Investigation of the Antitumoral Activity of Syringic Acid on HT-29 Cells: An *In Vitro* Study

Feyza Burul[3], Öznur Altunlu[1*], Irmak Ferah Okkay[4], Cemil Bayram[2], Fatma Yeşilyurt[1], Ufuk Okkay[1]

[1]Department of Medical Pharmacology, Faculty of Medicine, Ataturk University, Erzurum, Türkiye

[2]Department of Pharmacology and Toxicology, Faculty of Veterinary, Ataturk University, Erzurum, Türkiye

[3]Department of Medical Pharmacology, Faculty of Medicine, Agri Ibrahim Cecen University, Agri, Türkiye

[4]Department of Pharmacology, Faculty of Pharmacy, Ataturk University, Erzurum, Türkiye

Corresponding Author: Öznur Altunlu, Department of Medical Pharmacology, Faculty of Medicine, Ataturk University, Erzurum, Türkiye, e-mail: altunluoznur@gmail.com

Received: December 11, 2023 **Accepted:** December 28, 2023 **Published:** December 31, 2023

To cite this article: Burul et al. (2023). Investigation of the Antitumoral Activity of Syringic Acid on HT-29 Cells: An In Vitro Study. Recent Trends in Pharmacology, vol 1, issue 3: 123-130.

Abstract

Colorectal cancer (CRC) is one of the most common cancers in the world with high mortality rates, and it is the second leading cause of cancer-related deaths worldwide and it is estimated that more than 930,000 deaths will occur due to CRC. In addition to genetic and environmental factors, oxidative stress, lipid peroxidation and parameter changes in the antioxidant system are among the risk factors. Syringic acid (SA) which contains polyphenolic compounds, has antioxidant, anti-lipid peroxidative and anti-cancer effects. Due to these effects, syringic acid may have a healing effect on HT-29 cells. In our study, we determined the effect of SA on cell viability on HT-29 cells with MTT, and its effects on antioxidant and oxidant parameters with TAC-TOS tests. Syringic acid decreased viability, increased TAC levels and did not cause a significant change in TOS levels in HT-29 cells. These findings may indicate that SA has curative effects on CRC.

Keywords: *Anticancer, Antioxidant activity, Colorectal cancer, HT-29 Cells, Syringic acid*

1. Introduction

Colon cancer or colorectal cancer (CRC) is a serious type of cancer with high incidence and mortality rates in developed countries (Pacal et al., 2020). It is the second leading cause of cancer-related deaths worldwide and it is estimated that more than 930,000 deaths will occur due to CRC (WHO, 2023). It is the second most common type of cancer in women and the third in men, and death rates are 25% higher in men than in women (Li et al., 2021). Risk factors for CRC, in which environmental and genetic factors play a role, include age, overweight and obesity, sedentary lifestyle, smoking, excessive alcohol intake, low-fiber and high-fat diet, and consumption of processed, burnt or charred red meat (Ahmed, 2020). Other risk factors such as personal or family history of colorectal polyps or CRC, inherited conditions such as Lynch syndrome, personal history of inflammatory bowel disease, racial and ethnic background, and the presence of type 2 diabetes also play a role in the development of CRC (Simon, 2016). Regular physical activity, a diet rich in fruits and vegetables, a high-fiber diet, a diet rich in folate, calcium, dairy products, vitamin D, vitamin B6, magnesium intake, fish consumption, garlic and regular aspirin use are protective factors associated with a reduced incidence of CRC (Thanikachalam & Khan, 2019). Gene mutations related to oncogenes, tumor suppressor genes and DNA repair mechanisms are involved in the pathogenesis of CRC. Depending on the origin of the mutation, colorectal carcinomas are classified as sporadic (70%), hereditary (5%) and familial (25%). Pathogenic mechanisms that lead to this situation; There are three types: chromosomal instability, microsatellite instability and methylator phenotype of CpG islands (I. Mármol et al., 2017). Tumors typically with chromosomal instability are known to have mutations in specific oncogenes and/or tumor suppressor genes such as APC, KRAS, PIK3CA, BRAF, SMAD4, or TP53 (Schmitt & Greten, 2021).

Standard treatments for CRC are surgery, chemotherapy, and radiotherapy, which can be used in combination (Johdi & Sukor, 2020). Chemotherapy for first-line treatment of metastatic disease is typically a combination of 5-Fluorouracil, folinic acid, and oxaliplatin (FOLFOX protocol) or irinotecan (FOLFIRI protocol). 5-Fluorouracil in the FOLFOX regimen can be replaced with capecitabine, but the combination of capecitabine with irinotecan is known to be more toxic than FOLFIRI. Double (two chemotherapeutic agents) and triple (three chemotherapeutic agents) chemotherapy regimens consisting of 5-fluorouracil, folinic acid, oxaliplatin and irinotecan (FOLFOXIRI protocol) have been shown to be effective (Kuipers et al., 2015). The Food and Drug Administration (FDA) and the European Medicines Agency (EMA) have approved monoclonal antibodies such as cetuximab, panitumumab, bevacizumab and ramucirumab (Smith & Desai, 2018). Although monoclonal antibodies show high specificity against the targeted antigens, their duration of action is short

and the risk that cells producing high levels of protein may escape from the T cell and survive in the host restricts their use. Although immune checkpoint inhibitors, one of the new trends in immunotherapy, are the most effective drug group in combination therapy, they may have negative effects such as systemic toxicity. Many patients succumb to disease relapse after treatment. Therefore, it is important to find alternative and effective treatments for the treatment of CRC patients (Johdi & Sukor, 2020). Syringic acid (SA), also known as 4-hydroxy-3,5-dimethoxybenzoic acid, is a phenolic compound from the dimethoxybenzene subfamily of benzoic acids and is abundant in some certain types of fruits and green trees. Recent studies have shown that, like other polyphenols, this substance has antioxidant, anti-inflammatory, anti-lipid peroxidative, anti-mitogenic and anti-cancer effects by affecting target molecules involved in the cell cycle (Mihanfar et al., 2021; Srinivasulu et al. ., 2018). It is stated that SA can modulate proteins, transcriptional factors, growth factors and signaling molecules, especially in various cancer cells (J. Pei et al., 2021). SA may be a promising therapeutic agent in the treatment of CRC through various mechanisms (Gheena & Ezhilarasan, 2019). For this purpose, in our study, the effects of SA on Human Colorectal Adenocarcinoma Cell Line (HT-29) were examined.

2. Material Method

2.1. Cell Culture Procedure

HT-29 cell line was incubated at 37°C in Roswell Park Memorial Institute Media (RPMI 1640) medium with stable L-Glutamine containing 10% Fetal Bovine Serum (FBS) and penicillin-streptomycin (100 U/ml-100 µg/ml) antibiotic. It was grown in an incubator containing 37 $^{\circ}$C temperature, 5% carbon dioxide (5%) and 95% humidity and in 25-75 cm^2 cell culture flasks. After sufficient confluency (min. 90%), the 75 cm^2 flask was washed with Phosphate Buffer Saline (PBS), and the cells were separated from the flask by adding Tyripsin-EDTA. After passaging, cells were transferred to 96-well plates. When the cells covered approximately 80% of the well surface, SA was applied to the wells at doses of 6.25 µg/mL, 12.5 µg/mL, 25 µg/mL, 50 µg/mL and 100 µg/mL. After 24 hours, cell viability was measured with 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) solution containing 5 mg/mL.

2.2. Cell Viability

MTT

MTT test was used to evaluate cell viability. 10 µL of MTT solution was added to each well and after the incubation period, the formazan crystals was dissolved in 100 µl of dimethyl sulfoxide (DMSO) (Sigma Aldrich). Finally, the absorbance value was determined at 570 nm in the spectrophotometer (BioTek Instruments, USA) (Okkay & Ferah Okkay, 2022).

2.3. Markers of Oxidative Stress

Total Antioxidant Capacity (TAC) and Total Oxidant Status (TOS)

TAC and TOS kits were used to determine oxidative stress levels (Rel Assay Diagnostic). For the TAC kit, 18 µl of sample and standards were added to each well. Then, 300 µl Reagent 1 was added and the contents of each well were mixed with Heidolph Microtiter Plate Shaker Titramax at 300 rpm for 30 seconds. After 30 seconds, the first reading was taken on the spectrophotometer at 660 nm. Then, 45 µl Reagent 2 was added to each well, a second mixing was made, and after 5 minutes of incubation at 37 °C, a second reading was taken at 660 nm. The results were calculated according to the formulas in the kit procedure. For the TOS kit, 45 µl of sample and standards were added to each well. After the addition of 300 µl Reagent 1, the wells were homogenized with Heidolph Microtiter Plate Shaker Titramax at 300 rpm for 30 seconds. Afterwards, 15 µl Reagent 2 was added and the mixing procedure was performed again. After 5 minutes of incubation at 37 °C, a second reading was taken at 530 nm. The results were calculated according to the formulas in the kit procedure (Sezen et al., 2023).

2.4. Statistical Analysis

Data were analyzed with the GraphPad 9.5 program. ELISA results were analyzed using the One-way analysis of variance (ANOVA) analysis of variance test. For Post-HOC analysis Dunnett test was performed.

3. Findings

3.1. MTT

MTT analysis results are shown in Figure 1. SA application at doses of 6.25 µg/mL, 12.5 µg/mL, 25 µg/mL, 50 µg/mL and 100 µg/mL significantly reduced the viability of the cells compared to the control group.

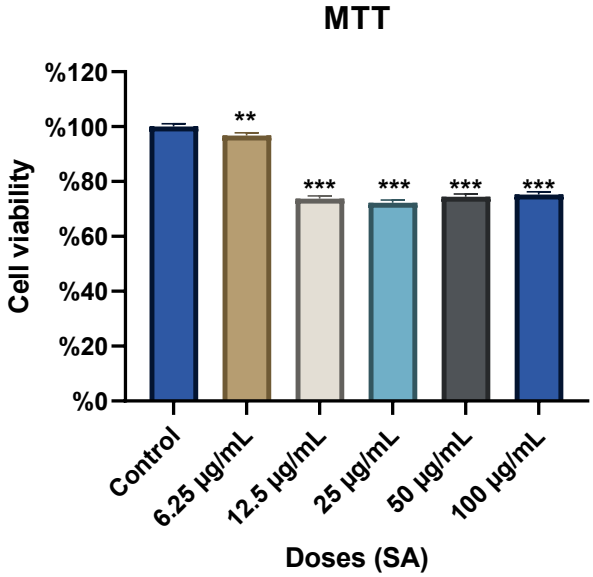

Figure 1. Effect of SA on viability in HT-29 cells ($p<0.05$, the difference between groups **; $p<0.001$, the difference between groups ***)

3.2. TAC-TOS

TAC-TOS analysis results are seen in Figure 2. In the TAC test, SA at doses of 6.25 µg/mL and 12.5 µg/mL significantly increased the antioxidant level, and at doses of 50 µg/mL and 100 µg/mL, it significantly decreased it. Considering the TOS test, a significant increase in oxidant levels was observed at doses of 6.25 µg/mL, 25 µg/mL, 50 µg/mL and 100 µg/mL.

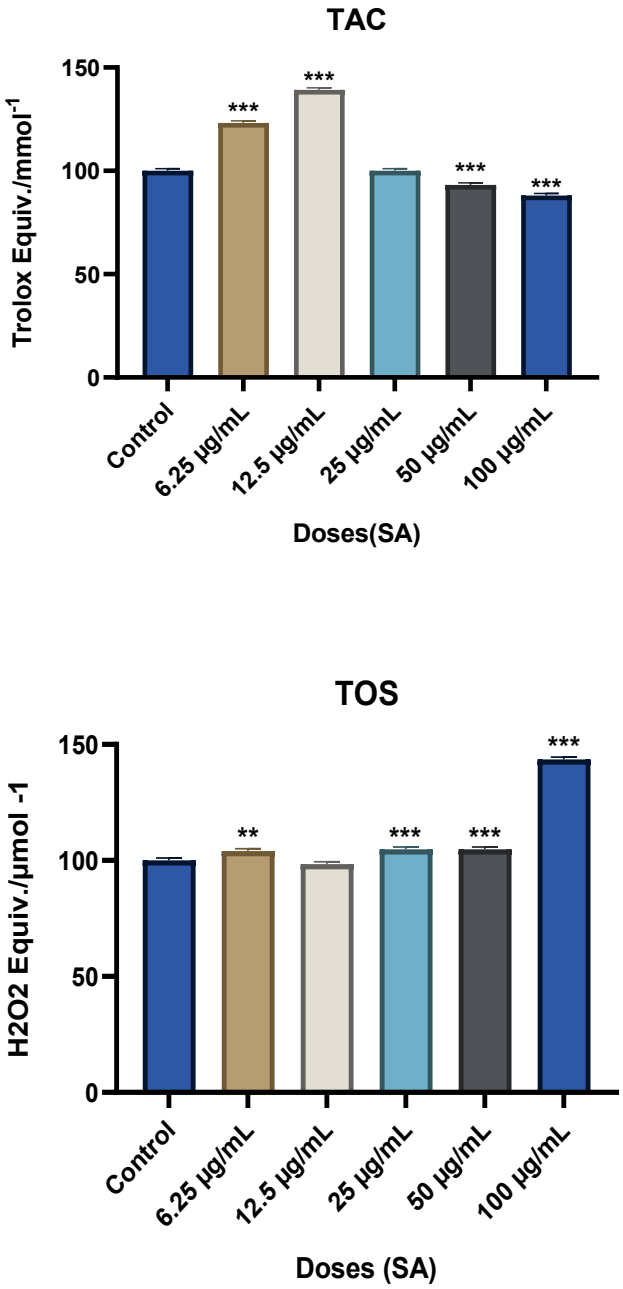

Figure 2. Effect of SA on TAC and TOS levels in HT-29 cells ($p<0.05$, the difference between groups **; $p<0.001$, the difference between groups ***)

4. Discussion

CRC is one of the most common cancers worldwide, with between one and two million new cases diagnosed each year, and its incidence has been increasing year by year (Inés Mármol et al., 2017). CRC; it is associated with biological damage caused by many pathological factors such as oxidative stress caused by reactive oxygen species (ROS) on DNA and RNA, parameter changes in the antioxidant system and lipid peroxidation (Yücel et al., 2012).

In the group of polyphenols, phenolic acids are compounds with antioxidant effects (Srinivasulu et al., 2018). SA, in its phenolic component structure, is a bioactive compound with antioxidant, anti-inflammatory, anti-lipid peroxidative and anti-cancer effects through its effects on target molecules involved in the cell cycle. It also functions as a free radical scavenger and acts against ROS (Ferah Okkay et al., 2022). It has been reported that ROS levels in CRC cells are generally higher than in normal cells and potential ROS-targeted therapies against CRC are promising (Lin et al., 2018). In a study conducted in 2019, it was stated that SA induced cytotoxicity by causing an increase in ROS on the Human Hepatoblastoma Cell Line (HEPG2). Thus, SA can have a positive therapeutic effect in anticancer research with its various effects on ROS (Gheena & Ezhilarasan, 2019).

Antioxidants are known to play a central role in preventing lipid peroxidation (Clemente et al., 2020). It has been stated that SA is a strong inhibitor of low-density lipoprotein oxidation, supports the scavenging of free radicals, and shows antioxidant activity by reducing malondialdehyde (MDA) production. Sancak et al., in his study, it was shown that SA significantly increased TAC levels and significantly decreased TOS levels in rats with renal ischemia reperfusion (Sancak et al., 2016).

In another study, it was shown that SA treatment applied to rats with acute pancreatitis significantly reduced TOS levels, which have an effect on oxidative stress (Cikman et al., 2015). In our current study, in the TAC test, SA at doses of 6.25 µg/mL and 12.5 µg/mL significantly increased the antioxidant level, and at doses of 50 µg/mL and 100 µg/mL, it decreased it. Considering the TOS test, a significant increase in oxidant levels was observed at doses of 6.25 µg/mL, 25 µg/mL, 50 µg/mL and 100 µg/mL. This suggests that SA at doses of 50 µg/mL and 100 µg/mL may be toxic to HT-29 cells. In our study, it can be said that the positive effects of SA on increasing antioxidant levels and reducing oxidative stress support the studies in the literature, and the ideal dose of its effect on HT-29 is 12.5 µg/mL.

In addition to oxidative stress and lipid peroxidation, inflammation is known to be an important trigger in CRC. Growing evidence reveals the potential role of inflammation in the development and progression of CRC types, as well as treatment with anti-inflammatory drugs (Long et al., 2017). A study has shown that SA inhibits the development of inflammatory mediators by modulating the AKT/mTOR signaling pathway on gastric cancer, thus it can induce apoptosis in various cancer cells (Jinjin Pei et al., 2021). For this reason, SA may be a candidate agent for use in CRC treatment with its anti-inflammatory effect.

SA sensitizes CRC cells to conventional chemotherapeutic drugs. It has been stated that this effect of SA may be related to its inhibition of molecular targets in cancer treatment (Abaza et al., 2013). According to our MTT results, the significant decrease in viability of HT-29 cells confirms the toxicity of SA on cancer cells. Further studies are needed to prove whether this toxicity is related to inhibition of molecular targets.

In a study conducted by Mihanfar et al., SA reduced the viability of SW-480 human colorectal cells within 48 hours (Mihanfar et al., 2021). In our study, the fact that SA significantly reduced HT-29 cell viability at doses of 6.25 µg/mL, 12.5 µg/mL, 25 µg/mL, 50 µg/mL and 100 µg/mL within 24 hours supports the information in the literature. While SA caused a significant decrease in cell viability at a dose of 12.5 µg/mL, it did not cause a significant decrease in TOS levels. For this reason, we think that the most ideal dose in terms of reducing cell viability and increasing antioxidant levels is 12.5 µg/mL.

5. Conclusion

Studies show that the antioxidant, anti-inflammatory, anti-lipid peroxidative and anticancer effects of SA on CRC. This investigate supports the evidence showing the curative effect of SA on CRC.

References

Abaza, M.-S., Al-Attiyah, R. a., Bhardwaj, R., Abbadi, G., Koyippally, M., & Afzal, M. (2013). Syringic acid from Tamarix aucheriana possesses antimitogenic and chemo-sensitizing activities in human colorectal cancer cells. Pharmaceutical

Biology, 51(9), 1110-1124. https://doi.org/10.3109/13880209.2013.781194

Ahmed, M. (2020). Colon Cancer: A Clinician's Perspective in 2019. Gastroenterology Res, 13(1), 1-10. https://doi.org/10.14740/gr1239

Cikman, O., Soylemez, O., Ozkan, O. F., Kiraz, H. A., Sayar, I., Ademoglu, S., Taysi, S., & Karaayvaz, M. (2015). Antioxidant Activity of Syringic Acid Prevents Oxidative Stress in l-arginine-Induced Acute Pancreatitis: An Experimental Study on Rats. Int Surg, 100(5), 891-896. https://doi.org/10.9738/intsurg-d-14-00170.1

Clemente, S. M., Martínez-Costa, O. H., Monsalve, M., & Samhan-Arias, A. K. (2020). Targeting Lipid Peroxidation for Cancer Treatment. Molecules, 25(21), 5144. https://www.mdpi.com/1420-3049/25/21/5144

Ferah Okkay, I., Okkay, U., Gundogdu, O. L., Bayram, C., Mendil, A. S., Ertugrul, M. S., & Hacimuftuoglu, A. (2022). Syringic acid protects against thioacetamide-induced hepatic encephalopathy: Behavioral, biochemical, and molecular evidence. Neuroscience Letters, 769, 136385. https://doi.org/https://doi.org/10.1016/j.neulet.2021.136385

Gheena, S., & Ezhilarasan, D. (2019). Syringic acid triggers reactive oxygen species–mediated cytotoxicity in HepG2 cells. Human & Experimental Toxicology, 38(6), 694-702. https://doi.org/10.1177/0960327119839173

Johdi, N. A., & Sukor, N. F. (2020). Colorectal Cancer Immunotherapy: Options and Strategies. Front Immunol, 11, 1624. https://doi.org/10.3389/fimmu.2020.01624

Kuipers, E. J., Grady, W. M., Lieberman, D., Seufferlein, T., Sung, J. J., Boelens, P. G., van de Velde, C. J., & Watanabe, T. (2015). Colorectal cancer. Nat Rev Dis Primers, 1, 15065. https://doi.org/10.1038/nrdp.2015.65

Li, J., Ma, X., Chakravarti, D., Shalapour, S., & DePinho, R. A. (2021). Genetic and biological hallmarks of colorectal cancer. Genes Dev, 35(11-12), 787-820. https://doi.org/10.1101/gad.348226.120

Lin, S., Li, Y., Zamyatnin, A. A., Jr., Werner, J., & Bazhin, A. V. (2018). Reactive oxygen species and colorectal cancer. J Cell Physiol, 233(7), 5119-5132. https://doi.org/10.1002/jcp.26356

Long, A. G., Lundsmith, E. T., & Hamilton, K. E. (2017). Inflammation and Colorectal Cancer. Current Colorectal Cancer Reports, 13(4), 341-351. https://doi.org/10.1007/s11888-017-0373-6

Mármol, I., Sánchez-de-Diego, C., Pradilla Dieste, A., Cerrada, E., & Rodriguez Yoldi, M. J. (2017). Colorectal Carcinoma: A General Overview and Future Perspectives in Colorectal Cancer. Int J Mol Sci, 18(1). https://doi.org/10.3390/ijms1801019

Mármol, I., Sánchez-de-Diego, C., Pradilla Dieste, A., Cerrada, E., & Rodriguez Yoldi, M. J. (2017). Colorectal Carcinoma: A General Overview and Future Perspectives in Colorectal Cancer. International Journal of Molecular Sciences, 18(1), 197. https://www.mdpi.com/1422-0067/18/1/197

Mihanfar, A., Darband, S. G., Sadighparvar, S., Kaviani, M., Mirza-Aghazadeh-Attari, M., Yousefi, B., & Majidinia, M. (2021). *In vitro* and in vivo anticancer effects of syringic acid on colorectal cancer: Possible mechanistic view. Chemico-Biological Interactions, 337, 109337. https://doi.org/https://doi.org/10.1016/j.cbi.2020.109337

Okkay, U., & Ferah Okkay, I. (2022). *In vitro* neuroprotective effects of allicin on Alzheimer's disease model of neuroblastoma cell line. Journal of

Surgery and Medicine, 6(2), 209-212. https://doi.org/10.28982/josam.1068336

Pacal, I., Karaboga, D., Basturk, A., Akay, B., & Nalbantoglu, U. (2020). A comprehensive review of deep learning in colon cancer. Computers in Biology and Medicine, 126, 104003. https://doi.org/https://doi.org/10.1016/j.compbiomed.2020.104003

Pei, J., Velu, P., Zareian, M., Feng, Z., & Vijayalakshmi, A. (2021). Effects of Syringic Acid on Apoptosis, Inflammation, and AKT/mTOR Signaling Pathway in Gastric Cancer Cells. Front Nutr, 8, 788929. https://doi.org/10.3389/fnut.2021.788929

Pei, J., Velu, P., Zareian, M., Feng, Z., & Vijayalakshmi, A. (2021). Effects of Syringic Acid on Apoptosis, Inflammation, and AKT/mTOR Signaling Pathway in Gastric Cancer Cells [Original Research]. Frontiers in Nutrition, 8. https://doi.org/10.3389/fnut.2021.788929

Sancak, E. B., Akbas, A., Silan, C., Cakir, D. U., Turkon, H., & Ozkanli, S. S. (2016). Protective effect of syringic acid on kidney ischemia-reperfusion injury. Renal Failure, 38(4), 629-635. https://doi.org/10.3109/0886022X.2016.1149868

Schmitt, M., & Greten, F. R. (2021). The inflammatory pathogenesis of colorectal cancer. Nat Rev Immunol, 21(10), 653-667. https://doi.org/10.1038/s41577-021-00534-x

Sezen, S., Ertuğrul, M. S., Balpınar, Ö., Bayram, C., Özkaraca, M., Okkay, I. F., Hacımüftüoğlu, A., & Güllüce, M. (2023). Assessment of antimicrobial activity and *In Vitro* wound healing potential of ZnO nanoparticles synthesized with Capparis spinosa extract. Environ Sci Pollut Res Int. https://doi.org/10.1007/s11356-023-30417-8

Simon, K. (2016). Colorectal cancer development and advances in screening. Clin Interv Aging, 11, 967-976. https://doi.org/10.2147/cia.S109285

Smith, K. M., & Desai, J. (2018). Nivolumab for the treatment of colorectal cancer. Expert Rev Anticancer Ther, 18(7), 611-618. https://doi.org/10.1080/14737140.2018.1480942

Srinivasulu, C., Ramgopal, M., Ramanjaneyulu, G., Anuradha, C. M., & Suresh Kumar, C. (2018). Syringic acid (SA) – A Review of Its Occurrence, Biosynthesis, Pharmacological and Industrial Importance. Biomedicine & Pharmacotherapy, 108, 547-557. https://doi.org/https://doi.org/10.1016/j.biopha.2018.09.069

Thanikachalam, K., & Khan, G. (2019). Colorectal Cancer and Nutrition. Nutrients, 11(1), 164. https://www.mdpi.com/2072-6643/11/1/164

WHO. (2023). Colorectal cancer. Retrieved 02.10 from https://www.who.int/news-room/fact-sheets/detail/colorectal-cancer#:~:text=Colorectal%20cancer%20is%20the%20third,people%20aged%2050%20and%20above.

Yücel, A. F., Kemik, O., Kemik, A. S., Purisa, S., & Tüzün, I. S. (2012). Relationship between the levels of oxidative stress in mesenteric and peripheral serum and clinicopathological variables in colorectal cancer. Balkan Med J, 29(2), 144-147. https://doi.org/10.5152/balkanmedj.2011.035