Effect of isolated bioactive flavonoid apigenin-7-O-β-D-glucuronide methyl ester on cyclooxygenase-2 gene expression in the breast cancer MCF-7 cell lines

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ABSTRACT

Introduction: Breast cancer is one of the most commonly diagnosed cancers worldwide. Apigenin-7-O-β-D-glucuronide methyl ester is a bioactive flavonoid and has been isolated from the ethyl acetate leaf extract of Manilkara zapota, shown in vitro anticancer activities. Objective: The present study was aimed to investigate the possible inhibitory activity of apigenin-7-O-β-D-glucuronide methyl ester on the cyclooxygenase (COX)-2 gene expression in the breast cancer MCF-7 cell lines. Materials and Methods: MCF-7 cell lines were cultured in monolayers in RPMI 1640 and antiproliferative activity of apigenin-7-O-β-D-glucuronide methyl ester was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), a yellow tetrazolium (MTT) assay with different concentrations, the levels of COX-2 gene expression were measured by reverse transcription real-time polymerase chain reaction (RT-PCR). Results: The results showed that apigenin-7-O-β-D-glucuronide methyl ester (1, 5, 10, 50, and 100 μg/mL) shown significantly exhibited a concentration-dependent cytotoxic effect on MCF-7 breast cancer cell with half maximal inhibitory concentration (IC₅₀) value of 40.17 µg/ml comparing with standard tamoxifen with IC₅₀ values of 7.72 µg/ml. From the RT-PCR analysis showed apigenin-7-O-β-D-glucuronide methyl ester significantly inhibited the COX-2 gene expression fold −2.5 and −10.31 in MCF-7 cell lines. Conclusion: From this study, it can be concluded that the apigenin-7-O-β-D-glucuronide methyl ester, significantly inhibits the growth and attenuates the COX-2 gene expression in MCF-7 breast cancer cells.

KEY WORDS: Anticancer activity, Apigenin-7-O-β-D-glucuronide methyl ester, Cytotoxicity, Manilkara zapota, MCF-7, Real-time polymerase chain reaction

INTRODUCTION

Breast cancer is one of the usually diagnosed cancers and the second leading cause of cancer death in women worldwide. The incidence of breast cancer is approximately 10.4%, and it is the most common cancer in women, showing annual 1–2% increases, and causes high morbidity and mortality. About 30% of the patients with early-stage breast cancer have recurrent disease. The prognosis of breast cancer depends on various biological and molecular factors. Cyclooxygenase (COX) group of enzymes is important for the conversion of arachidonic acid to prostaglandins and thromboxane, COX-1 is constitutively expressed at a constant level throughout the cell cycle in most of the tissues for maintain normal functioning of gastrointestinal tract and COX-2, an inducible enzyme, plays a key role in pathophysiological processes of inflammation as well as several cancers including breast, prostate, lung, liver, cervical, intestinal, and skin. From the recent studies suggest that COX-2-derived metabolites such as PGE₂ and TXA₂ contribute to the maintenance of tumor viability, premalignant hyperproliferation, tumor growth, transformation, invasion, and metastatic spread, and COX-2 has been
shown to be overexpressed in many human malignant tumors. Prostaglandins intensification the expression and activation of aromatase, an enzyme that converts androgen to estrogen. Estrogen can stimulate the growth of cancer cells through the activation of the estrogen receptor (ER) and its target genes. COX-2 expression is associated with angiogenesis and lymph node metastasis in human breast cancer. Breast cancer over time acquires different mutations and the proportion of ER-negative cells in tumor increases.

Medicinal plants scientifically used for the treatment of breast cancer; however, theoretically, medicinal plants have the potential to constitute a very important aspect of drug discovery due to the fact that, if they are properly harnessed, they could address the problem of synthetic drugs being largely out of reach to poorer populations in developing countries. Apigenin-7-O-β-D-glucuronide methyl ester a bioactive flavonoid isolated from the Manilkara zapota ethyl acetate leaf extract had significant in vitro PLA2 inhibitory activity, and exhibited a wide range of in vitro antiproliferative activity against HT-29 (colon cancer cell line), MCF-7 cell line (breast cell line) and HEP G2 (liver cancer cell line).

It also showed dose-dependent significant inhibitory effect on proinflammatory enzymes such as 5-lipoxygenase (5-LOX) and COX-2, pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β) in dose-dependent manner and shown in vivo anti-inflammatory potential at late phase of carrageenan induced inflammation in Wistar rats. Hence, the present study was aimed to evaluate the inhibitory effect of 5-LOX and COX-2 dual inhibitor apigenin-7-O-β-D-glucuronide methyl ester on COX-2 gene expression in the MCF-7 cell line for the development of potent plant-derived anticancer drugs for the treatment of breast cancer.

MATERIALS AND METHODS

The apigenin-7-O-β-D-glucuronide methyl ester (isolated from the M. zapota ethyl acetate leaf extract) was dissolved in 10% dimethyl sulfoxide (DMSO) to give a final concentration of DMSO not >0.5% and did not affect cell survival. Roswell Park Memorial Institute (RPMI) 1640 with L-glutamine were purchased from GibcoBRL (Life Technologies, Grand Island, NY, USA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and DMSO were purchased from Sigma Chemical Co. (St. Louis, MO, USA), Trizol reagent, SYBR Green-I reagent.

**MCF-7 (Breast Cancer Cell Line) Cell Culture**

MCF-7, cell lines were obtained from American type culture collection (Manassas, VA, USA) and maintained with RPMI 1640 with L-glutamine supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 U/ml penicillin (in vitro gen), maintained at 37°C in a 5% CO₂ incubator.

**MCF-7 Cell Treatment**

MCF-7 cell lines in the presence or absence of the isolated compound apigenin-7-O-β-D-glucuronide methyl ester and analyzed for gene expression as studied by real-time polymerase chain reaction (RT-PCR) analysis. The effect of the apigenin-7-O-β-D-glucuronide methyl ester derivative was studied by employing two doses, namely 50 and 100 μg/ml in MCF-7 cell line, and then analyzed for COX-2 gene expression fold.

**Total RNA Extraction and cDNA Synthesis**

Total RNA was extracted by the TRIZOL Reagent according to the manufacturer’s instructions. The concentration of prepared RNA was measured by a nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and its integrity was confirmed by electrophoresis on a 1.2% agarose gel containing 1% formaldehyde. After the RNA was prepared, cDNA was synthesized by the First-Strand Synthesis Kit according to the manufacturer’s instructions. The synthesized cDNA was immediately used in RT-PCR or stored at −70°C for later use.

**RT-PCR**

For gene expression, RT was performed using PrimeScript™ 1st strand cDNA Synthesis Kit (TAKARA BIO INC.) following the manufacturer’s instructions. RT reactions contained 1 μg of total RNA samples, 1 μl of Oligo dT Primer (50 μM), 1 μl of dNTP Mixture (10 mM each), 4 μl of ×5 PrimeScript Buffer, 0.5 μl of RNase Inhibitor (40 U/μl), and 1 μl of PrimeScriptRTase (200 U/μl) and were topped off to 20 μL with DEPC-treated water. The thermal profile for RT consisted of incubation at 42°C for 60 min and termination of the reaction at 95°C at 5 min. One microliter of undiluted cDNA was used in the RT-quantitative PCR (RT-qPCR) protocol. RT-qPCR was performed with the QuantiNova SYBR Green PCR kit (Qiagen, Valencia, CA, USA). Quantitative RT-PCR was performed using a Rotor-Gene Q 2PLEX HRM RT-PCR system (Qiagen). The qPCR reactions, which were prepared to a final volume of 25 μl, included 12.5 μl of 2 × KAPA SYBR® FAST qPCR master mix (×2) kit (Sigma), 10 μmol forward/reverse primers, and cDNA sample. The amplification protocol involved denaturation at 95°C for 3 min, followed by 40 cycles at 95°C for 10 s and 60°C for 34 s. Beta-actin was used as an internal control. The primer sequences, length, and annealing temperatures are listed [Table 1].

**Statistical Analysis**

All the results were expressed as the mean ± standard error mean of triplicate analysis (n = 3). The statistical analysis was carried out by one-way ANOVA followed by Dunnett’s test and antiproliferative activity of apigenin-7-O-β-D-glucuronide methyl ester was reported as half maximal inhibitory concentration (IC₅₀) values (defined as the concentration of the compound required to inhibit cell proliferation by 50%) using GraphPad Prism software version 6.0. *P ≤ 0.05,
**Results and Discussion**

**Cytotoxic Effect of Apigenin-7-O-β-D-glucuronide Methyl Ester on MCF-7 (Breast Cancer) Cell Lines**

The cytotoxic effect of apigenin-7-O-β-D-glucuronide methyl ester [Figure 1] was evaluated on MCF-7 cancer cell lines with different doses, namely 1, 5, 10, 50, and 100 µg/ml. As shown in Figure 2, a dose-dependent significant anticancer activity of apigenin-7-O-β-D-glucuronide methyl ester was observed at 100 µg/ml by showing 70.37% of cell viability with the significant IC$_{50}$ of 40.17 µg/ml. Tamoxifen was employed as positive control whose IC$_{50}$ value was 8.21 µg/ml.

**Inhibitory Effect of Apigenin-7-O-β-D-glucuronide Methyl Ester on COX-2 gene Expression**

As shown in Table 2, the RT-PCR showed that with an increase in the concentrations at 50 and 100 µg/ml of apigenin7-O-β-D-glucuronide methyl ester in MCF-7 cell lines a concentration depended decreased in the COX-2 mRNA gene expression in fold −2.5 and −10.31 in treated cells, compared with the control cells [Figures 3 and 4]. The difference between the control and treated cells was statistically significant ($P < 0.05$). Krishna Chaithanya et al. reported that the RT-PCR analysis showed that 50 µg/ml of mesuaferrin-A attenuated the COX-2 mRNA expression at the transcription level induced by LPS in RAW 264.7 cells.

The present study was conducted to investigate the possible anticancer activity of apigenin7-O-β-D-glucuronide methyl ester on COX-2 gene expression in the MCF-7 breast cancer cell line. *In vitro* and *in vivo* studies on anticancer activity of flavonoids isolated from herbal formulation revealed that the bioactive flavonoid showed promising anticancer activity against MCF-7 breast cancer cell line with IC$_{50}$ of 24.948 with dose-dependent inhibitory effect on hepatocellular carcinoma in mice.

From the recent *in vivo* and *in vitro* anticancer studies demonstrated that the bioactive secondary metabolites

![Figure 1: Apigenin-7-O-β-D-glucuronide methyl ester](image)

![Figure 2: Dose-dependent effect of apigenin-7-O-β-D-glucuronide methyl ester on MCF-7 (breast cancer cell line) proliferation. Values are expressed as mean ± standard error *m* $P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ represent significant difference compared with control group by Student’s $t$-test ($n = 3$).](image)

![Figure 3: Inhibitory effect of apigenin-7-O-β-D-glucuronide methyl ester on cyclooxygenase-2 gene expression in MCF-7 cell line](image)

![Figure 4: Cyclooxygenase-2 gene expression studies in MCF-7](image)

<table>
<thead>
<tr>
<th>Name of gene</th>
<th>Primers</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>COX-2</td>
<td>F.P</td>
<td>5'-CACTACATCCCTGACCCACTT-3'</td>
</tr>
<tr>
<td></td>
<td>R.P</td>
<td>5'-ATGCTCTCGTGTGATATGT-3'</td>
</tr>
<tr>
<td>Beta-actin</td>
<td>F.P</td>
<td>5'-ATGCTGAGACCCACTTCAACAC-3'</td>
</tr>
<tr>
<td></td>
<td>R.P</td>
<td>5'-CACGTCACACTTCATGATGG-3'</td>
</tr>
</tbody>
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RT-PCR: Real-time polymerase chain reaction, F.P: Forward primer, R.P: Reverse primer, COX: Cyclooxygenase
isolated from medicinal plants such as kaempferol, apigenin, curcumin, resveratrol, and pterostilbene downregulate the nuclear factor kappa, mitogen-activated protein kinases and phosphatidylinositol 3-kinase/AKT (AK strain of mice T’ representing its transforming capabilities in cancer cells) cancer, and inflammatory signaling pathways.\cite{11}

CONCLUSION

The results of the present study demonstrated that apigenin7-O-β-D-glucuronide methyl ester belongs to flavonoid isolated from the M. zapota ethyl acetate leaf extract exhibited significant antiproliferation activity on MCF-7 breast cancer cell line, by downregulating cyclooxygenase-2 mRNA expression. Our findings suggest that apigenin7-O-β-D-glucuronide methyl ester is a functional constituent in the M. zapota medicinal plant and further developed as a novel anticancer agent for curing cancer-related disease without causing any side effects.

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REFERENCES

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