RESEARCH ARTICLE

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Tempeh Extract and HSC-3 Cell Migration: An *In Vitro* Study

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Abstract

Objective: To compare the migration capacity of HSC-3 cells following treatment with various concentrations of tempeh extract. **Methods:** This laboratory-based study using HSC-3 cell lines, which were treated with tempeh extract (SNI-3144:2015) dissolved in either 70% ethanol or water at concentrations of 25, 50, 100, 200, and 400 µg/mL. Cell migration was assessed using a scratch assay at 6 and 24 hours, conducted in triplicate. Data were analyzed using ANOVA and Kruskal–Wallis tests. **Result:** At the 6-hour observation point, no significant differences in migration were observed between groups for either solvent ($p>0.05$). However, after 24 hours, the 50 µg/mL group (6.88±11.92) demonstrated the largest scratch area compared with other concentrations ($p<0.033$). **Conclusion:** Tempeh extract showed no inhibitory effect on HSC-3 cell migration when compared to doxorubicin, particularly when water was used as the solvent. Further research is needed with higher concentrations of tempeh extract to evaluate its potential inhibitory effects on HSC-3 cell migration.

Keywords: Oral squamous cell carcinoma- Tempeh extract- HSC-3- Migration

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Introduction

Oral cancer is a significant global health concern, with a particularly high burden in Southeast Asia due to risk factors such as betel quid chewing [1], tobacco use [2], and alcohol consumption [3, 4]. According to GLOBOCAN 2022, there were 389,846 new cases of oral cancer worldwide, with Indonesia accounting for 6,515 of those cases [5]. Oral cancer lesions are often asymptomatic in the early stages, contributing to delayed diagnosis and poor prognosis [6, 7].

Oral squamous cell carcinoma (OSCC) is the most prevalent malignancy of the oral cavity [8]. It arises from the squamous epithelial cells of the mucosa and is characterized by its potential to infiltrate and metastasize to surrounding tissues. OSCC can develop in various anatomical regions, including the lateral borders of the tongue, the floor of the mouth, the hard and soft palate, and the gingiva [9]. Notably, approximately 70% of oral cancer cases are diagnosed at an advanced stage, underscoring the urgent need for preventive strategies and early detection to improve prognosis and survival rates [10].

Current standard treatments for oral cancer chemotherapy, radiotherapy, and surgery often result in significant adverse effects, compromising patients' quality of life [11]. Therefore, natural substances are increasingly being investigated as alternative or complementary therapeutic agents.

Tempeh, a traditional Indonesian food made from fermented soybeans, is rich in isoflavones, particularly genistein and daidzein. Genistein, in particular, has demonstrated a range of bioactive properties, including anticancer, antitumor, and antioxidant effects. Previous studies have shown that genistein can inhibit metastasis and angiogenesis in various cancer types [12]. Isoflavones found in tempeh have been reported to suppress the progression of several cancers, including colon, cervical, pancreatic, liver, and lung cancers [13]. Given these properties, this study was to compare tempeh extract particularly for its ability to inhibit cancer cell migration in oral cancer HSC3 cell line.

Materials and Methods

Sample Preparation

This study is an in vitro laboratory-based experimental research. The migration ability of HSC-3 oral squamous carcinoma cells was evaluated using a scratch assay. The experimental design consisted of eight treatment groups: a solvent control group (DMSO), a negative control group (no treatment), a positive control group (3 µM doxorubicin), and five groups treated with tempeh extract

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at concentrations of 25, 50, 100, 200, and 400 µg/mL. Each treatment was conducted in triplicate. The HSC-3 cell line was obtained from the laboratory's biobank. HSC-3 cells were obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank, originally established from a metastatic lymph node of a tongue squamous cell carcinoma. The cell line is catalogued under JCRB0623 and registered with RRID:CVCL_1288. Mycoplasma testing for in vitro model was not performed because the cell lines used in the experiment were obtained from a reputable cell bank that routinely performs mycoplasma testing and certifies their cultures as mycoplasma-free. Therefore, additional testing was not deemed necessary at the initial stages. Ethical approval for this study was obtained from the Ethical Committee, Faculty of Dentistry, Universitas Trisakti (758/S1/KEPK/FKG/6/2024).

Tempeh Extraction

Tempeh, fermented soya bean derived from Glycine max (L.) Merr., was obtained from the Indonesian Tempe House and prepared according to the Indonesian National Standard (SNI 3144:2015). The tempeh was dried at 50°C for 3 hours, ground into flour, and sieved using a 60-mesh filter. Extraction was carried out via maceration using 100 g of tempeh flour with either 70% ethanol or distilled water for 3 hours. The resulting extract was filtered using Whatman No. 42 filter paper and concentrated using a rotary evaporator to obtain a thick extract. Quantitative phytochemical analysis was performed to determine the total phenolic and flavonoid content. The concentrated extract was subsequently diluted to the required concentrations using 70% ethanol or distilled water, depending on the treatment group.

Cell Culture

The culture medium was prepared using Dulbecco's Modified Eagle Medium (DMEM) supplemented with fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution (Penicillin–Streptomycin and Amphotericin B). HSC-3 cells were sub-cultured and harvested approximately one week after initial seeding. Cells were seeded into 24-well plates at a density of 0.05×10^6 cells per well and incubated until reaching confluency. A linear scratch was made across the cell monolayer using a 10 µL micropipette tip to simulate a wound. The medium was then removed, and the wells were rinsed with 250 µL of phosphate-buffered saline (PBS) to eliminate cellular debris. Subsequently, the cells were treated with the respective tempeh extracts. Cell migration was observed at 0, 6, and 24 hours using a fluorescence microscope, and the scratch area was quantified using T-Scratch software (v1.0, 2008), a freely available image analysis tool developed by the CSE Lab, ETH Zurich (Switzerland), designed for automated quantification of scratch wound healing assays.

Data Analysis

Data analysis (unit in percentage) was performed using one-way ANOVA and Kruskal–Wallis tests, depending on data distribution. Post hoc analysis was conducted using the Mann–Whitney U test. Statistical analyses were carried out using SPSS version 22. A p-value of <0.05 was considered statistically significant.

Results

Quantitative phytochemical analysis revealed that the total phenolic and flavonoid contents of the tempeh extract prepared with 70% ethanol were 68.6 µg/mL and 6.0 µg/mL, respectively. In contrast, the extract prepared with water contained higher levels, with total phenolic and flavonoid contents of 87.2 µg/mL and 11.3 µg/mL, respectively (Table 1).

The migration ability of HSC-3 cells was evaluated using a scratch assay, and the resulting data were expressed as the percentage of the remaining open scratch area. The study involved eight treatment groups: a negative control, a positive control (3 µM doxorubicin), a solvent control (DMSO), and five groups treated with tempeh extract at concentrations of 25, 50, 100, 200, and 400 µg/mL, diluted in either 70% ethanol or water. Scratch images were captured at 0, 6, and 24 hours using a fluorescence microscope at 4× magnification, and the open area was analyzed using T-scratch software (Figure 1).

After 6 and 24 hours, a reduction in the open scratch area was observed in all groups treated with tempeh extract, regardless of the solvent used (Tables 2 and

Table 1. Total Phenolic and Flavonoid Content of Tempeh Extracts Using Different Solvents

No	Extract Type	Absorbance (Phenolic)	Phenolic Content (µg/mL)	Phenolic Content (µg/g extract)	Absorbance (Flavonoid)	Flavonoid Content (µg/mL)	Flavonoid Content (µg/g extract)
1	Tempeh extract with 70% ethanol solvent	0.638	68.6	68.6	0.039	6	6
2	Tempeh extract with water solvent	0.792	87.2	87.2	0.047	11.3	11.3

Table 2. Comparison of each Groups after 6 hours Treatment

Solvent	Average Open Area, % (SD)								p-value
	Positive Control	Solvent Control	Negative Control	400 µg/mL	200 µg/mL	100 µg/mL	50 µg/mL	25 µg/mL	
Water	32.23 (1.82)	29.19 (5.75)	32.86 (5.94)	28.79 (2.21)	29.04 (6.45)	32.51 (3.29)	28.24 (5.15)	33.01 (1.94)	0.708
70% Ethanol	28.15 (4.28	24.86 (2.58)	28.14 (4)	31.24 (4.99)	33.79 (5.01)	28.26 (3.33)	34.11 (4.85)	33.74 (4.41)	0.213

* $p<0.05$

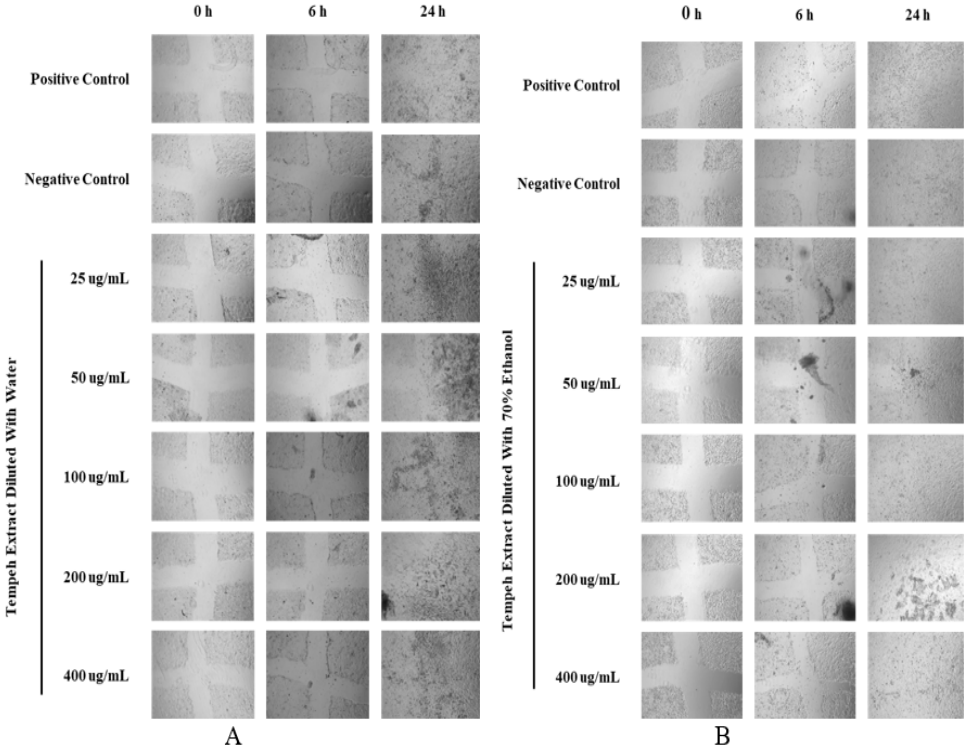

Figure 1. Scratch assay of Tempeh Extract with Water (A) and 70% ethanol (B) solvent.

Table 3. Comparison of each Groups after 24 hours Treatment

Solvent	Average Open Area, % (SD)								p-value
	Positive Control	Solvent Control	Negative Control	400 µg/mL	200 µg/mL	100 µg/mL	50 µg/mL	25 µg/mL	
Water	31.1 (1.08)	4.87 (8.44)	0	0	0.97 (1.68)	0	6.88 (11.92)	0	0.033*
70% Ethanol	0	0	0	0	0	0	2.55 (4.42)	0.31 (0.44)	0.147

3). At the 24-hour mark, the group treated with water-diluted tempeh extract at 50 µg/mL exhibited the largest remaining open area, with a mean of 6.88%, followed by the 200 µg/mL group with 0.97% (Table 3). In contrast, the 25, 100, and 400 µg/mL groups showed complete closure of the scratch area (Figure 1A). Post hoc analysis using the Mann–Whitney U test indicated a statistically significant difference between the positive control and all other groups ($p<0.05$) (Table 4).

For the tempeh extract prepared with 70% ethanol, the 50 µg/mL group showed the largest remaining open area at 24 hours (mean 2.55%), followed by the 25 µg/mL group (mean 0.31%). The 100, 200, and 400 µg/mL groups exhibited complete closure of the scratch area (Figure 1B).

Discussion

Oral cancer remains a significant global health

Table 4. Post-hoc Analysis for 24 hours Treatment Dissolve in Water Solvent (p-value)

	Solvent Control	Negative Control	400 µg/mL	200 µg/mL	100 µg/mL	50 µg/mL	25 µg/mL
Positive Control	0.046*	0.037*	0.037*	0.046*	0.037*	0.046*	0.037*
Solvent Control		0.317	0.317	0.796	0.317	0.796	0.317
Negative Control			1	0.317	1	0.317	1
400 µg/mL				0.317	1	0.317	1
200 µg/mL					0.317	0.796	0.317
100 µg/mL						0.317	1
50 µg/mL							0.317

* $p<0.05$

issue due to the increasing incidence of new cases each year. The disease's aggressiveness lies in the ability of cancer cells to migrate and invade surrounding tissues, contributing to poor prognosis and high mortality. Despite significant advancements in cancer therapies—including chemotherapy, radiotherapy, and surgical procedures—these treatments are often associated with undesirable side effects. Doxorubicin, a widely used chemotherapeutic agent, was utilized as the positive control in this study. It has demonstrated effectiveness in treating various cancers, such as breast and hematologic malignancies [14]. However, its clinical use is limited by adverse effects, including allergic reactions, alopecia, nausea, and cardiomyopathy [15]. This highlights the need for alternative therapies, particularly those derived from natural sources, which are anticipated to offer therapeutic benefits with fewer side effects.

Tempeh, a fermented soybean product widely consumed in Indonesia, contains isoflavones natural compounds classified as flavonoids with well-documented antioxidant properties (Table 1). Isoflavones, particularly genistein, have been shown to inhibit cancer progression by modulating gene expression related to apoptosis and cell cycle regulation [16]. Although various types of tempeh exist (e.g., black soybean, mung bean), soybean-based tempeh is the most widely consumed. One of the strengths of this study is the quantitative phytochemical analysis conducted on the extracts, revealing significant levels of flavonoids: 6.0 µg/mL in 70% ethanol extract and 11.3 µg/mL in the water extract. Prior studies have also reported the anticancer effects of soy-derived isoflavones, particularly in reducing the risk, recurrence, and metastasis of breast cancer [17, 18].

In this study, tempeh extract was diluted in two different solvents: 70% ethanol and distilled water. Ethanol is a commonly used solvent in phytochemical extraction and has shown efficacy in previous studies [19]. Water was also used to better simulate physiological conditions, as tempeh is consumed orally and water is the primary solvent in the human body.

Figure 1 presents the results of the scratch assay on HSC-3 cells. No significant differences were observed after 6 hours in either solvent group ($p > 0.05$; Table 2). However, after 24 hours, the positive control (doxorubicin) exhibited the highest remaining open area compared to all other treatment groups ($p < 0.05$; Tables 3 and 4). Post hoc analysis using the Mann–Whitney test revealed significant differences between the positive control and all five water-diluted tempeh extract groups, suggesting that tempeh extract does not inhibit HSC-3 cell migration as effectively as doxorubicin. Interestingly, both solvents yielded similar trends, with the 50 µg/mL concentration group demonstrating the largest open area after 24 hours. Despite increasing concentrations, the inhibitory effect on cell migration was not optimal, indicating the need for higher or more refined concentrations to achieve effective inhibition.

These findings contrast with several previous studies reporting the anticancer effects of isoflavones in breast cancer. This discrepancy may be attributed to biological differences between oral squamous cell carcinoma (OSCC) and breast cancer. For instance, ethanol-extracted tempeh has been reported to exhibit an IC_{50} of 5.20 ± 1.01 µg/mL against MCF-7 breast cancer cells [20]. This may be explained by the estrogen-dependent nature of breast cancer, which makes it more responsive to phytoestrogens such as genistein [21]. Phytoestrogens mimic the structure of endogenous estrogen and can bind to estrogen receptors (ERα and ERβ) [22]. While ERα promotes cell proliferation via growth factor and cytokine induction, ERβ acts as a negative regulator, suppressing ERα-mediated activity [23]. Genistein has been shown to preferentially bind to ERβ, thereby exerting a protective, anti-proliferative effect in breast tissue [24]. In contrast, OSCC is not an estrogen-dependent cancer. Its pathogenesis is more strongly linked to environmental and behavioral risk factors, including tobacco use, alcohol consumption, betel quid chewing, and human papillomavirus (HPV) infection [25]. Therefore, the mechanism of action of isoflavones may be less pronounced in OSCC compared to estrogen-sensitive malignancies.

This study has certain limitations. Unlike breast cancer cells, HSC-3 cells are not influenced by estrogen signaling pathways, which may explain the reduced responsiveness to isoflavone-based interventions. Although tempeh extract shows some potential in inhibiting HSC-3 cell migration, the concentrations used in this study were insufficient to produce a robust effect. Future studies should explore higher concentrations or fractionated active compounds to better elucidate their anticancer potential against OSCC.

In conclusion, Tempeh extract (SNI-3144:2015) demonstrated no significant inhibitory effect on HSC-3 cell migration when compared to doxorubicin, particularly when using water as the solvent. Although a significant difference was observed between the positive control and the water-diluted tempeh extract groups, no significant differences were found among the various concentrations of tempeh extract prepared with either water or 70% ethanol. Interestingly, both solvents at a concentration of 50 µg/mL yielded the largest remaining scratch areas after 24 hours, suggesting a potential inhibitory trend. While tempeh extract did not achieve comparable efficacy to doxorubicin, these findings indicate its potential to inhibit HSC-3 cell migration. Further research using higher concentrations and purified compounds is recommended to fully assess its anticancer properties

Author Contribution Statement

RA, RAH, FK, ST contributed to conceptualization, data curation, formal analysis, investigation, methodology, project administration, and resources. RA contributed to supervision and validation. ST contributed to writing the original manuscript. IG contributed to formal analysis, methodology, software, visualization, validation, and review/editing of the manuscript.

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Ethical Declaration

The study received approval from the Research Ethics Commission of the Faculty of Dentistry, Trisakti University under the reference number 758/S1/KEPK/FKG/6/2024.

Conflict of Interest

None.

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