, and solvent type for plant extraction (10,27). The present *in vitro* anti-breast cancer study revealed that the CF pod shell and leaf extracts induced MCF-7 cell death by concentration- and time-dependent manners (Figure 1), alongside inhibiting cell growth (Figure 2), suppressing cell migration (Figure 3), and inducing cell apoptosis (Figure 4). ROS generation in extract-treated cells has been confirmed as a mechanism for cell apoptosis. The mechanism underlying the CF downregulation of EGFR protein expression was then determined. The results showed that the CF pod shell and leaf extracts significantly inhibited the level of EGFR protein expression, which was related to increased proliferation and resistance to cancer cell apoptosis. Our findings confirmed the data obtained from Kopustinskiene et al (22) who demonstrated that flavonoids modulated the enzymes activities that could be scavenged the ROS levels, involved in the stop of cell cycle distribution, activating apoptosis, stimulating autophagy, and reducing the growth/proliferation and invasion of cancer cells. It has been reported that the ethanolic extract from the leaves of *Trigonella erythema* contained flavonoid contents of 0.7 mg/mL and exerts cytotoxicity on MCF-7 cells by inhibiting cell viability by ~48% (28). This could be confirmed by our results, as the obtained extracts contained flavonoid contents of 30.6 ± 14.0 and 904.1 ± 89.2 mg RE/g for the CF pod shell and leaf crude extract, respectively. Rhein significantly inhibited cell proliferation, apoptosis, and migration in colorectal cancer cells by directly targeting mammalian target of rapamycin (mTOR) (21). The present findings confirmed these results. The CF pod shell extract exerted a higher cytotoxicity on MCF-7 cells and inhibited EGFR expression more effectively than the CF leaf extract. This might be due to the higher rhein content found in the CF pod shell extract when compared to the CF leaf extract. On the contrary, the CF leaf extract inhibited MCF-7 cell growth more effectively than the CF pod shell extract. This might be due to the higher flavonoids content found in the CF leaf extract. These results were consistent with those of the previous studies showing the cytotoxic effects of CF leaf extract on various cancer cell lines (29). However, the ethanolic CF leaf extract in the present study could be safer than the ethyl acetate CF leaf extract used on cancer patients. Regarding our results, this is the first study on the mechanism underlying the CF downregulation levels of EGFR protein expression. This study provided novel insights into the anticancer effects of the CF pod shell and leaf extracts on breast cancer cell lines and their effective uses in anti-breast cancer therapy.

Conclusion

The CF pod shell and leaf extracts decrease breast cancer cell growth, activate apoptosis, suppress migration, and induce ROS formation in MCF-7 cells. Both extracts also decrease EGFR expression. These findings may indicate that the CF pod shell and leaf extracts are potential sources of chemotherapeutic agents for breast cancer treatment. The CF pod shell extract exerted higher cytotoxicity on MCF-7 cells and inhibited EGFR expression more effectively than the CF leaf extract. However, the CF leaf extract inhibited the MCF-7 cell growth more effectively than the CF pod shell extract.

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Authors' contribution

SB designed the study and the experiments, conducted the project, and prepared the manuscript. BB was responsible for MCF-7 cell study. PJ, SP, KJ, TL, and KS prepared and characterized the extract. TP assisted in experimental work and appraised the manuscript.

Conflict of interests

The authors declare no conflict of interest.

Ethical considerations

Ethical issues related to plagiarism have been carefully observed by the authors. All the experiments were performed in accordance with relevant guidelines and regulations. The protocol was approved by the Research Committee of the Faculty of Medicine, Mahasarakham University, Thailand.

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References

1. Siegel RL, Miller KD, Fuchs HE, Jemal A. Cancer statistics, 2022. CA Cancer J Clin. 2022;72(1):7-33. doi: 10.3322/caac.21708.
2. Azamjah N, Soltan-Zadeh Y, Zayeri F. Global trend of breast cancer mortality rate: a 25-year study. Asian Pac J Cancer Prev. 2019;20(7):2015-20. doi: 10.31557/apjcp.2019.20.7.2015.
3. Sliva D, Jedinak A, Kawasaki J, Harvey K, Slivova V. *Phellinus linteus* suppresses growth, angiogenesis and invasive behaviour of breast cancer cells through the inhibition of AKT signalling. Br J Cancer. 2008;98(8):1348-56. doi: 10.1038/sj.bjc.6604319.
4. Longatto Filho A, Lopes JM, Schmitt FC. Angiogenesis and breast cancer. J Oncol. 2010;2010:576384. doi: 10.1155/2010/576384.
5. Kong FT, He CX, Kong FL, Han SF, Kong XS, Han WQ, et al. Grape seed procyanidins inhibit the growth of breast cancer MCF-7 cells by down-regulating the EGFR/VEGF/MMP9 pathway. Nat Prod Commun. 2021;16(2):1-8. doi: 10.1177/1934578x21991691.
6. Mitra S, Dash R. Natural products for the management and prevention of breast cancer. Evid Based Complement Alternat Med. 2018;2018:8324696. doi: 10.1155/2018/8324696.
7. Nickerson NK, Mohammad KS, Gilmore JL, Crismore E, Bruzzaniti A, Guise TA, et al. Decreased autocrine EGFR signaling in metastatic breast cancer cells inhibits tumor growth in bone and mammary fat pad. PLoS One. 2012;7(1):e30255. doi: 10.1371/journal.pone.0030255.
8. Limtrakul P, Yodkeeree S, Thippraphan P, Punfa W, Srisomboon J. Anti-aging and tyrosinase inhibition effects of *Cassia fistula* flower butanolic extract. BMC Complement Altern Med. 2016;16(1):497. doi: 10.1186/s12906-016-1484-3.
9. Kaur S, Pandit K, Chandel M, Kaur S. Antiproliferative and apoptogenic effects of *Cassia fistula* L. n-hexane fraction against human cervical cancer (HeLa) cells. Environ Sci Pollut Res Int. 2020;27(25):32017-33. doi: 10.1007/s11356-020-08916-9.
10. Bahorun T, Neergheen VS, Aruoma OI. Phytochemical constituents of *Cassia fistula*. Afr J Biotechnol. 2005;4(13):1530-40. doi: 10.4314/ajfand.v4i13.71772.
11. Gupta M, Mazumder UK, Rath N, Mukhopadhyay DK. Antitumor activity of methanolic extract of *Cassia fistula* L. seed against Ehrlich ascites carcinoma. J Ethnopharmacol. 2000;72(1-2):151-6. doi: 10.1016/s0378-8741(00)00227-0.
12. Duraipandiyan V, Baskar AA, Ignacimuthu S, Muthukumar C, Al-Harbi NA. Anticancer activity of rhein isolated from *Cassia fistula* L. flower. Asian Pac J Trop Dis. 2012;2 Suppl 1:S517-S23. doi: 10.1016/s2222-1808(12)60213-8.
13. Irshad M, Mehdi SJ, Al-Fatlawi AA, Zafaryab M, Ali A, Ahmad I, et al. Phytochemical composition of *Cassia fistula* fruit extracts and its anticancer activity against human cancer cell lines. J Biol Act Prod Nat. 2014;4(3):158-70. doi: 10.1080/22311866.2014.933084.
14. Santhi R, Saravanan V. Assessment of in vitro antibacterial, anti-inflammatory, antioxidant and anti-proliferative activity of *Cassia fistula* Linn. methanolic extract. Indo Am J Pharm Sci. 2015;2(2):609-17.
15. Sakulpanich A, Gritsanapan W. Extraction method for high content of anthraquinones from *Cassia fistula* pods. J Health Res. 2008;22(4):167-72.
16. Chewchinda S, Wuthi-udomlert M, Gritsanapan W. HPLC quantitative analysis of rhein and antidermatophytic activity of *Cassia fistula* pod pulp extracts of various storage conditions. Biomed Res Int. 2013;2013:821295. doi: 10.1155/2013/821295.
17. Yingngam B, Zhao H, Baolin B, Pongprom N, Brantner A. Optimization of ultrasonic-assisted extraction and purification of rhein from *Cassia fistula* pod pulp. Molecules. 2019;24(10):2013. doi: 10.3390/molecules24102013.
18. Yuan YF, Hu XY, He Y, Deng JG. Synthesis and anti-tumor activity evaluation of rhein-aloe emodin hybrid molecule. Nat Prod Commun. 2012;7(2):207-10.
19. Antonisamy P, Agastian P, Kang CW, Kim NS, Kim JH. Anti-inflammatory activity of rhein isolated from the flowers of *Cassia fistula* L. and possible underlying mechanisms. Saudi J Biol Sci. 2019;26(1):96-104. doi: 10.1016/j.sjbs.2017.04.011.
20. Henamayee S, Banik K, Sailo BL, Shabnam B, Harsha C, Srilakshmi S, et al. Therapeutic emergence of rhein as a potential anticancer drug: a review of its molecular targets and anticancer properties. Molecules. 2020;25(10):2278. doi: 10.3390/molecules25102278.
21. Zhang H, Yi JK, Huang H, Park S, Park S, Kwon W, et al. Rhein suppresses colorectal cancer cell growth by inhibiting the mTOR pathway in vitro and in vivo. Cancers (Basel). 2021;13(9):2176. doi: 10.3390/cancers13092176.
22. Kopustinskiene DM, Jakstas V, Savickas A, Bernatoniene J. Flavonoids as anticancer agents. Nutrients. 2020;12(2):457. doi: 10.3390/nu12020457.
23. Boontha S, Taowkaen J, Phakwan T, Worauaicha T, Kamonnate P, Buranrat B, et al. Evaluation of antioxidant and anticancer effects of *Piper betle* L (Piperaceae) leaf

extract on MCF-7 cells, and preparation of transdermal patches of the extract. Trop J Pharm Res. 2019;18:1265-72. doi: 10.4314/tjpr.v18i6.17.
24. Amith SR, Wilkinson JM, Fliegel L. Assessing Na+/H+ exchange and cell effector functionality in metastatic breast cancer. Biochim Open. 2016;2:16-23. doi: 10.1016/j.biopen.2016.01.001.
25. Buranrat B, Noiwetch S, Suksar T, Ta-Ut A. Inhibition of cell proliferation and migration by *Oroxylum indicum* extracts on breast cancer cells via Rac1 modulation. J Pharm Anal. 2020;10(2):187-93. doi: 10.1016/j.jpha.2020.02.003.
26. Shehat MG, Tigno-Aranjuez J. Flow cytometric measurement of ROS production in macrophages in response to FcγR cross-linking. J Vis Exp. 2019(145):10.3791/59167. doi: 10.3791/59167.
27. Scarano A, Chieppa M, Santino A. Looking at flavonoid biodiversity in horticultural crops: a colored mine with nutritional benefits. Plants (Basel). 2018;7(4):98. doi: 10.3390/plants7040098.
28. Farhan H, Rammal H, Hijazi A, Annan H, Daher A, Reda M, et al. Chemical composition, in vitro cytotoxicity and anti-free radical properties of six extracts from Lebanese *Trigonella berythea* Boiss. Pak J Pharm Sci. 2013;26(6):1157-63.
29. Kaur S, Kumar A, Thakur S, Kumar K, Sharma R, Sharma A, et al. Antioxidant, antiproliferative and apoptosis-inducing efficacy of fractions from *Cassia fistula* L. leaves. Antioxidants (Basel). 2020;9(2):173. doi: 10.3390/antiox9020173.