Food Research 5 (3) : 121 - 128 (June 2021)
Journal homepage: http://www.myfoodresearch.com

FOOD RESEARCH

FULL PAPER

Tempeh extract reduces cellular ROS levels and upregulates the expression of antioxidant enzymes

[1,*]Surya, R., [1]Romulo, A. and [2]Suryani, Y.

[1]*Food Technology Department, Faculty of Engineering, Bina Nusantara University, Jakarta 11480, Indonesia*

[2]*Centre for Immunobiology, Institute of Infection, Immunity and Inflammation, University of Glasgow, Glasgow, United Kingdom*

Article history:
Received: 3 October 2020
Received in revised form: 19 November 2020
Accepted: 21 January 2021
Available Online: 23 May 2021

Keywords:
Soybean,
Tempeh,
Isoflavones,
Reactive oxygen species,
Antioxidant

DOI:
https://doi.org/10.26656/fr.2017.5(3).560

Abstract

Tempeh is an Indonesian traditional food produced from soybeans through a mould fermentation involving *Rhizopus oligosporus*. It is rich in bioactive phytochemicals, including isoflavones that are known to exhibit antioxidant activities. This study aimed to investigate the ability of tempeh ethanol extract to reduce cellular reactive oxygen species (ROS) levels in HepG2 cells *in vitro*. Tempeh extract exhibited greater total phenolics, total flavonoids and free radical inhibition capacity than soybean extract. Both tempeh extract and soybean extract reduced the basal levels of cellular ROS in the cells, but tempeh extract induced higher expression of antioxidant enzymes [catalase, superoxide dismutase-2 (SOD2) and superoxide dismutase-3 (SOD3)] compared to soybean extract. This study provides novel evidence suggesting the ability of tempeh to tackle cellular oxidative stress by upregulating the expression of antioxidant enzymes. These findings would give an insight into the potential of tempeh to be developed as a functional food beneficial for human health.

1. Introduction

Soybeans (*Glycine max*) are legumes that have long been recognized as sources of high-quality proteins and healthful fat. They are rich in bioactive phytochemicals that are potentially beneficial towards human health, such as isoflavones, phytosterols, phytic acids, saponins and bioactive peptides. Numerous studies have reported the implication of these phytochemicals in preventing various diseases including cardiovascular disease, atherosclerosis, hypercholesterolemia, diabetes, osteoporosis, infertility and even cancer (Isanga and Zhang, 2008; Messina, 2016).

In Indonesia, soybeans are often processed into a traditional food called tempeh. The production of tempeh involves mould fermentation of soybeans by using *Rhizopus oligosporus*. The mycelia of the mould bind soybeans, thus forming a compact cake form with an earthy flavour. Tempeh possesses higher nutritional qualities compared to soybeans due to the fermentation process including higher protein bioavailability, reduced antinutritional factors and even the formation of vitamin B12 usually found only in foods of animal origin (Astuti *et al.*, 2001; Handoyo and Morita, 2006; Cao *et al.*, 2019). Tempeh is believed to procure health benefits, mainly owing to its phytochemicals, one of which is an isoflavone that exerts antioxidant activities (Karyadi and Lukito, 1996). During tempeh fermentation, soybean isoflavones present in conjugated forms with sugar molecules (glycoside forms) are bioconverted into free isoflavones (aglycone forms) with higher bioavailability and activities (Lee *et al.*, 2005; Zaheer and Humayoun-Akhtar, 2017).

One of the primordial roles of antioxidant at the cellular level is to neutralize reactive oxygen species (ROS). ROS are chemically reactive molecules and free radicals derived from molecular oxygen, such as hydrogen peroxide (H_2O_2), superoxide ($^{\bullet}O2^-$), hydroxyl radical (OH), singlet oxygen ($^1O_2$) and alpha-oxygen (α-O). Cellular ROS can be formed endogenously from various processes including respiration (particularly during oxidative phosphorylation in mitochondria) or exogenously by a variety of agents such as pollutants, heavy metals, tobacco, smoke, drugs, xenobiotics, or radiation (Ray *et al.*, 2012). High levels of cellular ROS can lead to oxidative stress, a state referring to a redox imbalance within cells due to excess ROS or oxidants over the cell capability to mount an effective antioxidant

*Corresponding author.
Email: *reggie.surya@binus.edu*

eISSN: 2550-2166

response. Oxidative stress alters normal cellular functions, causes macromolecular damage and is implicated in various diseases such as atherosclerosis, diabetes, neurodegeneration, cancer and ageing (Liguori *et al.*, 2018; Liu *et al.*, 2018). Several cellular antioxidant enzymes exist naturally to counteract ROS and oxidative stress, such as superoxide dismutase and catalase (Birben *et al.*, 2012).

Due to the potential of tempeh in human health and disease prevention, it has been considered as a functional food (Karyadi and Lukito, 2000; Nout and Kiers, 2005). However, very few studies have been conducted to explore the health benefits of tempeh. Until now, there has been no study regarding the antioxidative properties of soybeans and tempeh in vitro. Therefore, this study aimed to investigate whether soybean extract and tempeh extract were able to reduce ROS levels in HepG2 cell line derived from human hepatocarcinoma *in vitro*. The expression levels of cellular antioxidant enzymes like catalase and superoxide dismutases were also analysed to understand the mechanisms underlying the phenomenon.

2. Materials and methods

2.1 Sample preparation

Fermentation of tempeh was done according to Surya and Rahayu (2012) with some modifications. Soybeans (var Anjasmoro, harvest age 85 days, produced by UD Sumber Makmur, Nganjuk, East Java, Indonesia) were purchased from a trustworthy online shop. The preparation steps applied to the soybeans prior to mould fermentation consisted of cleaning, boiling (30 mins), soaking in water for 48 h at room temperature (25^{o}C), manual husking, boiling (30 min), leaching and cooling. Starter (brand Unggul, produced by PD Sukma Jaya, Tegal, Central Java, Indonesia, 5 g/kg soybean) was mixed with the soybeans along with white vinegar (5 mL/kg soybean). The inoculated soybeans were then wrapped in a holed plastic bag and left fermented in an incubator (35^{o}C) for 24 hrs and at room temperature (25^{o}C) for another 24 hrs to produce tempeh.

Following the fermentation, tempeh was mixed with ethanol 95% with a ratio of 1:3 (w/v) by using a blender, resulting in a puree. The puree was then filtered by using a cheesecloth to separate the pulp from the filtrate. The filtrate was collected and sterilized by nylon syringe filter (pore size 0.22 µm) for further analyses. Soybean extract was prepared using the same method as tempeh extract.

2.2 Total phenolics

Total phenolics were analysed according to Wojdyło *et al.* (2007) with some modifications. Soybean extract or tempeh extract (100 µL) were mixed with Folin-Ciocalteu reagent (0.2 mL) and distilled water (0.2 mL) prior to incubation at room temperature for 3 mins. Following the addition of sodium carbonate (1 mL, 20%) to the mixture, total phenolics were determined after 1 hr of incubation at room temperature. The absorbance of the mixture was measured at 765 nm with a UV–VIS spectrophotometer. Quantification was done with respect to the standard curve of gallic acid and the results were expressed as gallic acid equivalents (GAE).

2.3 Total flavonoids

Total flavonoids were analysed according to Chang *et al.* (2007) with some modifications. Soybean extract or tempeh extract (0.5 mL) were mixed with ethanol (1.5 mL, 95%), $AlCl_3$ (0.1 mL, 10%), CH_3COOK (0.1 mL, 1 M) and distilled water (2.8 mL). After incubation at room temperature for 30 min, the absorbance of the mixture was measured at 415 nm with a UV–VIS spectrophotometer. Quantification was done with respect to the standard curve of quercetin and the results were expressed as quercetin equivalents (QE).

2.4 Free radical inhibition assay

The free radical inhibition assay was done based on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging analysis according to Barus *et al.* (2019) with some modifications. Into each cuvet, an aliquot of extract (1 mL) was added to methanol (7 mL) and DPPH solution (2 mL). The mixture was kept at room temperature in the dark for 20 min, then the absorbance was measured at 520 nm by UV-Vis spectrophotometer. The percentage of free radical inhibition was calculated by using the following equation:

$$\text{Free Radical Inhibition (\%)} = [(A_{cont} - A_{test}) / A_{cont}] \times 100\%$$

Where A_{cont} is the absorbance of the control reaction and A_{test} is the absorbance of the extract.

2.5 Cell culture and treatments

HepG2 cells were purchased from American Type Culture Collection (ATCC, USA). The cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL amphotericin B at 37° C in humidified air with 5% CO_2. During the actual experiments using soybean extract/tempeh extract/ paraquat, dilution was done in supplemented DMEM but without the addition of foetal bovine serum to prevent any interaction with the tested molecules. Soybean extract and tempeh extract in ethanol were diluted in supplemented DMEM with a ratio of 1:50. We have

previously performed preliminary toxicity experiments and found that 2% ethanol was not toxic for our cells even after 24 hrs of exposure. Paraquat (Fisher Scientific, 5 ppm) was used as a positive control for ROS formation.

2.6 Immunofluorescence staining of mitochondrial ROS

Cells seeded in 12-well plates were incubated for 4 hrs of contact with soybean extract/tempeh extract/ paraquat at 37°C. After washing with PBS, the cells were incubated with MitoSOX™ Red Mitochondrial Superoxide Indicator (Thermo Fisher Scientific, 2 µM) for 10 mins at 37°C, protected from light according to manufacturer's instructions. Cells were gently rewashed with warm buffer prior to imaging by fluorescence microscopy according to the manufacturer's guidelines (Bio-Rad ZOE Fluorescent Cell Imager).

2.8 RT-qPCR

RT-qPCR was performed according to Surya *et al.* (2016) with some modifications. Briefly, cells were incubated for 4 hrs of contact with soybean extract/ tempeh extract/paraquat at 37°C. RNA was isolated using Tri Reagent (Eurogenetec) according to the manufacturer's instructions. Two-step RT-qPCR was performed using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. Following cDNA synthesis from 1 µg RNA, real-time PCR was conducted using QuantiTect SYBR Green RT-PCR kit (Qiagen) with the following thermocycling conditions: initial denaturation at 95°C (10 min), denaturation at 95°C (15 s), annealing at 60°C (30 s) and extension at 72°C (30s). The sequences of the primers (5′ –3′) used are listed below: Catalase (F-GTG CGG AGA TTC AAC ACT GCC A, R-CGG CAA TGT TCT CAC ACA GAC G), SOD1 (F-CTC ACT CTC AGG AGA CCA TTG C, R-CCA CAA GCC AAA CGA CTT CCA G), SOD2 (F-CTG GAC AAA CCT CAG CCC TAA C, R-AAC CTG AGC CTT GGA CAC CAA C), SOD3 (F-ACG CTG GCG AGG ACG ACC TG, R-GCT TCT TGC GCT CTG AGT GCT C) and GAPDH (F-GTC TCC TCT GAC TTC AAC AGC G, R-ACC ACC CTG TTG CTG TAG CCA A).

2.9 MTT viability assay

The viability assay was done according to Senthilraja and Kathiresan (2015) with some modifications. Cells were seeded in a 96-well plate and treated with soybean extract/tempeh extract/paraquat for 4, 8, or 24 hrs at 37° C. After the incubation time was reached, cells were washed with PBS and incubated in 200 µL PBS containing 5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) at 37°C for 2 hrs. The presence of viable cells was visualized by the development of purple colour due to the formation of formazan crystals. The optical density (OD) value of each well was read at 595 nm by a microplate reader (Tecan). The percentage of viable cells was calculated by using the following equation:

$$\text{Viable Cells (\%)} = (OD_{test}/OD_{cont}) \times 100\%$$

Where OD_{cont} is the OD of untreated well and OD_{test} is the OD of well treated with the sample solution.

2.10 Statistical analysis

The obtained data ($n \geq 3$) were analysed by using software GraphPad Prism 4 for Windows. The student's t -test was done to analyse the mean difference between soybean extract and tempeh extract with regard to total phenolics, total flavonoids and percentage of free radical inhibition. One-way ANOVA was performed to compare the relative expression of antioxidant enzymes (catalase and superoxide dismutases) and the percentage of viable cells following exposure to soybean extract, tempeh extract and paraquat. When ANOVA showed a statistically significant effect ($p < 0.05$), comparison among data was done by using Tukey's HSD Post-hoc test. All data were expressed as mean ± SD.

3. Results

Table 1 summarizes the profiling of antioxidant activities of soybean extract and tempeh extract used in this study. Tempeh extract exhibits higher total phenolics and total flavonoids, thus rendering it more potent to inhibit free radicals (DPPH) compared to soybean extract.

Table 1. Antioxidant profile and activities of soybean extract and tempeh extract

Parameter (per g db)	Soybean	Tempeh
Total phenolics (mg GAE)	417.80±34.62[a]	1015.26±44.24[b]
Total flavonoids (mg QE)	155.62±30.71[a]	969.64±95.98[b]
% free radical inhibition	6.38±1.01[a]	21.23±3.42[b]

Values are expressed as mean±SD. Values with different superscripts in the same row are statistically different ($p<0.05$)

Figure 1A shows the basal levels of ROS in HepG2 cells. Exposure to paraquat, a ROS inducer, increased ROS levels in the cells, as shown by higher fluorescence intensity compared to the basal levels (Figure 1B). Interestingly, treatments with soybean extract and tempeh extract significantly reduced the basal levels of ROS in the cells (Figure 1C and 1D).

Figure 2A-D highlights the upregulation in the expression of four cellular antioxidant enzymes following exposure to soybean extract and tempeh

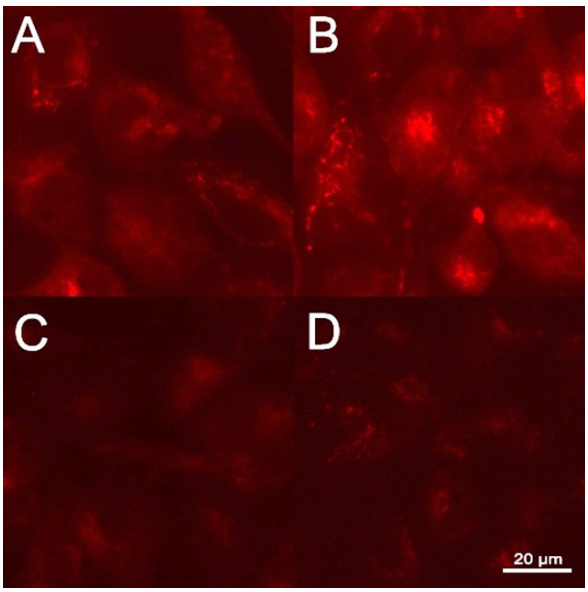

Figure 1. Fluorescent imaging of mitochondrial ROS in HepG2 cells under basal conditions (A) and upon exposure to paraquat (B), soybean extract (C) and tempeh extract (D). Higher fluorescent intensity of red colour indicated higher levels of mitochondrial ROS. Images are representative of three independent experiments.

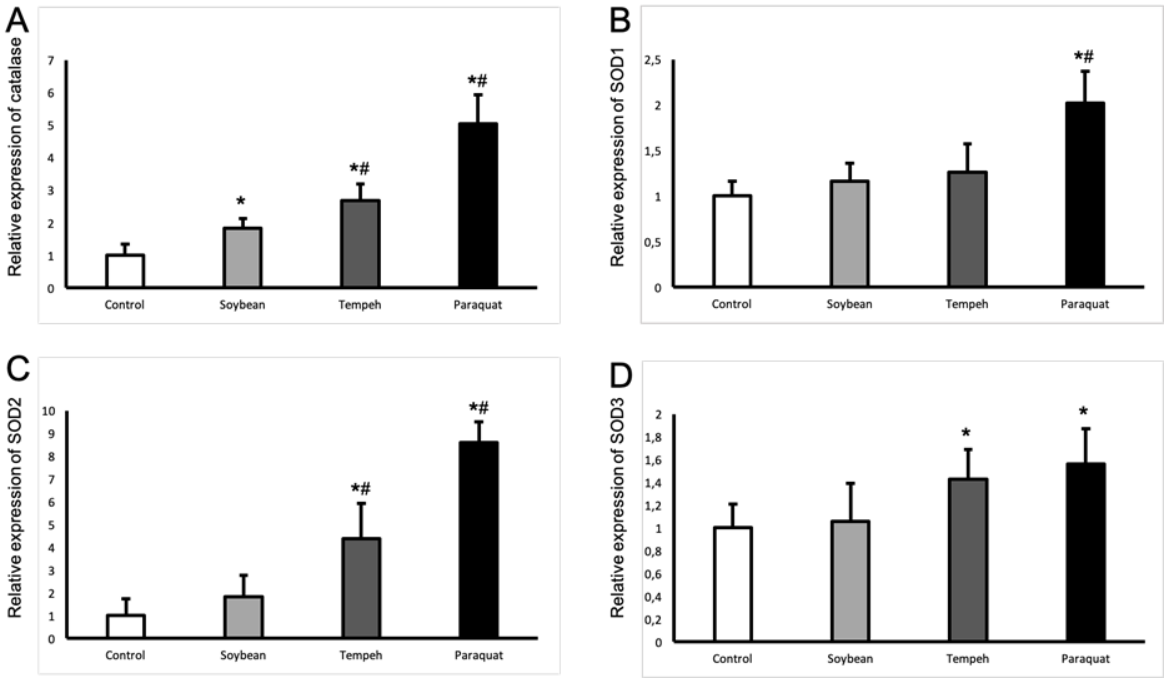

Figure 2. Relative expression of catalase (A), SOD1 (B), SOD2 (C) and SOD3 (D) in HepG2 cells under basal conditions and upon exposure to soybean extract, tempeh extract and paraquat. Asterisk (*) indicate a significant difference with control ($p<0.05$). Hash (#) indicate a significant difference with soybean ($p<0.05$).

extract for 4 hrs. The antioxidant enzymes comprised catalase, superoxide dismutase-1 (SOD1), SOD2 and SOD3. Paraquat induced significantly the expression of the four enzymes ($p < 0.05$). Tempeh extract upregulated significantly the expression of catalase, SOD2 and SOD3 to a lesser extent compared to paraquat. In all cases, tempeh extract exhibited a higher upregulation of antioxidant enzymes when compared to soybean extract. This latter gave rise only to the expression of catalase.

As presented in Figure 3, exposure to soybean extract and tempeh extract for 24 hrs did not cause cell demise. However, paraquat appeared to be harmful to the cells since treatment with paraquat killed almost 90% of

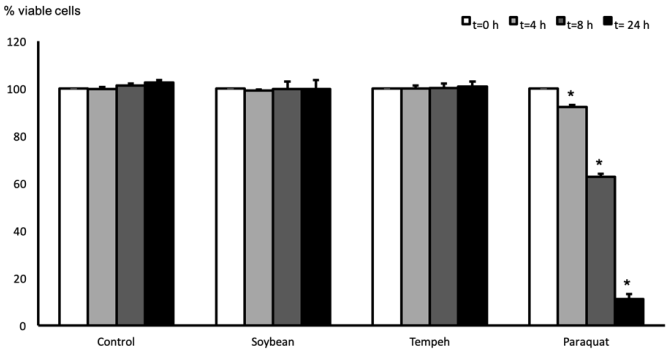

Figure 3. Percentage of HepG2 cell viability under basal conditions and upon exposure to soybean extract, tempeh extract and paraquat for 4, 8 and 24 hrs. Asterisk (*) indicate a significant difference with control paraquat at t = 0 hrs ($p<0.05$).

the cell population after 24 hrs.

4. Discussion

Soybeans are rich in bioactive phytochemicals that could contribute to human health (Alghamdi *et al.*, 2018). The most well-known phytochemicals in soybeans exhibiting antioxidative properties are mainly isoflavones. Soybean isoflavones have been reported to be potential antioxidants but their activities are much lower compared to tea epicatechin or alpha-tocopherol (Lee *et al.*, 2005). The major isoflavones found in soybeans are genistein, daidzein and glycitein (Xu *et al.*, 2015). Isoflavones are present in soybeans in two forms: isoflavone glycosides (conjugated to sugar molecules) and free isoflavones are known as aglycones. Both forms differ in activities and bioavailability (Zubik and Meydani, 2003; Miladinovic *et al.*, 2019).

In this study, soybeans and tempeh were extracted in 95% ethanol according to Chang *et al.* (2009) that reported that ethanol extracts of tempeh exhibited higher antioxidant activities compared to hexane, petroleum ether, ether and water extracts. We also obtained extremely low antioxidant activities with tempeh water extract (data not shown). Table 1 shows higher antioxidant activities in tempeh extract compared to soybean extract. This finding is in accordance with other studies revealing greater antioxidant activities in tempeh than in unfermented soybeans (Watanabe *et al.*, 2007; Chang *et al.*, 2009; Kuligowski *et al.*, 2017; Hashim *et al.*, 2018). Tempeh fermentation involves the bioconversion of isoflavone glycosides into isoflavone aglycones (Ferreira *et al.*, 2011). Lee *et al.* (2005) reported that isoflavone aglycones exerted higher antioxidant activities compared to isoflavone glycosides in LDL oxidation assay. In addition, during tempeh fermentation, there is also de novo formation of strong antioxidant compounds known as 3-hydroxyanthranilic acids (HAA) (Esaki *et al.*, 1996). These might explain the higher antioxidant activities in tempeh compared to soybeans.

At the cellular level, the main role of antioxidants is to inhibit oxidation, a chemical chain reaction producing free radicals (e.g. ROS) that may damage the cells. Antioxidants are expected to reduce the levels of cellular ROS, thus minimizing oxidative stress. High levels of ROS may impair the redox balance within cells, leading to cellular damage, cell senescence, cell death and, to a broader extent, chronic inflammation and cancer (Liu *et al.*, 2018). Figure 1B shows the induction of ROS formation by paraquat. Paraquat (N,N′-dimethyl-4,4′-bipyridinium dichloride) is a toxic organic compound often used as a herbicide. Paraquat generates ROS through a redox cycling mechanism by reacting with oxygen (O_2) and transforming it into superoxide anion ($^{\bullet}O2^{-}$) (Blanco-Ayala *et al.*, 2014). Soybean extract and tempeh extract reduced the basal levels of cellular ROS (Figure 1C and 1D). This phenomenon was likely to be due to the antioxidant activities of isoflavones that enable them to neutralize cellular ROS.

While high levels of ROS are often considered detrimental by provoking oxidation, the high basal level of ROS, particularly in cancer cells, has been hypothesized to exert a dual role: either protumorigenic or antitumorigenic. On one hand, ROS can provoke cellular oxidation leading to disruption of cellular activity and eventually cell death. On the other hand, cancer cells have the ability to maintain ROS homeostasis to hyperactivate signalling pathways necessary for tumorigenesis, one of which is the expression of protective cellular antioxidant enzymes to tackle oxidative stress such as superoxide dismutase (SOD) and catalase (Reczek and Chandel, 2017; Wang *et al.*, 2018). SOD is a group of enzymes that alternately catalyse the dismutation (or partitioning) of superoxide radicals into ordinary molecular oxygen (O_2) and hydrogen peroxide (H_2O_2). SOD exists in three different isoforms: SOD1, SOD2 and SOD3. SOD1 is distributed throughout the cytoplasm, nucleus and lumen between the outer and the inner membranes of mitochondria. SOD2 is located in the matrix of mitochondria while SOD3 is found mostly extracellularly (Miao and St Clair, 2009; Campos-Shimada *et al.*, 2018). Catalase is a cellular antioxidant enzyme responsible for decomposing toxic hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2) (Glorieux and Calderon, 2017). SOD and catalase are important enzymes protecting the cell from oxidative damage induced by ROS.

In this study, we analysed the expression of SOD1, SOD2, SOD3 and catalase in HepG2 cells following exposure to soybean extract and ethanol extract (Figure 2A-D). The expression of these four enzymes was upregulated upon exposure to paraquat. The sudden spike of cellular ROS induced by paraquat might facilitate the expression of these enzymes through the activation of nuclear factor erythroid 2-related factor 2 (Nrf2). Nrf2 is a ubiquitous protein acting as a transcription factor that regulates the expression of cellular antioxidant enzymes, including SOD and catalase (Dong *et al.*, 2008; Joshi and Johnson, 2012; Ma, 2013). Under basal conditions, Nrf2 is bound to Kelch-like ECH-associated protein 1 (Keap1) and is constantly degraded by ubiquitination. ROS may oxidize cysteine residues in Keap1, thus modifying its conformation and liberating Nrf2 to undergo gene expression (Sporn and Liby, 2012; Tonelli *et al.*, 2018).

Tempeh extract appeared to be more powerful in upregulating the expression of cellular antioxidant enzymes compared to soybean extract (Figure 2A, 2C, 2D). Tempeh extract induced mainly the expression of catalase, SOD2 and SOD3 while it was previously shown to reduce the basal levels of cellular ROS (Figure 1D). This indicates that the expression of these enzymes was not facilitated by ROS but rather by certain bioactive compounds in tempeh extract, presumably isoflavones. Indeed, isoflavones have been reported to protect HepG2 cells against oxidative stress by activating Nrf2. Isoflavones are able to bind directly into Keap1 to facilitate Nrf2 activation (Liang *et al.*, 2019). We hypothesized that isoflavones exerted two roles in reducing cellular ROS levels: direct role as antioxidants that neutralize ROS directly and indirect role as Nrf2 activator that facilitates the expression of antioxidant enzymes.

Interestingly, the expression levels of cellular antioxidant enzymes were not in accordance with cell viability. Despite the upregulation of SOD and catalase expression in HepG2 cells treated with paraquat, almost 90% of the cells died after being exposed for 24 hrs (Figure 3). Treatment with ethanol extracts of soybean and tempeh did not kill the cells. These findings highlight that even though ROS are required for ensuring signalling pathways in cellular transformation and tumorigenesis, their amount in the cells should be controlled under homeostatic conditions. When the amount of ROS in the cells is excessive, the cells would eventually die due to oxidative imbalance (Schieber and Chandel, 2014). Indeed, ROS are involved in the mechanism of apoptosis, a type of programmed cell death. ROS play an important role in the release of cytochrome c and other pro-apoptotic proteins, thus triggering caspase activation and apoptosis (Ott *et al.*, 2007).

5. Conclusion

Taken together, this study gives an insight into the potential of tempeh to be developed as a functional food that is potentially beneficial for human health. The findings obtained in this study provide for the first-time novel evidence at the cellular level regarding the potential of soybean and tempeh in counteracting cellular oxidative stress by neutralizing ROS. One of the means by which such protective effects are attained is through, but not limited to upregulating the expression of cellular antioxidant enzymes. Nevertheless, it remains unclear at which point the increase in enzyme expression can protect the cells from oxidative stress-induced by ROS since we previously observed that the cells exposed to paraquat still continued to die despite the augmented expression of antioxidant enzymes. In addition, the constituents of the ethanol extract of tempeh are yet to be characterized in order to identify the compounds responsible for cellular ROS reduction. Further studies are still required to establish the benefits of tempeh for human health to develop tempeh as a functional food.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgement

This study was a part of a project entitled "Antioxidant Profile and Activities of Tempeh and Oncom, Indonesian Traditional Functional Foods" funded by Binus University in the framework of Binus International Research (code PIB-72). The authors gratefully thanked The Research and Technology Transfer Office (RTTO) of Binus University for the opportunity and help given during the realization of this project.

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