ORIGINAL ARTICLE
Iran J Allergy Asthma Immunol
June 2022; 21(3):313-321.
Doi: 10.18502/ijaai.v21i3.9804

Eugenol: A New Option in Combination Therapy with Sorafenib for the Treatment of Undifferentiated Thyroid Cancer

Pedram Talezadeh Shirazi[1,2], Shirin Farjadian[3], Mohammad Hossein Dabbaghmanesh[2], Hossein Jonaidi[1], Ali Alavianmehr[3], Mehdi Kalani[4], and Ladan Emadi[1]

[1] Department of Basic Sciences, Faculty of Veterinary Medicine, Shahid Bahonar, University of Kerman, Kerman, Iran
[2] Shiraz Endocrine and Metabolism Research Center, Shiraz University of Medical Sciences, Shiraz, Iran
[3] Department of Immunology, Shiraz University of Medical Sciences, Shiraz, Iran
[4] Professor Alborzi Clinical Microbiology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran

Received: 2 July 2021; Received in revised form: 25 December 2021; Accepted: 11 February 2022

ABSTRACT

Thyroid cancer (TC) is the most common endocrine malignancy. Thyroidectomy and radiotherapy are common treatment modalities for patients with undifferentiated TC (UTC), and sorafenib is usually recommended to prevent a recurrence. However, malignant cells may evade chemotherapy-induced apoptosis, and combination therapy was developed to achieve better outcomes. This study investigated whether eugenol in combination with sorafenib was more effective than either substance individually in triggering apoptosis in the UTC.

The IC_{50} of sorafenib and eugenol was determined in a UTC cell line (8305C) by MTT assay, and their synergistic effect in combination therapy was investigated. Flow cytometry was used to evaluate the rate of apoptosis in treated cells. To confirm that cell death occurred through apoptosis, immunoblotting was used to determine the relative cleavage of caspase-8 and caspase-9.

The IC_{50} of sorafenib was 20 μM, and that of eugenol was 2100 μM. The sorafenib-eugenol combination (1:105) showed synergistic effects at concentrations equal to or less than their IC_{50}. The rate of apoptosis induction was higher in cells treated with eugenol or the eugenol-sorafenib combination compared to sorafenib-treated cells. The relative intensity of cleaved/uncleaved forms of caspase-8 increased in eugenol-treated cells compared to sorafenib-treated cells.

Sorafenib and eugenol at concentrations equal to or less than their IC_{50} had a synergistic effect in 8305C cells. The most potent apoptotic effect was achieved with sorafenib and eugenol at their IC_{50}. Lower doses of sorafenib could be used with eugenol to improve its efficacy while reducing its side effects.

Keywords: Apoptosis; Drug synergism; Eugenol; Sorafenib; Thyroid neoplasms

Corresponding Author: Ladan Emadi, DVM, PhD; Department of Basic Sciences, Faculty of Veterinary Medicine, Shahid Bahonar University of Kerman, Kerman-Iran. Tel/Fax: (+98 34) 3325 7747, E-mail: emadil@uk.ac.ir

- the first and second authors have equally contributed to this study

INTRODUCTION

Apoptosis is one of the natural mechanisms for homeostasis, and any defect or dysregulation in this process may lead to malignancy.[1] Thyroid cancer (TC),

the most common endocrine malignancy, is a public health concern worldwide.[2] Histologically, TC is classified as differentiated (DTC), poorly differentiated (PDTC), and undifferentiated (UTC).[3,4] Thyroidectomy and radiotherapy are common treatment modalities for patients with UTC; however, these patients are at high risk of cancer recurrence. Tyrosine kinase inhibitors such as sorafenib are usually recommended to prevent TC recurrenc.[5] These inhibitors trigger apoptosis through the intrinsic pathway by interfering with the phosphorylation of the pro-apoptotic proteins necessary for apoptotic factor activation.[6] Despite its benefits, sorafenib has side effects such as hand-foot syndrome, diarrhea, skin rash, weight loss, and fatigue; patients may thus be reluctant to continue taking this medication.[7] Furthermore, because malignant cells may develop mechanisms to evade chemotherapy-induced apoptosis, combination therapy was designed to achieve better outcomes.[8]

In recent years the use of herbal therapy in cancer treatment has attracted the public's attention.[9] Among herbal options, eugenol, a phenolic substance extracted from the cinnamon leaf, honey, and clove,[10] has shown potent anticancer effects in several *in vitro* and *in vivo* studies of colon cancer,[11] breast cancer,[12] acute promyelocytic leukemia,[13]and squamous cell carcinoma.[14] In addition to its conventional medical application as an antiseptic and anesthetic,[11] pain killers,[15] antiviral,[16] antioxidant,[17] and anti-inflammatory compounds,[18] it was considered to trigger apoptosis through the mitochondrial pathway.[19] The present study was designed to determine whether eugenol in combination with sorafenib increases apoptosis in a UTC cell line. Ultimately, the findings can shed light on whether it may be possible to reduce the side effects of sorafenib by using lower doses in combination with eugenol.

MATERIALS AND METHODS

The protocol of this study in applying the correct principles of working with human cancer cell lines and the proper disposal of a toxic substance was approved by the Ethics Committee of the Faculty of Veterinary Medicine, Shahid Bahonar University of Kerman (approval number: IR.UK.VETMED.REC.1400.014).

Cytotoxicity Assay

Cells of the 8305C UTC cell line (Pasteur Institute of Iran, Tehran, Iran) were cultured in CM10 containing Dulbecco's modified Eagle medium (DMEM, Gibco, Billings, MT, USA), 10% FBS (fetal bovine serum, Gibco), 1% penicillin/streptomycin (Sigma Aldrich, Darmstadt, Germany), and 1% L-glutamine at 37°C in a humidified incubator with 5% CO_2. Sorafenib powder (Merck, Mannheim, Germany) was reconstituted in DMSO (Merck) and then diluted in DMEM to make a 1 mM solution. Eugenol (Sigma Aldrich) was diluted 1:1 in DMSO and then diluted in DMEM to make a 10 mM solution.

To determine the $IC_{50,}$ an MTT assay was used. Cells were seeded into 96-well plates at 2×10^4 cells/well in 100 µL CM10 and incubated for 24 h in a 5% CO_2 incubator. Then 50 µL of each well supernatant was replaced with the desired concentrations of 2×eugenol (100–5000 µM), sorafenib (5–64 µM), or a combination of both prepared in CM10. After 24 h, 50 µL of the 5 mg/mL MTT solution [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide, Sigma Aldrich] was added to each well, and the plates were incubated for a further 4 h. Subsequently, 50 µL DMSO was added to each well, and the absorbance of the wells was determined at 570/660 nm. This test was conducted three times, each time in triplicate. The IC_{50} - a concentration of the drug that inhibits the growth of 50% of cells was determined by plotting log drug concentrations against cell growth inhibition with GraphPad PRISM version 6.

To determine the pharmacodynamic interaction of eugenol and sorafenib, combination index (*CI*) and fraction affected (*Fa*) (inhibition ratio) were calculated with CompuSyn software version 1.0.[20] For this purpose, the absorbance effects were tested in seven different concentrations, including the IC_{50} of sorafenib and eugenol. According to the instructions of this software, $CI<1$, $CI=1$, and $CI>1$ represent a synergistic, additive, and antagonistic effect, respectively (https://www.combosyn.com/dataEI.html).

Apoptosis Assay

Flow cytometry was used to evaluate the apoptosis rate in target cells. Cells were grown and treated with different concentrations of sorafenib and eugenol (at concentrations equal to or less than the IC_{50}), or their combination (sorafenib/eugenol at 1:105) for 24 h, washed with PBS, harvested by a scraper, and adjusted to 1×10^6 cell/mL. Then the cells were incubated with 5 µL PE-annexin V and 5 µL 7-AAD (BD Biosciences, San Diego, CA, USA) for 15 min at room temperature in the dark. Lastly, 400 µL 1× binding buffer was

added to each tube, and the cells were immediately analyzed by BD FACSCalibur flow cytometry (eBioscience, San Diego, CA, USA). In this assay, cells treated with 3% DMSO were used as a positive control, and untreated cells cultured in CM10 were used as a negative control. Flow cytometry tests were repeated in four independent runs, and the data were analyzed with FlowJo software version 7.6.

Immunoblotting

To confirm that cell death occurred through apoptosis, immunoblotting was used to determine the relative cleavage of caspase-8 and caspase-9. For this purpose, cells were grown in 75-mL tissue culture flasks and treated with different concentrations of sorafenib, eugenol, or their combination based on flow cytometry data. After incubation for 24 h, the cells were washed with PBS, scraped, and dispersed in lysis buffer (150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris–HCl, pH 7.5) supplemented with 10% protease inhibitor cocktail, and then sonicated for 20 min on ice. The lysates were centrifuged at 13,000 g and 4°C for 5 min, and the supernatants were stored at −20°C for immunoblotting. For western blot analysis, the untreated 8305C cells and etoposide-treated Jurkat cells were prepared under the same conditions as negative and positive controls. Etoposide is a chemotherapeutic drug with cytotoxic effects on cancer cell lines e.g., Jurkat, a human T lymphocyte cell line.

Protein concentration in each lysate was determined with the Bradford method. SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) was performed in a mini gel (Bio-Rad, Hercules, CA, USA) with 6% stacking and 12% separating gels, and 50 µg of each lysate was loaded in each well. Electrophoresis was done for 15 minutes at 80 V and 45 minutes at 120 V. Then, the protein bands were transferred onto PVDF (polyvinylidene difluoride) membrane with a semi-dry system (Bio-Rad) for 90 min at a 22 V constant current. The membrane was incubated in a blocking solution of 5% albumin in PBS-T (phosphate buffer solution with 0.1% Tween 20) at 4°C overnight with shaking. Then the membranes were cut into three pieces. The right part was incubated with primary mouse anti-human caspase-8 (1:1000 in PBS-T), the left part was incubated with mouse anti-human caspase-9 (1:500 in PBS-T), and the lower part was incubated with mouse anti-human GAPDH antibody (1:1000 in PBS-T) (BD Pharmingen, San Diego, CA, USA) at 4°C overnight. The membranes were washed several times with PBS-T and then incubated with HRP-conjugated goat anti-mouse secondary antibody (1:1500 in PBS-T) (BD Pharmingen) for 4 h at room temperature. Then they were washed with PBS-T and subsequently with PBS, and the bands were developed with 0.1 mL ECL substrate (Bio-Rad) per cm^2 of the membrane according to the manufacturer's instructions. The bands were detected with the ChemiDoc system (Bio-Rad) after incubation for 1 min in the dark, and the intensity of each band was determined with Image Lab software version 5.2.

Statistical Analysis

The comparison of flow cytometry data regarding the apoptosis percentage and immunoblotting data concerning the relative intensity of cleaved/uncleaved caspases among groups was made with one-way ANOVA, and Tukey's post hoc test was used to determine which differences were significant. All statistical analyses were done with SPSS-21, and $p<0.05$ was considered important.

RESULTS

To determine whether eugenol in combination with sorafenib could trigger apoptosis in 8305C cells better than either substance individually, the IC_{50} of each was determined by MTT assay, and the rate of apoptosis was determined by flow cytometry and confirmed with immunoblotting.

Calculation of IC_{50} Concentration and Synergistic Effects Sorafenib and Eugenol

The results of MTT assays yielded an IC_{50} of 20 µM for sorafenib and 2100 µM for eugenol (Table 1).

The combination analyses showed that sorafenib and eugenol had a synergistic effect at concentrations equal to or less than their IC50. A combination of both substances at their IC_{50} was the most effective in terms of synergistic effect, with the highest *Fa* value (i.e., the highest % inhibition) and highest $CI<1$ (i.e., the most significant degree of synergism) (Table 2).

Determination of Apoptosis Rate Induced by Sorafenib, Eugenol, and their Combination

As shown in Figure 1, the results of flow cytometry analysis showed the highest apoptosis rates at the IC_{50} of sorafenib and eugenol. Significantly higher

apoptosis rates (p<0.0001) were induced in cells treated with eugenol or the eugenol-sorafenib combination than in sorafenib-treated cells.

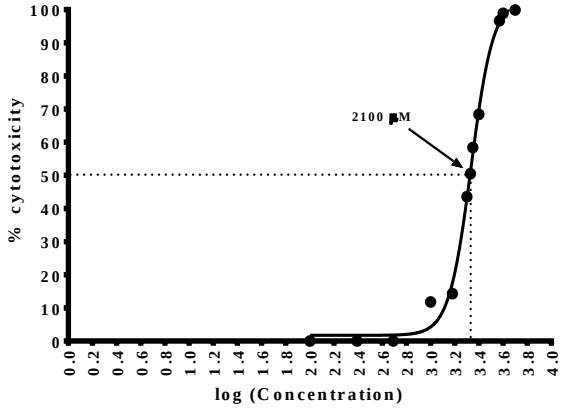
The viable cell rate was highest in cells treated with sorafenib alone and much lower in cells treated with eugenol alone or combining both substances. The necrosis rate was standard in all three groups, with no significant differences.

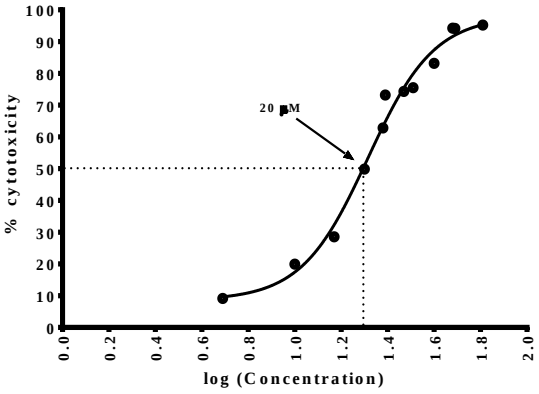
Evaluation of Activated Caspase-8 and Caspase-9

The results of immunoblotting are shown in Figure 2A. After normalization to the intensity of the GAPDH band as a reference, caspase-9 showed no significant differences among groups (p=0.33). In contrast, a significantly higher amount of cleaved versus uncleaved caspase-8 was observed in eugenol-treated cells compared to sorafenib-treated cells (p=0.029) (Figure 2B).

Table 1. Cytotoxic effect of different concentrations of eugenol and sorafenib in 8305C cells after 24 h (left), IC_{50} concentrations (right)

substance	Concentration (µM)	Cytotoxicity %
Eugenol	5000	99.9
	4000	98.9
	3750	96.65
	2500	68.42
	2250	58.39
	2125	50.48
	2000	43.58
	1500	14.30
	1000	11.81
	500	0
	250	0
	100	0
	IC_{50} = 2100 µM	
Sorafenib	64	95.19
	50	94.17
	48	94.23
	40	83.17
	32	75.49
	30	74.31
	25	73.19
	24	62.81
	20	49.86
	15	28.54
	10	19.92
	5	9.13
	IC_{50}=20 µM	



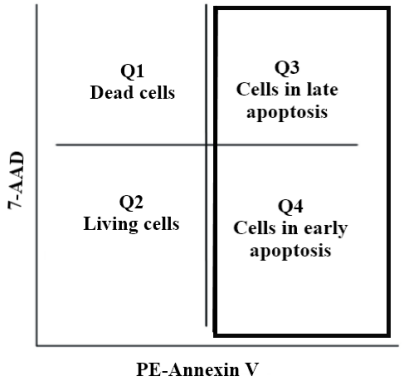


Published by Tehran University of Medical Sciences (http://ijaai.tums.ac.ir)

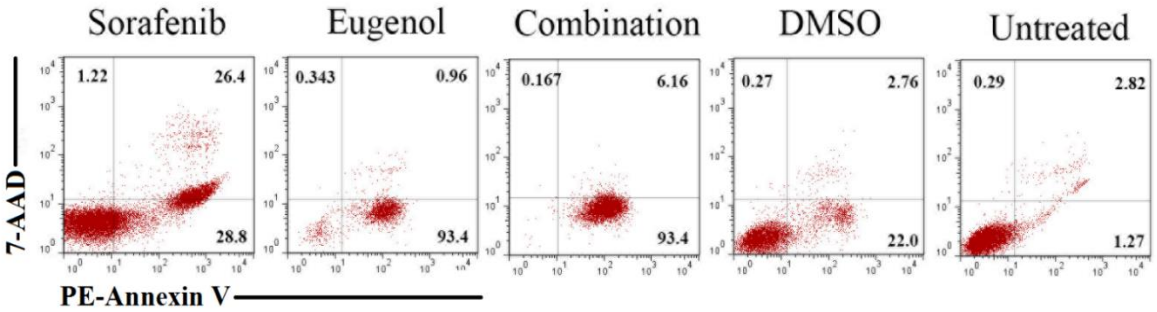
Table 2. Pharmacodynamic interaction of eugenol and sorafenib in 8305C cells after 24 h

Effect	Sorafenib + Eugenol (Fa)	Combination index CI)	Sorafenib (µM)	Eugenol (µM)
Synergism	0.05	0.51699		
	0.1	0.60392		
	0.15	0.66694		
	0.2	0.71988		
	0.25	0.76764		
	0.3	0.81265		
	0.35	0.85645		
	0.4	0.90015		
	0.45	0.94472		
	0.5	**0.99112**	**20**	**2100**
Antagonism	0.55	1.04042		
	0.6	1.09395		
	0.65	1.15350		
	0.7	1.22170		
	0.75	1.30273		
	0.8	1.40388		
	0.85	1.53975		
	0.9	1.74632		
	0.95	2.15695		
	0.97	2.52170		

A)



B)



C)

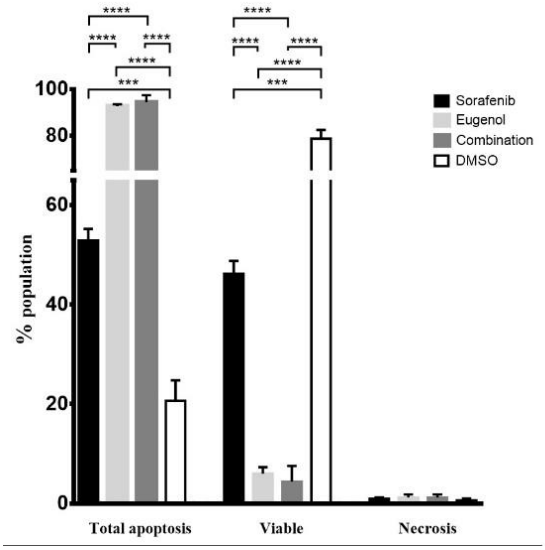
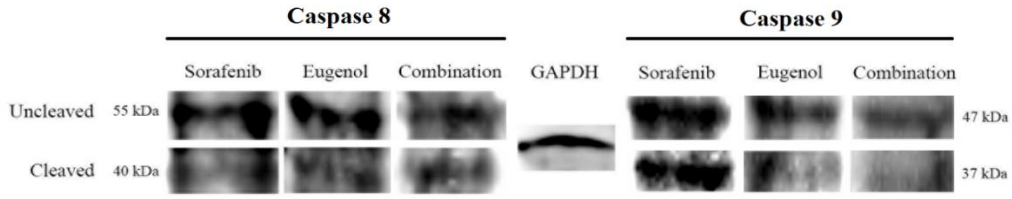


Figure 1. A) Flow cytometry quadrant in cell viability assessment: total apoptosis=Q3+Q4. B) Representative apoptosis rate induced by IC_{50} concentration of Sorafenib, Eugenol, and their combination in 8305C cells. C) Comparison of flow cytometry data among groups. All quantities are shown as the mean±SEM. ***p<0.001, ****p<0.0001.

A)



B)

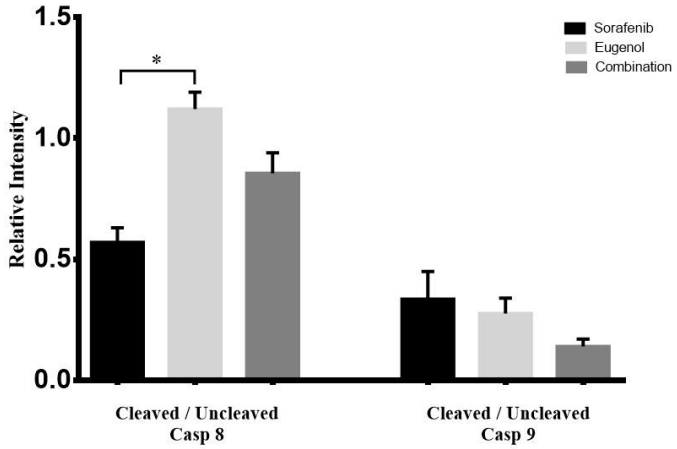


Figure 2. Cleavage of caspase-8 and caspase-9 in 8305C cells treated with sorafenib, eugenol, or their combination at IC_{50}. A) Immunoblotting results. B) Comparison of the relative amount of cleaved/uncleaved caspases in treated cells. Relative intensities are presented as the mean±SEM. *p<0.05.

DISCUSSION

The present study investigated the potential synergistic effect of sorafenib and eugenol in triggering apoptosis in 8305C cells, a model for UTC. All experiments were done with concentrations less than or equal to the IC_{50}.

The results of the MTT assay with 8305C cells yielded an IC_{50} of 20 µM for sorafenib and 2100 µM for eugenol. Cervello et al, reported that the IC_{50} for sorafenib in HepG2 and Huh7 cell lines was about 10 µM.[21] In another study by Tomonari et al, the IC_{50} was reported as 9.2±0.47 µM in PLC/PRF5-R1 cells and 25±5.1 µM in PLC-PRF5-R2 cells.[22] Fujisawa et al, reported that eugenol's cytotoxicity concentration (CC50) in HSG cells was 285 Mm.[23] In a study with HEK-293 cells, eugenol's effective dose (EC50) was 261.5 µM.[24] In another study, the IC_{50} of eugenol in five cancer cell lines was reported as 15.09 µM in MDA-MB-231, 22.75 µM in MCF-7, 18.31 µM in SIHA, 7.201 µM in SK-MEL-28, and 12.17 µM in A2058 cells.[25] Differences in the behavior on exposure to these two substances are likely related to the diverse origin of the cell lines used in these studies.

According to our analysis, the most potent apoptotic effect, in which 97% of cells showed early and late apoptosis, was achieved with a combination of sorafenib and eugenol at their IC_{50}. Unlike sorafenib, when tested alone, eugenol showed the same apoptotic potential as the combination of both substances. Few reports have appeared on the effects of sorafenib or eugenol alone on different cell lines. In a study of HepG2 cells, flow cytometry results showed increased apoptosis after sorafenib treatment.[26] Bonelli et al, related the induction of apoptosis by sorafenib to the release of cytochrome c into the cytosol, activation of caspase-9 and caspase-7, and cleavage of PARP-1.[27] Liang et al, investigated human glioblastoma cells with flow cytometry and western blotting and reported that eugenol induced apoptosis through the release of cytochrome c from mitochondria and the activation of caspase-9 and caspase-3.[28] Sarkar et al, also used western blot to investigate caspase-8 and caspase-9 cleavage in AGS (human gastric cancer) cells, and demonstrated that eugenol induced apoptosis through both intrinsic and extrinsic pathways.[29] Al-Sharif et al, showed that eugenol at a low dose (2 µM) was able to generate the intrinsic apoptosis pathway in three different breast cancer cell lines, e.g., MCF7, T47-D, and MDA-MB-231.[12]

Because removing malignant cells through necrosis releases dangerous signals which lead to inflammation, and chronic inflammation plays an important role in metastasis,[30] killing cancerous cells by apoptosis may be preferable. This points toward a significant potential benefit of using eugenol in combination therapy to induce apoptosis as an approach to treatment for recurrent TC.

Apoptosis or programmed cell death is responsible for maintaining homeostatic cellular balance in physiological conditions. Apoptosis can be induced through two distinct but interconnected pathways: intrinsic and extrinsic. Through their activation, caspases play a crucial role in apoptosis.[31] To investigate both apoptosis pathways, we evaluated the activation of caspase-8 as the mediator of the extrinsic pathway and caspase-9 as the initiator of the intrinsic pathway.[31] Western blot data showed that the ratio of cleaved/uncleaved caspase-8 was higher in eugenol- and eugenol/sorafenib-treated cells than in sorafenib-treated cells. However, this difference was significant only in the former pair of groups. In a study by Sarkar et al, caspase-3, caspase-8, and caspase-9 were measured to determine the effects of eugenol on the induction of apoptosis in AGS cells.[29] Increased amounts of cleaved caspase-3 and caspase-9 in HCC (hepatocellular carcinoma) cells exposed to a combination of sorafenib and C-ceramide were also reported by Jiang et al.[32]

Combination therapy in cancer treatment may maintain the benefits of a more significant apoptotic effect with less medication tolerance. A study by Yao et al, in two human liver tumor cell lines found that the combination of sorafenib and artesunate exerted a synergistic antiproliferative effect and induced synergistic apoptosis in HCC cells.[33] In addition, Eskiler et al, reported a synergistic effect of sorafenib in combination with nobiletin in decreasing the viability of PC-3 cells (a metastatic prostate cancer cell line), with less toxicity for normal cells than each drug alone.[34]

Using an apoptosis-inducing drug in combination with an anticancer drug that acts through an alternative mechanism may improve the efficacy of the latter agent at lower doses and thus reduce its side effects. Moreover, this type of combined treatment may also prevent tumor progression by reducing the induction of

inflammation. In this connection, Yi et al, reported that combination therapy with myricetin and methyl eugenol increased the number of apoptotic HeLa cells compared to single-drug treatment and increased caspase-3 activity in combination therapy compared to single-drug treatment.[35]

It is worth recalling that drug resistance is a significant problem in cancer treatment and is responsible for most cancer recurrences. Because resistance to apoptosis by the increased expression of anti-apoptotic proteins allows tumor cells to escape, the addition of drugs with alternative pathways is potentially helpful in overcoming resistance.[8]

The present *in vitro* study showed a synergistic effect of sorafenib and eugenol at concentrations lower than or equal to their IC_{50} in a UTC cell line. The most potent apoptotic effect was achieved by combining sorafenib and eugenol at their IC_{50}. Lower doses of this drug may be used in combination with eugenol to improve the efficacy of sorafenib while reducing its side effects. However, further studies are needed to determine the clinical significance of this combination.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

ACKNOWLEDGEMENTS

This work was done in partial fulfillment of the requirements for the Ph.D. degree in Physiology awarded to Pedram Talezadeh Shirazi and was financially supported by a grant from Kerman University. We thank A. Ebrahimpour and S. Nejabat for their kind assistance with the laboratory work, Dr. S. Zaeri for his help with the pharmacodynamic analysis, and K. Shashok (Author AID in the Eastern Mediterranean) for improving the use of English in the manuscript.

REFERENCES

1. Carneiro BA, El-Deiry WS. Targeting apoptosis in cancer therapy. Nat Rev Clin Oncol. 2020;17(7):395-417.
2. Kim J, Gosnell JE, Roman SA. Geographic influences in the global rise of thyroid cancer. Nat Rev Endocrinol. 2020;16(1):17-29.
3. Seo H, Li HY, Perez-Reyes E, Lee J-H. Effects of eugenol on T-type Ca2+ channel isoforms. J Pharmacol Exp Ther. 2013;347(2):310-7.
4. Haugen BR, Alexander EK, Bible KC, Doherty GM, Mandel SJ, Nikiforov YE, et al. 2015 American Thyroid Association management guidelines for adult patients with thyroid nodules and differentiated thyroid cancer: the American Thyroid Association guidelines task force on thyroid nodules and differentiated thyroid cancer. Thyroid. 2016;26(1):1-133.
5. Nguyen QT, Lee EJ, Huang MG, Park YI, Khullar A, Plodkowski RA. Diagnosis and treatment of patients with thyroid cancer. Am Health Drug Benefits. 2015;8(1):30.
6. Montor WR, Salas AROSE, de Melo FHM. Receptor tyrosine kinases and downstream pathways as druggable targets for cancer treatment: the current arsenal of inhibitors. Mol Cancer. 2018;17(1):1-18.
7. Thomas L, Lai SY, Dong W, Feng L, Dadu R, Regone RM, et al. Sorafenib in metastatic thyroid cancer: a systematic review. Oncologist. 2014;19(3):251.
8. Leary M, Heerboth S, Lapinska K, Sarkar S. Sensitization of drug-resistant cancer cells: a matter of combination therapy. Cancers. 2018;10(12):483.
9. Yin S-Y, Wei W-C, Jian F-Y, Yang N-S. Therapeutic applications of herbal medicines for cancer patients. Evid Based Complement Alternat Med. 2013;2013.
10. Nejad SM, Özgüneş H, Başaran N. Pharmacological and toxicological properties of eugenol. Turkish J Pharm Sci. 2017;14(2):201.
11. Jaganathan SK, Supriyanto E. Antiproliferative and molecular mechanism of eugenol-induced apoptosis in cancer cells. Molecules. 2012;17(6):6290-304.
12. Al-Sharif I, Remmal A, Aboussekhra A. Eugenol triggers apoptosis in breast cancer cells through E2F1/survivin down-regulation. BMC Cancer. 2013;13(1):1-10.
13. Yoo CB, Han KT, Cho KS, Ha J, Park HJ, Nam JH, et al. Eugenol isolated from the essential oil of Eugenia caryophyllata induces a reactive oxygen species-mediated apoptosis in HL-60 human promyelocytic leukemia cells. Cancer Lett. 2005;225(1):41-52.
14. Varadarajan S, Narasimhan M, Balaji TM, Chamundeeswari DP, Sakthisekaran D. In vitro anticancer effects of cinnamomum verum J. Presl, Cinnamaldehyde, 4 Hydroxycinnamic acid and Eugenol on an oral squamous cell carcinoma cell line. J Contemp Dent Pract. 2020;21:1027-33.
15. Maghbool M, Khosravi T, Vojdani S, Chaijan MR, Esmaeili F, Amani A, et al. The effects of eugenol nanoemulsion on pain caused by arteriovenous fistula

cannulation in hemodialysis patients: A randomized double-blinded controlled cross-over trial. Complement Ther Med. 2020;52:102440.
16. Benencia F, Courreges M. In vitro and in vivo activity of eugenol on human herpesvirus. Phytother Res. 2000;14(7):495-500.
17. Gülçin İ. Antioxidant activity of eugenol: A structure–activity relationship study. J Med Food. 2011;14(9):975-85.
18. Barboza JN, da Silva Maia Bezerra Filho C, Silva RO, Medeiros JVR, de Sousa DP. An overview on the anti-inflammatory potential and antioxidant profile of eugenol. Oxid Med Cell Longev. 2018;2018.
19. Fathy M, Fawzy MA, Hintzsche H, Nikaido T, Dandekar T, Othman EM. Eugenol exerts apoptotic effect and modulates the sensitivity of HeLa cells to cisplatin and radiation. Molecules. 2019;24(21):3979.
20. Chou T-C. Theoretical Basis, Experimental Design, and Computerized Simulation of Synergism and Antagonism in Drug Combination Studies. Pharmacol Rev. 2006;58(3):621-81.
21. Cervello M, Bachvarov D, Lampiasi N, Cusimano A, Azzolina A, McCubrey JA, et al. Novel combination of sorafenib and celecoxib provides synergistic anti-proliferative and pro-apoptotic effects in human liver cancer cells. PloS One. 2013;8(6):e65569.
22. Tomonari T, Takeishi S, Taniguchi T, Tanaka T, Tanaka H, Fujimoto S, et al. MRP3 as a novel resistance factor for sorafenib in hepatocellular carcinoma. Oncotarget. 2016;7(6):7207.
23. Fujisawa S, Atsumi T, Kadoma Y, Sakagami H. Antioxidant and prooxidant action of eugenol-related compounds and their cytotoxicity. Toxicology. 2002;177(1):39-54.
24. Chung G, Im S, Kim Y, Jung S, Rhyu M-R, Oh S. Activation of transient receptor potential ankyrin 1 by eugenol. Neuroscience. 2014;261:153-60.
25. de Sa Junior PL, Câmara DAD, Costa AS, Ruiz JLM, Levy D, Azevedo RA, et al. Apoptotic effect of eugenol envolves G2/M phase abrogation accompanied by mitochondrial damage and clastogenic effect on cancer cell in vitro. Phytomedicine. 2016;23(7):725-35.
26. Shi Y, Mo X, Hong S, Li T, Chen B, Chen G. Studying the role and molecular mechanisms of MAP4K3 in sorafenib resistance of hepatocellular carcinoma. Biomed Res Int. 2020;2020.
27. Bonelli MA, Fumarola C, Alfieri RR, La Monica S, Cavazzoni A, Galetti M, et al. Synergistic activity of letrozole and sorafenib on breast cancer cells. Breast Cancer Res Treat. 2010;124(1):79-88.
28. Liang W-Z, Chou C-T, Hsu S-S, Liao W-C, Shieh P, Kuo D-H, et al. The involvement of mitochondrial apoptotic pathway in eugenol-induced cell death in human glioblastoma cells. Toxicol Lett. 2015;232(1):122-32.
29. Sarkar A, Bhattacharjee S, Mandal DP. Induction of apoptosis by eugenol and capsaicin in human gastric cancer AGS cells-elucidating the role of p53. Asian Pac J Cancer Prev. 2015;16(15):6753-9.
30. Greten FR, Grivennikov SI. Inflammation and cancer: triggers, mechanisms, and consequences. Immunity. 2019;51(1):27-41.
31. Elmore S. Apoptosis: a review of programmed cell death. Toxicol Pathol. 2007;35(4):495-516.
32. Jiang S, Wang Q, Feng M, Li J, Guan Z, An D, et al. C2-ceramide enhances sorafenib-induced caspase-dependent apoptosis via PI3K/AKT/mTOR and Erk signaling pathways in HCC cells. Appl Microbiol Biotechnol. 2017;101(4):1535-46.
33. Yao X, Zhao C-r, Yin H, Wang K, Gao J-j. Synergistic antitumor activity of sorafenib and artesunate in hepatocellular carcinoma cells. Acta Pharmacol Sin. 2020;41(12):1609-20.
34. Guney Eskiler G, Deveci AO, Bilir C, Kaleli S. Synergistic effects of nobiletin and sorafenib combination on metastatic prostate cancer cells. Nutr Cancer. 2019;71(8):1299-312.
35. Yi J-L, Shi S, Shen Y-L, Wang L, Chen H-Y, Zhu J, et al. Myricetin and methyl eugenol combination enhances the anticancer activity, cell cycle arrest and apoptosis induction of cis-platin against HeLa cervical cancer cell lines. Int J Clin Exp Pathol. 2015;8(2):1116.