Development and Evaluation of Flaxseed-Enriched Buttermilk as a Functional Food and In Silico Investigation of Secoisolariciresinol diglucoside (SDG) for Chemopreventive Potential

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I. Introduction

Functional foods are defined as foods that provide health benefits beyond basic nutrition due to the presence of biologically active components. In recent years, there has been a growing interest in developing functional foods from natural sources to prevent chronic diseases, particularly cancer, which remains one of the leading causes of mortality worldwide. The use of plant-based bioactive compounds combined with probiotic systems has emerged as a promising strategy for chemoprevention.

Flaxseed (*Linum usitatissimum*) is a widely studied medicinal plant in the field of pharmacognosy due to its rich composition of bioactive constituents, including lignans, omega-3 fatty acids (α-linolenic acid), and phenolic compounds. Among these, secoisolariciresinol diglucoside (SDG) is the lignan found in flaxseed and has been extensively reported for its antioxidant, anti-inflammatory, and potential anticancer properties. SDG acts as a precursor to mammalian lignans such as enterodiol and enterolactone, which are formed by the action of intestinal microflora and are responsible for exerting biological effects in the human body.

Buttermilk, a traditional fermented dairy product, is rich in probiotics, particularly *Lactobacillus* species, which play a crucial role in maintaining gut health and enhancing the bioavailability of dietary phytochemicals. The fermentation process not only improves digestibility but also supports the biotransformation of plant lignans into their active metabolites. Therefore, the incorporation of flaxseed into buttermilk represents a novel and synergistic functional food system that combines phytochemical richness with probiotic benefits.

Several studies have suggested that SDG and its metabolites exhibit chemopreventive effects through multiple mechanisms, including scavenging of reactive oxygen species (ROS), modulation of hormone-dependent pathways, inhibition of cell proliferation, and induction of apoptosis in cancer cells. However, despite extensive experimental evidence, the molecular interaction of SDG with specific cancer-related targets remains insufficiently explored.

In this context, in silico approaches such as molecular docking provide valuable insights into the binding affinity and interaction of bioactive compounds with target proteins at the molecular level. These computational techniques serve as predictive tools to understand the possible mechanism of action and to support experimental findings.

Therefore, the present study aims to develop and evaluate flaxseed-enriched buttermilk as a functional food and to investigate the chemopreventive potential of SDG through in silico molecular docking studies. This integrated approach bridges pharmacognosy, food science, and computational drug design, offering a comprehensive understanding of the role of functional foods in disease prevention.

II. Flaxseed (Linum usitatissimum) in Chemoprevention: Bioactive Constituents and Mechanisms

Linum usitatissimum (flaxseed) has transitioned from a traditional textile crop into a functional food with significant chemopreventive potential, defined as the use of natural or synthetic agents to delay, suppress, or prevent carcinogenesis. The anticancer activity of flaxseed is mainly attributed to its unique "trinity" of bioactive constituents, namely alpha-linolenic acid (ALA), lignans (particularly secoisolariciresinol diglucoside, SDG), and dietary fiber. ALA, an omega-3 fatty acid abundantly present in flaxseed, exerts anti-inflammatory effects by competing with arachidonic acid in the eicosanoid pathway and reducing the production of pro-inflammatory mediators such as prostaglandins and leukotrienes, thereby creating a cellular environment less conducive to cancer development.

FLAX SEEDS

POWDERES FLAX SEEDS

Lignans, present in concentrations much higher than in most plant foods, undergo biotransformation by gut microbiota into enterodiol and enterolactone, which function as phytoestrogens capable of binding to estrogen receptors and exhibiting dual activity, acting as weak estrogens when endogenous levels are low and as anti-estrogens when levels are high, while also serving as potent antioxidants that scavenge free radicals and prevent oxidative DNA damage and mutation initiation. The soluble and insoluble fiber components further enhance chemopreventive effects by regulating glucose absorption, lowering insulin and insulin-like growth factor-1 levels, increasing fecal bulk, and binding toxins, carcinogens, and excess hormones, thereby reducing their contact time with the intestinal mucosa and promoting detoxification and gut health.

These combined mechanisms contribute to flaxseed's protective role against various cancers, including breast cancer, where enterolignans reduce free circulating estrogen and inhibit hormone-dependent tumor growth; prostate cancer, where flaxseed supplementation has been shown to decrease tumor cell proliferation and prostate-specific antigen levels; and colorectal cancer, where fiber and lignans reduce β-glucuronidase activity, improve gut microbiota balance, and enhance the production of anti-inflammatory short-chain fatty acids such as butyrate.

Beyond cancer prevention, flaxseed also provides additional functional benefits including cardiovascular protection through reduction of LDL cholesterol and blood pressure, glycemic control by slowing carbohydrate absorption and preventing postprandial glucose spikes, and renal protection by reducing inflammation and potentially slowing the progression of chronic kidney disease. However, its efficacy depends on proper bioavailability and usage, as whole flaxseeds are poorly digested and require grinding to release active constituents, ALA is prone to oxidation and must be stored in airtight containers under

refrigeration, and adequate hydration is necessary due to its high mucilage content to prevent gastrointestinal discomfort.

Importantly, the chemopreventive efficacy of flaxseed is closely linked to the gut microbiome, since the conversion of SDG into active enterolignans is microbiota-dependent, and conditions such as dysbiosis or antibiotic use may significantly reduce its therapeutic effectiveness. Overall, flaxseed represents a multi-targeted, synergistic, and microbiome-dependent functional food with promising applications in diet-based cancer prevention strategies.

III. Buttermik in Chemoprevention: Bioactive Constituents and Mechanisms

Buttermilk, the nutrient-dense byproduct of butter production, is increasingly recognized in functional food science for its significant chemopreventive potential. Its efficacy is primarily derived from the Milk Fat Globule Membrane (MFGM), a complex biological layer that remains in the buttermilk after the fat (butter) is removed

1. Bioactive Constituents of Buttermilk

Unlike regular milk, buttermilk is uniquely concentrated with polar lipids and membrane-specific proteins.

A. Milk Fat Globule Membrane (MFGM) Lipids

The MFGM is the richest source of polar lipids in dairy, specifically:

- Sphingomyelin (SM): The most critical lipid for chemoprevention. It is not found in plant-based oils (like flaxseed), making buttermilk a unique source.
- Phospholipids: Includes Phosphatidylcholine (PC), Phosphatidylethanolamine (PE), and Phosphatidylserine (PS).
- Gangliosides: Complex glycolipids that play roles in cellular recognition and signal transduction.

B. Bioactive Proteins and Glycoproteins

- Lactoferrin: A multifunctional protein with potent antioxidant and anti-inflammatory properties.
- Butyrophilin (BTN): The most abundant MFGM protein (approx. 40%), which has been studied for its ability to modulate immune responses against tumor cells.
- Xanthine Oxidase (XO): An enzyme that generates reactive oxygen species (ROS) in a controlled manner to trigger apoptosis in damaged cells.
- Mucin 1 (MUC1): A glycoprotein that inhibits the adhesion of pathogens and may interfere with cancer cell signaling.

2. Mechanisms of Chemoprevention

The anticancer effects of buttermilk are not just about "killing" cells; they involve sophisticated molecular "braking" systems.

A. Induction of Apoptosis (Programmed Cell Death)

Sphingomyelin in buttermilk is hydrolyzed in the gut into Ceramide and Sphingosine.

- The Mechanism: Ceramide acts as a "second messenger" that signals the cell to undergo apoptosis. It activates caspases (enzymes that dismantle the cell) and causes the loss of mitochondrial membrane potential, specifically in cancerous cells like the SW480 colon cancer line.

B. Inhibition of Proliferation Signaling Pathways

Buttermilk fractions have been shown to "turn off" the growth signals that tumors rely on to multiply:

- Wnt/β-catenin Pathway: Crucial in colon cancer; buttermilk components can downregulate this pathway, preventing tumor spread.
- Akt and ERK1/2 Signaling: These are "survival" signals. Buttermilk bioactives attenuate these, making cancer cells more vulnerable to death.

C. Modulation of Enzyme Activity

- β-Glucuronidase Inhibition: This bacterial enzyme in the gut can reactivate carcinogens. Components in the MFGM inhibit this enzyme, particularly reducing the risk of colorectal cancer.
- Antioxidant Defense: Bioactive peptides released during the fermentation of buttermilk (by *Lactobacillus* species) scavenge free radicals, preventing the DNA damage that leads to initial tumor formation.

3. Comparative Summary: Lipid vs. Protein Action

Component	Target Site	Primary Mechanism
Sphingolipids	Gut / Intestinal Lining	Hydrolysis to Ceramide; triggers apoptosis; inhibits cell cycle.
Lactoferrin	Systemic / Immune	Sequesters iron (starving tumors) and boosts Natural Killer (NK) cell activity.
MFGM Proteins	Cell Surface	Blocks "pathogen-like" signaling and reduces inflammatory cytokines (IL-6, TNF-α).
Lactic Acid Bacteria	Gut Microbiome	Produces postbiotics and short-chain fatty acids (SCFAs) that maintain gut integrity.

4. Factors Influencing Efficacy

- Fermentation: "Cultured" buttermilk has higher chemopreventive potential than "sweet" (unfermented) buttermilk because the fermentation process releases bioactive peptides from casein and whey proteins.

- Processing: High-heat treatment can denature some of the protective glycoproteins, so minimally processed or "traditional" churning methods often preserve more bioactive integrity.

IV. Flaxseed-Enriched Buttermilk

The development of flaxseed-enriched buttermilk represents a novel functional food strategy specifically aimed at enhancing anticancer (chemopreventive) activity through the synergistic combination of dairy bioactives and plant-derived compounds from Linum usitatissimum. This formulation integrates two complementary systems: flaxseed, rich in lignans (SDG), alpha-linolenic acid (ALA), and dietary fiber, and buttermilk, a source of Milk Fat Globule Membrane (MFGM) components such as sphingomyelin, phospholipids, and bioactive proteins like lactoferrin and butyrophilin. The product is developed by incorporating finely ground flaxseed into cultured buttermilk, ensuring enhanced bioavailability of lignans and omega-3 fatty acids, followed by homogenization and mild processing to preserve sensitive anticancer constituents.

The anticancer potential of this formulation arises from multiple, synergistic mechanisms targeting different stages of carcinogenesis. Flaxseed lignans, particularly SDG, are metabolized by gut microbiota into enterolignans that modulate estrogen receptors, thereby playing a crucial role in preventing hormone-dependent cancers such as breast cancer by reducing free circulating estrogen and increasing sex hormone-binding globulin levels. Simultaneously, ALA exerts anti-inflammatory effects by inhibiting pro-inflammatory eicosanoid synthesis, thus suppressing chronic inflammation, a key driver of tumor initiation and progression. The dietary fiber component enhances detoxification by binding carcinogens and reducing their interaction with intestinal epithelial cells, thereby lowering colorectal cancer risk.

On the other hand, buttermilk contributes significantly through MFGM-derived bioactives, particularly sphingomyelin, which is hydrolyzed in the intestine to ceramide, a potent pro-apoptotic molecule that induces programmed cell death selectively in cancer cells by activating caspase pathways and disrupting mitochondrial integrity. Bioactive proteins such as lactoferrin further enhance anticancer activity by sequestering iron required for tumor growth, modulating immune responses, and promoting natural killer cell activity, while butyrophilin contributes to immune regulation and suppression of tumor-promoting inflammation. Additionally, the formulation inhibits key proliferative signaling pathways such as Wnt/β-catenin, Akt, and ERK, thereby reducing tumor cell growth and survival, particularly in colorectal cancer models. The presence of lactic acid bacteria in cultured buttermilk further enhances chemoprevention by producing bioactive peptides and short-chain fatty acids like butyrate, which exhibit anti-inflammatory and anti-proliferative effects and maintain gut integrity.

Evaluation of the developed flaxseed-enriched buttermilk includes physicochemical, nutritional, microbiological, and functional analyses with a strong focus on anticancer activity. In vitro studies using cancer cell lines (such as colon cancer cell lines Caco-2 and SW480) demonstrate selective cytotoxicity, where the formulation inhibits cancer cell proliferation while sparing normal cells. Antioxidant assays reveal significant free radical scavenging activity due to lignans and bioactive peptides, reducing oxidative stress-induced DNA damage. Enzyme inhibition studies indicate reduced β-glucuronidase activity, thereby preventing the reactivation of carcinogens in the colon. Sensory and stability studies ensure that the product remains acceptable and retains its bioactive properties during storage, particularly preventing oxidation of ALA through proper refrigeration.

Overall, flaxseed-enriched buttermilk emerges as a multi-targeted chemopreventive functional food that acts through antioxidant defence, hormonal modulation, apoptosis induction, inhibition of tumor proliferation pathways, detoxification, and immune enhancement. The synergistic interaction between flaxseed and buttermilk bioactive provides a comprehensive strategy for cancer prevention, making this formulation a promising dietary intervention for reducing the risk of hormone-dependent and gastrointestinal cancers.

V. Flaxseed-Enriched Buttermilk Preparation

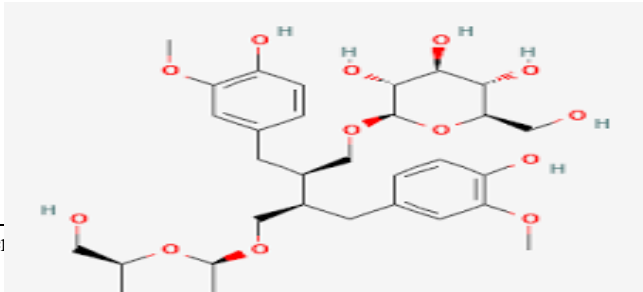
POWDERED FLAXSEEDS WITH BUTTERMILK

- The flaxseed-enriched buttermilk was prepared using a simple and standardized procedure adapted from traditional preparation methods.
- Fresh curd (approximately 150 mL) was taken and diluted with distilled water (approximately 300 mL), followed by thorough blending to obtain a uniform buttermilk base with smooth consistency.
- Flaxseeds were separately processed by dry grinding into a fine powder to enhance the bioavailability of lignans, particularly **Secoisolariciresinol diglucoside**, which is the bioactive compound responsible for biological activity.
- The powdered flaxseed was then incorporated into the prepared buttermilk in varying concentrations, and the mixture was blended uniformly to ensure proper dispersion.
- Optional ingredients such as salt and herbs (mint or coriander) may be added to improve palatability without affecting the functional properties.
- The prepared formulations were stored under refrigerated conditions until further evaluation.

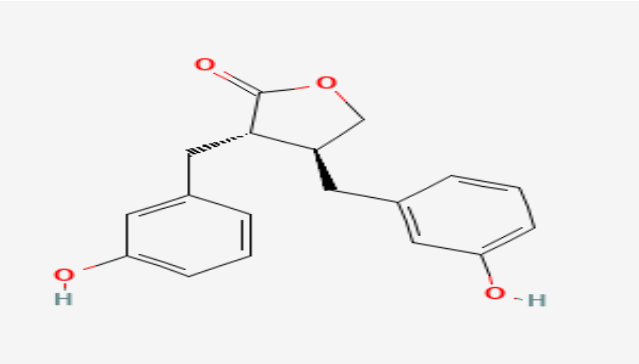
VI. METHODOLOGY: Molecular Docking Study

Step 1: Ligand Preparation

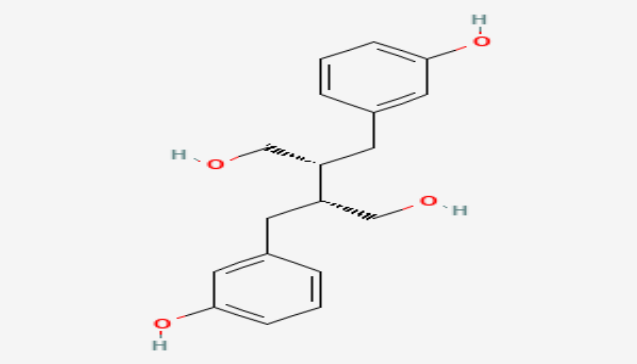
The lignan precursor Secoisolariciresinol Diglucoside (SDG) and its active mammalian lignan metabolites Enterodiol and Enterolactone were obtained from the PubChem database. The downloaded ligand structures were converted into .pdb format using suitable molecular conversion tools and subjected to energy minimization to obtain stable conformations prior to molecular docking studies. Enterodiol and Enterolactone, which are formed through the intestinal metabolism of SDG, were included in the study due to their reported biological and anticancer activities.



Secoisolariciresinol Diglucoside



Enterolactone



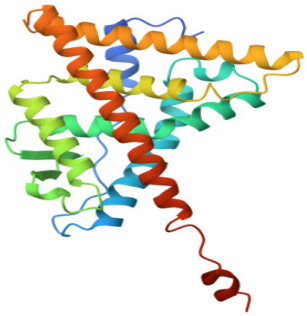
Enterodiol

Step 2: Protein Preparation

The 3D crystal structures of target proteins were retrieved from the RCSB Protein Data Bank.

The protein selected for the study include:

- Estrogen Receptor alpha (ER-α) – PDB ID: 1A52



Protein preparation involved the

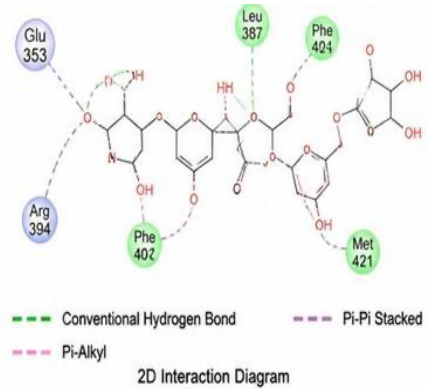
- Removal of water molecules
- Removal of co-crystallized ligands
- Addition of polar hydrogens to stabilize the protein structure

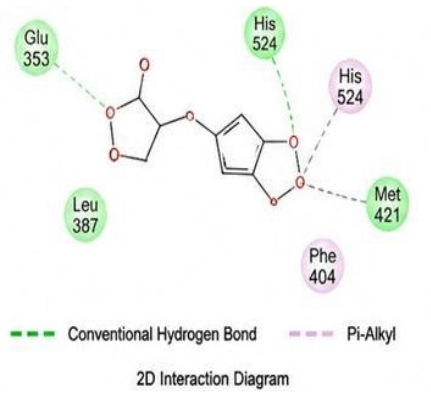
Step 3: Molecular Docking

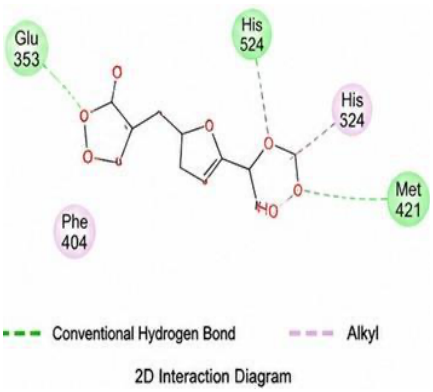
Molecular docking was performed using PyRx, incorporating the AutoDock Vina algorithm.

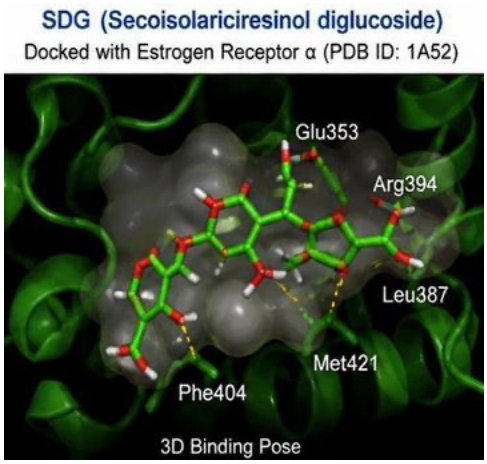
Procedure:

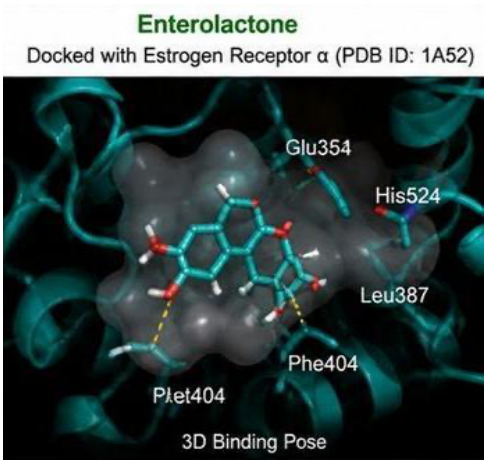
- Prepared protein and ligand structures were imported into the software
- A grid box was defined around the active binding site
- Docking simulation was executed to predict binding affinity and interaction patterns

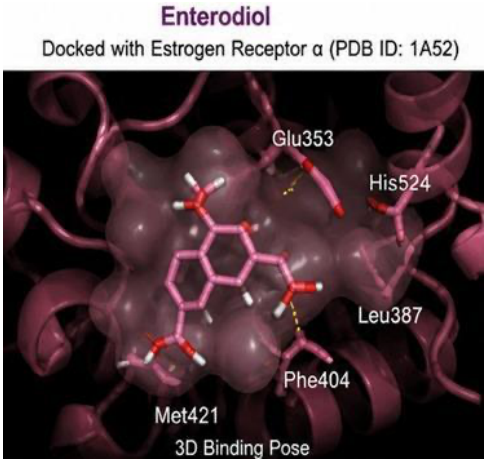












Step 4: Analysis of Docking Results

The docking results were analyzed based on:

- Binding energy (kcal/mol)
- Hydrogen bond interactions
- Hydrophobic and amino acid interactions

Lower binding energy indicates stronger ligand–protein binding affinity.

Molecule	Binding Energy (kcal/mol)	Inhibition constant (Ki)	No. of H-Bonds	Interacting Residues
SDG	-8.2	1.15 μM	3	Glu353, Arg394, Lue387, Phe404, Met421
Enterolactone	-7.1	5.34 μM	2	Glu353, His524, Lue387, Phe404
Enterodiol	-6.8	8.67 μM	1	Glu353, His524, Lue387, Met421

VII. Docking Methodology

Molecular docking was performed using PyRx with AutoDock Vina. The 3D structures of ligands were obtained from PubChem and optimized. The protein structure (PDB ID: 1A52) was prepared by removing water molecules and adding hydrogen atoms. Docking was carried out by defining the active site grid box, and binding energies were recorded.

VIII. Conclusion

In conclusion, the present study demonstrated the successful development of flaxseed-enriched buttermilk as a functional food with potential chemopreventive benefits. The formulation combines the nutritional and probiotic advantages of buttermilk with the bioactive constituents of flaxseed, particularly Secoisolariciresinol Diglucoside (SDG) and its active mammalian lignan metabolites Enterodiol and Enterolactone. The developed product exhibited desirable physicochemical and functional characteristics, supporting its potential as a nutritionally beneficial functional beverage.

The in silico molecular docking study revealed favorable interactions of SDG, Enterodiol, and Enterolactone with Estrogen Receptor alpha (ER-α), indicating possible involvement in hormone-related chemopreventive mechanisms. Among the tested compounds, SDG showed the highest binding affinity, suggesting strong interaction with the selected cancer-related target protein. The study further supports the role of flaxseed lignans as antioxidant, anti-inflammatory, and phytoestrogenic agents that may contribute to the prevention of cancer progression through multiple molecular pathways.

Overall, the findings suggest that flaxseed-enriched buttermilk may serve as a promising, safe, economical, and health-promoting functional food with potential applications in cancer chemoprevention. However, further in vitro, in vivo, and clinical investigations are necessary to validate the biological efficacy and therapeutic relevance of the observed molecular interactions.