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Evaluation of the Antiglycation Effect of Branched Chain Amino Acids and Phytochemical Compounds on RAW 264.7 Cell Line and their Synergistic Effect on Colorectal Cancer Cell Line Panel

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ABSTRACT

Flavonoids and branched chain amino acids (BCAAs) have been recognized as attractive agents for research due to their role in diabetes and cancer. The present study investigates the antiglycation effect of BCAAs and flavonoid compounds and their cytotoxic effect on different colorectal cancer cell lines individually and in combination. This study was conducted by using RAW 264.7 cell line (murine monocyte-macrophage cell and Methylglyoxal (MGO) for inducing the glycation effect. The Sulforhodamine B (SRB) assay was used to study the cytotoxicity of BCAAs and flavonoids compounds using colorectal cell lines SW480, SW620 and CACO2.

Flavonoids showed significant antiglycation effect compared to Aminoguanidine (AMG), while none of the BCAAs compounds displayed antiglycation effect. All flavonoids and branched-chain amino acids showed less degree of cytotoxicity against SW620, CACO2 and SW480 as compared to cisplatin. The synergetic effects was only shown against SW620 and CACO2 cell lines by using the combination of Apigenin and BCAAs while antagonistic effect appeared when combination of Quercetin and Hesperidin were used against SW480, SW620 and CACO2 cell lines.

Flavonoids such as Quercetin, Apigenin and Hesperidin could be used as a remedy for diabetes due to their surpassing effect upon antiglycation action of AMG.

Keywords: Flavonoids, BCAAs, Antiglycation, Cytotoxicity, Co-incubation.

Introduction

Glycation is a reaction between reducing sugar and the amino group of lipid, protein and nucleic acids. Which can occur in *vitro* and in *vivo*.[1] Furthermore, the outcome of the glycation is the production of Advanced Glycation End-products (AGEs) such as carboxymethyllysine, pentosidine, fluorescent and bridged structures. These AGEs are accumulated in the tissues leading to the change in the conformation of protein and its function leading eventually to the emergence of different diseases such as inflammation, atherosclerosis, arthritis, renal failure, diabetes and cancer.[2]

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Although the links between AGEs and certain disease such as cardiovascular disease, diabetic and cancer is understudied.[3] It was reported that AGE was first found in colon, prostate, and breast cancer. Furthermore, it was indicated that AGE could enhance migration, invasion, and growth of the cancer cells, through affecting the basement membrane of the cells.[4] Taking into consideration, several compounds have been identified as antiglycation agents; nevertheless, most of them have not been proven safe or efficient. Therefore, a growing body of research has aimed to minimize the deleterious effects of glycation[5]. Isoleucine (Ile), leucine (Leu), valine (Val) known as branched chain amino acids (BCAAs), have several physiological functions, including protein synthesis, improving insulin resistance, proliferation of lymphocyte, and reducing the apoptosis of hepatocyte.[6-10] Moreover, BCAAs supplements enhance aerobic exercise and decreases the rate of muscles fatigue.[11] The high BCAA plasma level in diabetic patients suggested that these acid could be involved in the development of T2DM.[9,12] Interestingly, it was reported that BCAAs could affect the PI3K/AKT/mTOR pathway where it can enhance their pivotal role in glucose homeostasis via activating mTORC1.[13] It was suggested that BCAAs could provoke negative feedback loop by mTORC1 activation causing inhibition of PI3K/AKT/mTOR pathway, which reverses the oxidative stress

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effect as well as suppressing the growth of cancer cells,[14] which could have a significant antiglycation activity as well.
On the other hand, flavonoids are found in all plants including medicinal plants and plant-based nutritional supplements. Furthermore, the purity of the bioactive compounds may affect the toxicity test results [15-18]. Moreover, flavonoids has a significant value due to their activity as a chemo-preventive agents against certain types of cancer[19] and as antidiabetic agents.[2] Thus, it has been suggested that it might also have an antiglycation effect. So in this study, the possible antiglycation effect of BCAAs and flavonoid compounds have been investigated using RAW 264.7 macrophage cell line and the antiproliferative effect of the combinations between BCAAs and flavonoids agents using different colorectal cancer cell lines.

Materials and Methods

Chemicals and biochemical
Dulbecco Modified Eagle Medium (DMEM) was obtained from Invitrogen (USA). Sulforhodamine B (SRB) dye was purchased from Promega (USA). The essential amino acids Ile, Leu and Val, Aminoguanidine (AMG), Methylglyoxal (MGO) as well as Quercetin, Apigenin and Hesperidin, were procured from Santa Cruz (USA). Unless stated otherwise all, other chemicals, and solvents used in this study were purchased at the analytical grade from Sigma-Aldrich (St. Luis, MO, USA).

The glycation assay
The RAW 264.7 cell line (murine monocyte-macrophage cell), were seeded at 10^4 cell/well in 96-well tissue culture plates and incubated at 37°C and 5% CO_2 overnight. After 12 h incubation, the cells were treated with the test tested amino acids; Ile, Leu, Val and BCAAs combination at concentrations (1-25 mM) as well as Quercetin, Apigenin and Hesperidin at concentrations (200-1 µM), and the glycating sugar "MGO" at 400 µM concentrations [6]. The AMG as a positive control was added 20 minutes prior to MGO [6,20]. The cells were then incubated for 48 h and assessed for viability using SRB test. The absorbance is measured at 570 nm.[21] The absorbance is proportional to cell viability.

The viability assay
The cytotoxicity measurements were determined using SRB colorimetric assay. Colorectal cell lines (SW480, SW620 and CACO2), were seeded in 96-well plates at a density of 5000 cells/well and cultured for 24 h, then cultured in the medium containing different bioactive compound such as: Quercetin, Apigenin and Hesperidin at concentrations (200-1 µM) as well as with the test tested amino acids; Ile, Leu, Val and BCAAs combination at concentrations (1-25mM) After 72 h, the SRB assay was performed [22]. All of the assays were performed in triplicate and the calculated IC50 antiproliferative activities were reported as the mean values ± SD (n = 3).

The antagonism and synergism analysis

In combination experiments, BCAAs were added with the bioactive compound at ½ of their IC50 value. To assess synergy and antagonism, experimentally and a combination index (CI) was determined according to the method of Mertens–Talcott.[23] The CI values consider as synergism if (CI < 1), additive effect if (CI = 1), and antagonism if (CI > 1).

Statistical analysis
The results were presented as means ± standard deviation (SD) of three independent experiments. Statistical differences between control and different treatment groups determined using GraphPad Prism ANOVA followed by Tukey test. For all statistical analyses, a p-value of less than 0.05 was considered statistically significant. P values of less than 0.0001 were considered of a highly significant statistical difference.

Results and Discussion

Effect of MGO on cell toxicity
Figure 1 shows the effect of different concentrations (100, 200, 300, 400 µM) of MGO on cell toxicity. It was observed that the use of different concentrations of MGO significantly affected cell toxicity in a concentration dependent manner ($p < 0.01$).

The antiglycation effect of BCAA and bioactive compounds
Table 1 shows the effect of BCAAs and different bioactive compounds on the antiglycation activity and viability of RAW 264.7 macrophage cell line. The antiglycation efficacy of AMG was also studied. Although the BCAAs displayed the *in vitro* antiglycation impact, nevertheless none of these acids can be considered as equally potent as AMG. Still, all of the bioactive compounds show significant antiglycation effect compared to positive control AMG.
Also, all of these acids and in all their used concentrations did not show any statistically significant differences when tested on RAW 264.7 cells, however, with the exception of Quercetin and Apigenin, Hesperidin showed a low decrease in cell viability after 48 hours of incubation.

Modulation of viability of colorectal cancer cell lines by bioactive compounds and BCAAs
Table 2 shows the antiproliferative effect of bioactive compounds (Quercetin, Apigenin, and Hesperidin). BCAAs and their combinations as well as the antiproliferative efficacy of Cisplatin against SW620, CACO2, and SW480 cancer cell lines. All the bioactive compounds as well as the BCAAs showed cytotoxicity against SW620, CACO2 and SW480 cell lines after 72 h of incubations. Although the flavonoids and BCAAs that were tested showed selectivity for toxicity against fibroblasts, however, these compounds (for flavonoids and BCAAs) did not show the same effect as cisplatin.
Results of combinations against SW620 cell lines showed suppression effect against SW620 cell lines. Most of the flavonoids exhibited also show an antagonistic effect against CACO2 cell lines except for Leu, Val, and BCAAs combinations with Apigenin that led to the synergistic toxicity. Moreover, the synergistic effect against SW480 cell lines was observed when Apigenin was combined with Ile, Leu, and Val and BCAAs. In fibroblasts, most of these combinations with each other showed an antagonistic effect except when Apigenin was combined with Ile, Leu, Val, and BCAAs groups.
All flavonoids compounds have antioxidant effects, therefore it was suggested that they have antiglycation properties, since oxidation promote the generation of AGEs by reacting with highly-reactive dicarbonyl compounds such as methylglyoxal with protein in cells.[6,24]
In the present study all the investigated flavonoids including Quercetin, Apigenin and Hesperidin led to the higher enhancement in antiglycation activity than the AGE effect as inhibitor of aminoguanidine. These results were in agreement with that of Wu and Yen[25] who reported the inhibition effect of different types of flavonoid including catechin, epicatechin, epicatechin gallate, epigallocatechin-3-gallate, luteolin, and kaempferol, on early and advance stage of glycation. Furthermore, it was reported that the aerial parts of *Retama sphaerocarpa*, which contains the different type of flavonoids have significant antiglycation effect.[26] Moreover, several works mentioned flavonoids' capability of inhibiting the generation of AGEs by the active site of the aromatic ring, which is the major site for trapping MGO.[27-29] In contrast, the ability of BCAAs to block the formation of AGEs was also investigated. The level of ROS could be reduced by BCAAs through increasing the expression of Nuclear factor erythroid2-related factor 2 (Nrf2) and inhibiting nuclear factor-kappa B (NF-κB) signaling pathway.[30,31] Nevertheless our result showed that BCAAs can inhibit glycation process with impact less than that of AGE inhibitor, aminoguanidine.
On the other hand, flavonoids showed antiproliferative activity against certain types of cancer cells, by causing cell cycle arrest then apoptosis.[6,32] In this study our results indicate the cytotoxicity of Quercetin, Apigenin, and Hesperidin against different colorectal

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cancer cell lines including SW480, SW620, and CACO2. However, it was not as effective as cisplatin on the same cell lines. In line with our results, several previous studies reported the antiproliferative effect of flavonoids against various types of cancer.[33]

The precise mechanism for cytotoxicity of flavonoids against cancer cell lines is not clear. Nevertheless, it was suggested that quercetin can suppress the survival signals, such as protein kinase C (PKC-α) and enhancement of death signals such as PKC-δ.[34] Moreover, quercetin can enhance the pro-apoptotic pathways by involving in the inhibitions of p53 gene and BCL-2 protein, the inhibition of expression of the BCL-2 gene prevent the suppressor activity of the BAD protein, which stimulate the intrinsic pathway of apoptosis.[35] Furthermore, quercetin have the ability to inhibit the PI3K/Akt and MAPK/ERK pathways in breast cancer cell lines, causing cell cycle arrest, apoptosis, inhibit invasion as well as suppresses the angiogenesis. [36] On the other hand, Apigenin have been known as anticancer agent *in vitro* and *in vivo*, by stimulating cell cycle arrest, autophagy, as well as apoptosis and migration for cancer cells, through modulating the activity of different pathways such as: Wnt/*β*-catenin in colorectal cancer, NF-κB in prostate cancer, JAK/STAT in Breast cancer, PI3K/AKT and MAPK/ERK in Melanoma.[37] Furthermore, Hesperidin can induce apoptosis in several types of cancers by changing the activity of different pathways. It can induce apoptosis and cell cycle arrest in colorectal cancer by decreasing the activity of PI3K/Akt/mTOR pathway and suppress the activity of MAPK/ERK in liver cancer.[38] In contrast, the cytotoxicity of BCAAs on colorectal cancer cell lines reported here is not significant as cisplatin does on the same cell lines. Our results were consistent with previous study [39] in which the effect of these acids in colorectal cancer HCT-116 cell line and liver cancer HepG2 cell line was publicized. It was indicated that these branched acid could modify the activity of PI3K/AKT pathway, which can induce apoptosis and autophagy.[39]

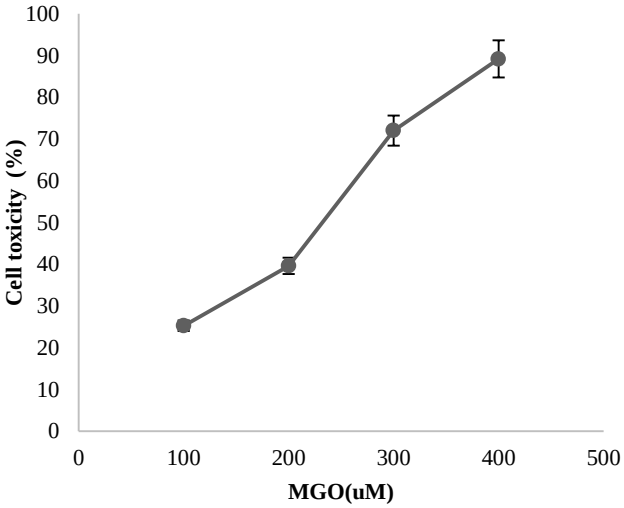

Figure 1: The effect of different concentrations of MGO on cell toxicity.
The results represent percentages of cytotoxity of treated cells, expressed as means of the three measurements ± SD of three independent replicates measurements.

Table 1: Antiglycation effect of BCAAs and bioactive compounds, using RAW 264.7 macrophage cell line

			Antiglycation (as of %Control) IC_{50} value			
(mM)	**Ile**	**Leu**	**Val**	**BCAA combinations**	**AMG (positive control)**	
	12.18 ± 2.18	11.02 ± 1.41	10.65 ± 2.44	14.86 ± 0.94	225x 10^{-3} ± 1.59	
p-value	NS	NS	NS	NS		
			RAW 264.7 cell line viability (as % control)			
(mM)	1	2	4	8	15	25
Ile	118.67 ± 2.29	109.55 ± 1.47	101.84 ± 1.27	95.7 ± 0.88	86.8 ± 1.46	85.6 ± 0.33
Leu	116.98 ± 0.50	101.66 ± 2.63	97.29 ± 2.33	92.4 ± 1.47	86.9 ± 1.23	85.7 ± 1.86
Val	129.65 ± 0.55	126.93 ± 0.55	120.79 ± 0.73	91.6 ± 1.89	89.2 ± 0.87	86.6 ± 0.53
BCAA combinations	134.86 ± 1.53	128.38 ± 1.16	94.98 ± 0.44	88.7 ± 2.63	83.4 ± 1.83	80.6 ± 0.93
			Antiglycation (as of %Control) IC_{50} value			
(µM)	Quercetin		Apigenin	Hesperidin	AMG (positive control)	
	52.7 ± 0.08		60.7 ± 0.02	195.02 ± 3.43	225 ± 1.59	
p-value	< 0.0001		< 0.0001	0.0002		
			RAW 264.7 cell line viability (as % control)			
(µM)	5	10	25	50	100	200
Quercetin	89.98 ± 1.03	82.25 ± 2.15	78.59 ± 1.72	69.94 ± 0.83	25.92 ± 1.72	16.53 ± 0.83
Apigenin	95.91 ± 2.21	85.51 ± 1.33	81.79 ± 2.36	66.65 ± 2.72	48.79 ± 1.54	14.55 ± 3.84
Hesperidin	96.69 ± 1.51	90.73 ± 3.23	88.20 ± 3.84	83.54 ± 2.73	72.26 ± 1.33	68.73 ± 1.94

Results are mean ± SD of three independent replicates. IC_{50} values (concentration at which 50% of glycated cell took place in comparison to non-glycated cells basal 48 h incubations). NS non-significant compared to AMG.

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Table 2: Modulatory effect of bioactive compounds and BCAAs, on the viability of colorectal cancer cell lines measured by SRB dye

	Cytotoxicity (as of %Control) IC_{25} value µM			
	SW620	**CACO2**	**SW480**	**Fibroblasts**
Quercetin	20.12 ± 1.38	NI	2.41 ± 0.20	1.21 ± 0.02
Apigenin	27.90 ± 1.86	85.05 ± 4.16	22.60 ± 3.33	3.26 ± 0.1
Hesperidin	107.37 ± 27.07	45.08 ± 3.69	47.92 ± 1.06	145.52 ± 1.64
Cisplatin	4.71 ± 0.14	1.46 ± 0.28	4.56 ± 0.11	4.13 ± 0.62
	The p-value compared to Cisplatin treatment			
	SW620	CACO2	SW480	Fibroblasts
Quercetin	NS	NS	NS	0.01
Apigenin	NS	NS	NS	NS
Hesperidin	NS	NS	NS	NS
	Cytotoxicity (as of %Control) IC25 value mM			
	SW620	CACO2	SW480	Fibroblasts
Ile	23.9 ± 0.16	NI	4.8 ± 0.07	16.7 ± 1.23
Leu	17.2 ± 9.23	10.3 ± 0.13	6.6 ± 0.19	16.9 ± 1.17
Val	NI	10.4 ± 0.28	12.5 ± 1.21	19.7 ± 1.09
BCAAs combinations	12.04 ± 3.25	8.3 ± 0.15	6.4 ± 0.14	16 ± 1.44
Cisplatin	4.71×10^{-3} ± 0.14	1.46×10^{-3} ± 0.28	4.56×10^{-3} ± 0.11	4.13×10^{-3} ± 0.62
	The p-value compared to Cisplatin treatment			
	SW620	CACO2	SW480	Fibroblasts
Ile	NS	NS	NS	NS
Leu	NS	NS	NS	NS
Val	NS	NS	NS	NS
BCAAs combinations	NS	NS	NS	NS
	CI value			
	SW620			
	Ile	Leu	Val	BCAAs combinations
Quercetin	1.91 ± 0.12	1.50 ± 0.24	NI	1.22 ± 0.03
Apigenin	1.94 ± 0.05	1.41 ± 0.41	NI	1.30 ± 0.24
Hesperidin	1.77 ± 0.13	1.21 ± 0.07	NI	1.12 ± 0.31
	CACO2			
	Ile	Leu	Val	BCAAs combinations
Quercetin	NI	NI	NI	NI
Apigenin	NI	0.24 ± 0.08	0.19 ± 0.01	0.21 ± 0.04
Hesperidin	NI	1.95 ± 0.11	1.94 ± 0.14	1.81 ± 0.08
	SW480			
	Ile	Leu	Val	BCAAs combinations
Quercetin	1.96 ± 0.12	2 ± 0.21	1.8 ± 0.13	1.86 ± 0.05
Apigenin	0.21 ± 0.04	0.11 ± 0.02	0.12 ± 0.03	0.17 ± 0.02
Hesperidin	2 ± 0.24	1.93 ± 0.08	2 ± 0.17	2.01 ± 0.16
	Fibroblasts			
	Ile	Leu	Val	BCAAs combinations
Quercetin	2.18 ± 0.21	1.95 ± 0.13	1.97 ± 0.18	2.21 ± 0.23
Apigenin	0.54 ± 0.04	0.47 ± 0.01	0.71 ± 0.06	0.64 ± 0.14
Hesperidin	1.96 ± 0.16	1.91 ± 0.08	2.04 ± 0.04	2.22 ± 0.02

Results are mean ± SD of three independent replicates. IC_{25} values (concentration at which 25% inhibition of cell proliferation took place in comparison to non-induced basal 72 h incubations). NI is non inhibitory. NS non-significant compared to cisplatin.

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In our study we revealed the combination effect of earlier flavonoids with these branched acids on the same colorectal cancer cell lines, and our result revealed the synergistic effect against CACO2 and SW480 as well as in fibroblast when BCAAs was co-incubated with only Apigenin. However, quercetin and Hesperidin show antagonistic effect in SW620, CACO2 and SW480 cell lines when co-incubated with BCAAs, suggesting that significant inhibition occur to either Wnt/β-catenin or PI3K/AKTpathways when BCAAs was co-incubated with Apigenin, and significant activation of PI3K/AKT and MAPK/ERK pathways when BCAAs was co-incubated with either quercetin or Hesperidin. However, further studies are necessary to determine the modulatory effect of several pathways when these acids are co-incubated with flavonoids in cancer cell lines.

Conclusion

This study has demonstrated the effect of BCAAs as well as Quercetin, Apigenin and Hesperidin as antiglycation agents *in vitro*. Furthermore, in this study we tested the cytotoxicity of these compounds on colorectal cancer cell lines separately and in combination, and it was clear that these flavonoids have higher antiglycation effect than BCAAs and Aminoguanidine. Moreover, it was also clear that flavonoids and BCAAs can reach a higher cytotoxic effect against colorectal cancer cell lines, particularly when Apigenin is co-incubated with these acids. However, further studies are necessary to determine their mechanism of action. Nevertheless, we suggested that flavonoids such as Quercetin, Apigenin and Hesperidin could be used as treatment for diabetes, and the co-incubation with BCAAs could be considered as a different therapeutic strategy against colorectal cancer.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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