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Original Article

2,3,4-Trihydroxybenzoic Acid, an Antioxidant Plant Metabolite Inhibits Cancer Cell Growth *in vitro*: Potential Role in Cancer Prevention

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ABSTRACT

Background: Colorectal Cancer (CRC) is the third most common cause of cancer death in the US and more than 1 million people are diagnosed with this disease every year worldwide. In this regard, flavonoids and their degradation products, namely Hydroxybenzoic Acids (HBAs) continue to be evaluated for the prevention of CRC. Hydroxybenzoic acids are also present as secondary plant metabolites in many fruits and vegetables. In the present study, we determined the ability of 2,3,4-THBA, which is present in fruits and vegetables and known to have antioxidant properties, to inhibit cancer cell growth in HCT-116 colon and MDA-MB-231 breast cancer cells. **Materials and Methods:** Cell proliferation assays and clonogenic assays were carried out to determine the effect of 2,3,4-THBA on cancer cell growth. Cell morphology was determined using light microscopy. Western Blot experiments were performed to determine the expression levels of Human Epidermal Growth Factor Receptor-type 2 (HER2), Fibroblast Growth Factor Receptor (FGF-R), Cyclin-Dependent Kinase (CDK) inhibitors p21 and p27. *In silico* studies were performed to determine the potential binding of 2,3,4-THBA to CDK1, CDK2, CDK4 and CDK6. **Results:** We demonstrate that 2,3,4-THBA dose-dependently inhibited HCT-116 colon and MDA-MB-231 cancer cell proliferation. It also significantly decreased the colony formation in both cancer cell lines. Treatment of cells with 2,3,4-THBA did not alter the levels of HER-2 and FGF-R; however, it induced the levels of expression of CDK inhibitors p21 and p27 in a dose-dependent fashion. *In silico* studies suggested that 2,3,4-THBA has the potential to bind to CDK1, CDK2, CDK4 and CDK6 through interactions with specific amino acids. **Conclusion:** The growth inhibitory effect of 2,3,4-THBA on cancer cells may occur partly through induction of CDK inhibitors p21 and p27, and through direct binding to CDKs involved in cell cycle regulation. These data suggest that 2,3,4-THBA from plant sources has the potential to be used in cancer prevention.

Keywords: Antioxidants, Cancer prevention, Cyclin dependent kinases, Molecular docking, Colon cancer 2,3,4-thba.

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INTRODUCTION

Colorectal Cancer (CRC), which comprises of colon and/or rectum cancers, is a significant health issue around the world. According to the world health organization, it is estimated that in the year 2020, more than 1.9 million people have been diagnosed with this disease worldwide, among whom 930,000 people have died.[1,2] Every year, over 150,000 cases of CRC are diagnosed and nearly 53,000 patients die of the disease in the United States, which makes it the second major cause of death from cancer when numbers for men and women are combined.[3,4] It is estimated that the number of new CRC cases worldwide will reach 3.2 million in 2040, based on the projection of population growth, aging and human development.[5] The predicted increase in CRC incidence is attributed to exposure to several risk factors and shifting lifestyles and diet toward western diet.[6] Contributing factors for the development of CRC include inflammatory bowel disease, family history of CRC, genetic syndromes such as Familial Adenomatous Polyposis (FAP), Lynch syndrome, and lifestyle factors such as lack of regular physical activity, obesity, regular alcohol consumption, diet high in processed meat, tobacco use, high fat diet and a diet low in fruits and vegetables.[7,8] The initiation of CRC starts with the appearance of an adenomatous polyp

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which develops into advanced adenoma leading to dysplasia and progression to invasive cancer.[9] Detection of these cancers at early stages, for example, at a stage confined to within the walls of colon are curable; however, once they spread to lymph nodes and metastasize, it is difficult to cure. In such stages, surgery and adjuvant chemotherapy options may help in treating cancer patients.

A large volume of published literature suggests that a diet rich in fruits and vegetables reduces the incidences of several types of cancers including CRC.[10,11] Their protective effects have been attributed to the presence of polyphenolic compounds (e.g., flavonoids) as well as simple phenolic compounds such as Hydroxybenzoic Acids (HBAs) in abundant amounts.[12,13] Interestingly, HBAs are also generated from the degradation of polyphenolic compounds by alkaline pH in the intestine and by the resident gut microbiota.[14-16] These polyphenolics and HBAs have excellent antioxidant and anti-inflammatory properties and are thought to contribute to their health benefits.[17,18] Owing to the rapid degradation of polyphenols in the intestine by microbiota, some researchers argued that the health benefits of the polyphenolics actually come from the degraded HBAs rather than the parent compounds.[19,20] Understanding of the role of HBAs therefore is important for their greater use in diet/supplements and is considered as beneficial to reverse common illnesses such as inflammation, cardiovascular diseases and cancer.

Hydroxybenzoic acids are compounds that contain a carboxylic group and one or more of OH groups, attached to the benzene ring. The most extensively studied HBA is salicylic acid (2-hydroxybenzoic acid; 2-HBA), which is abundantly present in plants such as the willow tree. The other plant derived HBAs studied include 2,3-dihydrobenzoic acid (2,3-DHBA, pyrocatechuic acid), 2,4-dihydroxybenzoic acid (2,4-DHBA, ↕-resorcyclic acid), 2,5-dihydroxybenzoic acid (2,5-DHBA, gentisic acid), 2,6- dihydroxybenzoic acid (2,6-DHBA, □-resorcyclic acid), 3,4-dihydroxybenzoic acid (3,4-DHBA, proto-catechuic acid), 3,5- dihydroxybenzoic acid (3,5-DHBA, α-resorcyclic acid), 3,4,5- Trihydroxybenzoic acid (3,4,5-THBA, gallic acid), and 2,4,6- Trihydrobenzoic acid (2,4,6-THBA). Previous studies carried out in our laboratory have demonstrated that 2,3-DHBA, 2,5-DHBA, 3,4-DHBA. 3,4,5-THBA, 2,4,6-THBA, all had the ability to inhibit colon cancer cell growth; however, these inhibitory properties were not observed when cancer cells were exposed with 2,4-DHBA and 2,6-DHBA.[21] This suggested that number and position of the OH group in the phenolic acid may be important in exerting the growth inhibitory properties. Among the different HBAs tested, 3,4-DHBA and 3,4,5-THBA were most effective compared to other HBAs. Also, the mechanisms of inhibition by these HBAs appeared to occur through different pathways, for example, 2,4-6-THBA-mediated suppression of cell growth occurred through inhibition of Cyclin-Dependent Kinases (CDK); whereas inhibition of cancer cell growth by 3,4,5-THBA was CDK-independent.[20]

In the present study, we investigated the ability of 2,3,4-Trihydroxybenzoic Acid (2,3,4-THBA) to inhibit cancer cell growth using HCT-116 human colon and MDA-MB-231 breast cancer cells using cell proliferation and clonogenic assays. The objective of this study was to determine the effect of 2,3,4-THBA on human cancer cell growth and identify the potential mechanisms by which it may suppress the growth through modulation of expression of CDK inhibitors p21 and p27, as well as to determine if it binds to CDKs. 2,3,4-THBA is a plant metabolite present in several fruits and vegetables including Adzuki beans.[22,23] This bean variety is a major cultivar in southeast Asia. It is also present in many plants including *Betula Pendula, Plinia cauliflora* and Phaseolus *vulgaris.*[24] Experimental evidence suggests that this plant metabolite has significant antioxidant properties. It is to be noted that the growth inhibitory properties of 2,3,4-THBA on human cancer cell lines have not been carried out till date. We demonstrate that 2,3,4-THBA dose-dependently inhibited the HCT-116 human and MDA-MB-231 breast cancer cell growth; however, no changes in the expression levels of Human Epidermal growth factor Receptor type 2 (HER2) or Fibroblast Growth Factor Receptor (FGF-R) were observed. Interestingly, it induced the levels of the Cyclin-Dependent Kinase (CDK) inhibitors p21 and p27, suggesting that inhibition of the cell growth may occur at least in part through the upregulation of these CDK inhibitors. *In silico* studies showed that 2,3,4-THBA efficiently interacts with CDK1, CDK2 and CDK4, which suggests that direct binding and inhibition of the CDKs may also contribute to the inhibition of cancer cell growth.

MATERIALS AND METHODS

Cell lines

HCT-116 and MDA-MB-231 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The HCT-116 cells were cultured in McCoy's 5A media, and MDA-MB-231 cells were cultured in RPMI media containing 10% Fetal Bovine Serum (FBS) with antibiotics for 24 hr before treatment with 2,3,4-THBA.

Reagents

2,3,4-THBA was obtained from Sigma Aldrich (St. Louis, MO, USA); anti-HER2 antibody, anti-FGF-R antibody, anti-p21 antibody and anti-p27 antibody were purchased from Cell Signaling (Danvers, MA, USA). Super Signal™ West Pico Chemiluminescent Substrate, protease inhibitor tablets, MacCoy's 5A media, RPMI media and FBS and all other chemicals were obtained from Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

Cell proliferation assay, Cell lysate preparation and Western blotting and Microscopy

For cell proliferation assays, cells treated with 2,3,4-THBA at different concentration for 48 hr were trypsinized and counted using the Nexcelom Cellometer Auto T4 cell counter. For Western blotting, cells were treated with 2,3,4-THBA at specified concentrations, they are washed with phosphate buffered saline and lysates were prepared as previously described.[20-21] Concentrations of the protein in lysates were determined by Bradford assay and 50 µg was separated on an 8 or 12% polyacrylamide gel, transferred to immobilon membrane and immunoblotted with antibodies. The protein bands were detected using Super Signal™ West Pico Chemiluminescent reagent. In some experiments, following treatment with 2,3,4-THBA for the indicated times, images were captured in Carl Zeiss inverted microscope (10X magnification).

Clonogenic assay

HCT-116 cells or the MDA-MB-231 cells were seeded at a density of 500 cells per 100 mm plate and allowed to grow for 48 hr. The cells were treated with 2,3,4-THBA at different concentrations. Once a week the spent media was replaced with the fresh media containing 2,3,4-THBA. The cells were allowed to grow for 14-21 days, fixed with 100% methanol for 20 min and stained with 0.5% crystal violet prepared in 25% methanol. The stained colonies were photographed and quantified using Image-J software (NIH, Bethesda, MD, USA) as previously described.

Molecular docking studies

A molecular docking study was conducted on different CDKs with 2,3,4-THBA. Compound was retrieved from Pubchem for their Three-Dimensional (3D) structures (http://pubchem.ncbi.nlm.nih.gov). Downloaded compound was prepared using prepare ligand option from small molecule tab from BIOVIA Discovery Studio 2022 (BIOVIA, SanDiego, CA, USA). The CHARMm force field was used to minimize compounds, generate conformations, and create different isomers. Protein structures of CDK were downloaded from website (http://www.rcsb.org/pdb). Docking was carried out on the crystal structures of CDK1 (PDB ID-4Y72), CDK2 (PDB ID-6GUE), CDK4 (PDB ID-3G33) and CDK6 (PDB ID-3NUP) using BIOVIA Discovery Studio (DS) 2022 (BIOVIA, San Diego, CA, USA) software. In addition to removing water molecules and bound ligands, CHARMm was used to minimize the protein's energy consumption. The binding pocket of the different CDK protein was identified using Discovery Studio's "define and edit binding site" protocol. The protein-ligand docking was carried out using CDOCKER tool in the DS.[25] CDOCKER interaction energies generated were used as tool to study binding efficiency as previously described.[26]

Statistical analysis

All experiments were performed in triplicates. One-way ANOVA followed by Tukey's post-hoc analysis was used to analyze group differences to the control, and significance was defined at $p<0.05$. All statistical analyses were performed using IBM SPSS version 28.0.0.

RESULTS

2,3,4-THBA inhibits HCT-116 colon cancer cell proliferation

We first determined the effect of 2,3,4-THBA on HCT-116 cells by exposing the cells with the compound at different concentrations for 48 hr, and then measuring the number of cells in the petri dish. For this, cells were seeded at a density of 250,000 cells per 100 mm plate and grown for 48 hr. Cells were then treated with different concentrations of 2,3,4-THBA for 48 hr, cells trypsinized and counted. We observed that 2,3,4-THBA was able to inhibit HCT-116 colon cancer cell proliferation in a dose-dependent fashion (Figure 1). Approximately 35% inhibition was observed beginning at 250 µM, at 500 µM, the inhibition was 55%, at 750 µM, the inhibition was 90% and at 1000 µM, inhibition was nearly 100%.

In another set of experiments, to determine the effect of 2,3,4-THBA on cell attachment to the petri plate, cells were seeded in a 100 mm plate at a density of 250,000 cells per plate, and left untreated (control) or immediately treated with 2,3,4-THBA at 50 µM and 100 µM. 24 hr and 40 hr after treatments, cell images were captured in a Carl Zeiss inverted microscope at 10X magnification. It is clear from Figure 2 that the number of attached cells is considerably less in the plate treated with 50 µM 2,3,4-THBA compared to the control plate. In plates treated with

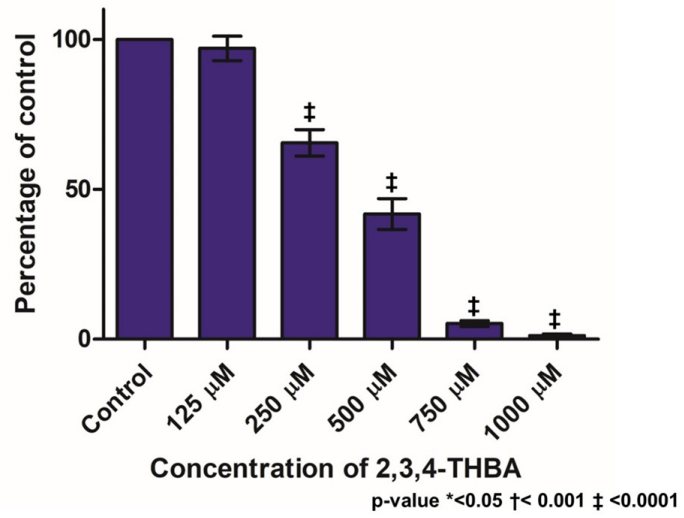

Figure 1: Effect of 2,3,4-THBA on HCT-116 colon cancer cell proliferation. Following treatment of cells with 2,3,4-THBA for 48 hr, cells were trypsinized and counted using a cell counter.

100 μM 2,3,4-THBA, only dead, floating cells were observed. This suggests that 2,3,4-THBA may interfere with attachment of cells to the plate and may cause cell cytotoxicity.

2,3,4-THBA inhibits colony formation in HCT-116 colon and MDA-MB-231 breast cancer cells

In these set of experiments, clonogenic assay was performed to demonstrate the effectiveness of 2,3,4-THBA against colony formation in HCT-116 and MDA-MB231 cells. For these, HCT-116 or MDA-MB-231 cells were seeded at a density of 500 cells per plate, treatment with 2,3,4-THBA was carried out as described in the methods section. Following 2-3 weeks of incubation, cells were fixed in methanol and stained with crystal violet. It is clear from Figure 3A that 2,3,4-THBA dose-dependently decreased the colony formation in HCT-116 cells. Quantification of the number-stained cells from an independent experiment showed that 2,3,4-THBA inhibited the colony formation by ~50% between 25 μM (Figure 3B). Assays carried out in MDA-MB-231 cells also showed that 2,3,4-THBA also dose-dependently inhibited colony formation (Figures 4A and 4B). In MDA-MB-231 cells, inhibition at 25 μM was 85%, and at 37.5 μM, the inhibition was 100%.

2,3,4-THBA does not affect the expression levels of HER2 and FGF-R; however, induces the expression levels of CDK inhibitors p27 and p21

To survive and grow continuously, cancer cells mutate growth factor receptors such as Human Epidermal growth factor Receptors (HER) and Fibroblast Growth Factor Receptors (FGF-R).[27-30] Among HER family, there are four members, namely HER1, HER2, HER3 and HER4. HER1 and HER2 proteins (~185 kDa molecular weight) are often over expressed in colon and breast cancers. Similarly, in many cancers FGF-R is over expressed and produces both mitogenic and angiogenic effects. There are 4 members in the FGF-R family, namely FGFR-1, FGFR-2, FGFR-3 and FGFR-4. The molecular weight of FGF-R ranges between 92 kDa to 140 kD. Binding of epidermal growth factor to HER2, or fibroblast growth factor to FGF-R are known to induce p42/p44 MAP kinase signaling pathway and leads to cell proliferation. To determine if 2,3,4-THBA affects the levels of expression of HER2 and FGF-R, we grew the HCT-116 cells in a 100 mm plate at a density of 250,000 cells per plate, and after 48 exposed the cells to 500 μM of 2,3,4-THBA for 48 hr. The cell lysates were prepared and immunoblotted with anti-HER2 antibodies or anti-FGFR antibodies. We observed that 2,3,4-THBA didn't affect the levels of HER2 (Figure 5A) or FGFR (Figure 5B) at the concentration tested.

We hypothesized that 2,3,4-THBA may mediate its inhibitory effects through induction of p21 and p27 proteins which are Cyclin-Dependent Kinase (CDK) inhibitors.[31-34] Both proteins are capable of inhibiting CDK1, CDK2, CDK4 and CDK6, and can cause inhibition of cell cycle progression and arrest cell growth. To determine if 2,3,4-THBA induces p27/p21, the lysate prepared for Figure 5 was analyzed in a 12% SDS-PAGE and immunoblotted with anti-p27 antibody (Figure 6A) or anti-p21 antibody (Figure 6B). It is clear from Figures 6 A and B that 2,3,4-THBA induces p27 and p21 expression in HCT-116 cells in a dose-dependent fashion. Quantification of the band showed that there was an approximately 2-fold increase in 27 protein levels at 500 μM THBA (Figures 6C). There was an increase of approximately 1.4-fold in p21 levels at 125 μM 2,3,4- THBA (Figures 6D). It is interesting to note that p27 protein levels kept increasing with increased concentrations of 2,3,4-THBA;

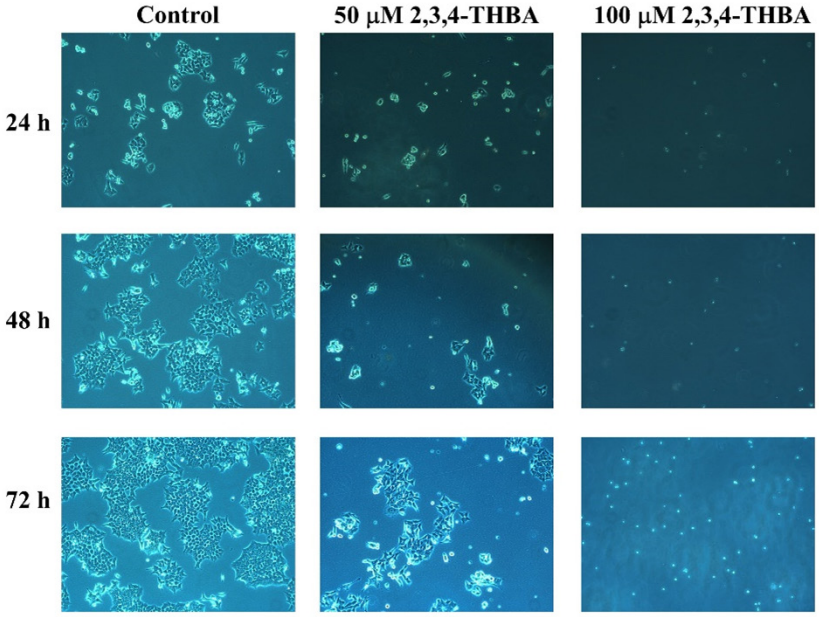

Figure 2: Microscopy images showing the effect of 2,3,4-THBA on HCT-116 colon cancer cell attachment (Images captured using Carl Zeiss inverted microscope at 10 X magnification).

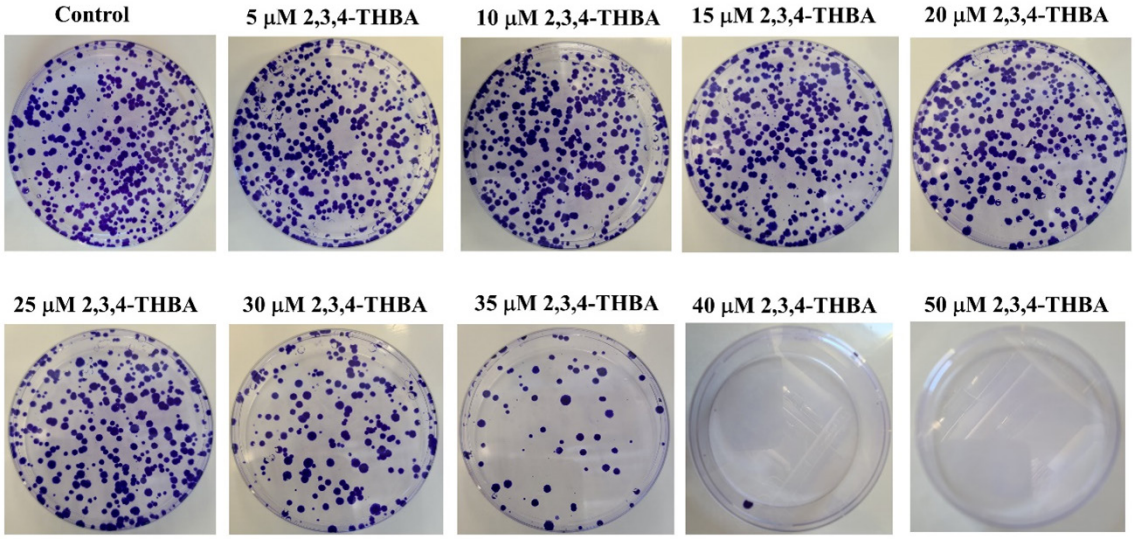

Figure 3: (A)-Colony formation assay showing the effect of 2,3,4-THBA on HCT-116 cells. Cells were seeded at a density of 500 cells per plate, treated with 2,3,4-THBA at various concentrations, following 14-21 days of incubation, cells stained with crystal violet dye and photographed.

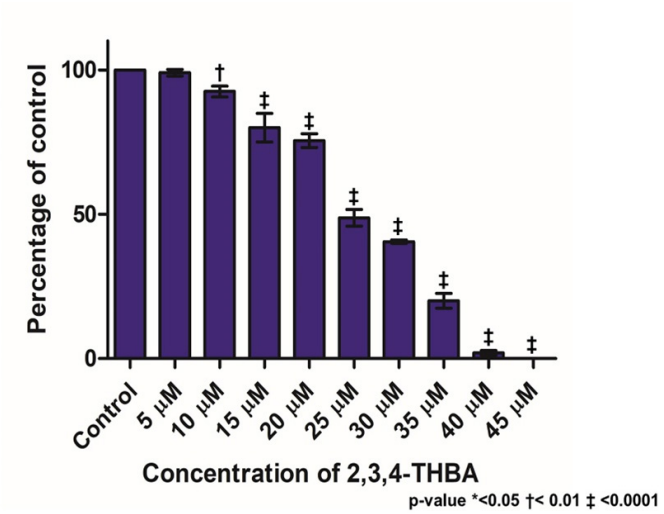

Figure 3: (B)-Quantification of the data (using Image-J software (NIH, Bethesda, MD) from Figure A.

however, p21 levels initially increased with 2,3,4-THBA (62.5 µM and 125 µM), but decreased at higher concentrations (500 µM).

In silico studies show potential interactions of 2,3,4-THBA with CDKs

Since CDK1, CDK2, CDK4 and CDK6 are involved in cell cycle regulation, we reasoned that 2,3,4-THBA may directly interact with specific amino acids on CDKs and this potentially may lead to inhibition of their enzyme activity and thereby contribute to inhibition of cancer cell growth. Therefore, *in silico* studies were performed to determine potential interactions of 2,3,4-THBA with CDK members. Table 1 summarizes the results of the docking of 2,3,4-Trihydroxy benzoic acid on the crystal structure of different CDKs. CDOCKER interaction energy represents interaction energy between the protein and ligand.[35] It is clear from Table 1 that 2,3,4-THBA interaction with CDK1 showed CDOCKER interaction energy of -29.09 Kcal/mol, with hydrogen bond interactions occurring at LYS A:33, ASP A:146, and hydrophobic interactions at TYR A:15, VAL A:18, LEU A:135, ALA A:145, PHE A:80, VAL A:64, PHE A:147, GLU A:51 (Figure 7A). 2,3,4-THBA interactions with CDK2 showed CDOCKER interaction energy of -25.58 Kcal/mol. Hydrogen bond interactions observed at THR A:47, LYS A:129; and hydrophobic interactions at LEU A:148, ASP A:127, ASN A:132, ASP A:145, TYR A:15, GLY A:147, THR A:47. It is interesting to note that these amino acids are within the active site of CDK2 (Table 1, Figure 7B). 2,3,4-THBA had a docking score of -32.43Kcal/mol with CDK4 forming several hydrogen bond interactions at LYS A:40, ASP A:163; and hydrophobic interactions at VAL A:101, VAL A:25, HIS A:100, ILE A:17, LEU A:152, GLU A:99, ALA A:38, VAL A:77, ALA A:162, PHE A:98 (Table 1, Figure 8A). 2,3,4-THBA docking with CDK6 showed docking score of -10.46Kcal/mol. It formed hydrogen bond interactions at ALA A:162, PHE A:164 residues, it formed hydrophobic interactions at PHE A:98, GLY A:165, LEU A:96, LEU A:166, LYS A:43, ASP A:163, VAL A:77, VAL A:76, LEU A:161 (Table 1, Figure 8B). However, the interaction energy for 2,3,4-THBA with CDK6 seemingly was the least among all the CDKs studied which depicts the low affinity interactions.

DISCUSSION

Cancer is a global disease, and every sixth death in the world is due to cancer. In the US, in 2024, it is estimated that there will be 2,001,140 new cancer cases and 611,720 cancer deaths, that is 5480 new cases and 1689 deaths every day.[36,37] The incidence of cancer is highest among countries with higher life expectancies, higher education and higher standard of living. In the past four

Table 1: Results of the docking of 2,3,4-Trihydroxy benzoic acid on the crystal structure of different CDKs. The interaction energy, hydrogen bond interactions and hydrophobic interactions are indicated [BIOVIA Discovery Studio 2022 (BIOVIA, SanDiego, CA, USA)].

Ligand and protein	CDOCKER interaction energy (KCAL/MOL)	Hydrogen bond interactions	Hydrophobic interactions
2,3,4-Trihydroxy benzoic acid CDK1 (PDB ID-4Y72)	-29.09	LYS A:33, ASP A:146	TYR A:15, VAL A:18, LEU A:135, ALA A:145, PHE A:80, VAL A:64, PHE A:147, GLU A:51
2,3,4-Trihydroxy benzoic acid CDK2 (PDB ID-6GUE)	-25.58	THR A:47, LYS A:129	LEU A:148, ASP A:127, ASN A:132, ASP A:145, TYR A:15, GLY A:147, THR A:47
2,3,4-Trihydroxy benzoic acid CDK4 (PDB ID-3G33)	-32.43	LYS A:40, ASP A:163	VAL A:101, VAL A:25, HIS A:100, ILE A:17, LEU A:152, GLU A:99, ALA A:38, VAL A:77, ALA A:162, PHE A:98
2,3,4-Trihydroxy benzoic acid CDK6 (PDB ID-3NUP)	-10.46	ALA A:162, PHE A:164	PHE A:98, GLY A:165, LEU A:96, LEU A:166, LYS A:43, ASP A:163, VAL A:77, VAL A:76, LEU A:161

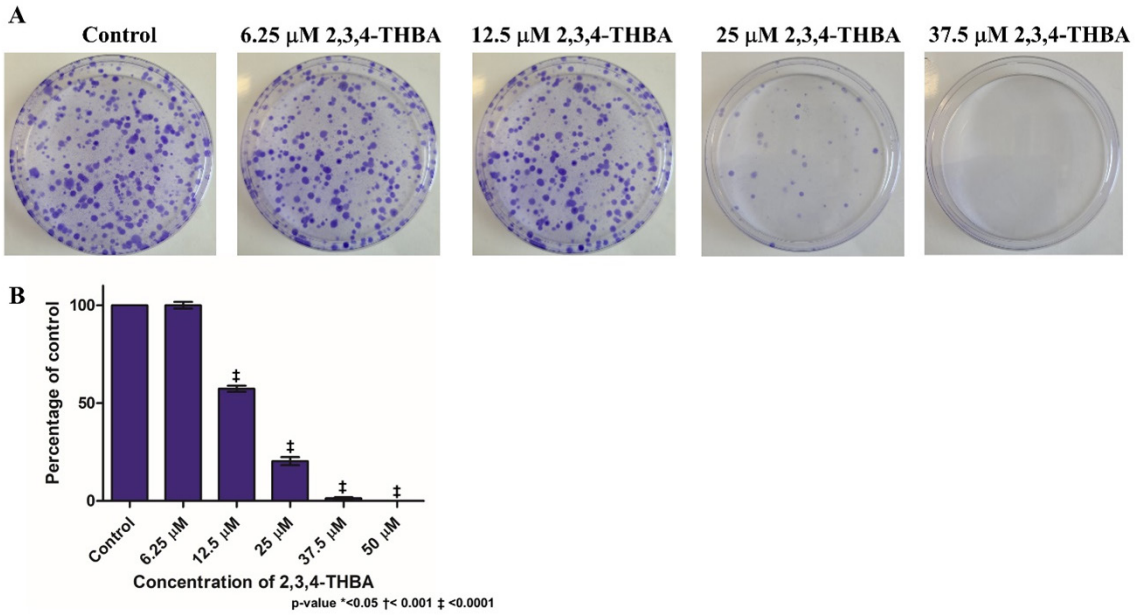

Figure 4: (A)-Colony formation assay showing the effect of 2,3,4-THBA on MDA-MB-231 cells. (B)-Quantification of the data from Figure 4A.

decades, efforts have been focused on developing drugs to treat cancer; however, very few drugs have been identified to prevent cancers. Examples of cancer prevention drugs identified include tamoxifen, raloxifene and aspirin.[38-40] Tamoxifen and raloxifene are most effective in lowering the risk of estrogen-receptor positive breast cancers,[38,39] aspirin is most effective in lowering the risk of colorectal cancers;[40] however, all three drugs have their own side effects. While tamoxifen/raloxifene administration can cause menopause-like symptoms, aspirin, when taken even at low doses, can increase gastro-intestinal bleeding in some individuals. Therefore, identification of natural compounds or synthetic drugs with lower side effects to prevent cancer should take precedence in future research.

In the present research project, using *in vitro* studies and in cultured cancer cells, we demonstrated that 2,3,4-THBA which exhibits antioxidant properties, dose-dependently inhibited the growth in HCT-116 colon cancer and MDA- MB-231 breast cancer cells. The inhibitory effect of 2,3,4-THBA was observed in both cell proliferation assays and clonogenic cell survival assays. It is important to point out that MDA- MB-231 cells were more sensitive to treatment with 2,3,4-THBA as compared to HCT-116 cells in clonogenic assays. For example, in MDA-MB-231 cells, 2,3,4- THBA reduced clone formation by 85% at 25 µM (Figure 4B); in HCT-116 cells it reduced the clone formation by ~50% at 25 µM (Figure 3B). The reason for the different degree of sensitivity of these two different cell lines to treatment with 2,3,4-THBA is not clear at this stage, it could be related to different

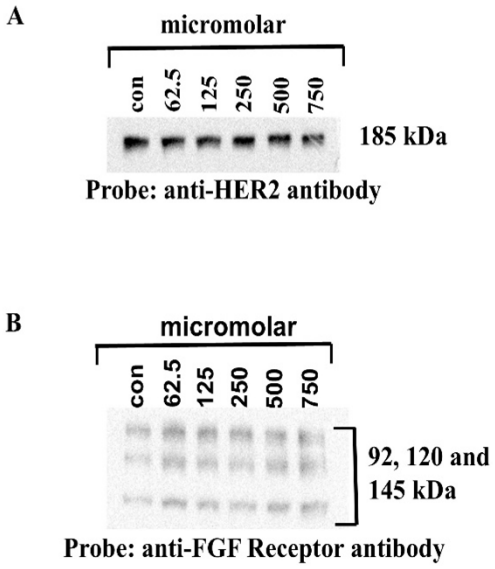

Figure 5: (A)-Western Blot showing the effect of 2,3,4-THBA on the expression levels HER2 (185 kDA) in HCT-116 cells. (B)-Western blot showing the effect of 2,3,4-THBA on the expression levels of FGF-R. The anti-FGF-R antibody detected three distinct bands with molecular weight 92 kDa, 120 kDa and 145 kDa, which represent the different isoforms of FGFR.

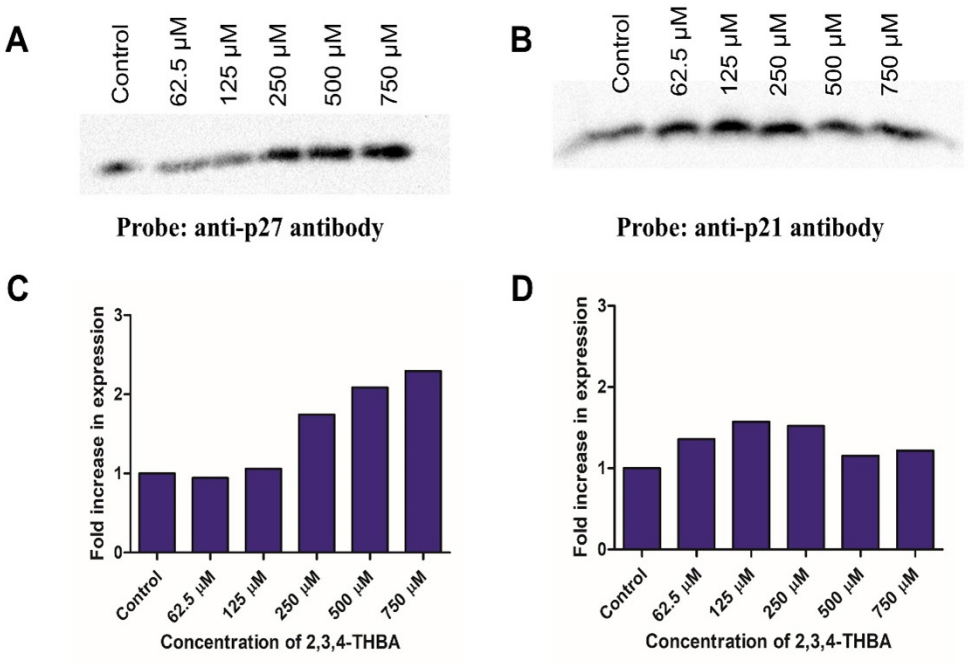

Figure 6: (A)-Western blot showing the dose-dependent effect of 2,3,4-THBA on p27 protein levels in HCT-116 cells. (B)-Western blot showing the dose-dependent effect of 2,3,4-THBA on p21 protein levels in HCT-116 cells. (C)-Quantification of the data from Figure 6A. (D)-Quantification of the data from Figure 6B.

target proteins for 2,3,4-THBA present in these two cell types. It is also not known if 2,3,4-THBA protein target is extracellular or intracellular. If the target protein is intracellular, one would assume that specific transporter protein(s) are required for the uptake of 2,3,4-THBA. In this context, it is important to note that 2,3,4-THBA is a monocarboxylic acid, and it is possible that members of Monocarboxylic Acid Transporter (MCT) family such as SLC5A8 may be involved.

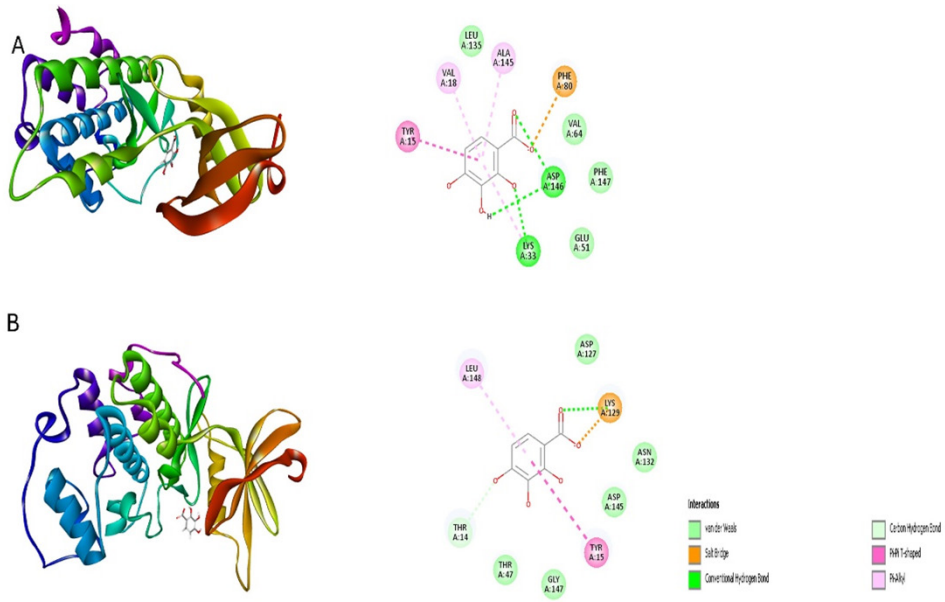

Figure 7: (A)-*In silico* studies showing the 2D and 3D interactions of 2,3,4-THBA with CDK1. (B)-*In silico* studies showing the 2D and 3D interactions of 2,3,4-THBA with CDK2.

An interesting observation in this research paper is that the degree of sensitivity of HCT-116 cells to treatment with 2,3,4-THBA differed drastically depending upon the way cells were treated and duration of treatment. For example, for Figure 1, which measured the effect of 2,3,4-THBA on cell proliferation, the treatment of cells with the compound was done 48 hr after seeding the cells on to the plate, followed by exposure with the compound for 48 hr. For Figure 2, which measured the effect of 2,3,4-THBA on cell attachment, treatment with compound was done while seeding the cells on to the plate, followed by exposure for 24 hr, 48 hr and 72 hr. For Figure 3, which measured the colony formation, treatment of cells was done 48 hr after seeding the cells, and in this case, the exposure time of cells with the 2,3,4-THBA was for 14-21 days. It is interesting to note that in Figure 1, 55% inhibition was observed around 500 μM THBA, however, in Figure 2, a greater than 50% inhibition was observed at 50 μM concentration of the compound. In Figure 3, ~50% inhibition in colony formation was observed at 25 μM 2,3,4-THBA. The reasons for differences in the degree of sensitivity of the 2,3,4-THBA in experiments of Figure 1, Figure 2 and Figure 3 are not clear at this stage. One possibility is that if the target of 2,3,4-THBA is extracellular, it may be more readily accessible if cells are treated with the compound immediately after trypsinization and before seeding onto the plate (as performed for Figure 2) in contrast to cells that were treated with the compound 48 hr after seeding onto the plate (Figure 1). The higher degree of sensitivity of HCT-116 cells as observed in Figure 3 compared to Figure 1 may be related to longer duration of exposure of 2,3,4-THBA, which may lead to an accumulation of the compound inside the cells over 14-21 days incubation period. Despite the observed differences in the degree of sensitivity in all experiments, it is clear that 2,3,4-THBA is effective in suppressing cancer cell growth.

In this research project, we also investigated if 2,3,4-THBA affects the expression levels of HER2 and FGFR. Many transmembrane receptors are upregulated in cancers and binding of ligands to their cognate receptors drive cell proliferation. We observed that 2,3,4-THBA did not affect the expression levels of both HER2 and FGFR (Figures 5 A and B). Following ligand binding, the HER2 is phosphorylated at Tyr877, in addition, it is also auto phosphorylated at Ty1211/ Tyr1222 and Tyr1248.[41,42] Phosphorylation of these sites couples HER2 to Ras-Raf-MAP kinase signal transduction pathway. We were also not able to detect changes in the phosphorylation status of HER2 as well as FGF-R (data not shown), and this may be related to poor affinity and quality of the phospho-specific HER2 and FGF-R antibody used in the study. Immunoblotting with higher affinity antibodies will establish if 2,3,4-THBA affects phosphorylation of FGFR. It will be also important to determine if 2,3,4-THBA inhibits phosphorylation of HER2 in the cytoplasmic domain at these sites using phospho-specific antibodies.

We observed that treatment of 2,3,4-THBA to HCT-116 cells induced both p21 and p27 proteins in a time dependent fashion (Figures 6A-D). p27 is a member of the Cip/Kip family of

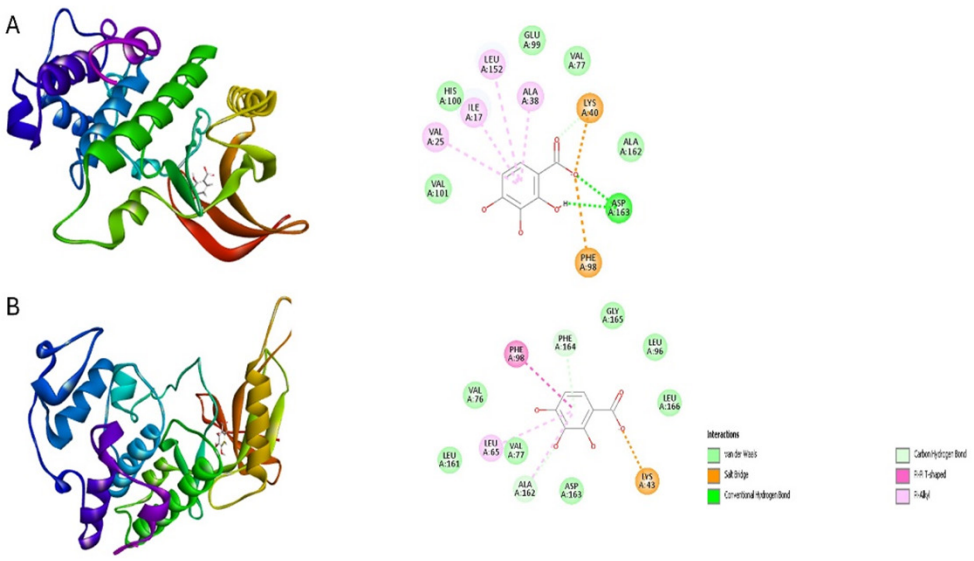

Figure 8: (A)-*In silico* studies showing the 2D and 3D interactions of 2,3,4-THBA with CDK4. (B)-*In silico* studies showing the 2D and 3D interactions of 2,3,4-THBA with CDK6.

Cyclin-Dependent Kinase (CDK) inhibitors. Its ability to inhibit G1 phase of the cell cycle at the G1 restriction point is derived from its capacity to inhibit CDK2/cyclin E, and other CDK/cyclin complexes. Expression levels of p27 are upregulated in quiescent cells treated with cAMP or other negative cell cycle regulators. Similar to p27, p21 also acts as an inhibitor of cell cycle progression. It forms heterotrimeric complexes with cyclins and CDKs, and in association with CDK2 complexes, it serves to inhibit kinase activity and block progression through G1/S phase of the cell cycle.[43] Interestingly, p21 can also bind to Proliferating Cell Nuclear Antigen (PCNA), a subunit of DNA polymerase and interfere with DNA replication.[44] In cells that contain DNA damage, p53 is phosphorylated and it upregulates p21 via p53-responsive DNA element.[45] Protein levels of p21 are downregulated through ubiquitination and proteasomal degradation.[46] While 2,3,4- THBA clearly induces both p21 and p27 protein levels, it is known how it causes upregulation of the protein levels. It could be at the level of transcription leading to increased synthesis of mRNAs and translation, or it is also possible that the upregulation is due to stabilization of the protein via inhibition of their degradation. Studies involving PCR of mRNAs isolated from 2,3,4- THBA with p21/p27 specific primers should resolve this issue.

Since CDKs regulate cell cycle, we reasoned that 2,3,4-THBA may directly bind to CDKs and modulate its activity. To ascertain the most pertinent CDK protein interactions for 2,3,4-THBA with cancer cell growth inhibitory potential, *in silico* studies were conducted.[35] A robust negative interaction energy of -29.09 kcal/mol was observed between 2,3,4-THBA and CDK1, accompanied by numerous hydrogen bonds and hydrophobic interactions (Table 1). This suggests a potent inhibitory effect on CDK1, given its pivotal role in cell cycle progression. Similarly, a strong negative interaction energy of -25.58 kcal/mol was observed with CDK2, indicating its potential for effective inhibition. Furthermore, the interaction between the compound and CDK4 exhibited an even stronger negative interaction energy of -32.43 kcal/mol, suggesting a significant / potential impact on cancer cell proliferation. In contrast, the interaction with CDK6 was less potent, characterized by a lower interaction energy and fewer interactions (Table 1). Consequently, 2,3,4-THBA interactions with CDK1, CDK2, and CDK4 appear to be the most promising for effectively targeting cancer cell growth, as these CDKs play crucial roles in regulating cell cycle progression. Further biochemical studies using CDK enzyme assays are required to establish the mechanism of action of 2,3,4-THBA in the inhibition of cancer cell growth. Future experiments directed at formulation of 2,3,4-THBA for enhanced local delivery (for example in the gastro intestine) will prove useful to establish effectiveness as a cancer preventing agent in *in vivo* studies.

CONCLUSION

In this research paper, we demonstrated that treatment of HCT-116 colon and MDA-MB-231 breast cancer cells with the plant metabolite 2,3,4-THBA inhibits cancer cell growth. The inhibition of cell growth appears to be mediated through induction of CDK inhibitors p21 and p27. Our results also suggest that inhibition of the cell growth may also occur via binding of 2,3,4-THBA to CDKs, particularly CDK1, CDK2, and CDK 4, leading to inhibition of their enzyme activity. These

observations suggest that 2,3,4-THBA has the potential for use in the prevention of CRC and other cancers.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

REFERENCES

1. Colorectal cancer. World Health Organization [cited Sep 13, 2024]. Available from: https://www.who.int/news-room/fact-sheets/detail/colorectal-cancer.
2. Genome-wide association study and Mendelian randomization analyses provide insights into the causes of early-onset colorectal cancer. World Health Organization [cited Sep 13, 2024]. Available from: https://www.iarc.who.int/cancer-type/colorectal-cancer.
3. Pankratz VS, Kanda D, Edwardson N, English K, Adsul P, Li Y, *et al.* Colorectal cancer survival trends in the United States from 1992 to 2018 differ among persons from five racial and ethnic groups according to stage at diagnosis: a SEER-based study. Cancer Control. 2022;29:10732748221136440. doi: 10.1177/10732748221136440, PMID 36264283.
4. Siegel RL, Wagle NS, Cercek A, Smith RA, Jemal A. Colorectal cancer statistics, 2023. CA Cancer J Clin. 2023;73(3):233-54. doi: 10.3322/caac.21772, PMID 36856579.
5. Xi Y, Xu P. Global colorectal cancer burden in 2020 and projections to 2040. Transl Oncol. 2021;14(10):101174. doi: 10.1016/j.tranon.2021.101174, PMID 34243011.
6. Keum N, Giovannucci E. Global burden of colorectal cancer: emerging trends, risk factors and prevention strategies. Nat Rev Gastroenterol Hepatol. 2019;16(12):713-32. doi: 10.1038/s41575-019-0189-8, PMID 31455888.
7. Eng C, Yoshino T, Ruíz-García E, Mostafa N, Cann CG, O'Brian B, *et al.* Colorectal cancer. Lancet. 2024;404(10449):294-310. doi: 10.1016/S0140-6736(24)00360-X, PMID 38909621.
8. Hossain MS, Karuniawati H, Jairoun AA, Urbi Z, Ooi J, John A, *et al.* Colorectal cancer: a review of carcinogenesis, global epidemiology, current challenges, risk factors, preventive and treatment strategies. Cancers. 2022;14(7):1732. doi: 10.3390/cancers14071732, PMID 35406504.
9. Markowitz SD, Bertagnolli MM. Molecular origins of cancer: molecular basis of colorectal cancer. N Engl J Med. 2009;361(25):2449-60. doi: 10.1056/NEJMra0804588, PMID 20018966.
10. Romagnolo DF, Selmin OI. Flavonoids and cancer prevention: a review of the evidence. J Nutr Gerontol Geriatr. 2012;31(3):206-38. doi: 10.1080/21551197.2012.702534, PMID 22888839.
11. Pyo Y, Kwon KH, Jung YJ. Anticancer potential of flavonoids: their role in cancer prevention and health benefits. Foods. 2024;13(14):2253. doi: 10.3390/foods13142253, PMID 39063337.
12. Bhosale PB, Ha SE, Vetrivel P, Kim HH, Kim SM, Kim GS. Functions of polyphenols and its anticancer properties in biomedical research: a narrative review. Transl Cancer Res. 2020;9(12):7619-31. doi: 10.21037/tcr-20-2359, PMID 35117361.
13. Juurlink BH, Azouz HJ, Aldalati AM, AlTinawi BM, Ganguly P. Hydroxybenzoic acid isomers and the cardiovascular system. Nutr J. 2014;13:63. doi: 10.1186/1475-2891-13-63, PMID 24943896.
14. Bermudezsoto M, Tomasbarberan F, Garciaconesa M. Stability of polyphenols in chokeberry (*Aronia melanocarpa*) subjected to *in vitro* gastric and pancreatic digestion. Food Chem. 2007;102(3):865-74. doi: 10.1016/j.foodchem.2006.06.025.
15. Hanske L, Engst W, Loh G, Sczesny S, Blaut M, Braune A. Contribution of gut bacteria to the metabolism of cyanidin 3-glucoside in human microbiota-associated rats. Br J Nutr. 2013;109(8):1433-41. doi: 10.1017/S0007114512003376, PMID 22906731.
16. Sankaranarayanan R, Sekhon PK, Ambat A, Nelson J, Jose D, Bhat GJ, *et al.* Screening of human gut bacterial culture collection identifies species that biotransform quercetin into metabolites with anticancer properties. Int J Mol Sci. 2021;22(13):7045. doi: 10.3390/ijms22137045, PMID 34208885.
17. Kalinowska M, Gołębiewska E, Świderski G, Męczyńska-Wielgosz S, Lewandowska H, Pietryczuk A, *et al.* Plant-derived and dietary hydroxybenzoic acids- A comprehensive study of structural, Anti-/Pro-oxidant, lipophilic, antimicrobial, and cytotoxic activity in MDA-MB-231 and MCF-7 cell lines. Nutrients. 2021;13(9):3107. doi: 10.3390/nu13093107, PMID 34578985.
18. Jideani AI, Silungwe H, Takalani T, Omolola AO, Udeh HO, Anyasi TA. Antioxidant-rich natural fruit and vegetable products and human health. Int J Food Proper. 2021;24(1):41-67. doi: 10.1080/10942912.2020.1866597.
19. Sankaranarayanan R, Kumar DR, Patel J, Bhat GJ. Do aspirin and flavonoids prevent cancer through a common mechanism involving hydroxybenzoic acids?-The Metabolite Hypothesis. Molecules. 2020;25(9):2243. doi: 10.3390/molecules25092243, PMID 32397626.
20. Sankaranarayanan R, Valiveti CK, Kumar DR, Van slambrouck S, Kesharwani SS, Seefeldt T, *et al.* The flavonoid metabolite 2,4,6-trihydroxybenzoic acid is a CDK inhibitor and an anti-proliferative agent: A potential role in cancer prevention. Cancers. 2019;11(3):427. doi: 10.3390/cancers11030427, PMID 30917530.
21. Sankaranarayanan R, Valiveti CK, Dachineni R, Kumar DR, Lick T, Bhat GJ. Aspirin metabolites 2,3-dhba and 2,5-dhba inhibit cancer cell growth: implications in colorectal cancer prevention. Mol Med Rep. 2020;21(1):20-34. doi: 10.3892/mmr.2019.10822, PMID 31746356.
22. Muhammad M, Zhu Y, Wen A, Liu N, Qin L. Phenolic profile, alpha-amylase inhibitory activity, and *in vitro* glycemic index of adzuki beans. Front Nutr. 2022;9:1063602. doi: 10.3389/fnut.2022.1063602, PMID 36618697.
23. Zhai H, Li C, Tang S, Duan H. Phenol constituents of *Pachysandra terminalis* and their antioxidant activity. Zhongguo Zhong Yao Za Zhi. 2010;35(14):1820-3. doi: 10.4268/cjcmm20101411, PMID 20939276.
24. National Center for Biotechnology Information. PubChem Compound Summary for CID 11874, 2,3,4-Trihydroxybenzoic acid [cited Sep 18, 2024]. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/2_3_4-Trihydroxybenzoic-acid.
25. Bhardwaj P, Biswas GP, Mahata N, Ghanta S, Bhunia B. Exploration of binding mechanism of triclosan towards cancer markers using molecular docking and molecular dynamics. Chemosphere. 2022;293:133550. doi: 10.1016/j.chemosphere.2022.133550, PMID 34999105.
26. Singh R, Bhardwaj VK, Sharma J, Das P, Purohit R. Discovery and *in silico* evaluation of aminoarylbenzosuberene molecules as novel checkpoint kinase 1 inhibitor determinants. Genomics. 2021; 113(1 Pt. 2):707-15. doi: 10.1016/j.ygeno.2020.10.001, PMID 33065246.
27. Moasser MM. The oncogene HER2: its signaling and transforming functions and its role in human cancer pathogenesis. Oncogene. 2007;26(45):646-6487. doi: 10.1038/sj.onc.1210477, PMID 17471238.
28. Hsu JL, Hung MC. The role of HER2, EGFR and other receptor tyrosine kinases in breast cancer. Cancer Metastasis Rev. 2016;35(4):575-88. doi: 10.1007/s10555-016-9649-6, PMID 27913999.
29. Wiedlocha A, Haugsten EM, Zakrzewska M. Roles of the FGF-FGFR signaling system in cancer development and inflammation. Cells. 2021;10(9):2231. doi: 10.3390/cells10092231, PMID 34571880.
30. Krook MA, Reeser JW, Ernst G, Barker H, Wilberding M, Li G, *et al.* Fibroblast growth factor receptors in cancer: genetic alterations, diagnostics, therapeutic targets and mechanisms of resistance. Br J Cancer. 2021;124(5):880-92. doi: 10.1038/s41416-020-01157-0, PMID 33268819.
31. Vermeulen K, Van Bockstaele DR, Berneman ZN. The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. Cell Prolif. 2003;36(3):131-49. doi: 10.1046/j.1365-2184.2003.00266.x, PMID 12814430.
32. Besson A, Dowdy SF, Roberts JM. CDK inhibitors: cell cycle regulators and beyond. Dev Cell. 2008;14(2):159-69. doi: 10.1016/j.devcel.2008.01.013, PMID 18267085.
33. Abukhdeir AM, Park BH. P21 and p27: roles in carcinogenesis and drug resistance. Expert Rev Mol Med. 2008;10:e19. doi: 10.1017/S1462399408000744, PMID 18590585.
34. Cheng M, Olivier P, Diehl JA, Fero M, Roussel MF, Roberts JM, *et al.* The p21Cip1 and p27Kip1 CDK inhibitors are essential activators of cyclin-D-dependent kinases in murine fibroblasts. EMBO J. 1999;18(6):1571-83. doi: 10.1093/emboj/18.6.1571, PMID 10075928.
35. Parthasarathy V, Kumar AK. Screening of potential GCMS derived antimigraine compound from the leaves of *Abrus precatorius* Linn. to target "calcitonin gene related peptide" receptor using in silico analysis. Food Sci Hum Wellness. 2019;8(1):34-9. doi: 10.1016/j.fshw.2019.01.001.
36. American Cancer Society. Cancer facts & figures. American Cancer Society [cited Sep 18, 2024]. Available from: https://www.cancer.org/content/dam/cancer-org/research/cancer-facts-and-statistics/annual-cancer-facts-and-figures/2024/2024-cancer-facts-and-figures-acs.pdf.
37. National Cancer Institute. Cancer statistics [cited Sep 18, 2024]. Available from: https://www.cancer.gov/about-cancer/understanding/statistics.
38. Gu KJ, Li G. An overview of cancer prevention: chemoprevention and immunoprevention. J Cancer Prev. 2020; 30;25(3): 127-35. doi: 10.15430/JCP.2020.25.3.127, PMID 33033707.
39. Shieh Y, Tice JA. Medications for primary prevention of breast cancer. JAMA. 2020;324(3):291-2. doi: 10.1001/jama.2020.9246, PMID 32692377.
40. Sankaranarayanan R, Kumar DR, Altinoz MA, Bhat GJ. Mechanisms of colorectal cancer prevention by aspirin-a literature review and perspective on the role of COX- dependent and-independent pathways. Int J Mol Sci. 2020;21(23):9018. doi: 10.3390/ijms21239018, PMID 33260951.
41. Muthuswamy SK, Gilman M, Brugge JS. Controlled dimerization of ErbB receptors provides evidence for differential signaling by homo- and heterodimers. Mol Cell Biol. 1999;19(10):6845-57. doi: 10.1128/MCB.19.10.6845, PMID 10490623.
42. Hazan R, Margolis B, Dombalagian M, Ullrich A, Zilberstein A, Schlessinger J. Identification of autophosphorylation sites of HER2/neu. Cell Growth Differ. 1990;1(1):3-7. PMID 1706616.
43. Pestell RG, Albanese C, Reutens AT, Segall JE, Lee RJ, Arnold A. The cyclins and cyclin-dependent kinase inhibitors in hormonal regulation of proliferation and

differentiation. Endocr Rev. 1999;20(4):501-34. doi: 10.1210/edrv.20.4.0373, PMID 10453356.

44. Flores-Rozas H, Kelman Z, Dean FB, Pan ZQ, Harper JW, Elledge SJ, *et al.* Cdk-interacting protein 1 directly binds with proliferating cell nuclear antigen and inhibits DNA replication catalyzed by the DNA polymerase delta holoenzyme. Proc Natl Acad Sci U S A. 1994;91(18):8655-9. doi: 10.1073/pnas.91.18.8655, PMID 7915843.
45. Wang Y, Prives C. Increased and altered DNA binding of human p53 by S and G2/M but not G1 cyclin-dependent kinases. Nature. 1995;376(6535):88-91. doi: 10.1038/376088a0, PMID 7596441.
46. Sheaff RJ, Singer JD, Swanger J, Smitherman M, Roberts JM, Clurman BE. Proteasomal turnover of p21Cip1 does not require p21Cip1 ubiquitination. Mol Cell. 2000;5(2):403-10. doi: 10.1016/s1097-2765(00)80435-9, PMID 10882081.

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