PJN
ISSN 1680-5194

PAKISTAN JOURNAL OF NUTRITION

ANSInet
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
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OPEN ACCESS Pakistan Journal of Nutrition

ISSN 1680-5194
DOI: 10.3923/pjn.2017.743.749

Research Article
The Potential of Tempeh as a Chemopreventive and Chemotherapeutic Agent Targeting Breast Cancer Cells

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Abstract

Background and Objective: The prevalence and incidence of breast cancer are much lower in Asian women than in Western women. However, data from various hospitals in Indonesia indicate that the incidence of breast cancer in the country has been rising by 2-8% over the last 10 years. Epidemiological studies have found that soy intake correlates to the low risk of breast cancer among Asian women. This study was conducted to prove the efficacy of tempeh consumption as a chemotherapy agent against breast cancer in DMBA-induced model animals. **Materials and Methods:** This study was a pre-clinical trial using a true experimental posttest-only control group design. The subjects were 25 white sprague-dawley rats that were induced with 7,12-Dimethylbenz[a]anthracene (DMBA). The observed parameters were AgNORs, BCl-2, Cas-3, p53 and VEGF. The test subjects were divided into a control group and 4 treatment groups that were given feed with modified tempeh starch contents of 1% (T1), 10% (T2), 50% (T3) and 75% (T4). **Results:** A tempeh diet had a significant effect on anti-proliferation and apoptosis markers in breast cancer cells ($p<0.05$). **Conclusion:** Of the four doses used in the trial, 50% tempeh flour was shown to be the most effective.

Key words: Anti-apoptotic, anti-proliferative, breast cancer, chemopreventive, chemotherapeutic, soybean, tempeh

Received: May 28, 2017 Accepted: September 11, 2017 Published: September 15, 2017

Citation: Siti Harnina Bintari and Kartika Nugraheni, 2017. The potential of tempeh as a chemopreventive and chemotherapeutic agent targeting breast cancer cells. Pak. J. Nutr., 16: 743-749.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Breast cancer is one of the most common cancers among women. Indonesia has the highest incidence of breast cancer among Asian countries[1] with 19,750 mortalities in 2012[2]. Numerous studies have failed to discover the principal cause of breast cancer. However, diet is suspected to be an important factor in the initiation, promotion, progression and prevention of breast cancer[3]. Prior research has generally focused on the impact of the diet of adult women on the incidence of breast cancer. However, some evidence suggests that childhood or adolescent diets could have greater impact than adult diets given that the development of breast tissues in childhood and adolescence is more sensitive towards dietary effects[4]. This evidence agrees with Lamartiniere *et al.*[5], who found that a woman's early life plays a very important role in predisposition for and protection against breast cancer.

The incidence of breast cancer among Asian women is lower than among women in the United States. Breast cancer can be triggered by diet-related factors such as the consumption of soft drinks, simple sugars, hydrogenated fat, refined grains and red and processed meat. On the other hand, diet can be a protective factor against breast cancer. Asian diets tend to be low in meat and fats but high in beans and pulses such as soybeans[6]. Soybeans can be fermented to produce a variety of traditional Asian products such as miso, soyu, tempeh and natto or processed into non-fermented products such as tofu and soy milk[7]. Soy-based foods can be cooked by frying, steaming, boiling or grilling. Tempeh is Indonesia's signature fermented soy product[8]. The consumption of soy and its derivatives from an early age can reduce the risk of breast cancer. Soybeans contain isoflavone, a diphenolic compound that can bind to oestrogen receptors and possesses an oestrogen-like chemical structure and activity. The mechanism by which soy consumption protects against breast cancer is suspected to be related to stimulation of breast gland differentiation, similar to the mechanism for raising oestrogen levels during pregnancy[4].

Epidemiological studies have indicated that high soy intake correlates to the low prevalence of breast cancer among Asian women[9] and isoflavone exposure in early life has been tentatively identified as a protective factor against the future likelihood of breast cancer[10,11]. Genistein is the principal soy isoflavone and exhibits protective effects against the initiation, promotion and progression of breast cancers in animal studies[5]. Building on this knowledge, this study seeks to prove the efficacy of tempeh consumption as a chemotherapy agent against breast cancer in DMBA-induced model animals.

MATERIALS AND METHODS

This study was a pre-clinical trial designed as a true experimental posttest-only controlled-group study. The research subjects were 25 female sprague-dawley white rats (*Rattus norvegicus*), separated into 5 groups. Breast cancer was induced with the carcinogen 7,12-dimethylbenz(a)anthracene (DMBA), provided orally beginning at the age of 47 days and then periodically 3 times per week for 16 weeks at a dose of 20 mg kg^{-1} b.wt., with an intragastric tube. After the DMBA induction period, the rats were randomly divided into 5 groups, one of which was the control group while the remaining 4 were treatment groups. The treatment groups were given feed with modified tempeh starch contents of 1% (T1), 10% (T2), 50% (T3) and 75% (T4).

During DMBA induction, the rats were given standard feed according to the AIN-93M composition in Reeves[12] and Reeves *et al.*[13]. After DMBA induction, the rats were fed with AIN-93M feed that was modified with tempeh flour. The compositions of unmodified AIN-93M feed and AIN-93M modified with tempeh starch are presented in Table 1. The

Table 1: Compositions of AIN-93M feed and AIN-93M modified with tempeh starch

Composition (g kg^{-1} diet)	AIN-93M	1% tempeh starch	10% tempeh starch	50% tempeh starch	75% tempeh starch
Cornstarch	465.692	455.692	365.692	165.692	0.00
Casein	140.000	140.000	140.000	140.000	110.692
Dextrinized cornstarch	155.000	155.000	155.000	155.000	0.00
Sucrose	100.000	100.000	100.000	100.000	0.00
Soybean oil*	40.000	40.000	40.000	40.000	40.000
Fibre	50.000	50.000	50.000	50.000	50.000
Mineral mix	35.000	35.000	35.000	35.000	35.000
Vitamin mix	10.000	10.000	10.000	10.000	10.000
L-cystine	1.800	1.800	1.800	1.800	1.800
Choline bitartrate	2.500	2.500	2.500	2.500	2.500
Tert-butylhydroquinone	0.008	0.008	0.008	0.008	0.008
Tepung tempeh	0.00	10.00	100.00	500.00	750.00

*For AIN-93M modified with tempeh starch, soybean oil was replaced with corn oil

modified AIN-93 feed was provided over 12 weeks. After the end of the intervention period, the rats were sacrificed using excessive chloroform inhalation and dissected to permit observation of breast tissues.

The cancer markers tested included AgNORS, VEGF (anti-proliferative), Cas-3 and Bcl-2 (apoptogenic), as well as p53, a tumour suppressor. The tests involved haematoxylin and eosin staining (HE) and immuno-histochemical staining of tissue. Inspection of staining results was performed by two independent experts. This study was undertaken at the following sites: The Food and Nutrition Laboratory, Inter-University Center, Gajah Mada University, Clinical Pathology Laboratory, Dr. Sardjito General Hospital and the Clinical Pathology Laboratory, Gajah Mada University.

Tempeh flour was made from fresh tempeh processed according to the method described by Bintari[14] and dried in the sun for 2×24 h. The dried tempeh was then ground into a flour. The soybeans used for tempeh production were a local Grobogan variety. The production of the tempeh and tempeh flour was conducted at the Microbiology Laboratory, Faculty of Mathematics and Natural Sciences, Semarang State University.

RESULTS

The DMBA induction through the intragastric route caused the onset of breast cancer, which could be observed through palpation of the mammary glands of test animals. None of the test subjects died during DMBA induction. Test animal body weights were monitored throughout the study. On average, they experienced weight loss during DMBA induction ($p<0.05$). Feeding with tempeh starch caused significant weight gain in the treatment groups relative to the control group ($p<0.05$) but there was no observable difference between different treatment groups ($p<0.05$).

Figure 1 shows the results of HE staining of mammary cells of the test animals. DMBA induction caused the appearance of cancer cells in the mammary glands. Cancer cells can be identified by their darker and more intense colour in the HE stained samples.

Figure 2 shows the results of immuno-histochemical staining for the markers AgNORs, Bcl-2, Cas-3, p53 and VEGF. AgNORs can be seen as dark spots localized along nucleolar areas. The control group shows more AgNORs staining than the treatment groups. AgNORs staining is more commonly seen in malignant cancers than in benign samples. Tempeh starch treatment appears to be successful in inhibiting the duplication of breast cancer cells.

Table 2: Average numbers of AgNORs, p53, Cas-3, Bcl-2 and VEGF spots on test animals' cancer cells

Group (n)[2]	Spots[1]	p*
AgNORs (5)		
K	84.7 ± 0.982^{A}	<0.05
T1	61.2 ± 1.979^{Ba}	
T2	64.8 ± 1.617^{Ca}	
T3	49.3 ± 0.515^{Db}	
T4	37.1 ± 2.487^{Ec}	
p53 (5)		
K	53.80 ± 0.889^{A}	<0.05
T1	45.8 ± 1.522^{Ba}	
T2	46.8 ± 0.3^{Ca}	
T3	42.1 ± 2.727^{Db}	
T4	24.9 ± 0.927^{Ec}	
Cas-3 (5)		
K	21.9 ± 0.967^{Aa}	<0.05
T1	24.9 ± 0.579^{Ba}	
T2	44.0 ± 1.061^{Cb}	
T3	57.4 ± 5.161^{Dc}	
T4	34.7 ± 0.846^{Ed}	
Bcl-2 (5)		
K	66.7 ± 1.3^{A}	<0.05
T1	47.3 ± 1.513^{Aa}	
T2	53.8 ± 4.813^{Bb}	
T3	41.4 ± 0.9^{Cc}	
T4	29.0 ± 0.88^{Dd}	
VEGF (5)		
K	87.0 ± 1.458^{A}	<0.05
T1	78.2 ± 0.943^{Ba}	
T2	57.0 ± 1.636^{Cb}	
T3	42.2 ± 1.722^{Dc}	
T4	28.10 ± 2.009^{Ed}	

[1]Average±SE [2]Number of test animals, *Significance according to ANOVA testing. Different capital superscripts within a column indicate significant differences ($p<0.05$) among the 5 groups by LSD's multiple comparison test. Different lower-case superscripts within a column indicate significant differences ($p<0.05$) among the 4 treatment groups by LSD's multiple comparison test

The expression of p53 in the treatment groups T1 to T4 differs significantly from expression in the control group. Treatment with 1 and 10% tempeh flour did not result in significant changes. This shows that cell damage persists at low doses of Tempeh flour.

Table 2 shows the percentage of immuno-histological spots for each cancer marker, from anti-proliferative to apoptogenic. Based on these data, feeding with low doses of tempeh flour (1 and 10%) did not provide satisfactory results. Larger doses of tempeh flour (50 and 75%) resulted in satisfactory prevention of proliferation and promotion of cancer cell death.

DISCUSSION

Results from this study demonstrate that tempeh consumption could prevent the incidence of breast cancer in rats induced by DMBA. Various tempeh concentrations in

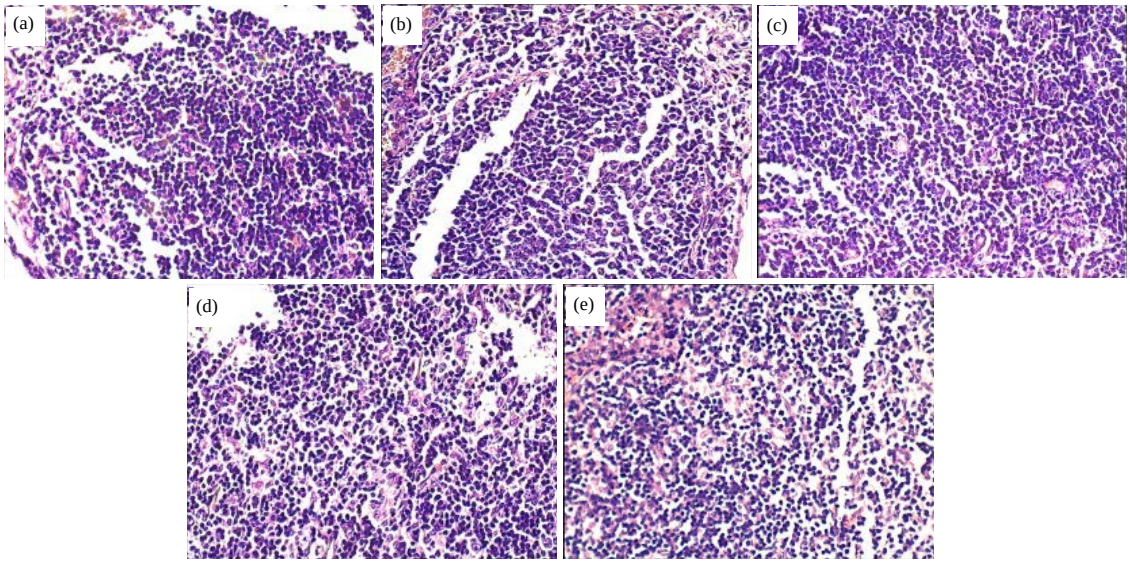

Fig. 1(a-e): HE staining of experimental groups (From left to right (a) Control group, (b) 1% tempeh flour, (c) 10% tempeh flour, (d) 50% tempeh flour and (e) 75% tempeh flour. Viewed under a microscope with 400× magnification)

the diet affect the incidence of breast cancer. This also strengthens the hypothesis that soybean consumption can prevent breast cancer. In this study, it is showed that large doses of tempeh (50 and 75%) are needed to generate such result, which are impractical to consume in daily life.

The prevalence and incidence of breast cancer among Asian women is much lower than among women in Western countries[15,16]. Even so, data from various Indonesian hospitals indicate that the incidence of cancer has been rising by 2-8% per year over the last 10 years. After cervical cancer, breast cancer is the second most common cancer among Indonesian women, with a prevalence of 17-19%[17,18].

Macro-and micronutrient intake plays an important part in breast cancer pathogenesis[18]. Diet is a factor that accounts for as much as 50% of the incidence of breast cancer. Soy is commonly found in Asian diets but rarely in Western diets. Some epidemiological studies have suggested that soy intake is correlated with lower risks of incidence of and/or morbidity from breast cancer[16].

Soy is a plant protein source consumed almost every day by many Indonesians. Tofu and tempeh are the most important soy products for Indonesians of all ages. Even though they both originate from soybeans, tempeh has far greater health benefits. The fermentation involved in tempeh production increases nutritional bioavailability, protein quality, antioxidant activity and isoflavone (aglycone) content in addition to reducing anti-nutrient concentration. All of these benefits can be applied by dietary treatment for a variety of diseases, whether metabolic, degenerative, or infectious[8].

Soy isoflavone supplementation has shown benefits for prostate cancer patients because genistein-a soy isoflavone-induces apoptosis and inhibits the growth of prostate cancer cells that are sensitive to androgen *in vitro*[19]. Consumption of genistein extracted from tempeh at doses of 1000 and 10,000 mg/day is capable of inhibiting proliferation and stimulating apoptosis of cancer cells in C_3H mice conditioned to develop breast cancer[14]. The Shanghai Breast Cancer Survival Study stated that among breast cancer survivors, the consumption of soy-based foods is closely correlated to reduced morbidity and mortality due to breast cancer[20].

Damage to the DNA of breast tissue cells due to DMBA induction causes the expression of the protein p53. If this DNA damage cannot be repaired, the cell remains in the S (synthetic) phase where it continues to synthesize new DNA for cell division. Mistakes in the S phase can lead to cell death or suicide. In the control group, excessive expression of p53 indicates damage to a large number of cells due to DMBA induction. Treatment groups provided with 1 and 10% tempeh starch did not exhibit appreciable improvement. The effects of dietary tempeh starch on p53 begins to become apparent at 50% tempeh in 42% of samples. 75% tempeh starch consumption induces less p53 expression (24% of samples). Expression of p53 is an initial step in signalling the cell to perform repairs or apoptosis.

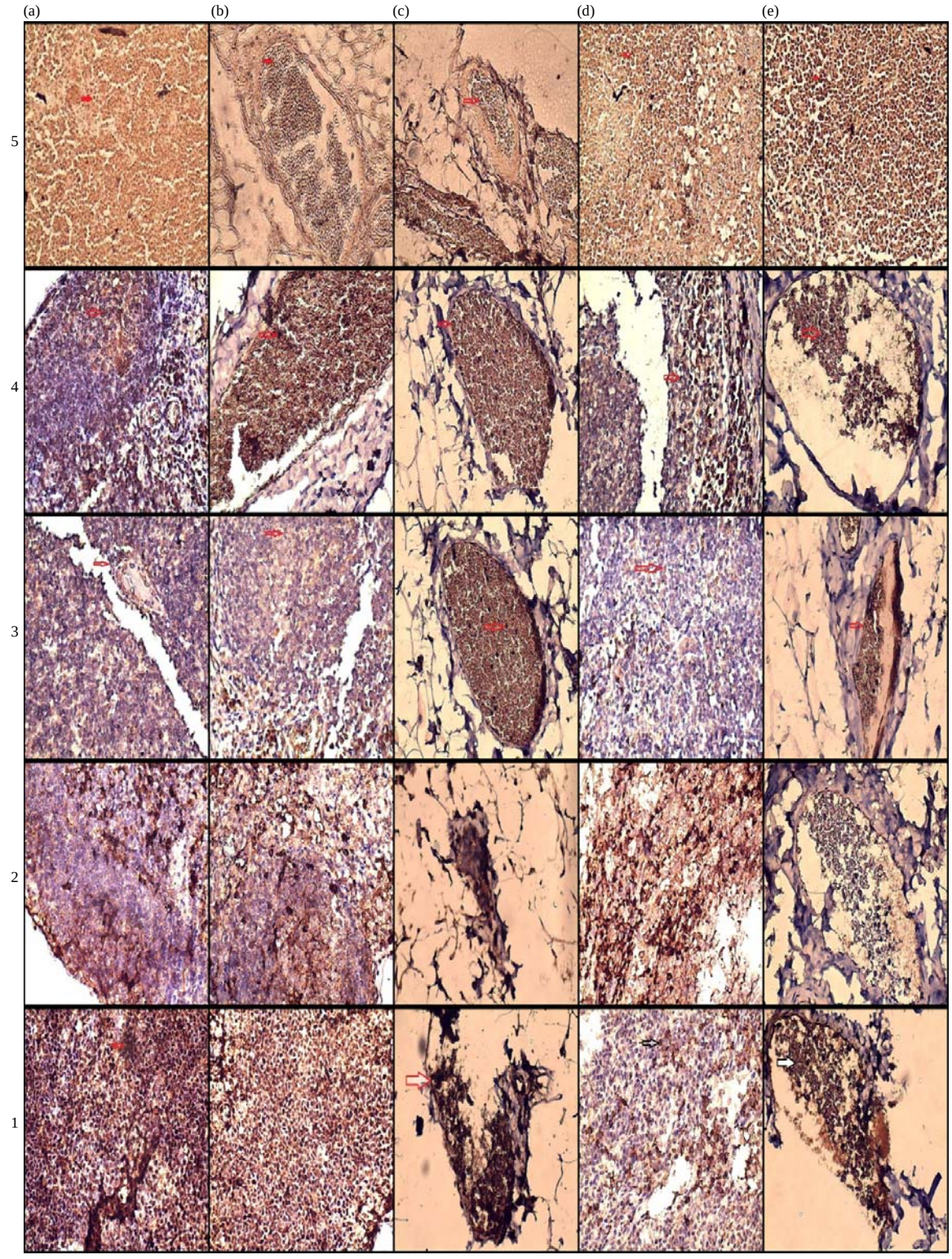

Fig. 2(a-e): Immuno-histochemical staining on breast cancer cells from test animals. From top to bottom: (1) AgNORs, (2) Bcl-2, (3) Cas-3, (4) p53 and (5) VEGF. From left to right (a) Control group, (b) T1, (c) T2, (d) T3 and (e) T4. Target cells are indicated with arrows. Seen under a microscope at 400× magnification

This signal for cell repair initiates proliferation activity. This can be seen by changes in VEGF and AgNORs markers, which exhibit parsimony between AgNORs spot numbers and cell repair activity. The control group shows AgNORs spots in 84.7% of cells, which indicates that many cells failed to make repairs. This stands in contrast to treatment with tempeh starch at doses of 1-75%, which produced progressively declining percentages of AgNORs spots, although there is no appreciable difference between doses of 1 and 10% tempeh. The VEGF protein promotes cell proliferation through the formation of new blood vessels[21]. Genistein has a suppressive effect upon breast cancer cells through the inhibition of angiogenesis, holding the cell cycle in the G2M phase and inducing apoptosis[22].

Angiogenesis plays an important role in cancer since the proliferation and metastasis rates of cancer cells both depend upon adequate supplies of oxygen and nutrients[23,24]. Kinoshita *et al.*[25] and Skobe *et al.*[26] found a positive correlation between VEGF expression and breast cancer prognosis. This study shows that tempeh can function as an angiogenesis inhibitor in breast cancer. The doses of tempeh flour used in this study have proven to be effective in inhibiting angiogenesis.

Excessive expression of the Bcl-2 protein, signified by brown cytoplasm and blue nuclei (Fig. 2), specifically inhibits cell apoptosis in response to certain stimuli. The presence of the Bcl-2 gene is a marker for the start of cell apoptosis. The activation of genes capable of inhibiting the Bcl-2 gene can induce apoptosis in some tumours. Crystal structures of Bcl-2 protein family members help to clarify the function of the Bcl-2 protein family, showing that domains of Bcl-2 homologues (BH1, BH2 and BH3) can form pockets capable of binding other Bcl-2 family members to form heterodimers[27-30].

Apoptosis is a process of regulated cell death and is a normal part of an organism's life. It can be triggered by several factors, including DNA damage beyond the capability of the repair system. Immuno-histochemical staining revealed that 80% of primary breast cancer preparations exhibited Bcl-2 expression. Breast cancer patients with pro-apoptotic Bcl-2 (i.e., cells that simultaneously express both Bcl-2 and Cas-3) have a better prognosis than patients with negative pro-apoptotic/anti-apoptotic Bcl-2. This study shows that high expression of Bcl-2 in the control group is not accompanied by high expression of Cas-3. This reveals that a considerable part of Bcl-2 in the control group is anti-apoptotic[27-29].

Tempeh flour, with its high genistein content (30% of tempeh's total isoflavone content), affects oxidative phosphorylation by inactivating the Bcl-2 anti-apoptosis gene. This leads to a co-expression of Bcl-2 and Cas-3, mediated by APAF, which expresses Bcl-2 in conjunction with Cas-3. This in turn reduces the number of cancer cells in breast tissue in the group treated with 50% tempeh starch[31].

It is concluded that tempeh consumption can help inhibit angiogenesis and the proliferation of cancer cells in breast cancer cases, thus leading to the death of cancerous cells. Tempeh contains active isoflavone compounds, mostly in the free forms of genistein and daidzein. Of the four doses used in the trial, 50% tempeh flour was shown to be the most effective. Consuming tempeh in such large amounts may be physiologically difficult, so it may be necessary to provide isoflavone intake through other foodstuffs or supplements in order to obtain the same results in humans.

In the meantime, there is still need for further research to analyze the effects of soy consumption (whether in fermented or non-fermented form) on breast cancer to scrutinise and understand the role of soy isoflavones upon the progression of cancer.

CONCLUSION

Tempeh has been shown to be capable of inhibiting proliferation and angiogenesis as well as triggering apoptosis in cancer cells in a dose-dependent manner.

SIGNIFICANCE STATEMENT

This study reveals the benefits of tempeh, a soybean fermented food from Indonesia, in a daily diet to prevent breast cancer. Previous epidemiological research suggested that early soybean consumption could reduce the prevalence of breast cancer. This study increases the understanding of how soybeans, particularly tempeh, lower the probability of breast cancer development.

ACKNOWLEDGMENTS

The authors would like to thank the Ministry of Research, Technology and Higher Education for its Penelitian Unggulan Perguruan Tinggi (PUPT) grant in 2016, which provided the funding for the study.

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