

Tangeretin's Anti-apoptotic Signaling Mechanisms in Oral Cancer Cells: In Vitro Anti-cancer Activity

Received 07/25/2023

Review began 09/19/2023

Review ended 10/09/2023

Published 10/22/2023

© Copyright 2023

GV et al. This is an open access article distributed under the terms of the Creative Commons Attribution License CC-BY 4.0., which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Venkatakarthikeswari GV ¹, Priyadharshini Ranganathan Jr. ², Sinduja Palati ²

¹. Pathology, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai, IND ². Oral and Maxillofacial Pathology, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai, IND

Corresponding author: Priyadharshini Ranganathan Jr., priyadharshinir.sdc@saveetha.com

Abstract

Introduction

Citrus fruit peels contain *Tangeretin*, a natural chemical flavonoid that reinforces plant cell walls and serves as a defense mechanism. Apoptosis, growth inhibition, anti-oxidant, anti-diabetic, and anti-cancer activities are only a few of its many qualities. *Tangeretin's* principal function is to shield healthy cells or tissues from the harmful effects of chemotherapy. The purpose of this study was to investigate the apoptotic activity of *Tangeretin's* impact on KB (oral cancer cells) cell lines.

Materials and method

This study employed *Tangeretin*, in investigating its effects on oral cancer cells. Oral cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM), with 10% fetal bovine serum at 37°C in a 5% CO₂ environment. Cell viability was assessed by seeding oral cancer cells in 96-well plates, exposing them to varying *Tangeretin* concentrations (50 μM, 100 μM, and 200 μM) with growth inhibition of KB cell viability in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and morphological changes in cells were observed under an inverted light microscope at 10x magnification. The results were reported as mean ± standard error mean (SEM) using one-way analysis of variance through IBM SPSS Statistics for Windows, Version 23 (Released 2015; IBM Corp., Armonk, New York, United States).

Result

MTT assay showed a significant reduction in KB cell viability when treated with *Tangeretin*. With a significant decrease in mRNA levels of the anti-apoptotic genes Bcl-2 and Bcl-xL. At 50 μM, 100 μM, and 200 μM, the levels of Bcl-2 were 0.85 ± 0.09, 0.62 ± 0.05, and 0.67 ± 0.05, respectively. Similarly, the mRNA expression of Bcl-xL was 0.82 ± 0.07 for 50 μM, 0.7 ± 0.06 for 100 μM, and 0.77 ± 0.06 for 200 μM. The mRNA expression levels of Bax were 1.1 ± 0.09 for 50 μM, 1.4 ± 0.12 for 100 μM, and 1.3 ± 0.11 for 200 μM, respectively.

Conclusion

Tangeretin showed a promising apoptotic activity in KB cells suggesting its utility as an anti-cancer compound. It prevented the growth and proliferation of cancer cells by acting on pro-apoptotic and anti-apoptotic genes. However, this conclusion is mostly based on the in vitro study. Therefore in vivo animal studies were needed to confirm the findings.

Categories: Genetics, Dentistry, Oncology**Keywords:** apoptosis, tangeretin, oral cancer, cell line, anti-cancer activity

Introduction

Tangeretin is a polymethoxylated flavone (PMF), primarily found in the peels of citrus fruits like tangerines, oranges, and mandarins. It serves as a protective mechanism in plants by strengthening their cell walls. This compound is abundant in citrus fruits and offers various beneficial effects, including apoptosis induction, growth inhibition, immune system enhancement, anti-angiogenic properties, and anti-cancer effects [1]. Extensive research, both in vitro and in vivo, supports its hypothesized bioactivities, such as anti-oxidant, neuroprotection, anti-microbial, anti-inflammatory, and efflux pump inhibition [2].

Tangeretin's role in inhibiting cell growth in the G1 phase by promoting the production of cyclin-dependent kinase inhibitors, specifically p27 and p21, has been demonstrated [3]. It has also been explored as a treatment for oxidative stress-related effects in cancer patients, such as DNA mutations and aberrant cell growth. In experiments with cancer cells, *Tangeretin* administration resulted in cell cycle arrest at

How to cite this article

GV V, Ranganathan P, Palati S (October 22, 2023) Tangeretin's Anti-apoptotic Signaling Mechanisms in Oral Cancer Cells: In Vitro Anti-cancer Activity. Cureus 15(10): e47452. DOI 10.7759/cureus.47452

G2/M/G1/S phases, reduced cell migration, and decreased cell proliferation [4].

Tangeretin has shown anti-cancer properties in breast cancer cells (MCF-7) by inhibiting cell growth and promoting apoptosis through mitochondrial disruption [5]. It also inhibits cancer cell migration and proliferation by causing chromatin condensation, apoptotic body formation, reduction in mitochondrial membrane potential (MMP), increased pro-apoptotic proteins, and nuclear shrinkage. Importantly, *Tangeretin* protects non-cancerous cells and tissues from damage caused by chemotherapy treatments [6].

Numerous in vitro and in vivo experiments consistently demonstrate *Tangeretin*'s ability to inhibit cancer cell growth and progression, highlighting its potential as an anti-cancer agent. However, there is a relative scarcity of research on the detailed mechanisms and efficacy of its anti-cancer properties [7]. This study aimed to evaluate the apoptotic activity of *Tangeretin* in oral cancer cells.

Materials And Methods

Procurement and culture of oral cancer cells and chemicals

Chemicals utilized for the study were described in Table 1. Oral cancer cell line was obtained from National Centre for Cell Science (NCCS), Pune, India. Cancer cells were grown in a Minimal Essential Medium (10% fetal bovine serum at 37°C under 5% CO₂).

Chemicals	Manufacturer details and location
<i>Tangeretin</i>	Sigma-Aldrich Chemicals Private Limited, Bangalore, India
Trypsin-EDTA	Gibco Enterprises, Ontario, Canada
FBS	Gibco Enterprises, Ontario, Canada
antibiotics-antimycotics	Gibco Enterprises, Ontario, Canada
DMEM	Gibco Enterprises, Ontario, Canada
PBS	Gibco Enterprises, Ontario, Canada
JC-1 (5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolylcarbocyanine iodide)	Invitrogen Corporation, Carlsbad, USA
Real-time PCR kit (MESA Green)	Invitrogen Corporation, Carlsbad, USA
MTT	Sigma-Aldrich Chemicals Private Limited, Bangalore, India

TABLE 1: Details of the chemicals used in the experiments carried out in the study.

EDTA: Ethylenediaminetetraacetic acid; FBS: Fetal bovine serum; DMEM: Dulbecco's modified Eagle's medium; PBS: Phosphate buffered saline; PCR: Polymerase chain reaction; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MTT assay, real-time polymerase chain reaction, and gene analysis

Oral cancer cells were introduced into 96-well plates at a concentration of 5x10⁵ cells per well and allowed to adhere overnight. Following this initial attachment, the cells were exposed to varying concentrations of *Tangeretin* in triplicate and then cultured at 37°C in a CO₂-rich environment for 24 hours. Next, we introduced 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into each well and continued the incubation for an additional four hours at 37°C. To dissolve the formazan compounds created from MTT, the cells were resuspended in 200 µl of dimethyl sulfoxide, and the optical density (OD) was measured using a spectrometer at a wavelength of 570 nm. This entire procedure was independently repeated three times. Subsequently, the mean OD ± standard deviation (SD) was computed for each set of replicates. The entire process was carried out in triplicate. To calculate the inhibition rate of cell growth, the following equation was employed: % growth inhibition = (1 - OD_{extract treated})/OD_{negative control} x 100 [8].

Real-time polymerase chain reaction (PCR) is used to measure the amounts of mRNA expression during gene expression studies. The TRI Reagent® (Sigma) was used to separate the total RNA. Using a commercial SuperScript™ III First-Strand cDNA Synthesis Kit (Invitrogen Corporation, Carlsbad, USA), total RNA (2g) from each sample was reverse transcribed in accordance with the manufacturer's instructions. Stratagene's MX3000p PCR machine (Stratagene California, San Diego, USA) was used to conduct real-time PCR. The MESA Green PCR Master Mix (Invitrogen Corporation, Carlsbad, USA), which includes SYBR green dye and all of the PCR components, was used to conduct the reaction. Melting curve analysis for each pair of

primers was used to determine the specificity of the amplified product. Utilizing CFX Manager Version 2.1 (Bio-Rad Laboratories, Inc., Hercules, USA), the data were examined using the comparative CT approach, and the fold change was determined using the 2CT method.

Statistical analysis

Cell viability at different concentrations of *Tangeretin* was recorded and mean ± SEM was calculated. Similarly fold change for Bcl-2, Bcl-xL, and Bax were obtained and mean ± SEM was derived. Further cell viability between three different concentrations of *Tangeretin* was compared using a one-way analysis of variance to analyze any significant difference in fold change at various concentrations.

Results

Tangeretin's viability was evaluated through an MTT assay to investigate its impact. After 48 hours of treatment, the effectiveness of *Tangeretin* exhibited a significant reduction in KB (oral cancer cells) cell viability caused by the treatment (Figures 1, 2). These findings showed the potent cytotoxic influence of *Tangeretin* on KB cells.

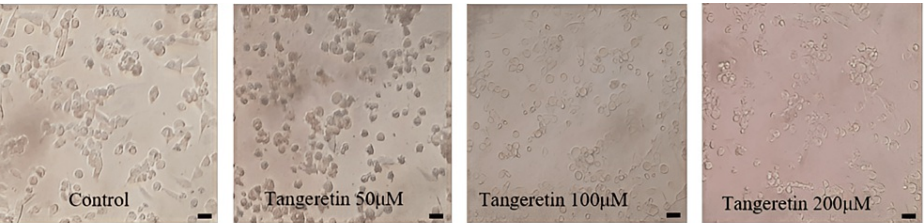


FIGURE 1: Tangeretin was treated with KB cells (50 µM, 100 µM, and 200 µM), and morphological changes in cells were observed under an inverted light microscope at 10x magnification.

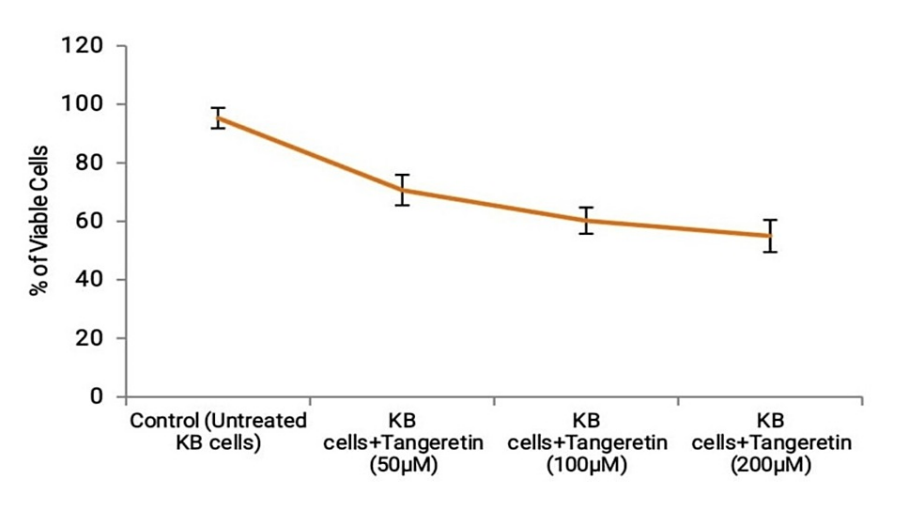


FIGURE 2: Cell viability assessment using MTT assay.
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Tangeretin at various concentrations in KB cell line, was used to assess the mRNA expression levels of Bcl-2, Bcl-xL, and Bax using real-time PCR. In comparison to the control group, *Tangeretin* demonstrated a significant decrease in mRNA levels of the anti-apoptotic genes Bcl-2 and Bcl-xL. At 50 µM, 100 µM, and 200 µM, the levels of Bcl-2 were 0.85±0.09, 0.62±0.05, and 0.67±0.05 (Figure 3). Similarly, the mRNA expression of Bcl-xL was 0.82±0.07 for 50 µM, 0.71±0.06 for 100 µM, and 0.77±0.06 for 200 µM (Figure 4).

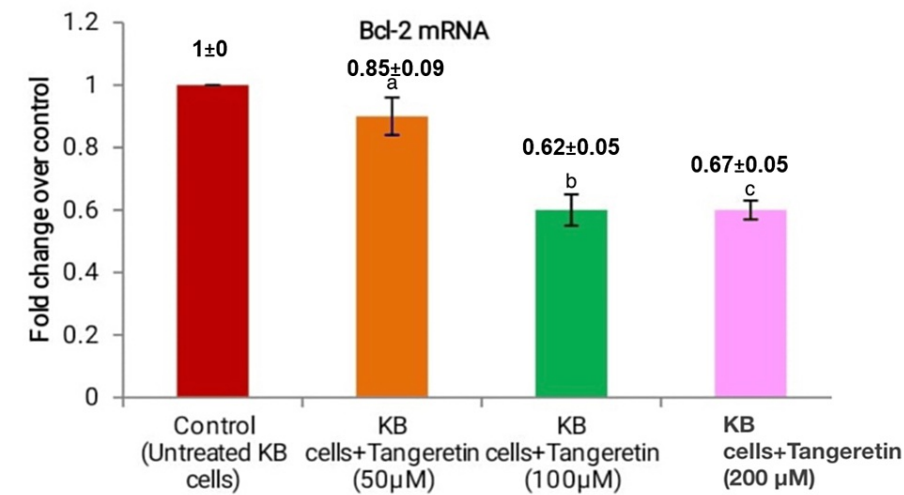


FIGURE 3: Tangeretin concentration at various micromoles in the KB cell line was indicated on the X-axis, and the fold change in Bcl-2 mRNA expression was indicated on the Y-axis. a) 50 µM of Tangeretin in KB cell line, b) 100 µM of Tangeretin, and c) 200 µM of Tangeretin treated KB cells. There was a significant decrease with a rise in Tangeretin concentration, inhibition of cancer cells was seen from 100 µM concentration indicating maximum anti-apoptotic activity.

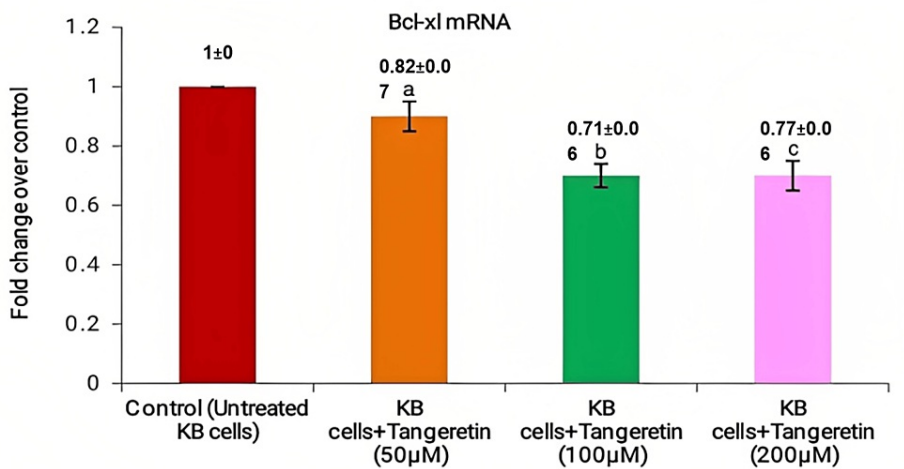


FIGURE 4: Tangeretin concentration at various micromoles in the KB cell line was indicated on the X-axis, and the fold change in Bcl-xL mRNA expression was indicated on the Y-axis. a) 50 µM of Tangeretin in KB cell line, b) 100 µM of Tangeretin, and c) 200 µM of Tangeretin treated KB cells. There was a significant decrease with a rise in Tangeretin concentration, maximum inhibition is seen from 100 µM concentration indicating maximum anti-apoptotic activity.

Moreover, the administration of *Tangeretin* resulted in the suppression of pro-apoptotic gene Bax expression. The mRNA expression levels of Bax were as follows: 1.1±0.09 for 50 µM, 1.4±0.12 for 100 µM, and 1.3±0.11 for 200 µM (Figure 5).

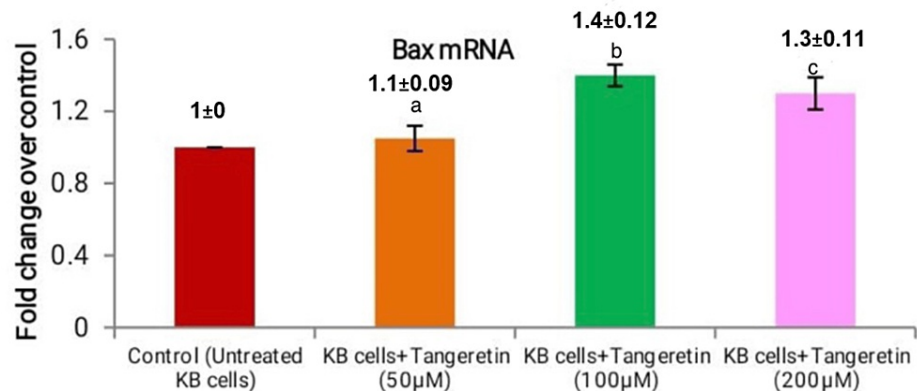


FIGURE 5: Tangeretin concentration at various micromoles in the KB cell line was indicated on the X-axis, and the fold change in Bax mRNA expression was indicated on the Y-axis. a) 50 µM of Tangeretin in KB cell line, b) 100 µM of Tangeretin, and c) 200 µM treated PC-3 cells. An increase in Tangeretin concentration levels showed a considerable rise in pro-apoptotic activity.

Discussion

Tangeretin as a natural flavonoid has good anti-cancer activity, with effective anti-apoptotic properties at higher concentrations. The results demonstrated a 50% reduction in viable cells at higher concentrations of *Tangeretin*. According to the current study, *Tangeretin* alone exhibits strong anti-cancer effects through pro-apoptotic mechanisms. According to a recent study, *Tangeretin* exerts growth-inhibitory activity by suppressing Cdk2 and Cdk4 kinase as well as by elevating p21 and p27 levels. In another study, it reduced COLO205 cell proliferation by arresting cell cycle progression in the G1 phase [9]. Similarly, Chen et al. reported tumor suppression activity by *Tangeretin* in human MCF-7/6 breast cancer cells cultured in female nude mice [10]. Further, *Tangeretin* showed better tumor suppression activity at low doses compared to low-dose cisplatin. This was due to the down-regulation of the PI3K/Akt signaling pathway [11]. These results indicate cytotoxic activity of *Tangeretin* was through apoptotic pathways through apoptotic genes such as Bcl-2, Bcl-xL, Bax, etc.

During programmed cell death, the upregulation of the anti-apoptotic gene Bcl-2 activates tumor suppressor genes through the mitochondria, thereby inducing caspase-9 activation, ultimately leading to apoptosis. However, in the context of carcinogenesis, cancer cells evade apoptosis and instead undergo uncontrolled cellular proliferation. Notably, the current investigation has revealed that *Tangeretin* can effectively target this pathway, leading to the induction of apoptosis. This is substantiated by the observed reduction in Bcl-2 expression levels within the study. These findings align with the work of Zheng et al., who demonstrated that *Tangeretin* disrupts hepatic cancer cell proliferation and migration in HepG2 cells by interfering with the Bcl-2 pathway, causing cell cycle arrest in the M/G2 phase [12]. Similarly, Arivazhagan et al. conducted research on mammary cancer in rats and observed that *Tangeretin* induces G1/S cell cycle arrest through the upregulation of p21 and p53 [13]. Furthermore, a study by Dong et al. on a gastric cancer cell line concluded that the upregulation of Bcl-2 primarily promotes apoptosis through mitochondrial dysfunction mediated by p53 and FasL/Fas signaling [14].

Similarly, Bcl-xL plays a regulatory role in the Akt/PI3K signaling pathway when exposed to *Tangeretin* in cancer cells. Guo et al. observed that *Tangeretin* induces DNA fragmentation by inhibiting anti-apoptotic genes like Bcl-xL, particularly in prostate cancer [15]. Study findings by Das et al. in the context of brain cancer elucidate how *Tangeretin* contributes to the downregulation of Bcl-xL, thereby promoting apoptosis [16]. In the present study, conducted on KB oral cancer cells, we found that increasing *Tangeretin* concentration leads to the inhibition of Bcl-xL expression. As previously mentioned, *Tangeretin* exerts its influence by inhibiting the expression of both Bcl-2 and Bcl-xL genes, consequently promoting the expression of the pro-apoptotic Bax gene. This multifaceted mechanism involves various pathways, including JNK, Akt/PI3K, p27, p53, and cyclins, and ultimately culminates in the activation of caspase 3,

promoting apoptosis as outlined in previous studies [14,16,17]. Previous research has provided evidence of TNF- α 's ability to increase the expression of the anti-apoptotic Bcl-2 protein in individuals afflicted with both high blood pressure [18] and oral cancer [19]. These results clearly highlight the utility of *Tangeretin* as a potential anti-cancer compound.

Limitations

Although *Tangeretin* showed potential apoptotic activity. The results were based on the in vitro study, based on the apoptotic gene expression. However, the study did not investigate the cell signaling pathway. Further, *Tangeretin* was a crude extract therefore specific compound for its apoptotic activity couldn't be ascertained.

Conclusions

Tangeretin showed a promising apoptotic activity in KB cells suggesting its utility as an anti-cancer compound. It prevented the growth and proliferation of cancer cells by acting on pro-apoptotic and anti-apoptotic genes. However, this conclusion is mostly based on the in vitro study. Therefore in vivo animal studies were needed to confirm the findings.

Additional Information

Disclosures

Human subjects: Consent was obtained or waived by all participants in this study. **Animal subjects:** All authors have confirmed that this study did not involve animal subjects or tissue. **Conflicts of interest:** In compliance with the ICMJE uniform disclosure form, all authors declare the following: **Payment/services info:** All authors have declared that no financial support was received from any organization for the submitted work. **Financial relationships:** All authors have declared that they have no financial relationships at present or within the previous three years with any organizations that might have an interest in the submitted work. **Other relationships:** All authors have declared that there are no other relationships or activities that could appear to have influenced the submitted work.

References

1. Agrawal KK, Murti Y: Tangeretin: a biologically potential citrus flavone . *Curr Tradit Med*. 2022, 8:31-41. [10.2174/221508380866220304100702](#)
2. Boye A, Ahmad I, Fakhri S, Hussain Y, Khan H: Incipient citrus polymethoxylated flavone tangeretin as anticancer drug candidate: mechanistic insights, limitations and possible solutions. *Adv Cancer Biol - Metastasis*. 2021, 3:100010. [10.1016/j.adcanc.2021.100010](#)
3. Iyappan P, Bala MD, Sureshkumar M, Veeraraghavan VP, Palanisamy A: Fucoxanthin induced apoptotic cell death in oral squamous carcinoma (KB) cells. *Bioinformation*. 2021, 17:181-91. [10.6026/97320630017181](#)
4. Arafa EA, Shurrab NT, Buabeid MA: Therapeutic implications of a polymethoxylated flavone, tangeretin, in the management of cancer via modulation of different molecular pathways. *Adv Pharmacol Pharm Sci*. 2021, 2021:4709818. [10.1155/2021/4709818](#)
5. Ko YC, Choi HS, Liu R, Kim JH, Kim SL, Yun BS, Lee DS: Inhibitory effects of tangeretin, a citrus peel-derived flavonoid, on breast cancer stem cell formation through suppression of Stat3 signaling. *Molecules*. 2020, 25:[10.3390/molecules25112599](#)
6. Dey DK, Chang SN, Vadlamudi Y, Park JG, Kang SC: Synergistic therapy with tangeretin and 5-fluorouracil accelerates the ROS/JNK mediated apoptotic pathway in human colorectal cancer cell. *Food Chem Toxicol*. 2020, 143:111529. [10.1016/j.fct.2020.111529](#)
7. Matsuzaki K, Ohizumi Y: Beneficial effects of citrus-derived polymethoxylated flavones for central nervous system disorders. *Nutrients*. 2021, 13:145. [10.3390/nu13010145](#)
8. Raj P, Priyadharshini R, Jayaraman S, Sinduja P: Molecular mechanisms underlying chemopreventive anticancer activity of stevioside on human prostate cancer cell line in vitro. *Clin Cancer Investig J*. 2023, 12:8. [10.51847/lvXDMvXRty](#)
9. Raza W, Luqman S, Meena A: Prospects of tangeretin as a modulator of cancer targets/pathways . *Pharmacol Res*. 2020, 161:105202. [10.1016/j.phrs.2020.105202](#)
10. Chen Q, Gu Y, Tan C, Sundararajan B, Li Z, Wang D, Zhou Z: Comparative effects of five polymethoxyflavones purified from *Citrus tangerina* on inflammation and cancer. *Front Nutr*. 2022, 9:963662. [10.3389/fnut.2022.963662](#)
11. Hasan AA, Kalinina E, Nuzhina J, Volodina Y, Shtil A, Tatarskiy V: Potentiation of cisplatin cytotoxicity in resistant ovarian cancer SKOV3/cisplatin cells by quercetin pre-treatment. *Int J Mol Sci*. 2023, 24:10960. [10.3390/ijms241310960](#)
12. Zheng J, Shao Y, Jiang Y, et al.: Tangeretin inhibits hepatocellular carcinoma proliferation and migration by promoting autophagy-related BECLIN1. *Cancer Manag Res*. 2019, 11:5231-42. [10.2147/CMAR.S200974](#)
13. Arivazhagan L, Sorimuthu Pillai S: Tangeretin, a citrus pentamethoxyflavone, exerts cytostatic effect via p53/p21 up-regulation and suppresses metastasis in 7,12-dimethylbenz(α)anthracene-induced rat mammary carcinoma. *J Nutr Biochem*. 2014, 25:1140-53. [10.1016/j.jnutbio.2014.06.007](#)
14. Dong Y, Cao A, Shi J, et al.: Tangeretin, a citrus polymethoxyflavonoid, induces apoptosis of human gastric cancer AGS cells through extrinsic and intrinsic signaling pathways. *Oncol Rep*. 2014, 31:1788-94. [10.3892/or.2014.3034](#)
15. Guo J-J, Li Y-J, Xin L-L: Tangeretin prevents prostate cancer cell proliferation and induces apoptosis via activation of notch signalling and regulating the androgen receptor (AR) pathway and the phosphoinositide

- 3-kinase (PI3k)/Akt/mTOR pathways. *Bangladesh J Pharmacol.* 2015, 10:937-47. [10.3329/bjpp.v10i4.23699](https://doi.org/10.3329/bjpp.v10i4.23699)
16. Das A, Miller R, Lee P, et al.: A novel component from citrus, ginger, and mushroom family exhibits antitumor activity on human meningioma cells through suppressing the Wnt/ β -catenin signaling pathway. *Tumour Biol.* 2015, 36:7027-34. [10.1007/s13277-015-3388-0](https://doi.org/10.1007/s13277-015-3388-0)
 17. Zhu WB, Xiao N, Liu XJ: Dietary flavonoid tangeretin induces reprogramming of epithelial to mesenchymal transition in prostate cancer cells by targeting the PI3K/Akt/mTOR signaling pathway. *Oncol Lett.* 2018, 15:433-40. [10.3892/ol.2017.7307](https://doi.org/10.3892/ol.2017.7307)
 18. Akifa Begum, Palati Sinduja, R Priyadarshini, Selvaraj Jayaraman: Estimation of clinicopathological correlation and comparison of salivary TNF- α among normal and post radiotherapy patients of oral cancer- a cross-sectional study. *J Res Med Dent Sci.* 2021, 9:92-7.
 19. Anushya P, Palati Sinduja, Selvaraj Jayaraman, R Priyadarshini: Clinico pathological correlation of salivary TNF-alpha level in hypertensive patients under treatment. *J Res Med Dent Sci.* 2021, 9:102-8.