Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcog.com | www.phcog.net

ORIGINAL ARTICLE

Ferruginol-induced Apoptosis in Human Colon Cancer Cells (HCT-116) through the Mitochondria-Mediated Apoptotic Pathway

Hua-Long Lin, Pei-Rui Chen, Chun-Chun Mao[1], Wei-E Zheng, Jia-Qi Wang

Departments of Medical Oncology and [1]Thyroid and Breast Surgery, Ruian People's Hospital, Rui'an, Wenzhou, Zhejiang, China

Submitted: 10-Mar-2020 Revised: 05-May-2020 Accepted: 05-Dec-2020 Published: 12-Jul-2021

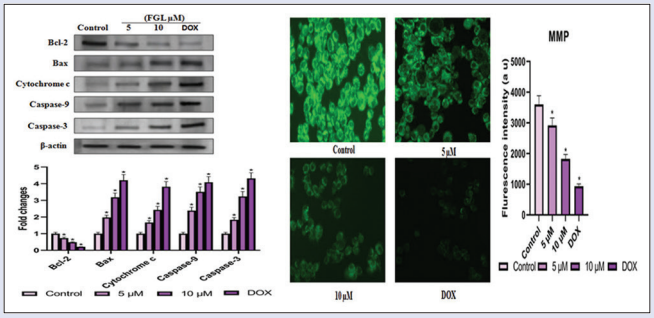
ABSTRACT

Background: Colon cancer is the third leading cause of cancer-related deaths globally. Despite advances in systemic therapies, colorectal cancer still remains one of the foremost challenges due to high mortality rate. **Objectives:** In this current investigation, we observed the anticancer and proapoptotic potentials of ferruginol (FGL) in human colon cancer (HCT-116) cells. **Materials and Methods:** We recognized that the potentials of FGL against cell viability in HCT-116 cells were determined by MTT assay. Production of reactive oxygen species (ROS), status of mitochondrial membrane potential (MMP), catalase (CAT), superoxide dismutase (SOD), diminished glutathione (GSH), and thiobarbituric acid-reactive substances (TBARS) were also evaluated. In addition, apoptotic protein expressions such as bax, caspase-9, cytochrome-c, Bcl-2, and caspase-3 were assessed through Western blotting analysis. **Results:** Our findings showed that FGL induced apoptosis as confirmed through the thrashing of cell viability, improved ROS formation and TBARS, and decreased antioxidants (SOD, CAT, and GSH) and MMP in HCT-116 cells. Further, the proapoptotic role of FGL was downregulated the Bcl-2 expression, whereas improved caspase-9, Bax, caspase-3, and cytochrome-c expressions in HCT-116 cells. **Conclusion:** Therefore, FGL stimulates apoptosis in HCT-116 cells through triggering oxidative damage and mitochondrial-mediated apoptotic pathway.

Key words: Antioxidants, apoptosis, colon cancer, ferruginol, HCT-116 cells, reactive oygen species

SUMMARY

- The mortality rate from colon cancer has dramatically reduced over the years due to the early diagnosis, effective treatment, and diet
- Ferruginol (FGL) has been considered among such natural remedial agents. FGL, a bio-active compound derived from the *Persea nubigena* and *Podocarpus andina*
- FGL activates the apoptosis through a mitochondrial-mediated pathway during the suppression of Bcl-2, whereas improved caspase-3, cytochrome-c, Bax, and caspase-9 expressions in HCT-116 cells.



Abbreviations used: FGL: Ferruginol; ROS: Reactive oxygen species; MMP: Mitochondrial membrane potential; SOD: Superoxide dismutase; CAT: Catalase; GSH: Glutathione; TBARS: Thiobarbituric acid reactive substances; DOX: Doxorubicin; FCS: Fetal calf serum; DMEM: Dulbecco's modified Eagle's medium.

Correspondence:

Dr. Jia-Qi Wang,
Department of Medical Oncology, Ruian People's Hospital, Rui'an, Wenzhou, Zhejiang 325200, China.
E-mail: beibei0116@163.com
DOI: 10.4103/pm.pm_53_20

Access this article online
Website: www.phcog.com
Quick Response Code:

INTRODUCTION

Colon cancer is the third most common cancer detected in women and men, which is fourth frequent cause of cancer-associated deaths globally.[1] The death rate from colon cancer has intensely reduced in the past 20 years due to the early detection, more successful treatments, and diet. The colon cancer was diagnosed highly in nowadays due to the prevalent availability of colonoscopy. As per the World Health Organization, in 2018, the 1.8 million new incidences of colon cancer were predictable and 0.86 million victims were died worldwide.[2] Nearly, 70% of colon cancer incidence was sporadic cases in which were influenced by numerous environmental factors and daily life habits such as smoking, dietary habits, excessive alcohol consumption, and loss of physical activity. About 25% of colon cancer victims have a genetic predisposition, and 5% of victims has inherited factors linked to the cancer progression.[3] It is familiar that a high intake of phytochemicals predominant in vegetables and fruits considerably reduces the risk of different chronic ailments such as diabetes,[4] coronary heart disease,[5] and carcinogenesis.[6] Medicinal herbs were considered to curing a range of ailments and have an extended history of established beneficial assistance. Because of the deprived alertness against presently existing chemotherapeutic agents and their harmful outcomes, the healing of colon cancer needs the natural beneficial agents.[7]

Phytochemicals were received growing interests for the cancer management because of their structural intricacy, inborn biological function, affordability, chemical diversity, effortless accessibility,

Cite this article as: Lin HL, Chen PR, Mao CC, Zheng WE, Wang JQ. Ferruginol-induced apoptosis in human colon cancer cells (HCT-116) through the mitochondria-mediated apoptotic pathway. Phcog Mag 2021;17:244-9.

less toxic outcomes, capacity to alter a range of signal transduction cascades, and cell mechanisms.[8] Numerous natural compounds have been earlier examined against human colon cancer cells such as nobiletin,[9] stevioside,[10] and arctigenin.[11] Herbs are well recognized for their therapeutic benefits because of the occurrence of natural phytocomponents. Ferruginol (FGL) was considered among such natural remedial agents. FGL is a bioactive compound derived from the *Persea nubigena* and *Podocarpus andina* (Podocarpaceae). FGL is a abietane diterpene that occurs in plants that belongs to the families of *Cupressaceae*, *Podocarpaceae*, *Verbenaceae,* and *Lamiaceae*. FGL has exposed hopeful therapeutic properties such as antibacterial, antifungal,[12] miticidal,[13] antiplasmodial,[14] cardioactive,[15] antiulcerogenic,[16] and antioxidative[17] properties.

Apoptosis is a programmed cell death, which sustains the cellular equilibrium. If this stability is concerned, it may eventually lead to unrestrained cell growth and division. Therefore, the invention of promising anticancer drugs, mostly aims on apoptosis-triggering agents through different cascades.[18] The extreme reactive oxygen species (ROS)-mediated cascade is regarded as an well-known molecular pathway that arbitrating to apoptosis. Normal cells susceptible to generate ROS continuously that is neutralized through the availability of antioxidant enzymes.[19] Equally, if ROS formation surpasses the limit, it results in the condition of oxidative stress. Such circumstances of oxidative stress can result in different illnesses such as cancer.[20] ROS controls numerous signaling cascades that are concerned in a typical performance of cells. Thus, modified ROS statuses can show the way to diminished functioning of the cellular mechanisms. The mitochondrial-regulated pathway is a well-explored signaling cascade controlled by ROS.[10] Although the function of FGL triggering mitochondrial-regulated apoptosis in human colon cancer cells remains unidentified. Thus, the current investigation intended to expose the mitochondrial-mediated apoptotic cascade of the FGL on HCT-116 cells.

MATERIALS AND METHODS

Chemicals

FGL and doxorubicin (DOX) were purchased from Sigma-Aldrich, USA. The phosphate buffer solution (PBS), Dimethyl sulfoxide (DMSO), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Dulbecco's Modified Eagle's Medium (DMEM), glutathione (GSH), and FBS were purchased from HiMedia Laboratory, USA. The primary antibodies for Bax, caspase-9, cytochrome-c, Bcl-2, caspase-3, and β-actin were attained from SantaCruz Biotechnology (SantaCruz, CA, USA). All other chemicals were employed of diagnostic range.

Cell culture

Human colon cancer (HCT-116) cells were obtained and sustained at 37°C with a moistened ambiance of 5% CO_2. HCT-116 cells were seeded in DMEM including with streptomycin (100 μg/mL), penicillin (100 U/mL), and 10% heat-inactivated fetal calf serum. DOX has been utilized as control.

Cell viability assay

The cytotoxic potential of FGL to the HCT-116 cells was evaluated through the MTT test.[21] The cells were added with FGL at numerous dosages (5, 10, 15, 20, 25, and 30 μM). Later than, 48 h, 50 μL of MTT (2 mg/mL) was treated to every well to take an entire reaction quantity of 200 μL. Later than 4 h incubation, the developed formazan crystals that occur in every well were liquefied through mixing of 150 μL of DMSO, and finally absorbance at 540 nm was resoluted on a scanning multiwell plate reader.

Mitochondrial reactive oxygen species measurement

The intracellular ROS accretion was determined in HCT-116 cells through the dichloro-dihydro-fluorescein diacetate (DCFH-DA) staining.[22] The DCFH-DA is an cell permeable non-fluorescent stain, which was utilized as a substrate for an quantity of intracellular ROS accretion status. After supplementing with diverse amounts (5 and 10 μM) of FGL, the DCFH-DA (10 μM) was introduced for 30 min (37°C) to stain the cells. The treated cells were examined at an wavelength of 485/535 nm in a microplate reader.

Detection of mitochondrial membrane potential

The alteration of mitochondrial membrane potential (MMP) was done as per the previously mentioned method.[23] Briefly, the cells were supplemented with diverse dosages of FGL (5 and 10 μM) and DOX for 18 h, were cleansed and suspended in chilled PBS. The 10 mM of Rh-123 was introduced to about 1×10^6 cells for 30 min at 37°C and then rinsed two times with PBS. The MMP was investigated beneath the floid cell imaging station (Invitrogen, USA) and determined at 485/530 nm in an spectrofluorometer (Schimadzu, USA).

Assays of antioxidants and lipid peroxidation status

HCT-116 cells were cultured in a cell culture plate at 1×10^5 cells/mL population and at 16 h later culturing cells were added with FGL (5 and 10 μM) and DOX and incubated. After incubation, the icy PBS was introduced to the cells for cleansing. Gathered cells were added in PBS (10 mM, pH-7.5) and lysed in ice through sonicating two times for 15 s. TritonX-100 buffer (1%) was amalgamated to lysates then kept for 10 min on ice, and then it was separated through centrifugation at 5000 × *g* for 10 min at 4°C to take out cellular remains, and the supernatant was analyzed for oxidative parameters. The status of catalase (CAT) was assessed through the method of Sinha.[24] GSH quantities were analyzed through the method of Ellman.[25] The thiobarbituric acid reactive substances (TBARS) concentrations were analyzed through the approach.[26] The superoxide dismutase (SOD) function was assessed through the approach.[27]

Western blot analysis

Gathered HCT-116 cells were lysed on ice for 30 min along with the addition of 100 μL of lysis buffer and centrifuged at 13,000 × *g* for 15 min. The upper phase was removed from the lysates and protein status was measured. Lysate (50 μg of protein) was heated (5 min), electrophoresed on sodium dodecyl sulphate-polyacrylamide gel (10%), and translocated into nitrocellulose membranes that were subsequently introduced with primary antibodies such as cytochrome-c, Bax, caspase-3, Bcl-2, and caspase-9 (SantaCruz Biotechnology, USA) at 4°C for overnight. Then, membranes were incubated with secondary antibodies. The formed protein bands were observed through an improved chemiluminescence kit (Amersham, Little Chalfont, Buckinghamshire, UK).

Statistics

The result was characterized as mean ± standard deviation. Statistical examination was implemented in the Graphpad Prism-8 tool (San Diego, CA, USA) using ANOVA later *post hoc* Tukey's assessment. The variations were evaluated as significant at $P < 0.05$.

RESULTS

Cytotoxic effect of ferruginol in HCT-116 cells

The cytotoxicity of FGL to the cell viability of HCT-116 cells was investigated using the MTT assay and IC_{50} (50% growth inhibitory

concentration) level was also measured. As shown in Figure 1, the cell viability was inhibited by FGL (5, 10, 15, 20, 25, and 30 μM) in a dose-dependent manner. Among the tested concentrations, 5 and 10 μM of FGL was selected for further investigations.

Effect of ferruginol on reactive oxygen species staining

HCT-116 human colon cancer cells exposed to FGL revealed the augmented ROS generation as confirmation by the improved DCF fluorescence strength [Figure 2]. In this investigation, FGL (5 and 10 μM) exposure significantly improved ROS production level in HCT-116 cell line in a dose-dependent manner. DOX, a positive control drug-treated cancer cells, wase also greatly enhanced ROS as evaluated with FGL-treated cells.

Effect of ferruginol on mitochondrial membrane potential staining

One of the promising processes involved in an apoptosis of HCT-116 cells induced by FGL was the loss of MMP [Figure 3]. The MMP was measured by rhodamine-123 fluorescence intensity. In this investigation, the control cells exemplified improved level of MMP, whereas FGL (5 and 10 μM) treatment depleted of MMP in HCT-116 cell lines. DOX, a positive control drug-treated cancer cells, was also significantly reduced MMP as evaluated with FGL-treated cells.

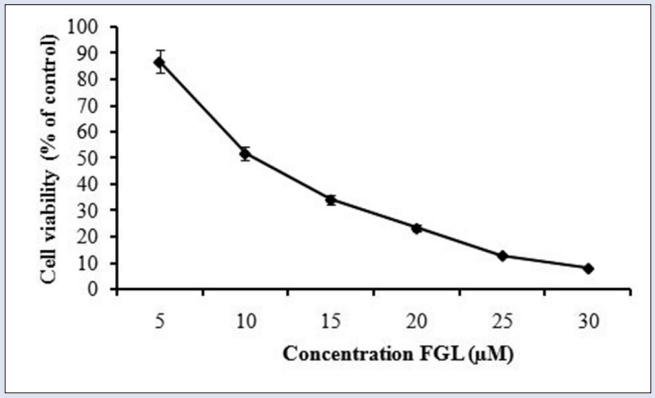


Figure 1: The cytotoxic effect of ferruginol on HCT-116 cells was measured by MTT assay

Effect of ferruginol on antioxidant lipid peroxidation status

The level of lipid peroxidation (TBARS) and antioxidants (CAT, SOD, and GSH) in control and FGL supplemented HCT-116 cells were shown in Figure 4. Control cells showed decreased status of TBARS as well as augmented antioxidants status in HCT-116 human colon cancer cells. Conversely, we recognized that increased status of TBARS and weakened functions of antioxidants in the FGL (5 and 10 μM) and DOX administered HCT-116 cells.

Effect of ferruginol on the Western blotting protein expression of apoptosis markers

We assessed that the apoptosis linked proteins (caspase-9, cytochrome, caspase-3, Bax, and Bcl-2) expressions in HCT-116 cells. The findings of Western blot analysis established that FGL (5 and 10 μM) treatment decreased Bcl-2 expression and augmented the expression of Bax, caspase-3, cytochrome-c, and caspase-9 on HCT-116 cells in a dose-reliant mode. In addition, DOX treatment also stimulated the better activation of apoptosis compared to the FGL [Figure 5].

DISCUSSION

The cell viability of the cancer cells was reduced with enhance in concentration of the anticancer drugs. It proposes the capability of the drugs as a successful anticancer medicine.[28] As per the earlier investigations, the supplementation of FGL in SK-Mel-28 cells results in reduced cell growth and augmented cytotoxicity using the MTT assay.[29] ROS is the mostly generated through mitochondria and performs as an secondary messenger for different cellular functions such as apoptosis and cell multiplication. However, the definite function of ROS remains unidentified, it can be implied that the continuous formation and elimination are essential to uphold and control the physiological stability of cells.[30] Elevated level of ROS has been exposed to be related with the lowering of the MMP.[31] In the current investigation, we observed high levels of ROS formation and reduced MMP in dose reliantly following to FGL supplementation, signifying that apoptosis was activated through a prominent ROS status in HCT116 cells. As reported earlier, the FGL improved the generation of ROS and decreased MMP in thyroid cancer cell line MDA-T32.[32]

ROS was recognized as formed from the responses of escaped electrons along with oxygen beneath different systems containing lipid

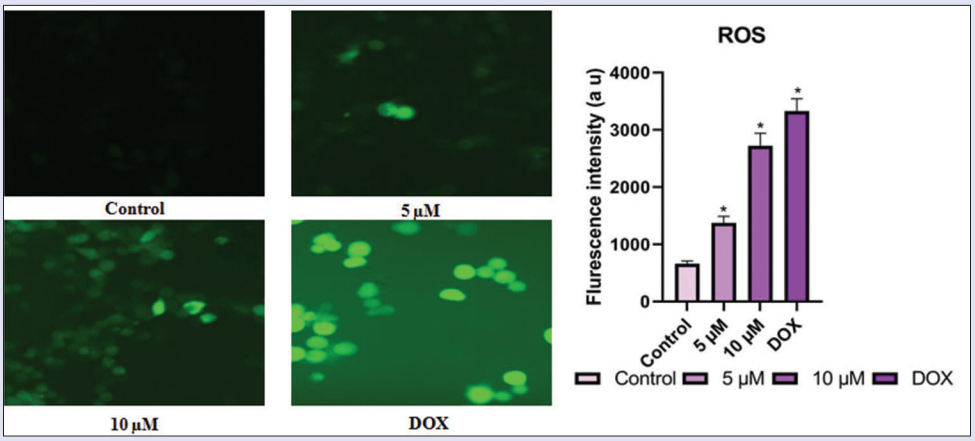


Figure 2: The effect of ferruginol on intracellular reactive oxygen species formation was determined with HCT-116 cells using DCFH-DA. The data represent mean ± standard deviation of triplicate, *$P < 0.05$ as compared with the control group

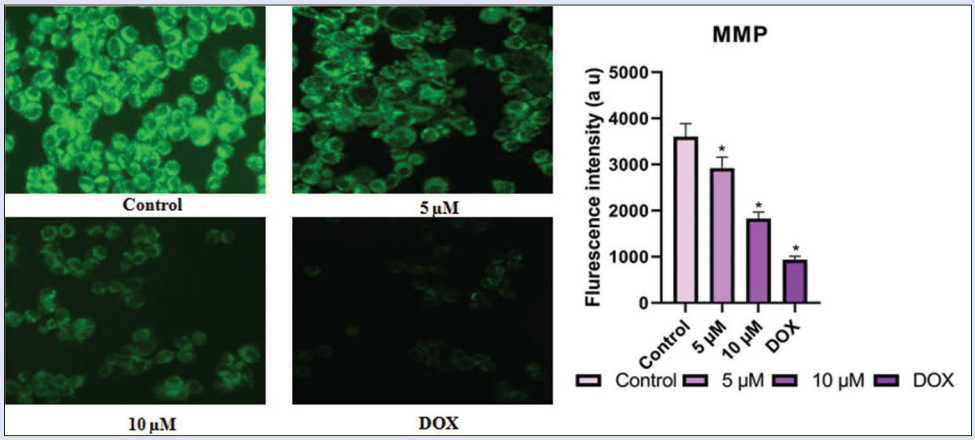


Figure 3: The effect of ferruginol on mitochondrial membrane potential status was analyzed with HCT-116 cells using the rhodamine-123. The data represent mean ± standard deviation of triplicate, *$P < 0.05$ as compared with the control group

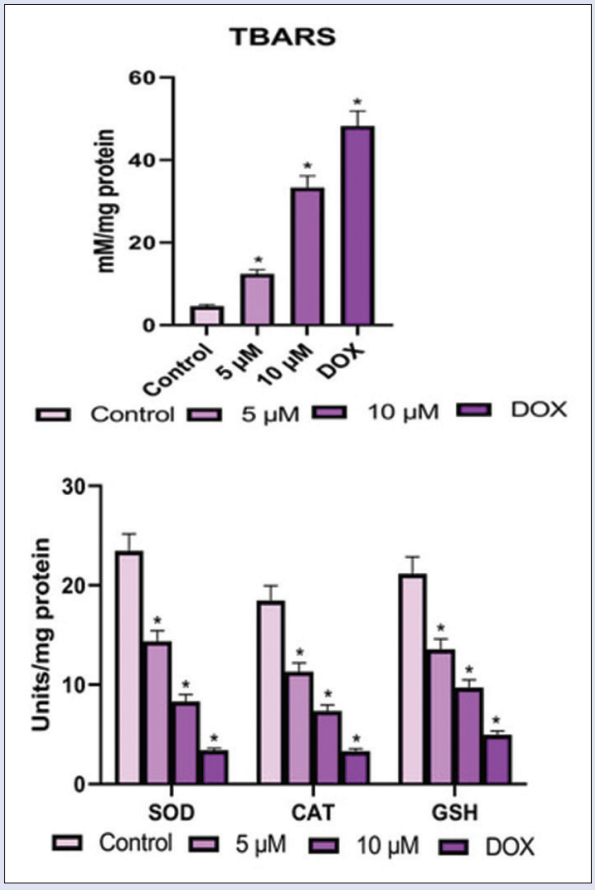


Figure 4: Effects of ferruginol increased lipid peroxidation and modulate cellular antioxidant status in HCT-116 cells. The data represent mean ± standard deviation of triplicate, *$P < 0.05$ as compared with the control group. Superoxide dismutase-enzyme concentration required for 50% inhibition of nitroblue tetrazolium reduction in 1 min. glutathione-mg/dl. Catalase-mmol of hydrogen peroxide consumed per minute

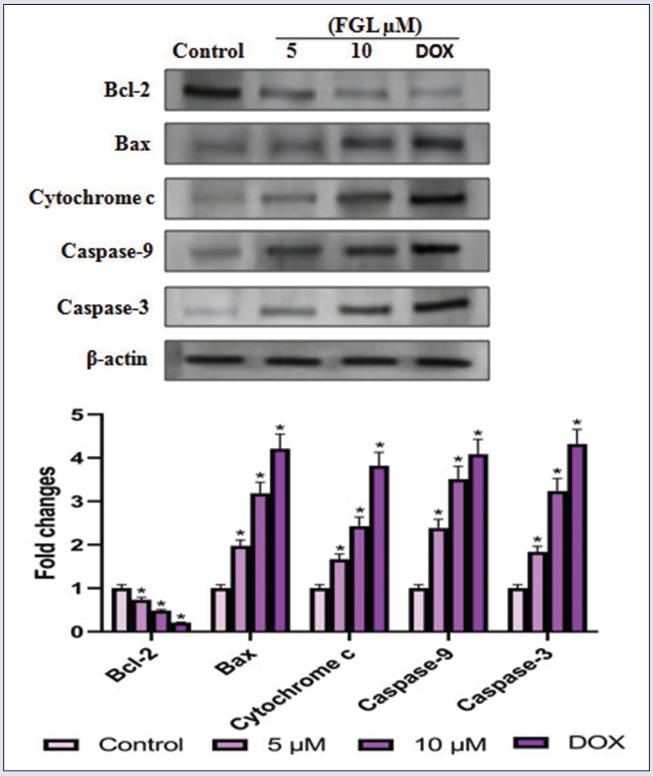


Figure 5: The anticancer effect of ferruginol of apoptotic signaling proteins in HCT-116 cell lines was investigated by Western blotting method. The cells were treated with ferruginol (5 and 10 µM) and doxorubicin for 24 h and the expressions of Bcl-2, Bax, caspase-3, cytochrome-c, and caspase-9 were analyzed. β-actin was used as a loading control. The data represent mean ± standard deviation of triplicate, *$P < 0.05$ as compared with the control group

peroxidation. Lipid peroxidation is an multifaceted procedure in that the primary developed lipid radicals are transformed to TBARS.[33] FGL treatment increased the levels of TBARS in HCT-116 cells were observed in this study. In this examination, antioxidant protection is a crucial device to deactivate the oxygen-free radicals regulated tissue damage.[34] Free radical hunting enzymes such as CAT, SOD, and GSH are the primary line to protect the cells toward the oxidative damage, decomposed O_2, and H_2O_2 before their relations to develop the most reactive hydroxyl radicals. FGL suggestively reduced the functions of SOD, CAT, and GSH in HCT-116 cells. Previously reported that *Dasyatis sephen* venom increased TBARS and decreased antioxidants on HeLa cell lines.[35]

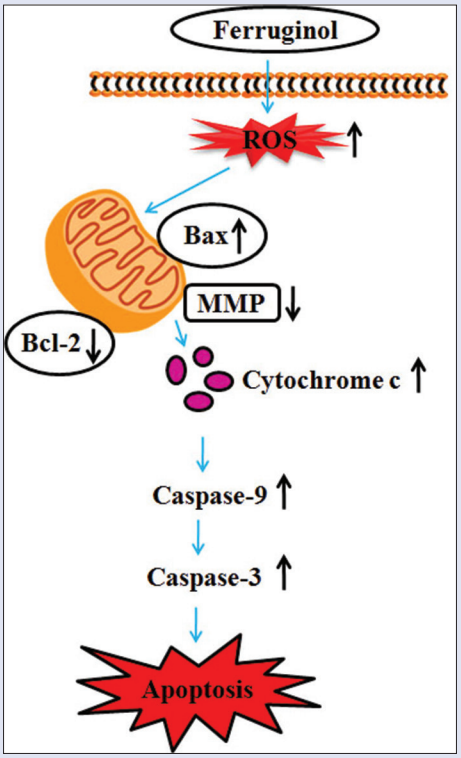


Figure 6: A schematic drawing proposed molecular mechanism and the overall possible signaling pathways for ferruginol-induced mitochondrial-mediated apoptosis in HCT-116 colon cancer cells

Bcl-2 protein is made up of diverse components that perform an imperative function in the apoptosis mediation. We observed that FGL triggered augmented Bax and diminished Bcl-2 expressions. This outcome concurs with a number of preceding investigations representing the mediation of Bcl-2 are implicated in the apoptotic mechanisms.[36,37] Protein cleavage may be stimulated by the induction of caspases that arbitrates to apoptosis. Caspase stimulation may take place by any one of the following two routes: (i) through the induction of death receptors in the surface of cells that trigger the upstream of caspase-8 and subsequently leads to the stimulation of the downstream of caspase-3 and-7; or (ii) by the mitochondria, through the discharge of cytochrome-c that triggers the apoptotic protease like apoptotic protease-activating factor-1, resulting to the caspase-9 induction.[38] Therefore, the improved concentration of caspase-9, cytochrome-c, and caspase-3 subsequent to the FGL supplement also confirmed the contribution of a mitochondrial-regulated intrinsic apoptotic cascade in the current investigation. In recent time, Ho *et al.*[39] revealed that FGL can trigger the apoptosis in lung cancer cells. Likewise, Xiong *et al.*[40] also reported that FGL-activated apoptosis leads to cell death of ovarian cancer cells.

CONCLUSION

The present investigation concluded that FGL is capable of acting antitumor effect in HCT-116 cells through the loss of cell viability, improved ROS, lipid peroxidation, decreased MMP, and antioxidant enzymes. In this manner, FGL activates the apoptosis through a mitochondrial-mediated pathway during the suppression of Bcl-2, whereas improved caspase-3, cytochrome-c, Bax, and caspase-9 expressions in HCT-116 cells. Overall, a possible mechanism of FGL-induced apoptosis was shown in Figure 6, and it has been used for the treatment of colon cancer as an anticancer drug. However, further research needed in future to clarify the precise mechanisms of FGL against the colon cancer.

Acknowledgements

Authors are thankful to the Department of Medical Oncology, Ruian People's Hospital, Ruian, Wenzhou, Zhejiang, China, 325200 for their support for this research.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Ahmed K, Zaidi SF, Cui ZG, Zhou D, Saeed SA, Inadera H, *et al.* Potential proapoptotic phytochemical agents for the treatment and prevention of colorectal cancer. Oncol Lett 2019;18:487-98.
2. Ahmad M. Colon cancer: A clinician's perspective in 2019. Gastroenterology Res 2020;13:1-10.
3. Mork ME, You YN, Ying J, Bannon SA, Lynch PM, Rodriguez-Bigas MA, *et al.* High prevalence of hereditary cancer syndromes in adolescents and young adults with colorectal cancer. J Clin Oncol 2015;33:3544-9.
4. Neuhouser ML. The importance of healthy dietary patterns in chronic disease prevention. Nutr Res 2019;70:3-6.
5. Aune D, Giovannucci E, Boffetta P, Fadnes LT, Keum N, Norat T, *et al.* Fruit and vegetable intake and the risk of cardiovascular disease, total cancer and all-cause mortality-a systemic review and dose-resonse meta-analysis of perspectives studies. Int J Epidemiol 2017;46:1029-56.
6. Wang C, Yang T, Guo X, Li D. The associations of fruit and vegetable intake with lung cancer risk in participations with different smoking status: A meta-analysis of proepective cohort studies. Nutrients 2019;11:1791.
7. Sasaki S, Samejima S, Uruga T, Anzai K, Nishi N, Kawakita E, *et al.* Synthetic studies of the spirocyclic cyclohexene part of versipelostatin, a novel GRP78/Bip molecular chaperone downregulator. J Antibiot (Tokyo) 2013;66:147-54.
8. Zhao Y, Hu X, Zuo X, Wang M. Chemopreventive effects of some popular phytochemicals on human colon cancer: A review. Food Funct 2018;9:4548-68.
9. Morley KL, Ferguson PJ, Koropatnick J. Tangeretin and nobiletin induce G1 cell cycle arrest but not apoptosis in human breast and colon cancer cells. Cancer Lett 2007;251:168-78.
10. Ren HP, Yin XY, Yu HY, Xiao HF. Stevioside induced cytotoxicity in colon cancer cells via reactive oxygen species and mitogen-activated protein kinase signaling pathways-mediated apoptosis. Oncol Lett 2017;13:2337-43.
11. Kim JY, Hwang JH, Cha MR, Yoon MY, Son ES, Tomida A, *et al.* Arctigenin blocks the unfolded protein response and shows therapeutic antitumor activity. J Cell Physiol 2010;224:33-40.
12. Búfalo J, Cantrell CL, Jacob MR, Schrader KK, Tekwani BL, Kustova TS, *et al.* Antimicrobial and antileishmanial activities of diterpenoids isolated from the roots of salvia deserta. Planta Med 2016;82:131-7.
13. Chang ST, Chen PF, Wang SY, Wu HH. Antimite activity of essential oils and their constituents from taiwania cryptomerioides. J Med Entomol 2001;38:455-7.
14. Areche C, Theoduloz C, Yáñez T, Souza-Brito AR, Barbastefano V, de Paula D, *et al.* Gastroprotective activity of ferruginol in mice and rats: Effects on gastric secretion, endogenous prostaglandins and non-protein sulfhydryls. J Pharm Pharmacol 2008;60:245-51.
15. Ulubelen A, Birman H, Oksüz S, Topçu G, Kolak U, Barla A, *et al.* Cardioactive diterpenes from the roots of salvia eriophora. Planta Med 2002;68:818-21.
16. González MA, Clark J, Connelly M, Rivas F. Antimalarial activity of abietane ferruginol analogues possessing a phthalimide group. Bioorg Med Chem Lett 2014;24:5234-7.
17. Roa-Linares VC, Brand YM, Agudelo-Gomez LS, Tangarife-Castaño V, Betancur-Galvis LA, Gallego-Gomez JC, *et al.* Anti-herpetic and anti-dengue activity of abietane ferruginol analogues synthesized from (+)-dehydroabietylamine. Eur J Med Chem 2016;108:79-88.
18. Pistritto G, Trisciuoglio D, Ceci C, Garufi A, D'Orazi G. Apoptosis as anticancer mechanism: Function and dysfunction of its modulators and targeted therapeutic strategies. Aging (Albany NY) 2016;8:603-19.

19. Son Y, Cheong YK, Kim NH, Chung HT, Kang DG, Pae HO, *et al.* Mitogen-activated protein kinases and reactive oxygen species: How can ROS activate MAPK pathways? J Signal Transduct 2011;2011:792639.
20. Chang YC, Chuang LM. The role of oxidative stress in the pathogenesis of type 2 diabetes: From molecular mechanism to clinical implication. Am J Transl Res 2010;2:316-31.
21. Leong CO, Suggitt M, Swaine DJ, Bibby MC, Stevens MF, Bradshaw TD. *In vitro, in vivo* and *in silico* analyses of the antitumor activity of 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazoles. Mol Cancer Ther 2004;3:1565-75.
22. Jesudason EP, Masilamoni JG, Jebaraj CE, Paul SF, Jayakumar R. Efficacy of DL-alpha lipoic acid against systemic inflammation-induced mice: Antioxidant defense system. Mol Cell Biochem 2008;313:113-23.
23. Johnson LV, Walsh ML, Chen LB. Localization of mitochondria in living cells with rhodamine 123. Proc Natl Acad Sci U S A 1980;77:990-4.
24. Sinha AK. Colorimetric assay of catalase. Anal Biochem 1972;47:389-94.
25. Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys 1959;82:70-7.
26. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979;95:351-8.
27. Kakkar P, Das B, Viswanathan PN. A modified spectrophotometric assay of superoxide dismutase. Indian J Biochem Biophys 1984;21:130-2.
28. Bhavana J, Kalaivani MK, Sumathy A. Cytotoxic and pro-apoptotic activities of leaf extract of croton bonplandianus baill. Against lung cancer cell line A549. Indian J Exp Biol 2016;54:379-85.
29. Jia Y, Wu C, Zhang B, Zhang Y, Li J. Ferruginol induced apoptosis on SK-Mel-28 human malignant melanoma cells mediated through P-p38 and NF-κB. Hum Exp Toxicol 2019;38:227-38.
30. Dickinson BC, Chang CJ. Chemistry and biology of reactive oxygen species in signaling or stress responses. Nat Chem Biol 2011;7:504-11.
31. Mortezaee K, Saehi E, Mirtavoos-Mahyari H, Motevaseli E, Najafi M, Farhood B, *et al.* Mechanisms of apoptosis modulation by curcumin: Implications for cancer therapy. J Cell Physiol 2019;234:12537-50.
32. Luo G, Zhou J, Li G, Hu N, Xia X, Zhou H, *et al.* Ferruginol diterpenoid selectively inhibits human thyroid cancer growth by inducing mitochondrial dependent apoptosis, endogenous reactive oxygen species (ROS) production, mitochondrial membrane potential loss and suppression of mitogen-activated protein kinase (MAPK) and PI3K/AKT signaling pathways. Med Sci Monit 2019;25:2935-42.
33. Ayala A, Muñoz MF, Argüelles S. Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. Oxid Med Cell Longev. 2014;2014:360438. doi:10.1155/2014/360438.
34. Cockfield JA, Schafer ZT. Antioxidant Defenses: A Context-Specific Vulnerability of Cancer Cells. Cancers (Basel). 2019;11(8):1208.
35. Rajeshkumar RK, Vennila R, Karthikeyan S, Prasad NR, Arumugam M, Velpandian T, *et al.* Antiproliferative activity of marine stingray dasyatis sephen venom on human cervical carcinoma cell line. J Venom Anim Toxins Incl Trop Dis 2015;21:41.
36. Chen Y, Huang K, Ding X, Tang H, Xu Z. Magnolol inhibits growth and induces apoptosis in esophagus cancer KYSE-150 cell lines via the MAP kinase pathway. J Thorac Dis 2019;11:3030-8.
37. Wu H, Medeiros LJ, Young KH. Apoptosis signaling and BCL-2 pathways provide opportunities for novel targeted therapeutic strategies in hematologic malignances. Blood Rev 2018;32:8-28.
38. Zhou Z, Zhu C, Cai Z, Zhao F, He L, Lou X, *et al.* Betulin induces cytochrome c release and apoptosis in colon cancer cells via NOXA. Oncol Lett 2018;15:7319-27.
39. Ho ST, Tung YT, Kuo YH, Lin CC, Wu JH. Ferruginol inhibits non-small cell lung cancer growth by inducing caspase-associated apoptosis. Integr Cancer Ther 2015;14:86-97.
40. Xiong WD, Gong J, Xing C. Ferruginol exhibits anticancer effects in OVCAR-3 human ovary cancer cells by inducing apoptosis, inhibition of cancer cell migration and G2/M phase cell cycle arrest. Mol Med Rep 2017;16:7013-17.