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Evaluation of Effects of Specific Bioactive Compounds in Plant, Propolis and Bee-Pollen on Crohn's Disease: A Food Nutrition Approach

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Abstract

Natural foods rich in bioactive compounds (BC) may provide an effective strategy for food and nutrition-based Crohn's disease (CD) management. However, their BCs' alone and combined effects have not been explored sufficiently. This study aims to evaluate the impact of specific BCs in plants, propolis, and bee pollen on CD by *in silico* and *in vitro* techniques. For this, the plants suggested in a clinical database were screened in another curated therapeutic database to obtain specific BCs with therapeutic use identifiers. In contrast, those of propolis and bee pollen were obtained from previously conducted publications. The identified BCs were subjected to *in silico* molecular docking simulations (MDS) to determine their binding affinity scores (BAS) with aryl hydrocarbon receptor (Ahr). Of them, two with the highest affinity score (AS) and the other two with the lowest AS were selected. Their cytotoxic effects on HCT-116 Human colon cancer cells were tested by *in vitro* MTS assay, while up- and downregulating effects on Ahr-related CYP1A1, CYP1B1, IDO1, and IDO2 genes by real-time qPCR. The findings demonstrated that MDS studies determined the highest BAS with Ahr to be β-carotene (β-C) (-8.99 kcal/mol) and biotin (B) (-6.39 kcal/mol). In comparison, the lowest BAS was to inositol (I) (-5.34 kcal/mol) and niacin (N) (-5.32 kcal/mol), respectively. *In vitro* MTS assay demonstrated that N and I were cytotoxic on HCT-116 cells, while β-C was noncytotoxic. But B did not exhibit any significant effect. The gene expression test showed that β-C downregulated IDO1 and IDO2, while B downregulated IDO1 only. On the other hand, N downregulated both CYP1A1 and IDO2, whereas I downregulated CYP1A1 only. β-C and B in combination upregulated all genes, but N and I downregulated them. In conclusion, proper selection of BC may effectively moderate CD pathogenesis and management with its protective and anti-inflammatory properties. We therefore suggest food and nutrition-associated research at preclinical and clinical levels.

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Introduction

Inflammatory bowel disease (IBD) is a chronic and recurrent inflammatory disorder mainly related to the intestinal tract. About 6.8 million people live with IBD worldwide, over 3.5 million of whom live in North America and Europe (Caban and Lewandowska, 2022). The prevalence of morbidity in Western countries remains high, at over 0.3% (Cai et al., 2021). Crohn's Disease (CD) is one of the two main IBDs (Alexandrescu et al., 2024). CD causes progressive bowel damage and disability, commonly affecting the terminal ileum and proximal colon with a discontinuous and patchy, segmental, and transmural inflammation (Dolinger et al., 2024). Its pathogenesis is associated with environmental, genetic, immunological, and microbial factors (Vanderstappen et al., 2024). Of these factors, food and nutritional patterns are important (Shehada and McMahon, 2024).

The pharmacological strategies currently used in CD management lack desirable efficacy and poor tolerability (Sigall Boneh et al., 2024). For instance, corticosteroids have substantial adverse effects (Geesala et al., 2024), and many patients have a suboptimal response to medical treatments (Christensen et al., 2024). Therefore, n integrative food and nutritional approaches are gaining significant attraction amongst the clinicians, to lower the complications of CD, such as the elimination of detrimental compoounds and processed foods in the dietary preferences (Chen et al., 2024),, for the management of CD-associated inflammation without adverse effects through downregulating inflammatory cytokines and enzymes, enhancing antioxidant defence, restoring the epithelial barrier, and modulating cellular signalling pathways (Wu et al., 2022).

Naturally occurring foods, including plants, herbs and many others, are an endless source of products and bioactive compounds (BCs), including phenolic and non-phenolic compounds, unsaturated fatty acids, dietary fibres and prebiotics, carotenoids, bioactive peptides, and vitamins, that promise alternative treatments for intestinal health, including (Laaroussi et al., 2020; Nascimento et al., 2021). Amongst the natural sources in CD management, propolis and bee pollen are valuable for human nutrition and health (Filannino et al., 2021). Propolis, also called bee glue or bee bread, is a resinous, natural, aromatic substance produced by bees from plants since ancient times. Over three hundred phytochemical compounds have been identified in propolis, mainly belonging to the flavonoid, terpene, and phenolic acid families, which can benefit intestinal barrier function and mucosal renewal (Chen et al., 2021). Recent works suggest propolis may be a candidate for intervening in CD with its high potential to modulate inflammatory pathways and immune response (Da Silva et al., 2028; Soleimani et al., 2021). Similarly, bee pollen can restore the function of the impaired intestinal barrier (Li et al., 2019). Thereby, food and nutrition approaches concerning CD have been implicated for a long time to identify (a) the food constituents associated with a higher risk of acquiring CD in susceptible individuals and (b) the BCs influencing CD's nutritional intervention, with many unanswered questions yet (Sila and Hojsak, 2024). Until now, the combined effect of plants, propolis, and bee pollen on CD has not been examined. Therefore, this study aimed to evaluate the impact of specific BCs in plants, propolis, and bee pollen on CD based on a food nutrition approach using *in silico* and *in vitro* methods.

Materials and Methods

Materials

The human colon cancer cell line (HCT-116) was obtained from the American Type Culture Collection (ATCC® CCL-247, Manassas, USA). β-carotene (β-C) (Lucarotin®) was purchased from BASF (Darmstadt, Germany), and niacin (N) (SOL-01860), inositol (I) (SOL-01450) and biotin (B) (SOL-00280) were from Solgar Inc. (New Jersey, USA), respectively.

Methods

Data sources and collection of bioactive compounds. The list of plants suggested for CD management (CD protective and anti-inflammatory) was initially selected from a clinical database, NIMBAL (www.nimbal.org). Subsequently, the selected plants were reviewed in a curated therapeutic use database, IMPPAT 2.0 (https://cb.imsc.res.in/imppat), to obtain the list of specific BCs with therapeutic use identifiers. Then, the chemical information of the BCs was retrieved from the PubChem database. Finally, the BCs in propolis and bee-pollen were collected from Komosińska-Vassev et al. (2015) and Przybyłek and Karpiński (2019).

In-silico studies

The BCs' three-dimensional (3D) structures were obtained from the DrugBank server (Wishart et al., 2006). Ligands were subjected to energy minimization (EM) in YASARA Structure Software utilizing the NOVA force field (Krieger and Vriend, 2002a). The biological assembly of aryl hydrocarbon receptor (Ahr) is dimeric. Ahr is widely expressed in gut immune cells, and its activation correlates with IBD's outcome, including CD (Pernomian et al., 2020). Thus, the two-chain (A and C) structure of the protein was extracted from the X-ray structure (Protein Data Bank entry 5NJ8) (Schulte et al., 2017). Different protein conformations were obtained from the CABS-flex 2.0 server, which utilizes Monte Carlo dynamics and provides different protein structures acquired by running coarse-grained molecular dynamics simulations (Kuriata et al., 2018). This approach mimics the dynamic structure of the protein, providing different protein orientations, and is widely used to obtain various conformations (Patra et al., 2019; Ortega et al., 2021). Three structures out of ten structures were selected according to the structural novelty.

Molecular docking simulations

Each ligand was docked to the three different protein conformations obtained from the CABS-flex server using a blind docking approach. Simulations were performed with the Autodock Vina program (Trott et al., 2010) embedded in YASARA Structure Software (Krieger and Vriend, 2002a). The grid box size for each docking simulation was 92.88 x 92.88 x 86.01 Å, which covered the whole protein. For three protein structures and four ligands, a total of twelve simulations were performed. Twenty-five different poses for each simulation were produced, and these poses were evaluated according to their binding affinity and hit population (number of hits) on the same binding sites. Lastly, the best pose with the highest binding affinity and hit population was chosen for each ligand to proceed into molecular dynamics simulations.

Molecular dynamics simulations

Molecular dynamic simulations for four ligand-protein complexes were performed at the YASARA Structure (Krieger and Vriend, 2002a) with a YASARA2 force field. Each complex was placed in a cubic box with dimensions 93.60 x 93.60 x 93.60 Å. These dimensions ensured that any protein atom remained in the box during the simulation. pH-dependent hydrogen bonding network optimization and pK_a prediction followed by protonation state assignment for each amino acid residue (Krieger et al., 2006; Krieger et al., 2012). The simulation medium was equilibrated with 0.9% NaCl (pH=7.4) water

molecules (0.997 g/mL) at 298 K. The length of the simulations was set to 50 ns, and molecular dynamics simulations were performed using the default settings of the YASARA Structure macro (http://www.yasara.org/md_run.mcr). After the simulations, root mean square deviation (RMSD) was performed to check the systems' equilibration and free energy of binding calculations. YASARA Structure molecular dynamics calculates binding free energies for ligands and analyses binding energy macro (Krieger et al., 2002b). This module calculates free energy without the entropy term from standard mode analysis (Equation 1).

$$\text{Binding Energy (kcal/mol)} = (G_{Receptor}+G_{Ligand}) + (G_{solvReceptor}+G_{solvLigand}) - (G_{Complex}+G_{solvComplex}) \quad (1)$$

Here, the first two terms are the potential energies of the receptor and ligand, the following are the solvation energies of the receptor and ligand, and the last is the potential and solvation energies of the complex. YASARA, as explained in its manual, provides positive binding energies (http://www.yasara.org/macros.htm). More positive values indicate stronger binding, whereas negative numbers indicate weak binding, but they do not mean non-binding ligands. However, in literature, binding affinities and binding free energies are always discussed with negative values. Thus, for clarity, the signs obtained from YASARA were reversed. The detailed discussion can be found in the YASARA manual.

In vitro citotoxicity (MTS) assay

HCT-116 Human colon cancer cell culture cytotoxicity test was performed according to the study by Abdik (2022). HCT-116 cell line is used in colon cancer biology, cancer stem cells, and the discovery of novel anti-cancer products, with its high invasive and metastatic potential *in vitro*, linked to an association between CD and colon cancer development (Kuehn et al., 2016). Cultivation of cells was conducted in T-75 flasks (Costar) at 37℃ in a humidified atmosphere (5% CO_2) in the incubator. Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Gibco, UK) with 4.5g/L D-glucose containing 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen) and 1% penicillin/streptomycin/amphotericin (PSA) (Invitrogen) were used to culture the cells. The subculturing was performed twice a week. The cells were passaged when they reached 80% using 0.25% trypsin/EDTA (Invitrogen). Upon achieving confluence, the cells were detached from the culture surface via trypsinization, followed by determining the cell count using a hemocytometer after centrifugation of the cell pellet. The cells were then exposed to *an in vitro* cytotoxicity MTS assay.

HCT-116 cells were seeded in 96-well plates at a density of $5*10^3$ cells per well, with the prepared samples. β-C, N, I and B were dissolved in Dulbecco's Phosphate Buffered Saline (1xDPBS) (Invitrogen) at a broad concentration range of 25, 50, 100, 200, and 400 µM because there was no standard protocol and sufficient evidence in the literature. The plates were then incubated at 37 °C for 24 and 48 h. The MTS assay (5-(3-carboxymethoxyphenyl)−2-(4,5-dimethylthiazol)−3-(4-sulfophenyl) tetrazolium) was used to determine cytotoxicity, adhering to the manufacturer's instructions. After adding 10 µL of MTS solution/100 µL 1X DPBS containing 4.5g/L glucose to each well, the plates were incubated at 37°C for 1 h. Absorbance readings were recorded at 490 nm using a BioTek 800 TS ELISA microplate reader. Each assay was performed five times. The CV data's mean and standard deviations (±) were calculated using the MS Office Excel Program (USA). The CV (%) results of HCT-116 cells for 24 and 48 h were statistically tested by One-way ANOVA with Tukey Post Hoc test ($p < 0.05$). A sample was classified as cytotoxic if CV (%) dropped to 70% or lower, whereas samples with CV (%) exceeding 70% were categorized as noncytotoxic.

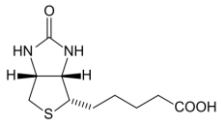
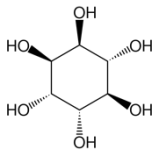
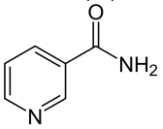
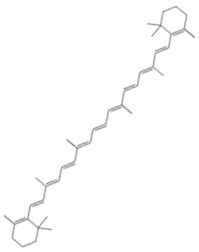
Gene expression analysis by real-time qPCR

Total RNA was isolated from HTC-116 cell lines using the innuPREP RNA Mini Kit 2.0 (845-KS-2040050 Analytik Jena, Germany). One µg of RNA was converted to cDNA following the instructions of the Wonder RT-cDNA Synthesis Kit (Euroclone EME037050, Italy), and the cDNAs were then incubated at 25°C for 10 min, 42°C for 15 min, and 85°C for 5 min, respectively. The concentration and purity of RNAs and cDNAs were determined at 280 nm using a BioSpec-Nano-Spectrophotometer (Shimadzu, Germany). The cDNA samples were stored at 4°C till real-time qPCR analysis. The primers of IDO1 and IDO2 were designed according

Table 1. The primers' sequences for the genes CYP1A1, CYP1B1, IDO1 and IDO2

Primer (f/r)	Sequences	T_m (°C)	bp
CYP1A1 (f)	CACAGACAGCCTGATTGAGCA	57.5	120
CYP1A1 (r)	GTGTCAAACCCAGCTCCAAAGA	57.6	
CYP1B1 (f)	ATGTCCTGGCCTTCCTTTATGA	56.0	90
CYP1B1 (r)	AGACAGAGGTGTTGGCAGTG	57.2	
IDO1 (f)	GGCACACGCTATGGAAAACT	55.8	164
IDO1 (r)	GAAGCTGGCCAGACTCTATGA	56.3	
IDO2 (f)	CTGATCACTGCTTAACGGCA	55.2	281
IDO2 (r)	TGCCACCAACTCAACACATT	55.2	
GAPDH (f)	CCTGACCTGCCGTCTAGAAA	56.5	276
GAPDH (r)	TACTCCTTGGAGGCCATGTG	56.7	

Table 2. Interactions and binding affinities obtained from molecular docking simulations

Compound	Interacting Residues		Binding Affinity (kcal/mol)
Biotin (B) 	Phe 56-C	Gly 115-C	-6.39
	Tyr 76-C	Glu 116-C	
	Ala 79-C	Leu 119-C	
	Phe 83-C	Phe 136-C	
	Lys 88-C	Tyr 137-C	
	Asn 111-C	Ser 139-C	
	Leu 112-C	Ile 154-C	
	Glu 114-C		
Inositol (I) 	Asp 48-C	Asp 65-C	-5.34
	Ala 51-C	Lys 66-C	
	Ile 61-C	Val 69-C	
	Asn 62-C	Leu 227-C	
	Leu 64-C	Leu 228-C	
Niacin (N) 	Val 129-C	Ile 258-C	-5.32
	Thr 130-C	Leu 259-C	
	Phe 170-C	Leu 263-C	
	Gln 173-C	Ala 264-C	
	Glu 211-C	Leu 265-C	
	Asn 212-C		
β-carotene (β-C) 	Tyr 76-C	Phe 136-C	-8.99
	Ala 79-C	Tyr 137-C	
	Lys 80-C	Asn 238-C	
	Phe 83-C	Gln 240-C	
	Arg 107-C	Lys 242-C	
	Asn 111-C	Phe 266-C	
	Gly 115-C	Ile 268-C	
	Leu 118-C	Ala 269-C	
	Leu 122-C	Thr 270-C	
	Val 126-C		

to Salazar et al. (2017), while those of CYP1A1, CYP1B1 and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (housekeeping gene) according to Divi et al. (2014) by Exim Life Sciences Ltd. Şti. (Istanbul, Türkiye) (Table 1). The qPCR was performed in an Agilent Strategene MxPro 3005 P qPCR System (Santa Clara, USA) with Euroclone FluoCcyle II SYBR Master Mix. Cycling was initiated at 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s, and a melting curve was achieved at the end. Samples were run in triplicate, and relative expression was calculated using the comparative threshold cycle ($\Delta\Delta C_{t)}$ method, normalized to GAPDH. All C_t values varied between 18 and 25; the dissociation curve was obtained between 83.5 and 91°C.

Data analysis

The collected dataset's mean and standard deviation were calculated in the MS Office Excel Program. One-way ANOVA, including Tukey HSD test, was applied to test for differences between the sample groups using SPSS 19 software (IBM Corporation, USA). The significance level was set at $p<0.05$.

Results and Discussion

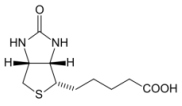
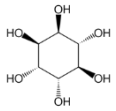
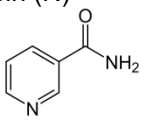
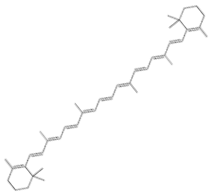
Selection of bioactive compounds

In the recent study, 29 plants with CD protective and anti-inflammatory properties suggested by the NIMBAL database were initially selected. Subsequently, 15 of them, including allspice, thyme, olive, watercress, rosemary, cranberry, elder (sambuca), pistachio, Trabzon persimmon, parsley, anise, basil, rocket, grape, and black pepper, were also found with their therapeutic use associated with CD in the IMPPAT 2.0 database. The propolis and bee-pollen associated BCs from Komosińska-Vassev et al. (2015) and Przybyłek and Karpiński (2019) were listed. After the duplicated BCs were deleted, the chemical information of each of the 26 BCs was retrieved from the PubChem database for *in silico* studies.

In-silico studies

The i*n silico* molecular docking simulations (MDS) determined the binding affinity scores (BAS) of the 26 BCs with Ahr. Of them, the two with the highest BAS (β-

Table 3. Protein-ligand interaction types and interacting residues were provided with the free energy of binding for each of the ligands

Compound	Interacting Residues and Interaction Type[a]		Free Energy of Binding (kcal/mol)
Biotin (B) 	Tyr 76^{C}-Hydrophobic	Phe 136^{C}-Hydrophobic	-117.27
	Ala 79^{C}-Hydrophobic	Tyr 137^{C}-Hydrophobic	
	Lys 80^{C}-Hydrophobic	Ala 138^{C}-Hydrophobic	
	Phe 83^{C}-Hydrophobic		
Inositol (I) 	Asp 48^{C} -Hydrophobic	Leu 64^{C}-H-bonding	-20.95
	Ala 51^{C}-Hydrophobic	Asp 65^{C}-Hydrophobic	
	Ile 61^{C}-Hydrophobic	Lys 66^{C}-Hydrophobic	
	Asn 62^{C}-Hydrophobic	Leu 228^{A}-H-bondig	
Niacin (N) 	Val 129^{C}-H-bonding	Asn 212^{C}-Hydrophobic	-65.00
	Thr 130^{C}-Hydrophobic	Ile 258^{C}-Hydrophobic	
	Phe 170^{C}-Hydrophobic	Leu 263^{C}-Hydrophobic	
	Gln 173^{C}-Hydrophobic	Ala 264^{C}-Hydrophobic	
	Glu 211^{C}-Hydrophobic	Leu 265^{C}-Hydrophobic	
β -carotene (β-C) 	Tyr 76^{C}-Hydrophobic	Leu 122^{C}-Hydrophobic	-359.89
	Ala 79^{C}-Hydrophobic	Val 126^{C}-Hydrophobic	
	Lys 80^{C}-Hydrophobic	Tyr 137^{C}-Hydrophobic	
	Phe 83^{C}-Hydrophobic	Gln 240^{C}-Hydrophobic	
	Leu 112^{C}-Hydrophobic	Lys 242^{C}-Hydrophobic	
	Glu 114^{C}-Hydrophobic	Gly 241^{C}-Hydrophobic	
	Glu 116^{C}-Hydrophobic	Phe 266^{C}-Hydrophobic	
	Leu 119^{C} -Hydrophobic	Ile 268^{C}-Hydrophobic	

[a] Superscript A and C define two distinct monomers (A and C).

C with -8.99 kcal/mol and B with -6.39 kcal/mol), and the two with the lowest AS (I with -5.34 kcal/mol and N with -5.32 kcal/mol) were selected for further simulations and *in vitro* tests. The other 22 BCs were excluded (Table 2).

Molecular recognition (i.e., binding specific molecules by noncovalent interactions) is fundamentally important in chemistry, such as recognizing chemical drugs and proteins when a chemical drug associates with its protein target (Huang et al., 2012). In this recent work, the binding sites and free energies of binding for each ligand are presented in Table 3, Figure 1 and Figure 2, respectively. The *in silico* findings indicated that the binding site of β was in the C chain of the protein, and was formed by Tyr 76, Ala 79, Lys 80 (sidechain hydrocarbons), Phe 83, Phe 136; Tyr 137, and Ala 138 residues, creating a highly hydrophobic cleft for the ligand (Figure 1-A). Additionally, the -NH group on the ureido ring of biotin formed an H-bond with Tyr 137. Regarding I, the binding pocket of I is located at the interface of the two monomers "A" and "C" chains (Figure 1-B). The highly hydrophobic pocket was formed by Asp 48, Ala 51, Ile 61, Asn 62, Asp 65 (sidechain hydrocarbons), Lys 66 (side chain hydrocarbons), and Val 69 residue of the C chain. Moreover, the ligand created two strong H-bonds with the Leu 64 residue of the C chain and the Leu 228 residue of the A chain. At the same time, N was buried in the hydrophobic pocket in the region with high β-sheet content in the C chain. It interacted with Thr 130, Phe 170, Gln 173, Glu 211, Asn 212, Ile 258, Leu 263, Ala 264, and Leu 265 residues through hydrophobic interactions, whereas it created an H-bonding interaction with Val 129 residue (Figure 1-C). Moreover, β-C, consisting of a long hydrocarbon chain (18 C atoms in the longest chain), was buried into highly hydrophobic pocket comprised of C chain aminoacids Tyr 76, Ala 79, Lys 80; Phe 83; Leu 112; Glu 114; Gly 115; Glu 116 (sidechain hydrocarbons), Leu118, Leu 119; Leu 122; Val 126; Tyr 137; Gln 240; Gly 241 Lys 242; Phe 266, and Ile 268 (Figure 1-D).

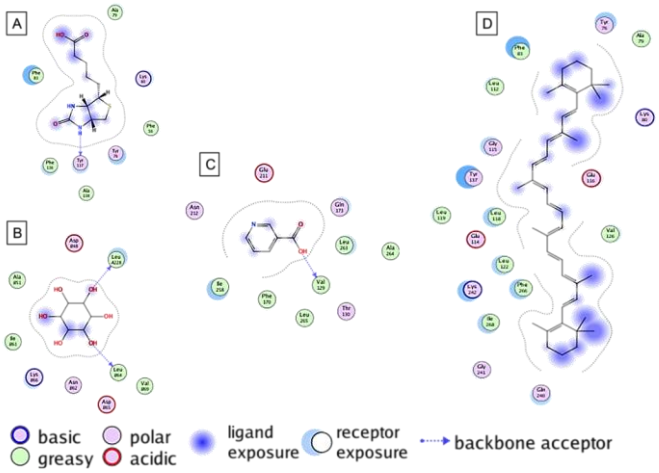


Figure 1. 2D protein-ligand interactions for: (A) biotin (B), (B) inositol (I), (C) niacin (N), and (D) β -carotene (β-C). Greasy legend defines hydrophobic interaction, and the B chain identifier represents a chain

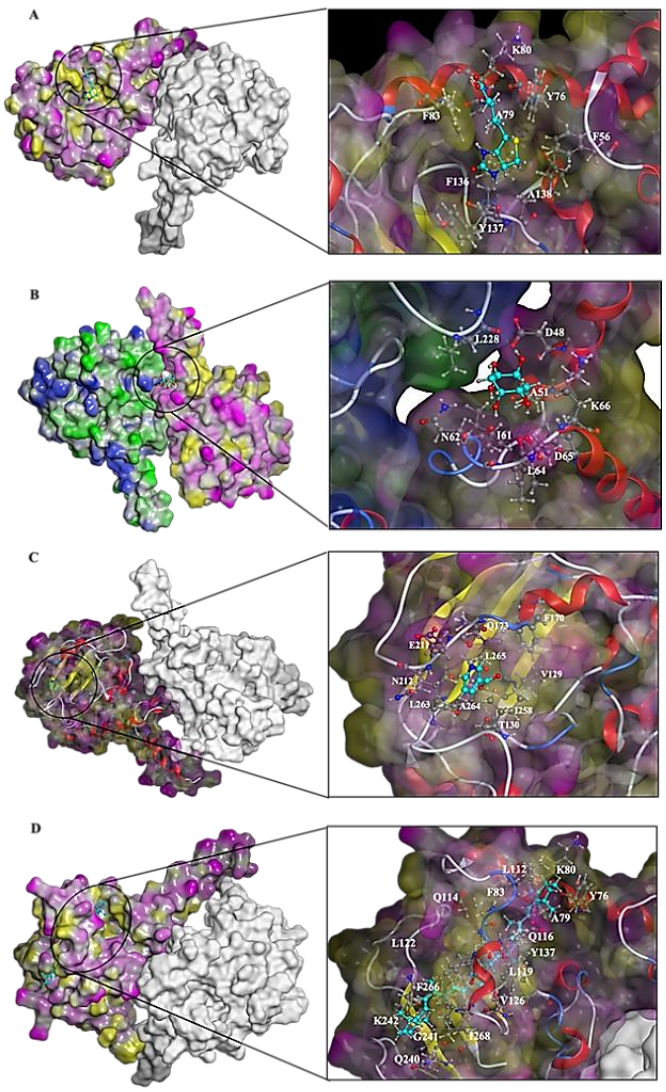


Figure 2. Binding sites observed after MD simulations for: (A) biotin (B), (B) inositol (I), (C) niacin (N), (D) β-carotene (-C). On the left side of the figures, binding sites on the overall dimeric structure of the protein and on the right zoomed in view of each site were provided. In the figure, yellow represents lipophilic, pink represents hydrophilic regions. The chain shown in white is the A chain. Since inositol is located between the A and C chains, chain A is colored, green for hydrophilic, blue for lipophilic regions.

Compared to the mean free energies of BAS values, β-C produced the highest value (-359.89 kcal/mol), followed by B (-117.27 kcal/mol), N (-65.00 kcal/mol), and I (20.95 kcal/mol), respectively (Table 3). The highest free binding energy for β-C might be attributed to a long hydrocarbon chain, including a long polyene chain, responsible for many hydrophobic interactions with the protein. In contrast, I was a tiny molecule with five hydroxyl (-OH) groups fused onto a cyclohexane ring. The time evolution of free energies of binding for each ligand is presented in Figure 3-A and Figure 3-B.

The mechanism of action of BCs in modulating the Ahr pathway in mitigating Cd-associated inflammatory reactions remains unclear (Bungsu et al., 2021). Ahr, a ligand-dependent transcription factor, exerts protective and anti-inflammatory effects in the gut's epithelial barriers as an attractive target for novel therapeutic interventions (Panda et al., 2023). The BCs, including quercetin, resveratrol, curcumin, indole-3-carbinol, and apigenin, may mediate Ahr based on agonist and antagonist activities, maintaining the gastrointestinal tract's mucosal integrity (De Juan and Segura, 2021; Goya-Jorge et al., 2021; Coelho et al., 2022). In the recent *in silico* study, β-C had the highest BAS with Ahr (-8.99 kcal/mol). β-C is a natural antioxidant with anti-inflammatory properties also found in the serum, and all body parts and tissues (Tufail et al., 2024; Su et al., 2024). The studies suggest β-C for gut health (Waldes et al., 2024). The other works investigating a correlation between Ahr and β-C indicated that β-C could TNFα-induced intestinal inflammation (Song et al., 2024), significantly recovered Ahr mRNA expression (Darwish et al., 2016), and decreased methylation in Ahr (Grieshober et al., 2020). Moreover, some other works suggest that the diet of CD patients was poorer in β-carotene (Labriola et al., 2022), whereas there was insufficient evidence for β-C concerning CD risk (Chen et al., 2023). Our *in silico* data suggest β-C for CD intervention through its modulatory effect on Ahr.

Our data demonstrated that B, a sulfur-containing water-soluble vitamin of the B group critical for human metabolism, produced the second-highest BAS with Ahr (-6.39 kcal/mol). It can attach to various proteins and nucleic acids (Green and Sambrook, 2021) and functions as a cofactor for enzymes in carboxylation reactions (Perry and Butterick, 2024). Its deficiency induces inflammation (Peterson et al., 2020). B has antagonistic characteristics on Ahr toxicity with high BAS (Kim et al., 2020). Gravina et al. (2023) showed that B decreased the expression of proinflammatory cytokines in IBD tissue, and Erbach et al. (2022) claimed that Routine assessment and supplementation of B may ameliorate IBD. According to our data, although B had the second-highest BAS with Ahr, it might be either the cause or effect of CD pathogenesis, requiring further research to clarify its pro- or anti-inflammatory effects on CD pathogenesis.

The recent *in silico* analysis determined that I exhibited the lowest two BASs with Ahr (-5.34 kcal/mol). Its main actions are linked to antioxidant and anti-inflammatory activities and neurotransmission (Chatree et al., 2020). The human liver and kidney can produce up to 4 g per day (Benvenga et al., 2021). It is absorbed in the gut (Dinicola et al., 2017) and influences the inflammatory process in the colon (Weinberg et al., 2020). Moreover, I can improve the CD's clinical conditions by decreasing bloating, flatulence and abdominal pain (Spagnuolo et al., 2017). Our data indicate that the link between I and Ahr needs further clinical research to clarify its mode of action on CD pathogenesis.

In this study, N, water-soluble protein, produced the lowest BAS with Ahr (-5.32 kcal/mol), almost close to I. Its derivatives nicotinamides, nicotinamide adenine dinucleotide, and nicotinamide adenine dinucleotide phosphate are crucial coenzymes responsible for multiple essential biologic effects in organs with a high cell turnover rate, such as gastrointestinal epithelium,

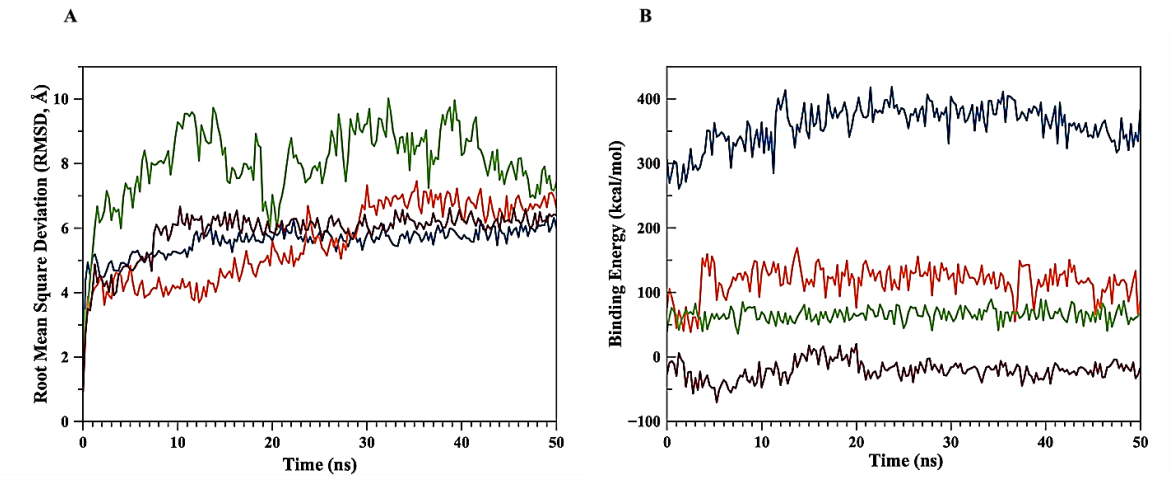


Figure 3. (A) Variation of RMSD values for protein backbone atoms from protein-ligand 50 ns MD simulations. (B) Free energy of binding values observed during MD simulations for protein-ligand complexes. (For both graphs, Blue: β-carotene (β-C)-Protein complex, Orange: Biotin (B)-Protein complex, Green: Niacin (N)-Protein complex, Brown: Inositol (I)-Protein Complex).

which is highly susceptible to its deficiency (He et al., 2019; Epstein, 2023). Niacin is a high-affinity Ahr ligand (Cella and Colonna, 2015). Biochemically, tryptophan is degraded to niacin, suppressing intestinal inflammation with butyrate (Badodi et al., 2021). Nikolaus et al. (2017), Friedman (2018), and Weinberg et al. (2020) reported that the serum levels of tryptophan were significantly lower in CD patients, inducing chronic inflammatory processes in the colon cancer formation in the long term. However, there is insufficient data on the correlation between N and CD pathogenesis, including Ahr. Therefore, our work makes a significant contribution to literature.

In-vitro cytotoxicity (MTS) assay

The *in vitro* MTS assay showed that β-C at 50-200 µM/24 h and 100-200 µM/48 h exhibited a significant increase in the CV (%) of the HCT-116 cell line ($p<0.05$), whereas B and the combined dose of β-C and B did not influence the CV (%) significantly ($p\geq0.05$) (Figure 4). On the other hand, I at 400 µM/48 h, N at 200 µM/48 h and 400 µM/24-48 h, their combined dose of 200 µM/48 h significantly decreased the CV (%) of the HCT-116 cell line, while ($p<0.05$) (Figure 5).

Patients with CD have an increased risk of developing colon cancer compared to the general population (Sato et al., 2023). Although our MTS study investigated a potential link between BC (β-C, N, I, and B) supplementation and the HCT-116 colon cancer cell line, indirectly, CD. Some works investigated the associations between the levels of β-C, B, I, and N and the risk of CD. (Wendland et al., 2001; Chen et al., 2023). For instance, β-C decreased intestinal inflammation in colonic epithelial cells (Cheng et al., 2021), B may ameliorate CD pathogenesis (Okabe et al., 1988; Erbach et al., 2022), I decreased bloating, flatulence and abdominal pain, improving the overall clinical condition of CD (Spagnuolo et al., 2017), and a deficiency of N adversely affects CD (Hui et al., 2017). However, there are inconsistent and limited evidence findings in the literature. Moreover, other studies on the cytotoxic effects of β-C at 5 µM β-C for 24 h (Palozza et al., 2008) and of N at 12.5, 25, 50 and 100 µM for 72 h (He et al., 2019) and 400, 800 and 1,600 µM for 12 h (Kim et al., 2016), respectively. , whereas Kim et al. (2016) showed the cytotoxic effect of N. In this study, our cytotoxic data align with those of Palozza et al. (2008) and Kim et al. (2016), whereas not with He et al. (2019), indicating the need for future research to predict the link between CD and cancer development.

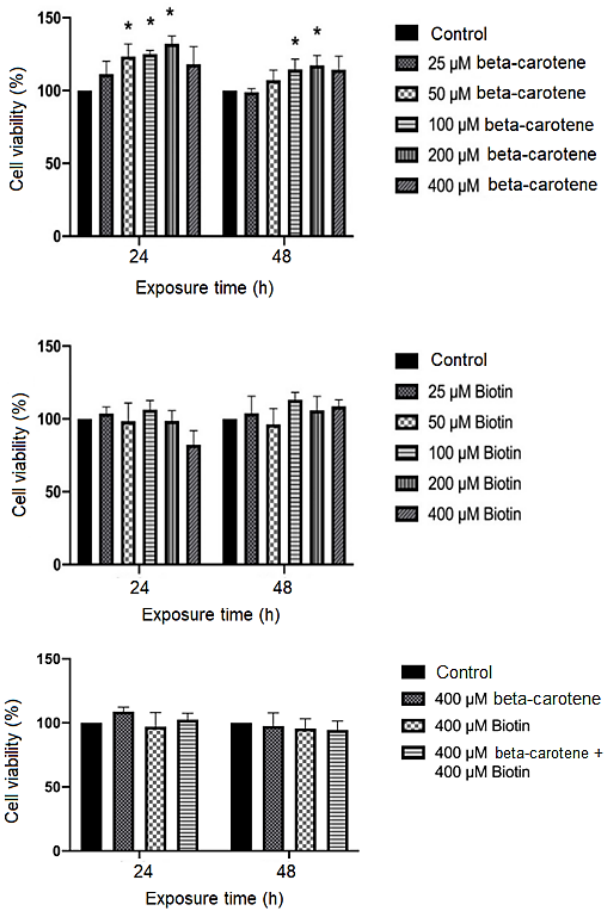


Figure 4. MTS assay, the CV results on HCT-116 colon cancer cell line to β-carotene (β-C) and biotin (B) after 24 h and 48 h of incubation. One-way ANOVA with Tukey Post Hoc test (*$p<0.05$)

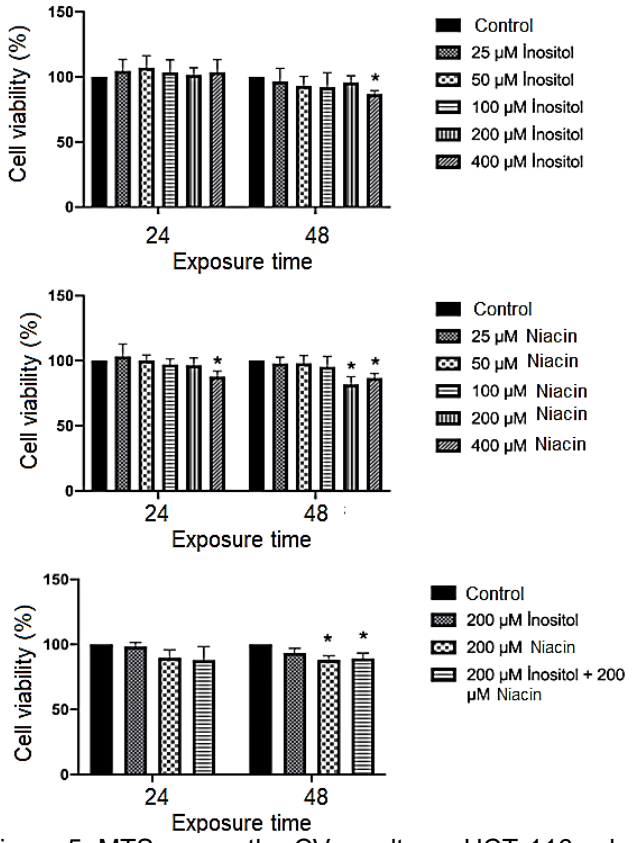


Figure 5. MTS assay, the CV results on HCT-116 colon cancer cell line to inositol (I) and niacin (N) after 24 h and 48 h of incubation. One-way ANOVA with Tukey Post Hoc test (*p <0.05)

real-time qPCR

The gene expression analysis demonstrated that β-C downregulated IDO1 and IDO2, B selectively downregulated IDO1, N downregulated both CYP1A1 and IDO2, and I specifically downregulated CYP1A1. Additionally, β-C and B, in combination, overexpressed four genes simultaneously, whereas N and I downregulated them (Table 4). No significant difference was found between the treatment groups (p>0.05).

Cytochrome P450 family 1 enzymes CYP1A1 and CYP1B1 are the effector molecules induced by Ahr. (Panda et al., 2023). The overexpression of CYP1A1 in human colon tumours activates Ahr (Lamas et al., 2018). In this study, β-C and B alone, combined with the highest BASs, upregulated Ahr CYCP1A1. In contrast, N and I alone and in combination downregulated CYP1A1, indicating specific alterations in the gut microbiota composition, as suggested by Rannug (2020). In the literature, there is insufficient evidence on the influence of BCs on CYP1A activity, which is exclusively regulated by Ahr (Ronquillo-Sánchez et al., 2013). Therefore, our data suggest that N and I may be utilized as Ahr-CYCP1A1 blockers in managing CD.

CYP1B1 catalyzes procarcinogen oxidation to carcinogenic reactive intermediates (Androutsopoulos et al., 2013). The overexpression of CYP1B1 in human colon tumours also activates Ahr (Lamas et al., 2018), activating procarcinogens into carcinogens (Dutour and Poirier, 2017). In this study, the CYP1B1 gene was upregulated by β-C and B supplementation alone and in combination. Although it has been speculated that CYP1B1 is involved in the action of β-C, the nature of this involvement needs further research (Satomi and Nishino, 2013). Another study indicated that a B supplementation of 10 nmol/L increased the transcriptional activity of CYP1B1 by 124% compared with 0.025 nmol/L (Rodriguez-Melendez et al., 2004). Our data suggest it might be a weak inhibitor of human P450 enzymes.

Ahr and IDO genes (IDO1 and IDO2) are closely interconnected: Ahr-mediated IDO genes produce kynurenine (Jaronen and Quintana, 2014). IDO-1 is expressed in notable dendritic cells, monocytes, and macrophages. However, less is known about IDO-2 (Savitz, 2020). IDO1 is the first and rate-limiting step in tryptophan catabolism along the kynurenine pathway (Lee et al., 2014), and one of the most overexpressed genes in CD (Wang et al., 2020). Host enzyme-catalyzed reactions are regulated by IDO1, leading to Ahr ligand metabolites such as N, serotonin, melatonin or n-acetyl serotonin (Ghiboub et al., 2020). Our study revealed that β-C and B alone downregulated IDO1, while β-C and B in combination overexpressed IDO1. Besides, N and I alone also upregulated IDO1, indicating both β-C and B can be considered blocking ligands with a high BAS with Ahr in CD management. IDO2 mediates the production of autoantibodies and IDO1-mediated T-cell regulation in inflammation (Wirthgen and Hoeflich, 2015). In this analysis, our data demonstrated that β-C and N alone downregulated IDO2, while β-C and B in combination downregulated IDO2. I alone overexpressed IDO2, indicating β-C, B, and I can be categorized as Ahr agonists closely interconnected to IDO genes in CD interventions.

Table 4. Expression levels of the genes CYP1A1, CYP1B1, IDO1 and IDO2

Reagent	CYP1A1			CYP1B1			IDO1			IDO2		
	Norm	ddCT	Fold	Norm	ddCT	Fold	Norm	ddCT	Fold	Norm	ddCT	Fold
C	5.18	0.00	1.00[a]	4.18	0.00	1.00[a]	2.45	0.00	1.00[a]	19.80	0.00	1.00[a]
B	5.85	0.66	1.59[b]	4.90	0.72	1.65[b]	3.26	0.81	1.76[b]	19.29	-0.51	0.70[c]
B-C	5.99	0.80	1.75 [b]	4.94	0.76	1.69[b]	2.35	-0.10	0.93[c]	18.39	-1.40	0.38[c]
B + β-C	5.89	0.71	1.63 [b]	4.74	0.55	1.47[b]	3.26	0.81	1.76[b]	20.56	0.77	1.70[b]
I	5.91	-0.50	0.71[c]	5.28	0.02	1.01[b]	20.32	0.74	1.67[b]	3.60	0.10	1.07[b]
N	6.27	-0.14	0.91[c]	5.27	0.00	1.00[b]	20.51	0.94	1.91[b]	3.14	-0.37	0.78[c]
I + N	5.68	-0.73	0.60[c]	4.76	-0.51	0.70[c]	18.35	-1.23	0.43[c]	3.09	-0.42	0.75[c]

[a] Control; [b] Upregulated; [c] Downregulated; Control; C, Biotin; B, β-carotene; β-C, Inositol; I, Niacin: N

Conclusion

Recently we evaluated the effects of specific BCs in plants, propolis, and bee pollen, based on a food nutrition approach to CD management, using *in silico* and *in vitro* methods. The findings suggested that CD management is a complicated process, and food nutrition interventions can effectively moderate the pathogenesis of CD. Besides, proper selection of BCs may promise a strategy with their protective and anti-inflammatory properties in CD management. Overall, the therapeutic potential of BCs deserves food and nutrition-associated research at preclinical and clinical levels.

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