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Article

Antiproliferative Activity of Soybean and Tempeh Extracts in Human Colorectal Cancer Cells

Rongjie Fan [1,†], Yong Hoon Joo [1,†], Made Astawan [2], Dmitriy Smolensky [3], Seong-Ho Lee [1,*] and Cheng-I Wei [1,*]

[1] Department of Nutrition and Food Science, College of Agriculture and Natural Resources, University of Maryland, College Park, MD 20742, USA
[2] Department of Food Science and Technology, Bogor Agricultural University, Bogor 16680, Jawa Barat, Indonesia
[3] Grain Quality and Structure Research Unit, Agricultural Research Service, United State Department of Agriculture, Manhattan, KS 66502, USA
* Correspondence: wei@umd.edu (S.-H.L.); slee2000@umd.edu (C.-I.W.)
† These authors contributed equally to this work.

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Abstract: Tempeh, a traditional Indonesian food, is made through fermentation of soybean and increases the bioavailability of beneficial nutrients, including phytoestrogens and protein. Recent research indicates that fermenting soybeans to make tempeh could improve bio functional properties including anticancer activity. This study aims to explore whether defatted soybeans and tempeh (fermented soybeans) extracts possess anti-proliferative activity of human colorectal (CRC) cancer cells. The defatted soybean and tempeh samples were extracted at a concentration of 35 g/100 mL using 70% ethanol, evaporated and then lyophilized. HCT116 cells were treated with soybean extract (SE) and tempeh extract (TE) for 24 h and MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay, flow cytometry, and Western blot analyses were performed. SE and TE exhibited inhibitory effects on cell viability, with TE showing a more significant dose-dependent inhibition compared to SE. Cell cycle analysis showed a significant increase in G1 arrest, along with a significant decrease in S and G2/M phases in both SE- and TE-treated cells. The induction of apoptosis was observed in cells treated with both SE and TE. Additionally, Western blot analysis showed increased Poly (ADP-ribose) polymerase cleavage for both treatments, indicating activation of apoptotic pathways in CRC cells treated with SE and TE. These findings indicate that soybeans and tempeh may be effective dietary options to help prevent colorectal cancer.

Keywords: soybeans; tempeh; fermentation; anticancer activity; colorectal cancer

1. Introduction

Despite significant progress in screening and therapeutic strategies, colorectal cancer (CRC) remains a major contributor to global cancer-related mortality and poses a substantial burden on healthcare systems. Risk factors for CRC include age, genetic predisposition, family history, inflammatory bowel diseases, and certain lifestyle choices such as a high-fat and low fiber diet, sedentary behavior, and smoking [1]. The molecular landscape of CRC is diverse, with various genetic mutations and alterations driving the initiation and progression of the disease. Common genetic alterations involve WNT signaling, APC gene mutations, and chromosomal instability [2]. The interplay of genetic and environmental factors contributes to the multifaceted nature of CRC, necessitating the need for identifying adjunctive treatments that can be integrated into daily diets to help mitigate cancer progression.

Soybeans (*Glycine max* (L.) Merr.) have been a staple food in many Asian countries for centuries and are recognized for their rich content in both macronutrients and micronutrients. Many studies revealed that isoflavones, primarily their aglycones (daidzein and genistein), are associated with a number of biological activities and health benefits, including lowering breast and prostate cancer incidence through modifying carcinogenic process [3], obesity [4], coronary heart disease [5], and neurodegenerative disease with memory improvement [6].

Fermentation is an effective method for enhancing the antioxidant compounds and activity in legume products [7]. Tempeh, a traditional Indonesian food, is produced by fermenting soybeans with *Rhizopus* spp. This fermentation process is known to enhance the nutritional profile of soybeans by improving digestibility and facilitating the bioconversion of isoflavone glycosides to their more bioactive aglycone forms [8]. The enzymatic activities of *Rhizopus* spp. during tempeh fermentation have been reported to induce alterations in the biological compounds of soybeans. Isoflavones such as daidzein, genistein, and glycitein can act as scavengers for free radicals, metal ion chelators, and inhibitors of human cancer cells [8]. Generally, phytoestrogens interact with nuclear estrogen receptors, ERα and ERβ, affecting the transcription of their target genes. [9]. Limited but promising research suggests that tempeh offers various health benefits, including improved gut health [10], enhanced cognitive function [11], and potential cancer prevention [12].

Although the majority of studies have focused on the health benefits of dietary soybeans, the differential bioactivity of soybeans and fermented soybeans remains unanswered. This study aims to investigate the anti-proliferative activity of unfermented and fermented soybean extracts using human adenocarcinoma CRC cells.

2. Materials and Methods

2.1. Tempeh Production

Non-GMO, Identity Preserved (IP) soybeans were sourced from Soymerica (Monterey Park, CA, USA). The starter culture, Raprima Ragi Tempeh Inoculum containing *Rhizopus microsporus* var. *oligosporus* (CBS 337.62), was obtained from LIPI Bandung and used at a concentration of 2 g per kg of dry soybeans. The tempeh production protocol is depicted in Figure 1A–C. Initially, soybeans were washed and soaked in water for 2 h at 24 °C. The beans were then boiled for 20 min, drained, and suspended in fresh water overnight. Following this soaking period, the soybeans were manually dehulled and boiled for an additional 10 min. After cooling and drying, the batch was divided into two. One-half was thoroughly mixed with the *Rhizopus* spp. inoculum to produce tempeh (fermented group). The other half, which did not receive the inoculum, served as the unfermented soybean control. Both groups were packed into perforated polypropylene bags (pierced at 1–2 cm intervals) to form 2 cm-thick cakes. Incubation was carried out at 27 °C for 12 h, after which the cakes were flipped, and the temperature was reduced to 25 °C for an additional 24 h. Six replicates were prepared for both fermented and nonfermented soybeans. The final products were evenly spread for freeze-drying (Harvest Right, LLC., North Salt Lake, UT, USA) and subsequently ground into a fine powder with a blender (CRANDDI, Zhongshan, China). The resulting powders were stored at −80 °C until extraction.

2.2. Extraction Method

To prepare the extracts, the powdered soybean or tempeh samples were first defatted. This was achieved by mixing the powder with n-hexane (1:3 *w*/*v*) and stirring at 300 rpm for 1 h at room temperature, a process repeated three times. The resulting defatted material was then subjected to ethanolic extraction. It was mixed with 100 mL of 70% ethanol, sonicated for 5 min (Misonix Sonicator, QSonica, LLC., Newtown, CT, USA), and stirred for an additional 50 min at 300 rpm. The resulting supernatant was clarified by sequential filtration through a Büchner funnel with Fisherbrand filter paper (25 μm, Fisher Scientific, Pittsburgh, PA, USA) and a Thermo Scientific Nalgene reusable filter unit (0.22 μm, Rochester, NY, USA). The collected filtrate was evaporated, and the resulting soybean ethanolic extract (SE) and tempeh ethanolic extract (TE) were lyophilized. Finally, SE and TE powders were stored at −80 °C until extraction. All powder samples were dissolved in DMSO, and the final concentration of DMSO in the culture medium did not exceed 0.1%.

2.3. Cell Culture and Measurement of Cell Viability

The HCT116 cell line was maintained in Dulbecco's Modified Eagle's Medium supplemented with fetal bovine serum (10% *v*/*v*) and antibiotics (penicillin-streptomycin, 1% *v*/*v*). Cell culture and MTT assay for cell viability were performed as we described previously [13]. Briefly, cells were seeded into 96-well plates at a density

of 1.0×10^4 cells/well. After 24 h of incubation, they were exposed to a range of concentrations (0, 100, 200, 300, 400, 500, and 600 μg/mL) of SE or TE for another 24 h. And then MTT assay was conducted for cell viability.

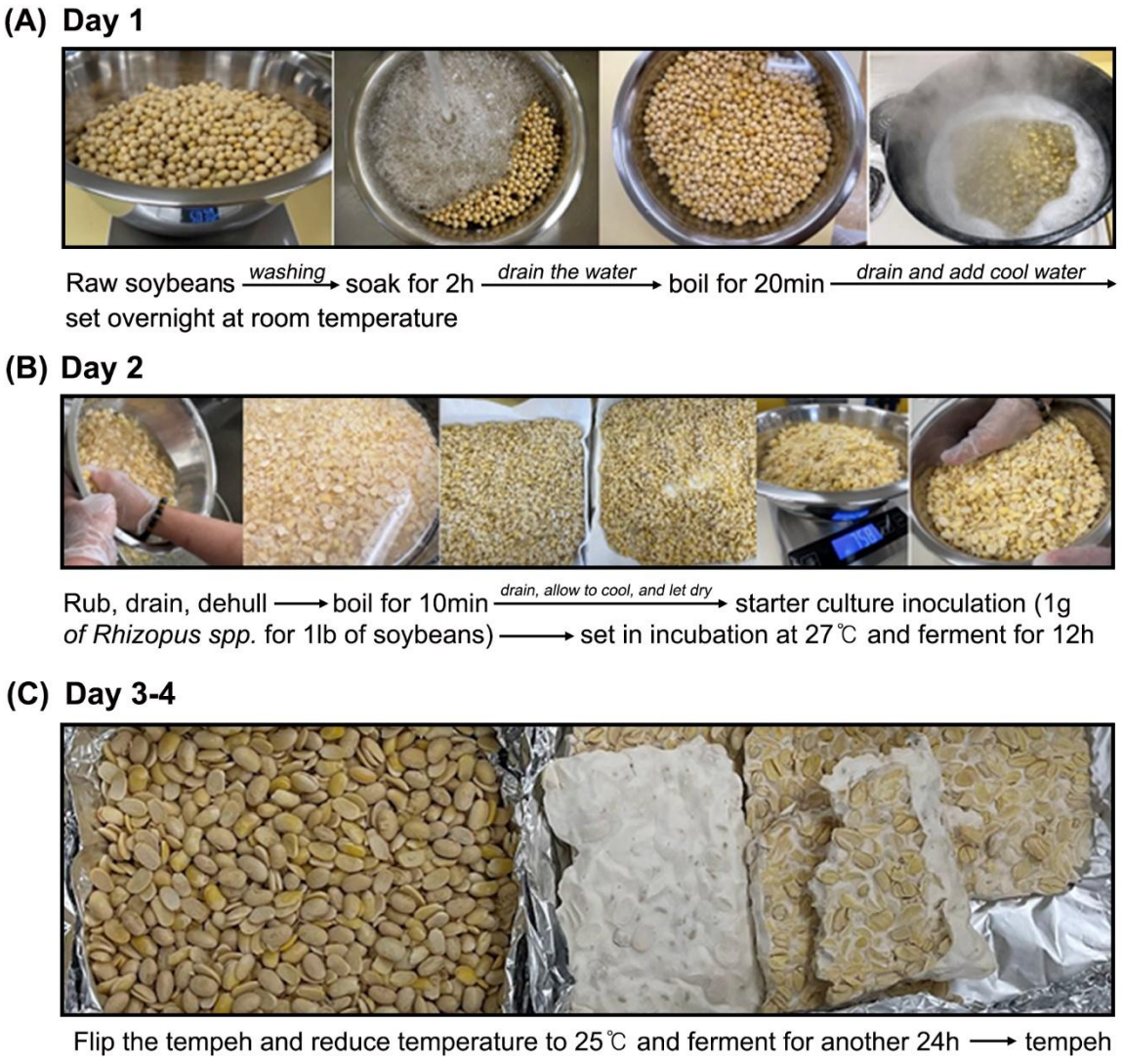

Figure 1. Tempeh production. Soybean and tempeh production on Day 1 (**A**), Day 2 (**B**), and Day 3 (**C**).

2.4. Cell Cycle Assay and Apoptosis Assay

Cell cycle and apoptosis assay were performed as we described previously [13]. Briefly, HCT116 cells were seeded in 100-mm plates at 1.5×10^6 cells/plate and allowed to attach for 24 h. The cells were then treated with SE or TE (0, 200, and 400 μg/mL) for 24 h. And cells were harvested and prepared for cell cycle and apoptosis assay.

2.5. SDS-PAGE and Western Blot

HCT116 cells, seeded and treated as described above, were used for protein analysis. SDS-PAGE and Western blot were performed as we described previously [13].

2.6. Statistical Analysis

All data presented are from a representative experiment and the total number of experiments performed is indicated. All results are presented as mean values (n = 3) ± standard deviation (SD). The data from all experiments were evaluated by one-way Analysis of variance (ANOVA) test to determine the differences among means, preceded by DUNCAN posthoc tests for intergroup comparisons (SPSS, version 21; SPSS Inc., Chicago, IL, USA). Differences were considered significant at $p < 0.05$.

3. Results

3.1. Production of Soybeans and Tempeh Extracts

The general steps of tempeh production include natural acidification during the soaking and boiling process. After dehulling and drying the soybeans, *Rhizopus* spp. are commonly used as starter organisms to prepare traditional fermented foods. With controlled humidity and temperature, tempeh forms and is bound by white mycelium. Although traditional tempeh production is similar, nutrient content and compound profiles can be affected by the source of soybeans and specific methods. For this study, tempeh was made following the protocol (Figure 1A–C) as previously described.

3.2. SE and TE Repressed Viability of Human CRC Cells

For biological assays, we examined if SE and TE affect viability of proliferating human CRC cells because cell survival is a key indicator in cancer progression. HCT116 cells were plated onto the cell culture flask and treated with different concentrations of SE and TE for 24 h. As shown in Figure 2A, the respective viability of HCT116 cells treated for 24 h with 0, 100, 200, 300, 400, 500, 600 μg/mL was 100, 55.2, 57.4, 57, 50.7, 50.6, 56% for SE; 100, 63.7, 57.6, 55, 47.1, 17.1, 0.8% for TE, respectively. The IC_{25}, IC_{50}, and IC_{75} values of HCT116 calculated for SE were 38.6 ± 37.2, and 517.3 ± 89.7 μg/mL, 669.0 ± 142.9 μg/mL, respectively. The IC_{25}, IC_{50}, and IC_{75} values calculated for TE were 116.8 ± 3.5, 291.3 ± 3 μg/mL, and 465.9 ± 9.1 μg/mL, respectively. The respective viability of SW480 cells treated for 24 h with 0, 100, 200, 300, 400, 500, 600 μg/mL was 100, 65.9, 66.8, 56.5, 62.5, 62.4, 54.2% for SE; 100, 59.4, 61.1, 56.1, 41.5, 0.6, 0.5% for TE, respectively. The IC_{25}, IC_{50}, and IC_{75} values of SW480 calculated for SE were 149.4 ± 38.2, and 622.5 ± 66.3 μg/mL, 1095.6 ± 95.8 μg/mL, respectively. The IC_{25}, IC_{50}, and IC_{75} values calculated for TE were 110.9 ± 8.7, 271.6 ± 6.4 μg/mL, and 432.3 ± 4.2 μg/mL, respectively. The respective viability of RKO cells treated for 24 h with 0, 100, 200, 300, 400, 500, 600 μg/mL was 100, 60.4, 68.3, 60.5, 58.7, 55, 54.9% for SE; 100, 47.1, 45.4, 45.5, 9.2, 0.2, 0.4% for TE, respectively. The IC_{25}, IC_{50}, and IC_{75} values of RKO calculated for SE were 110.2 ± 71, and 585.7 ± 36.7 μg/mL, 1061.2 ± 144.5 μg/mL, respectively. The IC_{25}, IC_{50}, and IC_{75} values calculated for TE were 41.1 ± 13.4, 204.5 ± 8.8 μg/mL, and 367.8 ± 4.7 μg/mL, respectively.

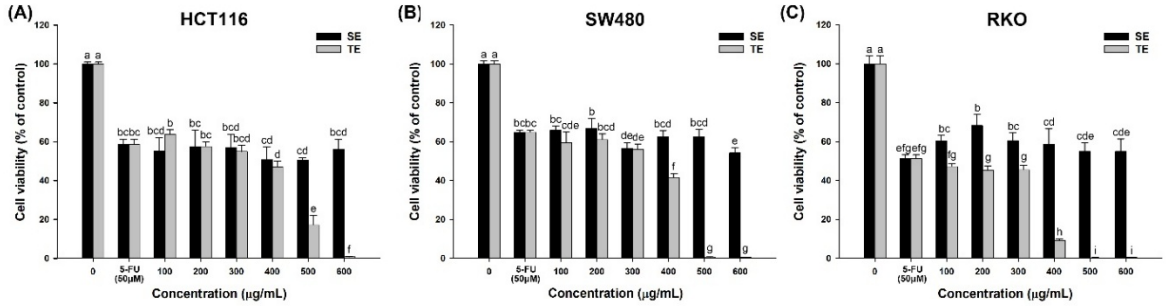

Figure 2. Soybean and tempeh ethanolic extract inhibited viability of HCT116, SW480 and RKO cells. (**A**) HCT116 (**B**) SW480 (**C**) RKO cells were plated onto a 96-well plate and treated with different concentrations of SE and TE at concentrations of 0, 100, 200, 300, 400, 500, and 600 μg/mL. Cell viability was assessed by the MTT test. Values are means ± SD (n = 3). Different letters indicate significant differences at $p < 0.05$ (between treatment and control). One-way ANOVA with Duncan's post-hoc test was used for statistical analysis.

3.3. SE and TE Induced G1 Arrest in Human CRC Cells

Since cell cycle arrest is one of the common mechanisms of anti-proliferative activity of cancer cells, cell cycle phase distribution of HCT116 cells was tested to provide more insights into the regulatory mechanisms controlling cell division. Flow cytometry analysis was performed using HCT116 cells treated for 24 h with 0, 200 and 400 μg/mL of SE and TE.

As shown in Figure 3A,B, proportions of each cell cycle phase in HCT116 cells after treatment of 0, 200 and 400 μg/mL were as follows: G1 phase was 39.6, 80.4 and 78.8% for SE, and 39.6, 76.1 and 71.9% for TE; S phase was 44.5, 10.8 and 10.5% for SE, and 44.5, 12.5 and 14.7% for TE; and G2 phase was 15.9, 8.7, and 10.7% for SE, and 15.9, 11.5 and 13.4% for TE. There were significant differences in all cell cycle phases between control and treatment groups. Cell cycle assay illustrated that both SE and TE led to cell cycle arrest at the G1 phase and hindered progress to S phase.

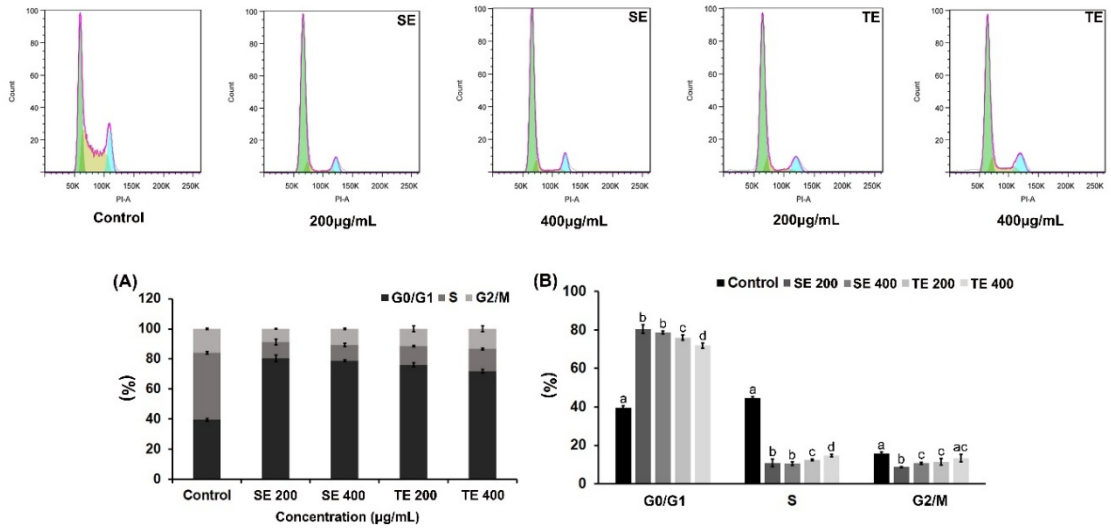

Figure 3. Cell cycle distribution based on the population percentage in HCT116 cells. (**A**,**B**) HCT116 cells were treated with SE and TE at 200 μg/mL and 400 μg/mL for 24 h. SE and TE significantly increased the G1 phase in HCT116 cells. Values are means ± SD (n = 3). Different letters indicate significant differences at $p < 0.05$ (between treatment and control). One-way ANOVA with Duncan's post-hoc test was used for statistical analysis.

3.4. SE and TE Induced Apoptosis in Human CRC Cells

In keeping with cell cycle regulation, apoptosis is another significant cellular event to determine tissue homeostasis and tumorigenesis and a promising target for cancer prevention and treatment. Since we observed a decrease of cell viability and an increase of G1 phase arrest in HCT116 cells treated with SE and TE, we compared the numbers of apoptotic cells using flow cytometry after annexin V-FITC/PI staining where a substantial amount of healthy, early and late apoptotic cells were detected.

As shown in Figure 4, the percentage of early apoptotic cells after treatment of 0, 200 and 400 μg/mL was 1.2, 4.3 and 5.6% for SE, and 1.2, 5.2 and 2.7% for TE. And the percentage of late apoptotic cells after treatment of 0, 200 and 400 μg/mL was 11.2, 13.4 and 13.0% for SE, and 11.2, 13.7 and 24.5% for TE. Overall, there is 17.8% total apoptosis for SE at 200 μg/mL and 18.9% total apoptosis for SE at 400 μg/mL. There is a total of 18.6% total apoptosis for SE at 400 μg/mL and 27.2% total apoptosis for TE. All taken together, the results indicate that treatment of 200 μg/mL and 400 μg/mL SE and TE significantly increased both early and late apoptotic cells. Western blot analysis was performed to measure PARP cleavage which is molecular marker of caspase-dependent apoptosis. As shown in Figure 5, treatment with SE and TE increased cleaved-PARP (89 KDa).

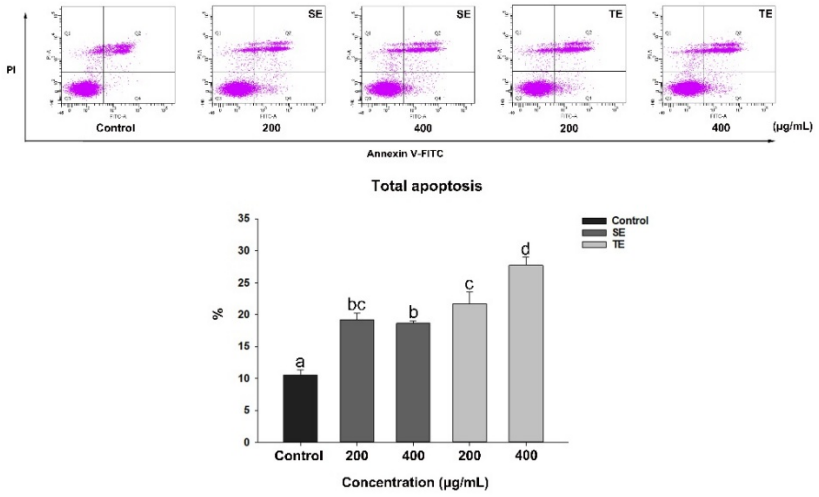

Figure 4. Induction of early and late apoptosis increased in Q2 (Early apoptosis cell) and Q4 (Late apoptosis cell) phases. HCT116 cells were treated with SE and TE at 200 μg/mL and 400 μg/mL for 24 h. Values are means ± SD (n = 3). Different letters indicate significant differences at $p < 0.05$ (between treatment and control). One-way ANOVA with Duncan's post-hoc test was used for statistical analysis.

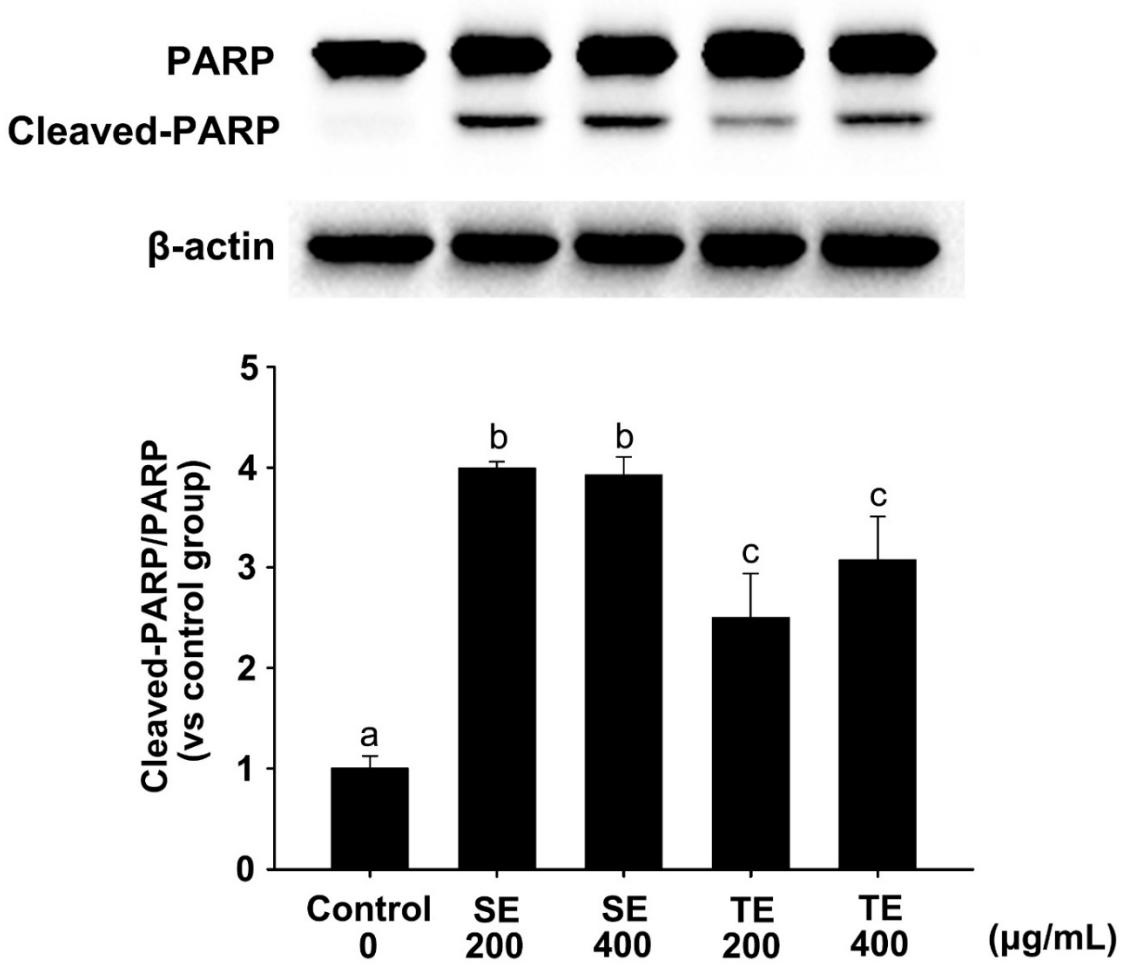

Figure 5. Expression of PARP and β-actin in HCT116 by Western Blot analysis. Quantification of PARP and β-actin normalized to the control group. Values are means ± SD (n = 3). Different letters indicate significant differences at $p < 0.05$ (between treatment and control). One-way ANOVA with Duncan's post-hoc test was used for statistical analysis.

4. Discussion

Most prior studies have documented the content and composition of phytochemicals, such as isoflavones, in soybean products, while there has been less research focused on fermented soybeans. Various factors, including fermentation, germination, cooking, different processing techniques, enzymatic hydrolysis, and heat treatment, can significantly influence the bioactivities of foods. It has also been observed that fermented products demonstrate higher antioxidative, anti-inflammatory, anti-obese, anti-atherosclerotic, and anti-carcinogenic activities compared to non-fermented products [14]. Tempeh has become a promising target food because the use of soybeans in the human diet is rapidly increasing and understanding the impact of processing techniques is essential.

The inheritance of sugar (sucrose, stachyose, raffinose), acids (propionic, formic, acetic) or total isoflavones, growing environment, and the variety of soybeans impact tempeh's quality [15]. Natural acidification during soaking creates a more acidic environment, facilitated by acid-producing bacteria, such as lactic acid bacteria [16]. This enhances tempeh's quality by converting soybean carbohydrate into lactic acid. Boiling reduces oligosaccharide levels, making tempeh more digestible and less likely to cause gastrointestinal discomfort [15]. Controlled temperature and relative humidity during the incubation phase promote the growth of dense mycelium biomass with the inoculation of *Rhizopus* spp. With all contributing factors considered, unfermented soybeans were carried out following the same procedure as fermented soybeans, except for the inoculation of *Rhizopus.* spp. Results have demonstrated that tempeh have been successfully produced, where bagged soybeans were covered with white mycelium.

Studies indicate a broad range of health benefits associated with tempeh consumption, including inhibition of in vivo angiogenesis, elevated levels of cecal *Bifidobacterium* and *Lactobacillus* promoting gut health, and increased short-chain fatty acid concentration [10]. Literature reviews have mentioned fermentation involves the

action of microbes that can break down conjugated isoflavones into their unconjugated forms, thus improving the digestibility and amino acid profiles of soybeans. During soybean fermentation, ACE-inhibitory peptides are produced through the proteolytic breakdown of soybean protein fractions (glycinin and β-conglycinin) [17]. Isoflavone glycosides are converted into more bioavailable aglycones by β-glucosidase, allowing to enhance the bioactivity of isoflavones [18]. Bioactive peptides in fermented soybeans have been studied for various health benefits, such as antioxidant [12], anticancer, antihypertensive, and antidiabetic [19]. Studies reported that the isolated antioxidant peptides contain methionine, proline, and lysine from water extract of tempeh [12]. An increase of quercetin by fermenting soybeans suggested as one of mechanisms for enhancement of total flavonoids properties. Glycitein was observed, while genistin and daidzin decreased, and genistein and daidzein increased with fermentation [20]. Recently, bioactive peptides from enzymatically hydrolyzed soy-based tempeh were identified and evaluated using in silico and in vitro analyses. The tempeh-derived peptides modulated key cancer-related proteins and selectively reduced the viability of pancreatic and colorectal cancer cells with minimal effects on normal cells. These findings support the potential of tempeh as a functional food component or nutritional adjunct for cancer prevention [21].

This research aims to investigate the potential antiproliferative activities of fermented soybeans compared with unfermented soybeans. The results indicate that all concentrations of SE and TE we tested reduced cell viability statistically. Cells exposed to TE exhibited a significantly lower cell viability compared to the same concentration of SE, suggesting tempeh is more potent in inhibiting CRC cell growth than unfermented soybeans. Since the only difference between TE and SE is fermentation with *Rhizopus* spp., we speculated that the soybean fermentation process may have produced certain types of compounds possessing anti-proliferative activity or decomposed certain types of proliferation-promoting compounds in the soybean. However, we do not exclude the possibility that unfermented soybeans contained bioactive compounds to potentially inhibit the growth of CRC cells, although the inhibition was lower than TE. Further study such as metabolite profiling needs to explain the difference cell viability of SE and TE, and observed plateau in soybean viability.

Cell cycle arrest is a crucial regulatory checkpoint and surveillance system that halts or delays the transition of the cell cycle, preventing the active proliferation of cancer cells. It is regulated by diverse cyclins and cyclin-dependent kinases (CDKs) depending on cell cycle phages. In particular, G1 arrest, a key player in the cell cycle machinery, has been the target of anti-cancer mechanisms by many types of dietary compounds and therapeutic drugs. Numerous phytochemicals can prevent cancer development by modulating signal transduction pathways such as cyclooxygenase-2, NF-κB, endothelin-1, and STAT3 and by directly regulating G1/S phase transition and arrest [22]. For example, genistein-enriched soybean caused G1 arrest in several types of cancer cells [23]. Genistein also led to epigenetic modification such as reversal of hypermethylation of $p16^{INK4a}$ or chromatin modeling [24] and downregulated Mdm2 at both transcriptional and post-translational levels, which removes phosphorylated p53 from the nucleus [25]. The predominant soy isoflavones increased Myt-1 and reduced the phosphorylation of both Myt-1 and Wee-1, protein kinases that transcriptionally repress cyclin B1 and the activation of p21 [26]. On the other hand, other flavonoids present in tempeh, such as quercetin, have exhibited G1 arrest in cancer cells through multiple mechanisms including suppression of cyclin D1 and CDK4 expression [27]. Resveratrol, another polyphenol in tempeh, has been associated with G1 arrest by modulating cyclin D1 [28].

Apoptosis is a programmed cell death and contributes to maintenance of tissue homeostasis. This process is precisely governed by caspases, a group of cysteine protease enzymes. Caspases play a central role in the execution of apoptosis by catalyzing the specific cleavage of DNA and many key cellular proteins. Studies have shown that a synthesized resveratrol analog suppressed the expression of oncogene c-Myc and cell cycle regulator cyclin D1, while also promoting p53 and p21 levels in MCF-7 cells, primarily by altering the expression of estrogen receptors (ER) α and β [28]. Regarding bioactive compounds in soybeans to induce apoptosis, Shafiee et al. claimed that lunasin abundant in soybeans activated apoptosis by regulating the enzyme activity of caspase-3 through a p38MAPK pathway in human prostate cancer cells [29]. Additionally, lunasin stimulated apoptosis in MCF-7 cells through the upregulation of the tumor suppressor phosphatase and tensin homolog (PTEN) deleted on chromosome ten [30], and by deactivating tumor suppressor proteins (such as Rb, p53, and pp32), which results in G1/S phase arrest and subsequent apoptosis. [31]. Defatted soy protein inhibited proliferation of P388D1 cells (mouse monocyte macrophage) with G2/M phase arrest [32]. Daidzein induced cell cycle arrest at the G1 and G2/M phases, and caspase-9 activity was significantly increased in human breast cancer MCF-7 and MDA-MB-453 cells [33]. Dietary isoflavone daidzein induced apoptosis in HBCCs and shown a down-regulated expression of proteins associated with cell survival, specifically PI3K, Akt, and mTOR [3]. Chan et al. discovered that tempeh increases the level of nuclear factor erythroid 2-related factor 2 (Nrf2) via the MAPK pathway, highlighting its potential anti-cancer effects. [34]. Moreover, a reduction in anti-apoptotic proteins Bcl-xL and Bcl-2, along with an increase in pro-apoptotic proteins (Bax, Bak, and Bad), caspases, and regulators related to endoplasmic reticulum stress,

has been reported. [35]. Poly (ADP-ribose) polymerase (PARP) is involved in DNA repair and is a characteristic feature of apoptosis [36]. According to an in vivo study, tempeh at a dosage of 900 mg/kg body weight elevated expression of anti-oxidative enzymes including SOD and CAT in senescent mouse models. Moreover, it also increased Nrf2 levels by downregulating p38 and JNK signaling [34].

Caspase-dependent apoptosis is mainly regulated by two distinctive pathways including external and internal pathway. The external pathway is stimulated by apoptotic ligands/receptors, activating caspsase-8 pathway. While internal pathway is stimulated by genomic instability and cell stress, leading to the release of cytochrome C from mitochondria and the subsequent activation of the caspase 9 pathway. We speculate that the increase of apoptosis and PARP cleavage by treatment of both SE and TE is attributed to the stimulation of those upstream apoptosis pathways, differential regulation of upstream components, or engagement of alternative signaling cascades. Further studies are required to elucidate which upstream pathway is associated with SE and TE-stimulated apoptosis in CRC cells. In addition, we speculate that variations in isoflavone profiles within the apoptotic pathways or the selectivity of bioactive compounds for specific proteins between soybeans and tempeh could contribute to their ability to cleave PARP. To address these questions, further analytical studies will be required to analyze compounds from SE and TE. In summary, these results clearly demonstrated that bioactive compounds present in SE and TE modulated key regulators of apoptosis by increasing cleaved-PARP protein expressions.

The methods used for extracting the bioactive compounds from SE and TE can vary based on the specific goals of the research and the properties of targeted compounds. Extraction methods often involve both defatting and the use of extraction solutions [7]. Extraction procedures typically encompass defatting and the utilization of solvents. Defatting, the removal of lipids from a given sample, plays a crucial role in refining extraction procedures. Considering the well-established therapeutic benefits of flavonoids in treating a range of diseases, including cancer, upcoming research may utilize High Performance Liquid Chromatography (HPLC) to determine the specific bioactive compounds that have been extracted. As detailed in the appendix, freeze-dried soybeans and tempeh samples extracted with 70% ethanol without prior defatting using n-hexane suggested different cellular responses. Both non-defatted soybeans ethanolic extract (SEE) and tempeh ethanolic extracts (TEE) demonstrated an inhibitory effect on HCT116 cells. SEE treatment samples plateaued, and TEE treatment samples exhibited a dose-dependent inhibition of HCT116 cells (Supplementary Figure S1A). Cell cycle assay results indicated the presence of the G1 phase (Supplementary Figure S2B,C). In the absence of defatting using n-hexane, a greater incidence of necrosis was observed with non-defatted tempeh samples (Supplementary Figure S3B,C). The differences in extracting bioactive compounds influenced cellular outcomes and suggested a potential link between lipid content and cellular response in the context of soybeans and tempeh extract-induced effects on HCT116 cells. However, a major limitation of this study is that the chemical constituents of the SE and TE were not identified or quantified. Without detailed metabolite profiling of fermentation, it is difficult to determine which specific compounds contribute to the observed biological effects. Future studies will include additional experiments to identify and quantify promising bioactive compounds using LC-Mass and optimize extraction methods based on efficacy of anti-cancer activities. Regarding selectivity index, additional experiments will need to be conducted using normal human colon cells (such as CCD-18Co and FHC). In addition, anti-cancer activity of SE and TE will need to be compared with well-known anti-tumorigenic natural compounds (such as resveratrol or carnosic acid) and expanded to in vivo studies using chemical (azoxymethane)-induced or genetic model (ApcMin mice).

5. Conclusions

We optimized soybean fermentation and tempeh production method in the lab and demonstrated that soybean and tempeh have anti-cancer effects by inducing G1 phase cell cycle arrest and apoptosis in human CRC cells (Figure 6). These results propose that tempeh, a traditional Indonesian food, could serve as a potential functional food for CRC prevention and treatment. However, chemical identification, metabolite profiling, and in vivo studies will be required to prove their safety and effectiveness.

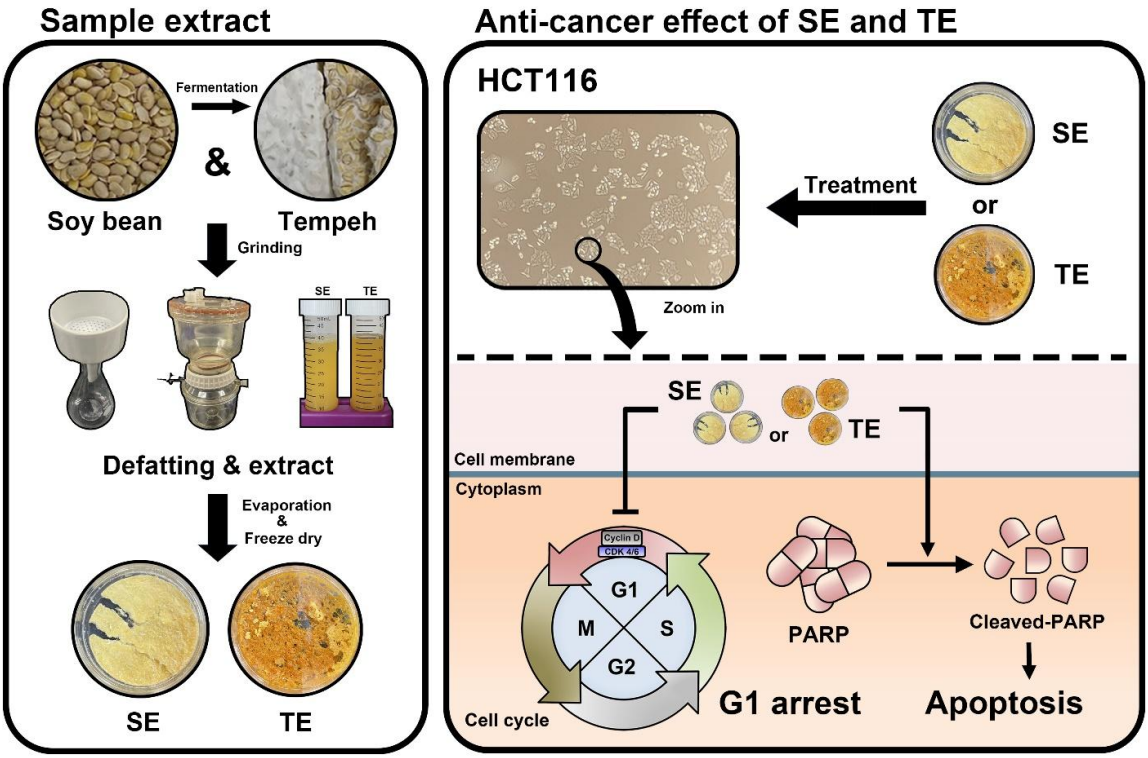

Figure 6. Sample preparation and proposed anti-cancer mechanisms of SE and TE in human CRC cells.

Supplementary Materials

The additional data and information can be downloaded at: https://media.sciltp.com/articles/others/26011915 41077877/FM-25090094-SI.pdf. Figure S1. Non-defatted soybean and tempeh ethanolic extract inhibited viability of HCT116 cells. HCT116 cells were plated onto a 96-well plate and treated with different concentrations of SEE and TEE at concentrations of 0, 100, 200, 300, 400, 500, and 600 μg/mL. Cell viability was assessed by the MTT test. Values are means ± SD (n = 3). Different letters indicate significant differences at $p < 0.05$ (between treatment and control). Figure S2. Cell cycle distribution based on the population percentage in HCT116 cells. (A and B). The HCT116 cells were treated with SEE and TEE at 0, 50 μg/mL, 100 μg/mL and 200 μg/mL. SEE and TEE significantly increased the G1 phase in HCT116 cells. Values are means ± SD (n = 3). Different letters indicate significant differences at $p < 0.05$ (between treatment and control). Figure S3. Induction of Q1 (Necrosis) in non-defatted soybean and tempeh ethanolic extract. (A and B) Induction of early and late apoptosis increased in Q2 (Early apoptosis cell) and Q4 (Late apoptosis cell) in non-defatted soybean ethanolic extracts. HCT116 cells were treated with SEE and TEE at 0, 50 μg/mL, 100 μg/mL and 200 μg/mL for 24 h. Values are means ± SD (n = 3). Different letters indicate significant differences at $p < 0.05$ (between treatment and control).

Author Contributions

R.F.: investigation, visualization, writing—original draft preparation, Y.H.J.: investigation, visualization, writing-original draft preparation, M.A.: conceptualization, writing—reviewing and editing, D.S.: writing—reviewing and editing. S.-H.L.: conceptualization, supervision, funding acquisition, writing—reviewing and editing, C.-I.W.: conceptualization, supervision, funding acquisition, writing—reviewing and editing. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

Use of AI and AI-Assisted Technologies

No AI tools were utilized for this paper.

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