http://pmjournal.ir Original Article
Spring 2021, Volume 6, Issue 23 (17-24)

DOI: 10.22034/pmj.2021.249038

Induction of Bax and Caspase-3 Genes Expression by Simultaneous Administration of Phycocyanin and Citrullus Colocynthis Extract in Human Colorectal Cell Line (HT-29)

Fatemeh Darabi[1], Halimeh Hassanpour[2], Parvaneh Maghami[1], Sohameh Mohebbi [3], Somayeh Arabzadeh[3*]

[1]Department of Biology, Faculty of Basic science, Science and Research Branch, Islamic Azad university
[2] Aerospace Research Institute, Ministry of Science Research and Technology, Tehran 834-14665, Iran
[3]Department of Biology, Faculty of Basic science, Ale Taha institute of higher education

**Corresponding author:* Somayeh Arabzadeh. Department of Biology, Faculty of Basic science, Ale Taha institute of higher education, *Email: sahar.arabzadeh@gmail.com*

Submitted: 2021-11-13
Accepted: 2021-12-15

Keywords:
Citrullus Colocynthis extract
phycocyanin
Bax
Caspase 3
Colon Cancer

©2021.Personalized Medicine Journal

Abstract:

Introduction: In this study the effect of *C. colocynthis* extract and phycocyanin on the growth of colon cancer cells, and the expression of Caspase 3 and Bax were investigated.

Materials & Methods: colon cancer cells (HT29) were treated with different concentrations of *C. colocynthis* and phycocyanin extracts for 24, 48 & 72 hours. The percentage of cell survival was then measured by MTT assay. Expression of Caspase 3 and Bax genes was investigated by real-time PCR. At the end results were analyzed by SPSS software.

Results: Significant differences were observed in the average percentage of dead cells with 2 to 30 mg/¼L concentration of extract, phycocyanin, extract + phycocyanin after 24, 48 and 72 hours ($p<0.05$). The combination of extract and phycocyanin as well as phycocyanin alone showed stronger inhibitory effects on growth of cancer cells compared to extract ($p <0.05$). The expression of Bax gene was significantly increased by treatment of combination of extract and phycocyanin (2.55-fold) and also *C. colocynthis* extract alone (1.67-fold) ($p <0.01$). In addition, the combination of extract and phycocyanin and Abujahl watermelon extract significantly increased the expression of caspase 3 gene (2.15 and 1.75), respectively ($p <0.01$).

Conclusion: The anticancer effect of *Citrullus Colocynthis* extract, as well as phycocyanin can be applied by increase the expression of Bax and caspase 3 genes and as a result, apoptosis induction in cancer cells.

INTRODUCTION

Most colorectal cancers are derived from non-benign small tumors (blood cells) or adenomatous polyps of colon. Although the polyps could be available in small size along with fewer symptoms, but gradually they can develop into colorectal cancer. Polyps are visible protrusions of mucosal epithelium, pathologically are classified into various types of non-neoplastic hamartoma, hyperplastic mucosal proliferation, and adenomatous polyps. Only adenomas are considered pre-cancerous lesions with the potential ability to transform into cancerous masses (1).

According to epidemiological evaluations, the global prevalence of colorectal cancer is higher in males than females and varies among the different parts of the world (2,3). According to an epidemiological study conducted in 2009, more than 50,000 new cancer patients were diagnosed in Iran each year and 38% of which are related to gastrointestinal tract anomalies (2). Thus, according to the previous studies, the prevalence of colorectal cancer in the Iranian population was ranked 3rd and 4th of males and females, respectively (3).

Therapeutic procedures of colorectal cancer depend entirely on the tumor region (colon or rectum) and the stage of the cancer progression. Although common

Copyright © The Author(s). This is an open-access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org /lic enses/by /4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

treatments for colorectal cancers include surgical procedures, chemotherapy, biological therapy, and radiation therapy, some patients have a combination of these therapeutic processes together (4). Surgery is the most common remedy used for colorectal cancer (5). The most advanced new aspect of modern medicine is in the field of pharmacogenomics, which means the effect of genetic structure of individuals in determining their response to drugs. Pharmacogenomics is also used instead of the word pharmacogenetic. Pharmacogenetics means the existence of variable sequences in specific candidate genes that cause different responses to a drug, and is in fact the relationship between individuals› genes and their response to the drug. Many researchers believe that the drugs on the market only affect a small number of patients and have no effect on other people and in some cases have an adverse effect. This problem is due to the fact that the so-called drugs are made as «one for all» (6).

By sequencing the human genome and discovering the genetic differences of individuals, it will be determined that the different responses of individuals to a drug are likely to be due to genetic differences. The tendency of individual medicine towards the design and preparation of drugs is commensurate with the molecular properties of individuals. In fact, variation in genes encoding metabolizing enzymes. Drugs, drug transporters, or drug targets can trigger different responses in individuals (7).

Induction of apoptosis, as the cell programmed death, is considered a crucial inhibitory mechanism in cancer cells proliferation. Several intracellular proteins are involved in the regulation of apoptosis, such as Cyt-*c*, caspase-3, and Bax (8). Caspases are a crucial part of the cysteine-aspartate protease enzymes family. Since these intracellular proteins are involved in disintegration of cellular protein structures, they have a pivotal role in apoptosis process. Functionally, the caspases are divided into two main types of initiators (including caspases of 8, 9, and 10) and the executioner types (including caspases of 3, 6, and 7). Both initiator and executioner caspases operate together convergently. Following activation of executioner caspases, the cascading consequences of cell destruction initiate, leading to apoptosis. Among various types of caspases such as caspases-1, 3, 4, and 9, caspase-3 plays a key role in programmed cell death. Caspase-3 is activated by type 9, leading to cell death through the process of intracellular organelles degradation. B-cell lymphoma-2 (Bcl-2) family proteins were primarily identified as proto-oncogene agents in B-cell follicular lymphoma with a crucial role in regulation of apoptosis process. Bcl2-associated X protein (Bax) is a member of pro-apoptotic Bcl-2 family. Increased levels of Bax gene expression can lead to an accelerated intracellular release of Cyt-*c* and consequently the activation of caspase-9 function. Thus, incremental trends of caspases and Bax proteins can potentially induce cancer cell death (8).

The application of cytostatic drugs or natural substances to inhibit cancer progression is an important subject of cancer research. Dried biomass products of Arthrospira have been categorized as "generally recognized as safe" (GRAS) by the US Food and Drug Administration (FDA). The ingredient of Arthrospira consist of phycocyanin (PHYC), a blue-red fluorescent, water-soluble and non-toxic biliprotein pigment. Phycocyanin is reported to be the main active ingredient of Arthrospira and was shown to have therapeutic properties, including antioxidant, anti-inflammatory, immune-modulatory and anticancer activities (9). Various studies showed that phycocyanin extract has an anticancer effect in a time and dose-dependent manner (10-11).

Moreover, *Citrullus colocynthis* (CIT) has been used in the treatment of several diseases comprising diabetes, asthma, constipation, toothache, leprosy, bronchitis, jaundice, joint pain, mastitis, and skin infections (12-13). *Citrullus colocynthis* is a species that belongs to the family cucurbitaceae (14). It has been known as a natural medicinal plant with wide biological activities, including antioxidant, anti-inflammatory, and antilipidemic effects (15).

This study aimed to investigate the synergistic anti-cancer effects of Phycocyanin (PHYC) and Citrullus Colocynthis (CIT) extract on the genes expression of Bax and caspase-3 in a colorectal cancer cell line (Ht29).

MATERIALS AND METHODS

Preparation of CIT extract and PHYC solution

PHYC solution and herbal extract of CIT were purchased from Barij Essential Pharmaceutical Company (Kashan, Iran). Different concentrations of each solution were prepared, including 1, 2, 5, 10, 20, 50, and 100 µg/ml in deionized water and stored in a refrigerator (4°C).

Treatment of HT-29 cells with CIT and PHYC extracts

Human colorectal cancer lineage (HT-29) was purchased from Pasteur Institute (Tehran, Iran), and the cells were cultured in the standard culture medium of DMEM (Gibco) with 10% inactivated fetal bovine serum (FBS), penicillin (100 unit/ml), and streptomycin (10 µg/ml) under standard conditions. Then, the HT-29 cells were treated with various prepared concentrations in treatment groups of CIT, PHYC, and CIT+PHYC (with the same ratio of 1 CIT: 1 PHYC). Following 24, 48, and 72 hrs of treatment, the rate of HT-29 cell death was assessed by MTT assay to obtain the best effective concentration with less toxicity.

Assessment of extracts cytotoxicity on HT-29 cancer cell line

Cytotoxic effect of the extracts was investigated by MTT [3- (4,5-dimethylthiazol-2-yl) -2, 5-diphenyl tetrazolium bromide] assay. First, the cell suspension (approximately 10^4 cells) was transferred to a 96-well plate. Then, the cells were treated with different concentrations of CIT extracts and PHYC. Following 24 hrs of incubation at 37 °C, the cell viability was assessed by MTT assay. This protocol was performed in triple replications for each concentration. In This process, the supernatant was removed from the 96-well plate and replaced with 200µl of MTT solution and incubated at 37 °C (2 hrs). Then, 125 ml of DMSO was added to the wells to dissolve the MTT crystals. The plates were placed on a shaker (15 min), and then the absorbance of the solution was read at 570nm using a spectrometer. Finally, the mean survival rate of HT-29 cells treated with CIT and PHYC were compared to the control group (untreated cells).

Total RNA extraction from HT-29 cancer cell line

The process of RNA extraction was performed from the cell suspension using the RNX-Plus kit (SinaClon; RN7713C) based on the manufacturer›s instruction. Then, the quality and quantity of the extracted RNA were assessed using NanoDrop and electrophoresis on the agarose gel. Finally, the cDNA was synthesized according to the standard protocol.

Real-time PCR assay

The primers used for the Real-time PCR technique for the genes of Bax, caspase-3 and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) as internal control genes were purchased from Sina Colon Co. (Table 1). Following 40 amplification steps, the levels of expressed mRNAs were calculated using the formula; [$\Delta CT = CT_{(target)} - CT_{(control)}$] compared to the GAPDH. Finally, gene expression levels were evaluated using the $2^{-\Delta Ct}$ method.

Statistical analysis

Shapiro–Wilk and Levene›s tests were used to determine the normal distribution of data and variances homogeneity, respectively. Quantitative data description was also performed based on central scattering indices, including the Mean ± Standard Deviation for the data of real-time PCR. One-way analysis of variance was used to investigate the significant differences among the groups. Following assessment of statistically significant differences, the ANOVA test was used to determine the differences among the groups. All data analysis was applied using SPSS (v. 20) software, and the p<0.05 was considered a significant level.

RESULTS

Inhibitory effects of CIT and PHYC on the proliferation of HT-29 cell line

A significant (p<0.05) difference was detected in the mean percentage of HT-29 dead cells following administration of 2-30 µg/µl extracts in CIT, PHYC, and CIT+PHYC groups after 24 and 48 hrs of treatment. While at concentrations of 1, 40, and 50 µg/µl extracts no significant (p>0.05) differences were observed among the treatment groups (Figures 1 and 2). The IC50 value was measured for CIT, PHYC, and CIT+PHYC groups respectively as 22.14±1.18, 5.2±0.86, and 6.8±1.06 µg/µl. These findings indicated higher cytotoxicity characteristics of PHYC and CIT+PHYC compared to the CIT group. Also, the IC50 value of PHYC and CIT+PHYC groups were found respectively 4.68±1.16 and 7.16±1.04 µg/µl after 48 hrs of treatment, indicating significant (p<0.05) higher cytotoxicity effects of these groups compared to CIT (19.23±1.12 µg/µl). In addition, 1-30 µg/µl concentration of extracts represented a significant (p>0.05) difference among the three treatment groups (CIT, PHYC, and CIT+PHYC groups) after 72 hrs of treatment. While concentrations of 40 and 50 µg/µl showed non-significant differences between the three treatment groups (Figure 3). A significant difference was found in the mean value of IC50 in all three treatment groups after 72 hrs of exposure to the extracts. IC50 of PHYC and CIT+PHYC were respectively as 5.25±0.61 and 6.25±1.01 µg/µl representing significant (p<0.05) higher cytotoxicity in comparison with CIT (20.08±0.5 µg/µl). Totally, there was a direct relationship between the concentration of treatments and inhibitory rate of cancer cell growth in which the lowest and the highest impacts were observed at the concentrations of 1 and

Table 1. Sequences of primers for each gene

Genes	Forward	Reverse
Bax	TCCCCCCGAGAGGTCTTTT	CGGCCCCAGTTGAAGTTG
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC
Caspase-3	GCCTGCCGTGGTACAGAACTGG	GCATACAAGAAGTCGGCCTCCA

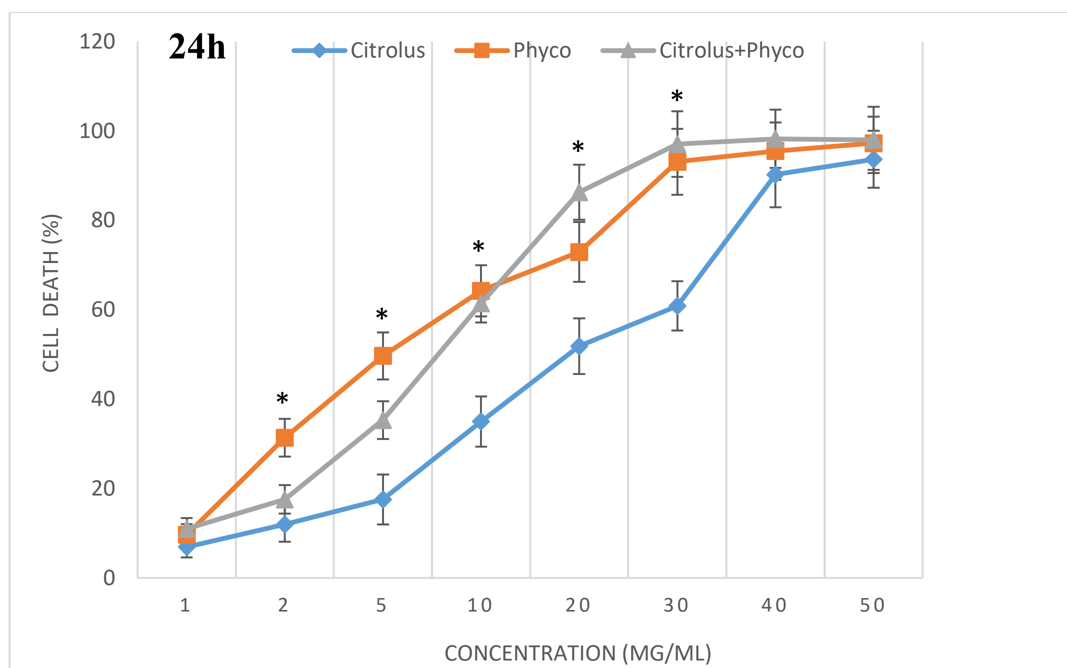

Fig 1. Effects of different concentrations of Phycocyanin and Citrullus Colocynthis on inhibition of cancer cells growth after 24 hrs of treatment in various groups. * represented the significant difference between treatment groups

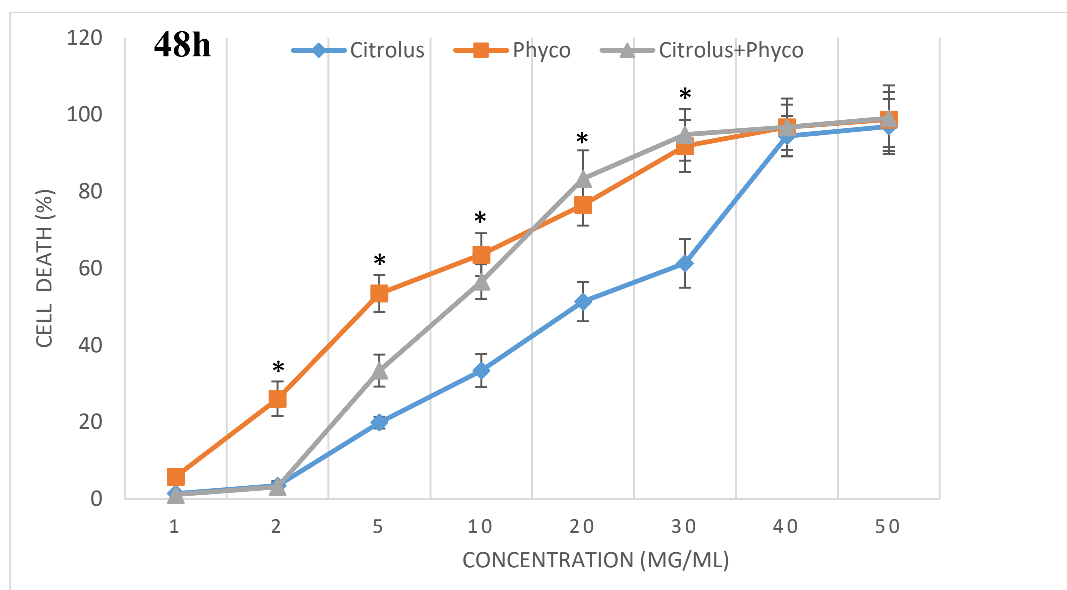

Fig 2. Effects of different concentrations of Phycocyanin and Citrullus Colocynthis on inhibition of cancer cells growth after 48 hrs of treatment in various groups. * represented the significant difference between treatment groups.

50 µg/µl, respectively. No significant differences were detected in the mean percentage of dead HT-29 cells at concentrations of 40 and 50 µg/µl in three treatment groups. However, the PHYC and CIT+PHYC showed a higher inhibitory effect on cancer cells growth at concentrations of 1-30 µg/µl compared to CIT group significantly ($p<0.05$).

Inhibitory effects of CIT and PHYC on the proliferation of HT-29 cell line in various exposure times

Assessments represented a significant ($p<0.05$) difference in cell death among treatment times (including 24, 48, and 72 hrs) at low concentrations of CIT (1 and 2 µg/µl) and PHYC (1 µg/µl). However, at the higher concentrations, no significant ($p>0.05$) differences were found in cell death among the various

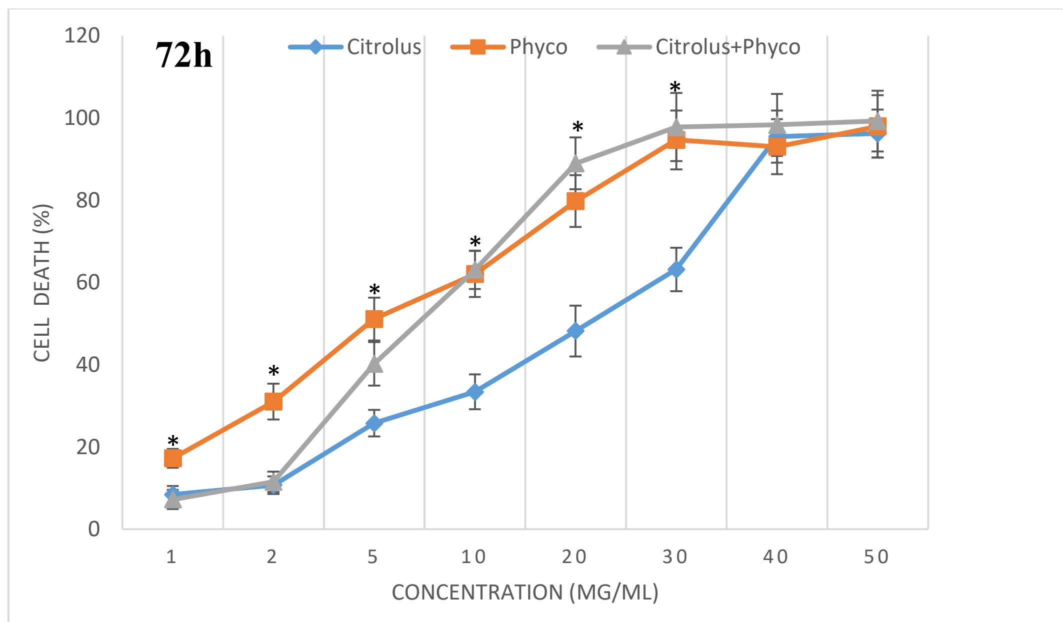

Fig 3. Effects of different concentrations of Phycocyanin and Citrullus Colocynthis on inhibition of cancer cells growth after 72 hrs of treatment in various groups. * represented the significant difference between treatment groups.

times of treatments with CIT and PHYC. Through the increased concentrations of CIT and PHYC, the effect of time showed a decremental trend indicating that the cell death rate was observed in 24 hrs after high dose treatment was such as the cell death rate was observed 48 and 72 hrs after low dose treatment. Low concentration of extracts needs more time to show their effects on cell death. Simultaneous treatment of CIT and PHYC with different concentrations of 1, 2, and 5 µg/µl showed a significant ($p<0.05$) alteration in apoptosis rate among treatment times of 24, 48, and 72 hrs. These results indicated that the apoptotic effects of the extracts are increased through the administration of PHYC.

Effects of CIT and PHYC on Bax gene expression

A significant ($p<0.05$) increased level (2.35-fold) was observed in Bax gene expression of HT-29 cancer cells following administration of CIT extract, while this value was found increased (1.2-fold) non-significantly ($p>0.05$) in PHYC group. Also, gene expression assessment showed that the most effective of extracts on Bax gene expression was seen in CIT+PHYC group significantly ($p<0.05$) by 35.2 times in the treatment groups compared to the control cells (Figure 4).

Effects of CIT and PHYC on caspase-3 gene expression

Expression of caspase-3 gene was increased (75.1-fold) significantly ($p<0.05$) following treatment with CIT extract, while this value was found non-significant ($p>0.05$) in PHYC group (1.19-fold). Simultaneous treatment of the extract and phycocyanin in CIT+PHYC group showed a significant ($P<0.05$) incremental effect (2.15-fold) on caspase-3 gene expression (Figure 5).

DISCUSSION

In general, cancers are heterogeneous diseases that differ in terms of incidence, pathogenicity, metastasis potential, as well as patients› response to treatment (16). The genetic heterogeneity of cancer has made it a very important goal for personalized medicine. Because cell proliferation and DNA replication take place rapidly in cancer cells, for a long time, cancer treatment was based on targeting DNA replication, which is done using chemotherapeutic drugs. but these toxic drugs, in addition to cancer cells, attach to cells that are in the tubular and normal cells of the normal genital tract. It also damages the digestive tract and hair follicles, and their side effects such as decreased blood cells, suppression of the immune system, inflammation in the gastrointestinal tract, and hair loss are inevitable.

Therefore, researchers have always been looking for new ways to diagnose and treat the disease and reduce the costs and injuries caused by it, and it has been more effective in targeting each tumor individually (17). However, cancer has two major differences from other diseases that are currently being treated through personal medicine: first, different patients have tumor-specific factors in cancer; while for other diseases, these factors are somatic genetic variants, and second, if personalized medicine can be used to predict the likelihood of disease. For other diseases, however, personalized medicine seeks to find factors on the basis of which it can design a drug tailored to the patient›s

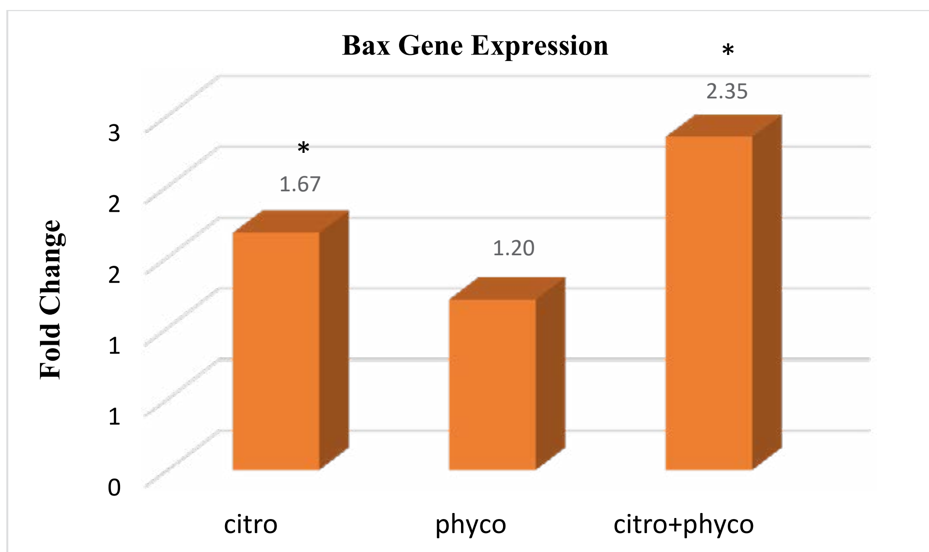

Fig 4. Effect of Phycocyanin and Citrullus Colocynthis on Bax gene expression. * represented significant difference compared to the control group

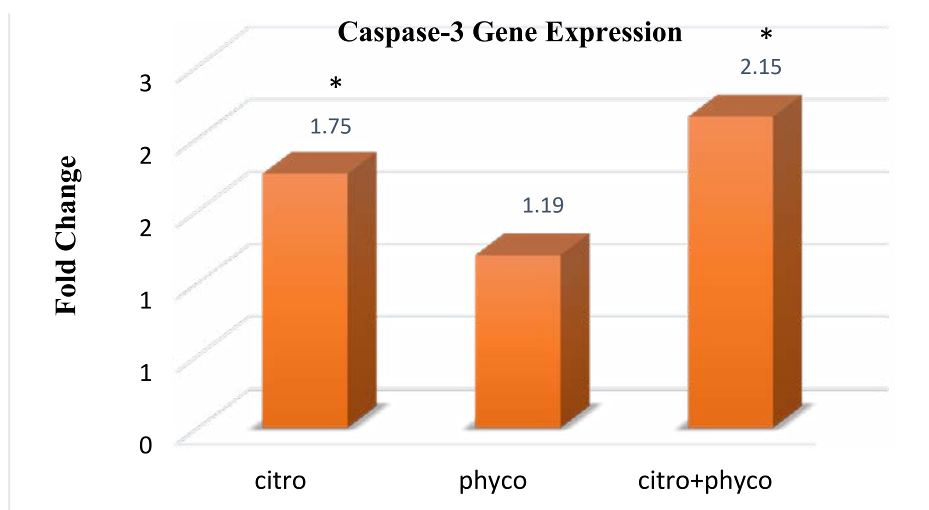

Fig 5. Effect of Phycocyanin and Citrullus Colocynthis on Caspase-3 gene expression. * represented significant difference compared to the control group

characteristics (18).

The importance of personalized medicine in the field of cancer is due to its many advantages. Proper prevention is used even before the onset of the disease, which delays the onset of the disease and reduces the severity of its symptoms. The creation of specific cancer subtypes causes a specific treatment to be performed for each subgroup according to the individual and molecular characteristics of the individual in order to create the best response to the treatment performed, thereby unwanted and toxic side effects resulting from ineffective treatments. , Especially for people who are resistant to this type of treatment,(19,20)

According to the results of this study, PHYC and the combination of CIT+PHYC in all three time periods (24, 48, and 72 hrs) represented the highest inhibitory effects on HT-29 cell line proliferation in comparison with CIT group. By the increased concentrations of extracts in all three treatment groups (CIT, PHYC, and CIT+PHYC), the inhibitory effects on HT-29 cell growth were also increased. Thus, at the concentration of 1 μg/μl extract, the lowest effect was observed and also the highest was found at the dose of 50 μg/μl of all extracts. At lower concentrations (1 and 2 μg/μl), the PHYC showed the highest inhibitory effects on cancer cells› growth compared to other groups. But at higher concentrations (40 and 50 μg/μl) of PHYC, no significant differences were detected. Thus, in all three

treatments groups, the higher concentrations had the most apoptotic effect, although PHYC and CIT+PHYC represented the highest levels of apoptosis induction due to the lower IC50 value.

The results of our study on the expression of Bax and caspase 3 genes showed that the combination of CIT+PHYC increased the expression of Bax gene by 35.2 times in cancer cells. CIT extract also increased the expression of Bax gene by 67.1 times. Besides, although the PHYC increased the expression of Bax gene 1.2-fold, this effect was not statistically significant. Since the Bax and caspase-3 proteins play a crucial role in induction of apoptosis, the results of our study showed that CIT extract as well as CIT+PHYC increased the expression of Bax and Caspase-3 genes resulted in apoptosis of cancer cells.

Although more research is needed, increased expression of Bax and caspase-3 genes seems to be one of the mechanisms of action of the CIT and PHYC on cell death. In this regard, several studies investigated the apoptotic and inhibitory effects of CIT and PHYC on various types of cancer cell lineages. In the study of Tavakol et al., the effects of CIT extract on inhibition of laryngeal cancer cell growth and normal mice fibroblast L-929 were approved. These cytotoxic features were concentration-dependent effects which can lead to apoptosis (21). In Liu›s study, the administration of CIT extract showed anticancer effects on HEPA-2 cells by induction of apoptosis and arrest of cell cycle (22). In another study by Lan et al., it was concluded that cocorbitacin E can potentially inhibit the growth of human breast cancer cells through concentration and time-based criteria. Subsequent analyses showed that cocorbitacin E could arrest the G2/M cell division phase, leading to cell apoptosis. They also showed that cocorbitacin E induced caspase-3 expression and upregulated P21 and P27. The results of our study were mainly in line with the findings of Lan et al (23).

In our study, CIT extract increased the apoptosis rate in HT-29 cell lines by increasing the expression of Bax and caspase-3 genes. Also, another study approved the effect of hydroalcoholic extract of CIT on the expression of caspase genes (especially caspases-3 and 8) in the MCF-7 breast cancer cell line. Treatment of cancer cells was associated with increased levels of expression of caspase-related genes and eventually induction of apoptosis. Also, with increasing the concentration and culturing time in the presence of CIT, the percentage of living cancer cells decreased significantly (24). The anti-cancer activity of CIT extract on breast cell lineages and gastric adenocarcinoma was also investigated, indicating the high inhibitory effects against these types of cancers. This inhibitory pathway induces the activity of caspases, Cyt-*c* and Bcl2/Bax leading to the apoptosis induction (25).

According to the anti-cancer effects of PHYC, the results of Hao et al. showed that the PHYC could induce apoptosis, arrest cell cycle, and inhibit cell migration, proliferation, and colonization of lung cancer which was parallel with our findings (26). We found that the PHYC in a dose-dependent pathway can increase the apoptosis rate in colon cancer cells. A study reported that the PHYC induced apoptosis and reduced the number of breast cancer cells in a dose-dependent manner (4). Our findings also approved these results. Although in our study, the PHYC represented high apoptosis, but showed non-significant impacts on Bax and caspase-3 genes expression. PHYC could induce apoptosis in colorectal HT-29 cell lines through various unknown mechanisms, which requires further studies. PHYC can also induce cell cycle arrest in G1 phase in various types of cancers, including colon cancer, breast cancer, and leukemia. On the other hand, other studies showed that the PHYC is able to arrest the progression of the cell cycle in the G2/M phase in pancreatic cancer cells, ovarian cancer cells, and HepG2 cancer cells leading to apoptosis (27-31). In another study by Jiang et al., the anticancer effects of PHYC against MDA-MB-231 cancer cells were examined (30). The results showed that PHYC inhibited the proliferation and colonic formation in cancer cells leading to cell cycle arrest in G0/G1 stage. The scientists also showed that the PHYC reduced the expression of cyclooxygenase-2 and arrest the migrations and metastasis in cancer cells. They also concluded that PHYC can induce apoptosis in cancer cells through activation of P53 MAPK and JNK signaling pathways and inhibition of ERK intracellular pathway (30). In a study conducted by Ravi et al. on 2015, they examined the anti-cancer properties of PHYC against breast cancer. They concluded that PHYC was sensitive to phycocyanin (IC50 = 5.98 μM) compared to normal cells. They also showed that PHYC reduced the proliferation and colonization of cancer cells (32). On the other hand, further studies showed that PHYC arrested cell cycle in G1 and decreased the level of cyclin E and CDK-2 mRNAs and also increased the level of p21. PHYC also reduced the cell migration and angiogenesis and increased cellular adhesion cancer cells. Interestingly, the PHYC reduced the expression of cyclooxygenase-2 leading to the production and secretion of prostaglandin E2 (PGE2). The researchers stated that all of these mechanisms of action of PHYC are mediated through the MAPK signaling pathway (32).

CONCLUSIONS

The results of this study showed that the CIT extract as well as the combined administration of CIT+PHYC could induce apoptosis in HT-29 colorectal cancer cells by increasing the expression of Bax and caspase-3 genes. These changes appear to be one of the mechanisms of apoptosis induction in human HT-

29 colon cancer cells.

REFERENCES

1. Aggarwal B, Prasad S, Sung B, Krishnan S. et al. Prevention and treatment of colorectal cancer by natural agents from mother nature. Curr Colorectal Cancer Rep. 2013; 9(1): 37-56. https://doi.org/10.1007/s11888-012-0154-1
2. Pourfarzi F, Whelan A, Kaldor J, Malekzadeh R. The role of diet and other environmental factors in the causation of gastric cancer in Iran--a population based study. Int J Cancer. 2009; 125: 1953-1960. https://doi.org/10.1002/ijc.24499 .
3. Fletcher R. The diagnosis of colorectal cancer in patients with symptoms: finding a needle in a haystack. BMC Med. 2009; 7: 18-29.https://doi.org/10.1186/1741-7015-7-18
4. Subhashini J, Mahipal SV, Reddy MC, Reddy MM. et al. Molecular mechanisms in C-Phycocyanin induced apoptosis in human chronic myeloid leukemia cell line-K562. Biochem pharmacol. 2004; 68(3): 453-462. https://doi.org/10.1016/j.bcp.2004.02.025
5. Fernandez E, La Vecchia C, Talamini R, Negri E. Joint effects of family history and adult life dietary risk factors on colorectal cancer risk. Epidemiology. 2002; 13: 360-363. https://doi.org/10.1097/00001648-200205000-00019
6. Hulot JS. Pharmacogenomics and personalized medicine: lost in translation? 2010, Available at: http://genomemedicine.com/content/2/2/13.
7. Rodriguez-Antona C, Gomez A, Karlgren M, Sim SC, Ingelman-Sundberg M. Molecular genetics and epigenetic of the cytochrome P450 gene family and its relevance for cancer risk and treatment. Hum Genet. 2010; 127: 1- 17.
8. Mahboubi E, Kmet J, Cook PJ, Ghadirian P. et al. Oesophegeal cancer studies in the Caspian Littoral of Iran: The Caspian Cancer registry. Br J Cancer. 1973; 28: 197-214. https://doi.org/10.1038/bjc.1973.138
9. Braune S, Krüger-Genge A, Kammerer S, Jung F and Küpper JH. Phycocyanin from Arthrospira platensis as Potential Anti-Cancer Drug: Review of In Vitro and In Vivo *Life* 2021, *11*(2),91; https://doi.org/10.3390/life11020091.https://pubmed.ncbi.nlm.nih.gov/33513794
10. Challouf R, Trabelsi L, Ben Dhieb R, El Abed O, Yahia A, Ghozzi K., Ben Ammar J, Omran H, Ben Ouada H. Evaluation of cytotoxicity and biological activities in extracellular polysaccharides released by cyanobacterium Arthrospira platensis. Braz. Arch. Biol. Technol. 2011, 54, 831–838. https://www.scielo.br/j/babt/a/ycrD5DMCKqHsVYKhcs64yFb/?lang=en
11. Liu Q, Huang Y, Zhang R, Cai T, Cai Y. Medical Application ofSpirulina platensisDerived C-Phycocyanin. Evid. Based Complement. Altern. Med. 2016, 7803846. https://pubmed.ncbi.nlm.nih.gov/27293463
12. Abo KA, Fred-Jaiyesimi AA, Jaiyesimi AEA. Ethnobotanical studies of medicinal plants used in the management of diabetes mellitus in Southwestern Nigeria. J Ethnopharmacol. 2008;115(1):67–71. https://pubmed.ncbi.nlm.nih.gov/17950547
13. Adam SEI, Al-Yahya MA, Al-Farhan AH. Response of Najdi sheep to oral administration of Citrullus colocynthis fruits, Nerium oleander leaves or their mixture. Small Rumin Res. 2001;40(3):239–44. https://pubmed.ncbi.nlm.nih.gov/11323208
14. Zaini NAM, Anwar F, Hamid AA, Saari N. a potential source for valuable nutrients and functional foods. Food Res Int. 2011;44(7):2368–76. https://www.tandfonline.com/doi/full/10.1080/10942912.2014.980946
15. Mohamed I. Alzarah, Abdulaziz A. Alaqil, Ahmed O. Abbas, Gamal M. K. Mehaisen, Gouda F. Gouda, Hanaa K. Abd El-Atty, Eman S. Moustafa. Inclusion of *Citrullus colocynthis* Seed Extract into Diets Induced a Hypolipidemic Effect and Improved Layer Performance. *Agriculture* 2021, *11*(9), 808; https://doi.org/10.3390/agriculture11090808
16. Fidler IJ. Tumor heterogeneity and the biology of cancer invasion and metastasis. Cancer Res.1978; 38:2651- 60.
17. Van't Veer LJ, Bernard R. Review article enabling personalized cancer medicine through analysis of gene expression patterns. Nature.2008; 452:564- 570.
18. Maddens CA. Personalized medicine facts, thoughts and future perspective. Master thesis, Cancer Genomics and Developmental Biology. Utrecht University.2011.
19. Ginsburg GS, and McCarthy JJ. Personalized medicine: revolutionizing drug discovery and patient care. Trends Biotechnol.2001; 19:491- 6.
20. Overdevest JB, Theodorescu D, Lee JK. Utilizing the molecular gateway: The path to personalized cancer management. Clin Chem.2009; 55:684-97.
21.Tavakkol Afshari J, Rakhshandeh H, Zamani AR, Mahdavi Sh. et al. Cytotoxicity effects of Citrullus colocynthis on Hep2 and L929 cell lines. Hakim Research Journal. 2005; .8(2): 47-54. https://www.semanticscholar.org/paper/Cytotoxicity-effects-of-Citrullus-colocynthis-on-Afshari-Rakhshandeh/95e58d4a2945254e0469d623fd79c2a2bca6d20c
22. Liu T, Zhang M, Zhang H, Sun C. et al. Inhibitory effects of cucurbitacin B on laryngeal squamous cell carcinoma. Eur Arch Otorhinolaryngol. 2008; 265: 1225-1232. https://doi.org/10.1007/s00405-008-0625-9
23. Lan T, Wang L, Xu Q, Liu W. et al. Growth inhibitory effect of cucurbitacin E on breast cancer cells. Int J Clin Exp Pathol. 2013; 6: 1799-1805.
24. Davoodi RS, Najafi M, Mazaheri M. Effect of Hydro Alcoholic Extract of Citrullus colocynthis fruit on Caspase 3 gene expression in MCF-7 breast cancer cell line. J Shahid Sadoughi Univ Med Sci. 2015; 23(5): 508-518.
25. Rezai M, Davoodi A, Asori M, Azadbakht M. Cytotoxic activity of Citrullus colocynthis Schrad fruit extract on gastric adenocarcinoma and breast cancer cell lines. Int J Pharm Sci Rev Res. 2017; 45(1).
26. Hao S, Yan Y, Li S, Zhao L. et al. The in vitro anti-tumor activity of phycocyanin against non-small cell lung cancer cells. Marine drugs. 2018; 16(6): 178. https://doi.org/10.3390/md16060178
27. Thangam R, SureshV, Princy WA, Rajkumar M. et al. C-Phycocyanin from Oscillatoria tenuis exhibited an antioxidant and in vitro antiproliferative activity through induction of apoptosis and G0/G1 cell cycle arrest. Food chemistry. 2013; 140(1-2): 262-272. https://doi.org/10.1016/j.foodchem.2013.02.060
28. Wang H, Liu Y, Gao X, Carter CL. et al. The recombinant β subunit of C-phycocyanin inhibits cell proliferation and induces apoptosis. Cancer letters. 2007; 247(1): 150-158. https://doi.org/10.1016/j.canlet.2006.04.002
29. Wang Z , Wang N, Chen J, Shen J. Emerging glycolysis targeting and drug discovery from chinese medicine in cancer therapy. Evid Based Complement Alternat Med. 2012; 12: 873175. https://doi.org/10.1155/2012/873175
30. Jiang L, Wang Y, Yin Q, Liu G. et al. Phycocyanin: A Potential Drug for Cancer Treatment. Journal of Cancer. 2017; 8(17): 3416. https://doi.org/10.7150/jca.21058
31. Zhang Y. The current status of treatment for colorectal cancer in China A systematic review. Medicine (Baltimore). 2017; 96: e824. https://doi.org/10.1097/MD.0000000000008242
32. Ravi M, Tentu S, Baskar G, Prasad SR. et al. Molecular mechanism of anti-cancer activity of phycocyanin in triple-negative breast cancer cells. BMC cancer.