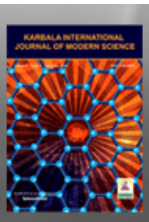

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Enhancement of Phenolic, Flavonoid, and Biological Activi-ties in Fermented Pea (Pisum sativum) Extracts via Frac-tionation

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Keywords

Pea, fermentation, antioxidant, phenolics, flavonoid, MCF-7.

RESEARCH PAPER

Enhancement of Phenolic, Flavonoid, and Biological Activities in Fermented Pea (*Pisum sativum*) Extracts via Fractionation

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Keywords: Pea, Fermentation, Antioxidant, Phenolics, Flavonoid, MCF-7

1. Introduction

Pulses, including peas, have been a part of human diet that contributes mainly to carbohydrate and protein intake. Dried peas, particularly green and yellow peas, are harvested as naturally dried seeds of *Pisum sativum*. They are well-known as inexpensive and widely available nutrient-dense food materials, making them an important food commodity. Studies on peas have been advancing to explore their benefits beyond merely fulfilling basic nutritional requirements [1]. In Indonesia, soup is a popular method of consuming peas, a practice that can be traced back to the Dutch colonial era. Peas are soaked in water prior to being added to soup [2]. Meanwhile, the fresh legumes of *P. sativum* are often cooked as a vegetable in the Indonesian spicy dish "sambal goreng" [3]. Recent developments aimed at incorporating peas into modern cuisine include the use of pea flour in the preparation of cookies and vegetarian sausages [4].

Another method for processing peas is through solid fermentation [5,6]. Traditional solid fermentation of pulses is complex because it incorporates multi-microorganisms rather than a single species or strain. The complexity increases due to differences in

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fermentation techniques and environmental conditions; thus, the results of fermented pulses vary. Nonetheless, fermentation is a promising approach to improve the profile of pulses, particularly in terms of the elimination of antinutritional factors and characteristic off-flavours, as well as the enhancement of techno-functional properties. The main microorganisms involved in traditional fermentation are fungi (e.g., *Rhizopus* spp. and *Aspergillus* spp.) and bacteria (e.g., *Bacillus* spp. and lactic acid bacteria) [7].

One of the notable solid fermentation methods for pulses in Indonesia is mould fermentation using either *Rhizopus oligosporus*, which is commonly used for making *tempe* [8], or *Aspergillus oryzae*, which is known for its role in soy sauce production [9]. *Rhizopus* is a genus of moulds that consists of ten species classified based on their morphological taxonomy. However, their existence is rather undesirable as their growth on agricultural products and food materials can lead to a decline in quality and a potential for toxicity [10]. Notably, certain species, such as *Rhizopus microsporus* var. oligosporus and *Rhizopus oryzae*, are well-regarded as starters in food fermentation processes throughout East and Southeast Asia [11]. *Rhizopus*-assisted fermentation delivers not only significant and favourable changes in flavour, taste, and texture but also enhances the nutritional profile of the substrates [6]. A recent study reported alterations in the fatty acid compositions of legumes due to fermentation with *R. oligosporus*, which increased the ratio of polyunsaturated fatty acids to saturated fatty acids, thereby benefiting human health, particularly in reducing the prevalence of heart disease [12].

Aspergillus is one of the influential mould genera in natural ecosystems and biotechnological applications [13]. *A. oryzae* is well-known for its ability to produce amylase and protease, enzymes that facilitate the degradation of proteins and starches into amino acids and sugars, respectively. Thus, *A. oryzae* plays a crucial role in fermented food production, including soy sauce, miso, and sake. Furthermore, *A. oryzae* is utilised as a biotechnological tool not only for enzyme production but also for the synthesis of fine chemicals through its secretion of secondary metabolites [14]. Lee et al. [15] observed changes in metabolites during the fermentation of several pulses and concluded that substrates played a more significant role than fermentation time in influencing the secondary metabolites and their subsequent antioxidant properties. Isoflavones and soyasaponins were more prominent in wild soybean (*Glycine soja*) and soybean (*Glycine max*) rather than in common bean (*Phaseolus vulgaris*) and hyacinth bean (*Lablab purpureus*). The increase in total phenolic content (TPC), total flavonoid content (TFC), and antioxidant properties was more pronounced in fermented wild soybeans. The inverse correlation between the consumption of fermented soy products and cardiovascular disease and cancer in Japanese women was revealed by Nozue et al. [16] through a population-based cohort study with a follow-up of at least 15 years. Meanwhile, Kyarchko et al. [17] utilised *A. oryzae*, either in mono or co-culture with *Lactobacillus plantarum*, to produce microbial protein from starch-rich pulse flour. This approach aimed to enhance the market value of the product and to hydrolyse pulse protein in order to increase the digestibility and/or bioactivity of the resulting peptides.

More scientists are interested in the exploration of *A. oryzae* due to the possibility of tailoring secondary metabolites by varying the culture medium and fermentation conditions, as well as the potential bioactivities of the resulting secondary metabolites [14].

A previous study investigating the fermentation of peas using *R. oligosporus* yielded noteworthy results, as the compactness of pea tempe was similar to that of soy tempe [6]. This similarity in appearance and texture between pea tempe and soy tempe was also reported by Rizwan et al. [18]. Furthermore, the macro- and micronutritional content of peas were examined both prior to and following fermentation with *R. oligosporus*. The protein content decreased from 19.78 to 17.68 g/100 g, the free amino acid content increased from 108 to 610 mg/100 g, and the phytate content decreased from 0.13 to 0.045 g/100 g following 48 h of fermentation [18].

In a separate investigation, Sánchez-Magaña et al. [19] reported that chickpea (*Cicer arietinum* L.) tempe prepared with *R. oligosporus* for 48 and 108 h exhibited an increase in ethanol-soluble phenolic content to 1.8 and 6.8 times that of unfermented chickpeas, respectively. Their research also indicated a significant enhancement in the Trolox equivalent of the ethanol extract of chickpea tempe against both 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and oxygen radical absorbance capacity (ORAC) by 4.6- and 3.3-fold, respectively, after 108 h of fermentation. An analysis of the ethyl acetate extract of chickpea tempe suggested an increase in phenolic content and inhibition against both ABTS and ORAC. Their findings implied that the enhanced bioactivities of chickpeas were likely attributed to both polar and semi-polar compounds generated during fermentation using *R. oligosporus* [19].

The fermentation of pea protein isolate using *A. oryzae* was investigated by Das et al. [20], revealing a

hydrolysis effect observable as early as 12 h of fermentation. The effect was evidenced by an increase in the percentage of low molecular weight peptides (<15 kDa) from 32 % to 44 % of the protein distribution, suggesting an enhancement in the overall water solubility of the proteins. Protein identification of both the pea protein isolate and *A. oryzae* displayed increased protein numbers. The identified proteins in the pea protein isolate increased from approximately 430 to 510 during 48 h of fermentation, while the number of identified proteins in *A. oryzae* rose from six to nearly 140 during the same fermentation period. Kumitch et al. [21] reported a hydrolysis effect during the initial 6 h of fermentation of pea protein-enriched flour using *A. oryzae*, which was indicated by 10.1 % degree of hydrolysis, an increase in crude protein by 2.4 % (d.b.), and a decrease in crude lipid and carbohydrate by 0.8 % (d.b.) and 1.6 % (d.b.), respectively. Additionally, low molecular weight peptides (<23 kDA) increased from 47.58 % to 58.84 % of the protein distribution during the 6-h fermentation period. Furthermore, the TPC increased by 44.7 %, which is expected to enhance antioxidant activity [22]. However, studies focusing on the bioactivity, including the antioxidant properties of fermented peas using *A. oryzae*, remain scarce.

The incubation stage of *R. oligosporus* in tempe preparation varies from 18 to 72 h at 25–38 °C. A simplified fermentation scheme has narrowed the fermentation window to 27–30 °C for 30–48 h [23]. Meanwhile, the first inoculation stage with *A. oryzae* in the preparation of koji lasted 48 h at 30 °C. The koji was later subjected to a second inoculation with brine and substrate for several months to produce miso [24]. Considering the typical fermentation used in the preparation of tempe and koji, this study focused on a fermentation period of less than 48 h. Additionally, Feng et al. [25] observed that barley tempe was covered by *R. oligosporus* mycelia after 18-h fermentation, while Chancharoonpong et al. [26] observed the growth of *A. oryzae* mycelia in soybean koji following 24-h fermentation. Both studies indicate that *R. oligosporus* and *A. oryzae* are fast-growing fungi. A previous study on pea fermentation using *R. oligosporus* showed that the water extract from 24-h fermented peas exhibited higher antioxidant activity than that of 48-h fermented peas [5]. Therefore, we chose to apply 24-h fermentation time in our study.

This present study aimed to further investigate the antioxidant activity of 24-h fermented peas using two different moulds: *R. oligosporus* and *A. oryzae*. Both filamentous moulds were selected due to their long-standing history in fermented food preparations in Asia [27], and both genera are generally considered safe [28]. *R. oligosporus* has begun to attract interest in occidental food systems concerning sustainability, waste valorisation, and functional food development [29]. Meanwhile, the utilisation of *A. oryzae* has expanded beyond food systems to include cosmetics and pharmaceuticals; furthermore, the approach to manipulating secondary metabolites has also advanced, incorporating gene editing [30]. Two solvents (i.e., water and methanol) were used to extract polar and less polar bioactive compounds. The resulting extracts were eluted through preparative thin-layer chromatography (TLC) and subsequently analysed for bioactivity and chemical composition. To the best of our knowledge, studies on pea fermentation using *Aspergillus* spp. are fewer than those using *Rhizopus* spp. The current study employed both cultures, followed by extraction and fractionation, while other studies analysed the whole extracts without fractionation using TLC.

2. Materials and methods

2.1. Material

Peas (*P. sativum*) were harvested in 2021 from a private garden in Bogor, West Java, Indonesia.

2.2. Preparation of inoculum

R. oligosporus C1 and *A. oryzae* pure isolates were obtained from the collection of the Research Center for Chemistry, BRIN. Semi-pure inocula were prepared by growing the isolates on cooked rice [31]. These isolates were taken from potato dextrose agar using an ose needle and diluted in sterile distilled water. A total of 50 mL of this diluted solution was combined with 500 g of cooked rice [32], which was prepared by cooking rice and water in a ratio of 1:0.75. The mixture of water and cooked rice was gently stirred until homogeneous prior to fermentation at room temperature. The fermentation process was stopped after 24 h due to the rapid growth of inoculum. The resulting fermented rice was dried in an oven at 50 °C for 24 h [32,33]. Finally, the dried fermented rice was ground to produce a fine powdered inoculum. The pulverisation was conducted in a clean but not sterile condition [33]. A viability study was not performed on the resulting inocula. Nevertheless, Nout and Rombouts [31] summarised that the semi-pure inoculum of *R. oligosporus* following drying at 40–55 °C contained 4×10^7–10^9 cfu/g mould and 10^4–10^5 cfu/g bacteria. The viable spores only represented 5–6 % of the total spores present in rice-grown starter [34]. On

the other hand, Uchida et al. [35] prepared a semi-pure inoculum of *A. oryzae* via simultaneous fermentation and drying at approximately 37 °C, resulting in 1.6 × 10^8 cfu/g mould and less than 10^5 cfu/g bacteria and yeast.

2.3. Preparation for fermentation

Peas (*P. sativum*) were soaked in water overnight, washed, and boiled [5]. The boiled peas were allowed to cool to room temperature and dehulled. The dehulled peas were then mixed with the powdered inoculum at a ratio of 5:1 and left at room temperature for 24 h to allow fermentation. Some of the dehulled peas were not mixed with the powdered inoculum and kept as unfermented samples.

2.4. Extraction

Unfermented and fermented peas were oven-dried (Ecocell 22, MMM Medcenter Einrichtungen GmbH, Planegg, München, Germany) at 50 °C for 24 h. Dried unfermented and fermented peas were ground to fine powders. The ground unfermented and fermented peas (10 g) were extracted with either aquadest or methanol (#822283, Merck Millipore, Burlington, MA, USA), respectively, in three stages, which each stage utilising 100 mL of solvent. Water and methanol were collected and dried in a vacuum oven at 50 °C, resulting in dried water and methanol extracts.

2.5. Gas chromatography-mass spectrometry

The volatile profiles of fermented pea extracts were analysed using a gas chromatograph (7890B GC System, Agilent Technologies Inc., Santa Clara, CA, USA) tandem mass spectrometry (MSD 5977A MSD, Agilent Technologies Inc., Santa Clara, CA, USA). The instrument was equipped with an HP-5MS Ultra-Inert column (30 m length × 0.25 mm i.d. × 0.25 μm thickness, Agilent Technologies Inc., Santa Clara, CA, USA) with helium as the carrier gas at a flow rate of 1 mL/min. The fermented pea methanol extracts were dissolved in n-hexane at a concentration of 1 mg/mL and filtered prior to splitless injection at 250 °C. The temperatures of the ion source and interface of the mass detector were set at 230 and 250 °C, respectively. Electron impact with ionisation energy of 70 eV was used.

2.6. DPPH assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay measures free radical scavenging activity. It is shown by a colour change from deep violet to light yellow when a DPPH radical is extinguished by a free radical scavenger that donates hydrogen atoms. The method described by Devi et al. [36] was employed with some modifications. A 1 mM methanolic DPPH was prepared by dissolving 4 mg of DPPH (#OF54G-GT, TCI, Tokyo, Tokyo Prefecture, Japan) in 10 mL of methanol. The solution was vortexed vigorously to obtain a homogeneous DPPH solution.

The methanol and water extracts of unfermented and fermented peas were dissolved in methanol to prepare samples at a concentration of 20,000 μg/mL. Five microliters of dissolved extract, 40 μL of DPPH solution, and 155 μL of methanol were transferred into a well plate, resulting in a final extract concentration of 500 μg/mL. A blank was prepared in a similar manner, except that the dissolved extract was replaced by methanol. All samples and blank were incubated at room temperature and in the dark for 30 min. The absorbance was measured at a wavelength of 518 nm (Varioskan™ Flash Multimode Reader, Thermo Fisher Scientific, Waltham, MA, USA). Each measurement was performed in triplicate. The percentage of inhibition was calculated using Equation (1).

$$Inhibition\ (\%) = \frac{A_b - A_s}{A_b} \quad (1)$$

Where A_b and A_s are the absorbance of the blank and the sample, respectively.

2.7. ABTS assay

The ABTS assay works based on the generation of ABTS radicals, which is blue-green in colour and becomes colourless as it is reduced by antioxidants. A 7 mM ABTS solution was prepared by dissolving 18 mg of ABTS (#A1888, Merck Millipore, Burlington, MA, USA) in 5 mL of deionised water. Additionally, a 140 mM of potassium persulphate solution was prepared by dissolving 189 mg of potassium persulphate (#216224, Merck Millipore, Burlington, MA, USA) in 5 mL of deionised water. Subsequently, 5 mL of 7 mM ABTS solution was mixed with 88 μL of 140 mM of potassium persulphate solution and kept in the dark at room temperature for 16 h to allow the generation of ABTS radicals. The resulting solution was diluted with deionised water at a ratio of 1:44 (v/v) to produce an ABTS reagent with an absorbance of 0.70 ± 0.02 at 734 nm. This ABTS reagent was ready for subsequent ABTS assay [37].

The unfermented and fermented pea methanol and water extracts were dissolved in methanol to

produce samples with a concentration of 20,000 μg/mL. Subsequently, 5 μL of dissolved sample, 100 μL of ABTS reagent, and 95 μL of aquadest were transferred to a 96-microwell plate, resulting in an overall sample concentration of 500 μg/mL. A blank was prepared in a similar manner, with the sample being substituted by methanol. The well plate was incubated at room temperature in the dark for 8 min before measuring absorbance at 734 nm (Varioskan™ Flash Multimode Reader, Thermo Fisher Scientific, Waltham, MA, USA). Each measurement was performed in triplicate. The percentage of inhibition was calculated using Equation (1).

For IC_{50} determination, the same amount (100 μL) of ABTS reagent was transferred to a 96-microwell plate. The amount of the sample and aquadest was varied to obtain final sample concentrations ranging from 100 to 500 μg/mL, while ensuring that the final reaction volume remained at 200 μL. The sample concentrations and the percentage of inhibitions were plotted on the x-axis and y-axis, respectively. The IC_{50} value was defined as the concentration that resulted in 50 % of inhibition.

2.8. Total phenolic content analysis

Total phenolic content was determined following the Folin-Ciocalteu method [38] with modification for the microplate. Fifty microliters of the sample were diluted with 110 μL of aquadest and subsequently mixed with 100 μL of Folin-Ciocalteu phenol reagent (#109001, Supelco Merck, Burlington, MA, USA). The mixture was incubated for 8 min. Later, 30 μL of 10 % sodium carbonate (#106549, Supelco Merck, Burlington, MA, USA) solution was added to the mixture, and the incubation was extended for an additional 2 h. The absorbance of the solution was measured at 765 nm, and the concentration was read against a standard curve that was previously prepared using gallic acid (#842649, Sigma-Aldrich, Burlington, MA, USA) solution at several concentrations. Each measurement was performed in duplicate.

2.9. Total flavonoid content analysis

Total flavonoid content was determined using the aluminium chloride ($AlCl_3$) colourimetric method [38]. Fifty microliters of sample were diluted with 58 μL of aquadest prior to mixing with 6 μL of sodium nitrite (#106392, Supelco Merck, Burlington, MA, USA) solution (5 % w/v). The mixture was incubated for 5 min at room temperature. Subsequently, 6 μL of $AlCl_3$ (#8.01081, Sigma-Aldrich, Burlington, MA, USA) solution (10 % w/v) was added to the mixture, and the incubation was extended for an additional 5 min. Following that, 80 μL of 1 M sodium hydroxide (#106498, Supelco Merck, Burlington, MA, USA) solution were added, and the reaction was allowed to continue for 30 min at room temperature. The absorbance of the solution was measured at 415 nm, and the concentration was read against a standard curve that was previously prepared using quercetin (#551600, Sigma-Aldrich, Burlington, MA, USA) solution at several concentrations. Each measurement was performed in duplicate.

2.10. Fractionation

The dried methanol extracts of fermented peas were subjected to subsequent fractionation using preparative TLC silica gel F254 (#1.05554.0001, Merck Millipore, Burlington, MA, USA). The dried extract, prior to fractionation, was diluted in methanol to obtain a 50,000 μg/mL sample. The TLC plates were cut to dimensions of 35 mm (width) × 70 mm (length). Each TLC plate was loaded with 250 μL of sample. The loading was done 5 mm from the bottom edge of the TLC plate with a 5 mm margin for each side. A TLC glass chamber with a lid was filled with 1 mL of methanol and 1 mL of ethyl acetate (#109623, Merck Millipore, Burlington, MA, USA). This solvent mixture ratio was chosen for its effectiveness in providing good band separation. The chamber was gently shaken to ensure a homogeneous mixture of solvents. The TLC plate was immediately dipped inside the TLC chamber and the lid was closed. Elution was performed until the solvent mixture reached 5 mm from the top edge of the plate. The TLC plate was removed from the TLC chamber using metal tweezers and left to dry at room temperature. This procedure was repeated once using a new TLC plate, resulting in a total elution of 500 μL of sample. The TLC plates were observed under ultraviolet (UV) light at either 254 or 365 nm, and each band was marked using a pencil. The resulting individual bands with the same band number from the two TLC plates were scrapped off the plates using a spatula and transferred into microtubes. Each scrapped band was added with 1000 μL of methanol, vortexed, and centrifuged at 6700×*g* for 4 min (Mini Spin Plus, Eppendorf, Hamburg, Germany). Each supernatant (600 μL) was transferred to a clean vial prior to subsequent analysis and was labelled according to the band number from Band 1 to Band 6.

All bands were subjected to DPPH and ABTS assays, following the methodologies outlined in Sections 2.6 and 2.7, respectively, with some

modifications. For the DPPH assay, each reaction well was filled with 100 μL of each band, 60 μL of methanol, and 40 μL of 1 mM methanolic DPPH. Meanwhile, for the ABTS assay, each well plate was filled with 50 μL of each band, 50 μL of aquadest, and 100 μL of ABTS reagent. Both assays were performed in duplicate.

The elution of 500 μL of methanol-dissolved extract and the extraction of the resulting bands were repeated twice in order to produce adequate fractionated samples for TPC/TFC and cytotoxicity analyses. The TPC and TFC analyses adhered to the methodologies outlined in Sections 2.8 and 2.9, respectively. Both analyses were performed in duplicate.

2.11. Cytotoxicity

The cytotoxicity of the fermented pea extract was determined using MCF-7 breast cancer cells with the alamarBlue™ method [33]. AlamarBlue™ is a blue and virtually nonfluorescent resazurin, which is converted to a red, highly fluorescent resofurin when it is reduced by living cells. The MCF-7 cell lines were obtained from the Research Center for Chemistry BRIN. These cell lines were cultured in Roswell Park Memorial Institute (RPMI) medium, which was prepared by diluting RPMI 1640 (Lot #1937557, Gibco™, Thermo Fisher Scientific, Waltham, MA, USA) and 2.2 g of sodium hydrogen carbonate (#K34791329526, Merck, Darmstadt, Hesse, Germany) in 1 L of reverse osmosis water. The RPMI medium was supplemented with 10 % foetal bovine serum (#1907413, Gibco™, Thermo Fisher Scientific, Waltham, MA, USA) and 1 % Anti-Anti (#1924798, Gibco™, Thermo Fisher Scientific, Waltham, MA, USA). The MCF-7 cells were maintained in a carbon dioxide (CO_2) incubator (Series 8000 Direct-Heat, Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C and 5 % CO_2.

The MCF-7 cells in RPMI solution (1×10^4 cells/mL) were transferred into a 96-well plate (100 μL per well) and incubated in a CO_2 incubator at 37 °C for 24 h. Subsequently, 4 mg of either fermented pea extract or fractionated band was dissolved in 200 μL of dimethyl sulfoxide (DMSO, #30.06.08, Merck, Darmstadt, Hesse, Germany). Samples in DMSO (2 μL of fermented pea extract or 5 μL of fractionated band) were added to the MCF-7 cells. An equivalent volume of DMSO was added to the cells to prepare the positive control. Meanwhile, an equivalent volume of DMSO was added to 100 μL of supplemented RPMI medium without any added cells to prepare the negative control.

The treated cells, along with the positive and negative controls, were incubated for the next 24 h. Later, 10 μL of alamarBlue™ (DAL1025, Invitrogen™, Waltham, MA, USA) was added to each well, and the incubation was continued for 4 h. The fluorescence intensity was measured using a microplate reader (Varioskan™ Flash Multimode Reader, Thermo Fisher Scientific, Waltham, MA, USA) at excitation and emission wavelengths of 560 and 590 nm, respectively. Cytotoxicity was calculated using Equation (2), with FI_{590} representing the fluorescence intensity at 590 nm emission. Each measurement was performed in duplicate.

$$Cytotoxicity\ (\%) = \left[1 - \frac{FI_{590\ of\ the\ treated\ cells} - FI_{590\ of\ negative\ control}}{FI_{590\ of\ positive\ control} - FI_{590\ of\ negative\ control}}\right] \times 100\ \% \quad (2)$$

2.12. Statistical analysis

Data were subjected to the Shapiro-Wilk normality test. When the p-value was greater than the level of significance ($\alpha = 0.05$), it implied that the null hypothesis H0 (i.e., the residuals follow a normal distribution) could not be rejected. As the data passed the normality test, the statistical analysis was continued with the analysis of variance (ANOVA) at a 95 % confidence interval, a common choice for such analyses [39]. The properties of unfermented and fermented pea extracts prior to fractionation were analysed using the standard ANOVA for independent samples. Following fractionation, their properties were analysed using nested ANOVA, with the band factor nested inside the fermentation culture factor. A post-hoc test was performed using Tukey's method. Additionally, a Spearman correlation test was employed to determine the correlation strength among the TPC, TFC, and biological properties. The mentioned tests were performed using Microsoft Excel 2019 version 2503 (Microsoft, Redmond, WA, USA) and XLSTAT version 2023.1.2.1406 software (Addinsoft, New York, NY, USA). Values are presented as mean ± standard deviation (SD) [40].

3. Results and discussion

3.1. Volatile profiles of unfermented and fermented pea extracts

Unfermented and fermented peas displayed different volatile profiles (Table 1). Fatty acids and fatty acid methyl esters (FAMEs) constituted the primary components of these profiles. They were detected in both unfermented and fermented peas but at different proportions. The predominant carboxylic acids identified were palmitic, linoleic, conjugated linoleic, and linolenic acids. According to Byrdwell and Goldschmidt [41], linoleic acid (46.1 %) was the main fatty acid of green split peas, followed by oleic acid (26.2 %), palmitic acid (15.2 %), and stearic acid (4.3 %). The difference in fatty acid composition between their report and the present study may be attributed to different

Table 1. Compounds identified and their respective percentage areas of unfermented and fermented peas previously prepared with either R. oligosporus *or* A. oryzae *subjected to extraction using methanol, with ≥90 % similarity compared to the suggested compounds from the library database.*

No.	Retention Time	Library/ID	CAS	Unfermented	*R. oligosporus*	*A. oryzae*
Fatty Acids						
1	10.138	Octanoic acid (*Caprylic acid*)	000124-07-2	1.42	0.00	0.00
2	12.933	n-Decanoic acid (*Capric acid*)	000334-48-5	1.17	0.00	0.00
3	15.481	Dodecanoic acid (*Lauric acid*)	000143-07-7	2.05	0.00	0.00
4	19.885	n-Hexadecanoic acid (*Palmitic acid*)	000057-10-3	8.70	9.18	17.52
5	21.582	10E,12Z-Octadecadienoic acid (*10,12-Conjugated linoleic acid*)	002420-56-6	15.01	12.34	0.00
6	21.639	9,12,15-Octadecatrienoic acid, (Z,Z,Z)- (*Linolenic acid*)	000463-40-1	10.93	0.00	0.00
7	21.807	9,12-Octadecadienoic acid (Z,Z)- (*Linoleic acid*)	000060-33-3	0.00	33.34	60.16
Fatty Acid Methyl Esters						
8	19.552	Hexadecanoic acid, methyl ester (*Methyl palmitate*)	000112-39-0	5.46	5.86	2.05
9	21.041	Methyl-gamma-linolenate (*Gamma linolenic acid, methyl ester*)	016326-32-2	0.00	2.46	0.00
10	21.192	9,12-Octadecadienoic acid, methyl ester	002462-85-3	22.60	0.00	15.47
11	21.212	9,12-Octadecadienoic acid (Z,Z)-, methyl ester (*Methyl linoleate*)	000112-63-0	0.00	11.70	00.00
12	21.265	9-Octadecenoic acid, methyl ester, (E)- (*Methyl elaidate*)	001937-62-8	14.65	15.63	0.00
13	21.476	Heptadecanoic acid, 14-methyl-, methyl ester	002490-23-5	0.00	0.00	1.16
14	21.484	Methyl stearate	000112-61-8	1.64	3.97	0.00
15	23.274	Eicosanoic acid, methyl ester (*Methyl arachidate*)	001120-28-1	0.00	0.46	0.00
16	24.898	Docosanoic acid, methyl ester (*Behenic acid, methyl ester*)	000929-77-1	0.00	0.28	0.00
17	26.415	Tetracosanoic acid, methyl ester (*Lignoceric acid, methyl ester*)	002442-49-1	0.00	0.41	0.00
Fatty Acid Ethyl Esters						
18	20.238	Hexadecanoic acid, ethyl ester (*Palmitic acid, ethyl ester*)	000628-97-7	1.01	0.00	0.00
19	26.164	9,12-Octadecadienoic acid (Z,Z)-, 2-hydroxy-1-hydroxymethyl) ethyl ester (*ß-Monolinolein*)	003443-82-1	11.27	0.00	0.00
Terpenoids						
20	27.019	Squalene	000111-02-4	0.00	0.35	2.70
21	27.049	Supraene	007683-64-9	1.19	0.00	0.00
Aldehydes						
22	23.074	9,17-Octadecadienal, (Z)-	056554-35-9	0.00	1.11	0.00
23	26.023	13-Octadecenal, (Z)-	058594-45-9	0.00	0.74	0.00
Alcohol						
24	26.233	9,12-Octadecadien-1-ol, (Z,Z)-	000506-43-4	0.00	1.20	0.00
Miscellaneous						
25	29.021	Gamma-tocopherol	007616-22-0	2.91	0.47	0.94
26	30.837	Ergosterol	000057-87-4	0.00	0.50	0.00
			Total	100.00	100.00	100.00

extraction methods. Linoleic acid was absent in the boiled and unfermented green peas in this study. However, a notable transformation was observed as linoleic acid was detected in substantial amounts following fermentation with either *R. oligosporus* or *A. oryzae* (Table 1).

The FAMEs in Table 1 are typically detected in palm oils [42] and various pulse oils [41], including oils prepared from fermented soybeans [37]. Methyl palmitate and methyl elaidate are two major FAMEs of palm oils [42]. In the current study, methyl palmitate was present in all samples. Conversely, methyl elaidate was detected only in unfermented and fermented peas with *A. oryzae*. Methyl linoleate, which constituted a significant proportion of FAMEs from soybean (55.67 %), corn (48.38 %), and sunflower (65.93 %) oils [43], was detected (11.70 %) in fermented peas with *R. oligosporus* but undetected in the other two samples. Meanwhile, its stereoisomer (i.e., 9,12-octadecadienoic acid, methyl ester), was not detected in fermented peas with *R. oligosporus*; however, it was present in both unfermented and fermented peas with *A. oryzae*. Methyl linolenate, which was reported (6.2 %) in split green peas [41], was also found in a small proportion in the green peas of the current study.

Only two fatty acid ethyl esters (FAAEs) were detected in unfermented peas, while none were found in both fermented peas. In comparison to FAMEs, the scientific literature on FAAEs is less extensive [44]. In the current study, only β-monolinolein was identified in unfermented peas in a significant percentage area. This compound, which is a derivative of linoleic acid, plays a role in lipid transport and metabolism, and it has applications in industry as a surfactant and emulsifier [45].

Squalene and supraene were detected in fermented and unfermented peas, respectively (Table 1). These compounds are stereoisomers. Squalene is present in various natural products, ranging from plants to animals, and it is abundantly found in shark liver oil [46]. The squalene content in various seeds, grains, and legumes was studied by Ryan et al. [47]. This compound has attracted the attention of scientists due to its bioactivities, such as anticancer and anti-hypercholesterolemia [46,47].

3.2. DPPH and ABTS assays of unfermented and fermented pea extracts

Comparing methanol and water for the extraction of fermentation products is important because these solvents have different polarities, extraction efficiencies, and selectivities for bioactive compounds. Methanol is an organic polar solvent commonly used for the extraction of natural products, including fermentation products. It has a lower polarity than water. Water is highly polar and can extract hydrophilic compounds such as proteins, polysaccharides, and certain glycosides, which are typically primary metabolites. In contrast, methanol is less polar and can dissolve both moderately polar and nonpolar compounds, including phenolics, alkaloids, and lipophilic substances, which are generally secondary metabolites known for their specific bioactivities [48–50].

Methanol extracts at 500 μg/mL prepared from both fermented peas displayed a significant increase in inhibition against both ABTS and DPPH radicals (Fig. 1A and B, respectively). Furthermore, water extracts at the same concentration showed high inhibition against ABTS radicals even prior to fermentation (Fig. 1A). The inhibition of the water extract following fermentation was greater according to both methods. Overall, both extracts exhibited better antioxidant activity under the ABTS protocol. Therefore, the investigation of the antioxidant

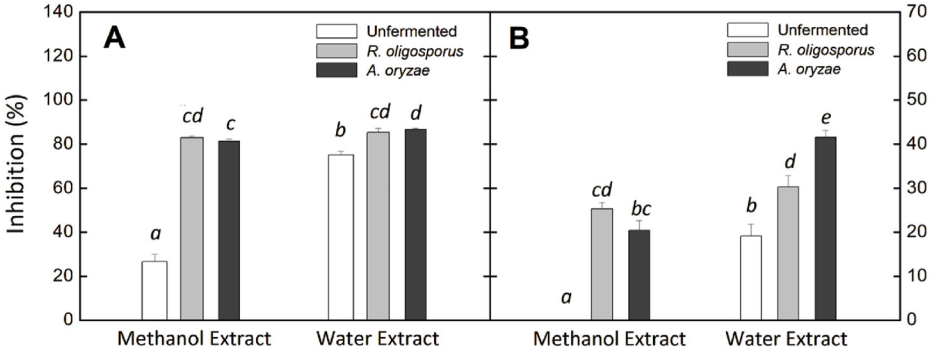


Fig. 1. Inhibition (%) against ABTS (A) and DPPH (B) radicals by unfermented and fermented pea extracts at 500 μg/mL prepared using R. oligosporus *and* A. oryzae *and extracted using methanol and water. Different superscript letters indicate significant differences at 95 % level of confidence. Values are expressed as mean ± SD.*

activity of fermented pea extracts against ABTS radicals was proceeded to obtain the IC_{50} values.

Pea water extracts did not exhibit significant changes following fermentation with *A. oryzae* (Table 2). Meanwhile, fermentation with *R. oligosporus* resulted in a decrease in the antioxidant capacity of water pea extract, as reflected by an increased IC_{50} value. In contrast, fermentation improved the IC_{50} of methanol extracts, regardless of the cultures. The antioxidant activity presented in Table 2 is reported as IC_{50} to facilitate comparison with other studies. It is important to provide the amount of ABTS radicals used for the assay [50]. For the IC_{50} values reported in Table 2, the initial concentration of ABTS radicals was 75 μM. The IC_{50} in Table 2 shows the extract concentration required to reduce the ABTS radicals from 75 to 37.5 μM. The IC_{50} of methanol extract decreased following pea fermentation, suggesting that the resulting fermented peas contained more active antioxidant compounds that could be extracted by methanol. Thus, a smaller concentration of fermented pea methanol extract was required (≤200.37 μg/mL) to reduce the ABTS radicals from 75 to 37.5 μM. In comparison, the methanol extract of unfermented peas comprised less active antioxidant compounds; therefore, a higher concentration (>500 of μg/mL) was needed to achieve the same ABTS reduction.

Fermentation enhances antioxidant activity by breaking down complex compounds into bioactive forms, thereby increasing bioavailability and producing new antioxidant metabolites. This process improves polyphenol conversion, releases bioactive peptides, boosts vitamin content, reduces antinutritional factors, and generates microbial metabolites with strong antioxidant properties [51,52].

A previous study by Saharan et al. [53] indicated that fermentation using *Aspergillus awamori* (MTCC 548) increased the antioxidant capacity of ethanolic extracts of various legumes, as investigated using ABTS and DPPH methods. The magnitude of antioxidant activity determined using the ABTS method was much greater in comparison to the activity determined using the DPPH method; however, the increase in antioxidant activity was more pronounced when evaluated using the DPPH method. This observation is almost similar to our findings. We also found that the effect of different cultures was significant ($p < 0.05$) when the fermented pea extracts were subjected to the DPPH assay, but no significant differences ($p > 0.05$) were observed when they were subjected to the ABTS assay.

Table 2. Determination of IC_{50} (μg/mL) for unfermented and fermented pea extracts prepared using R. oligosporus *and* A. oryzae *and extracted using methanol and water via the ABTS method.*

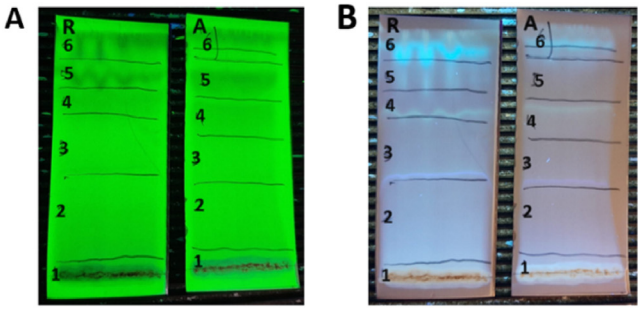
IC_{50} (μg/mL)	Unfermented	*R. oligosporus*	*A. oryzae*
Methanol extract	>500 ^{a}	146.65 ± 17.02 ^{c}	200.37 ± 19.55 ^{b}
Water extract	99.65 ± 17.53 ^{d}	174.92 ± 4.92 ^{bc}	94.21 ± 7.11 ^{d}

Different superscript letters indicate significant differences at 95 % level of confidence. Values are expressed as mean ± SD.

3.3. Fractionation of fermented pea extracts and their evaluations

The fractionation of fermented pea methanol extracts using TLC as the stationary phase and a mixture of methanol and ethyl acetate (1:1) as the mobile phase resulted in six bands for each extract (Fig. 2). The more polar bands were located in the lower part of the TLC and vice versa. The lower bands were more soluble in methanol compared to ethyl acetate.

The bands were scrapped, extracted in methanol, and subjected to both ABTS and DPPH assays. Different fermentation cultures exhibited significant effects ($p < 0.05$) only on the inhibition against DPPH radicals of the resulting bands (Fig. 3C). They did not significantly ($p > 0.05$) affect the ABTS inhibition (Fig. 3A).



Bands	Retention Factor (Rf)	
	R. oligosporus	*A. oryzae*
6	0.952	0.976
5	0.857	0.929
4	0.738	0.655
3	0.548	0.476
2	0.261	0.238
1	0.036	0.048

Fig. 2. Preparative TLC of methanol extracts of fermented peas after elution with a mixture of methanol and ethyl acetate at the ratio of 1:1, as observed under UV light at either 254 nm (A) or 365 nm (B). The extracts were prepared from peas fermented with either R. oligosporus *or* A. oryzae *(R and A, respectively), as indicated at the top of the TLC plates.*

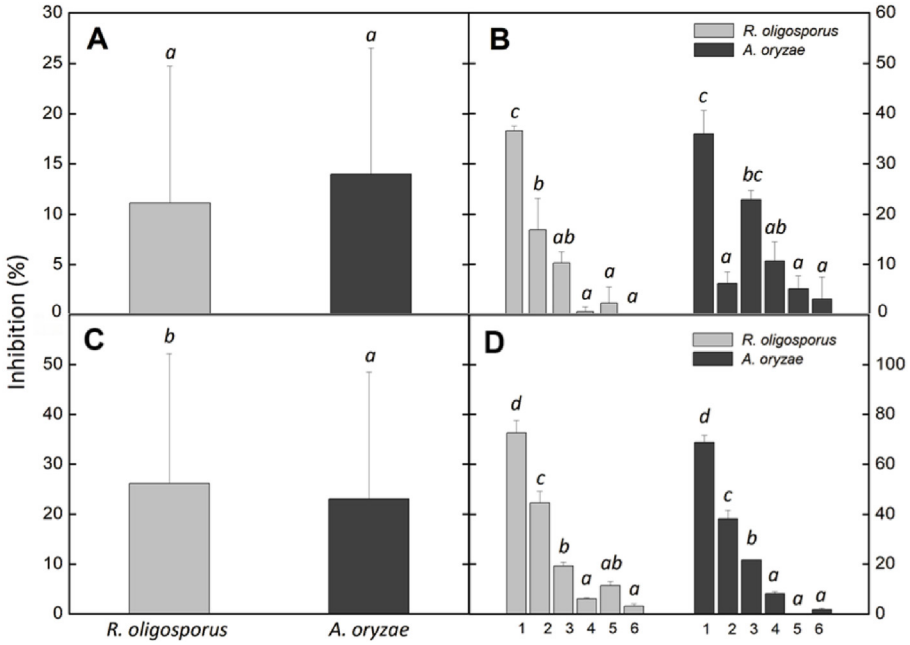


Fig. 3. Inhibition data for ABTS (B) and DPPH (D) of methanol extracts of fermented peas after elution with a mixture of methanol and ethyl acetate at the ratio of 1:1. The ABTS and DPPH assays were conducted at 2000 and 4000 μg/mL, respectively. The extracts were prepared from peas fermented with either R. oligosporus *or* A. oryzae*. The ABTS inhibition was not significantly ($p > 0.05$) affected by the fermentation cultures (A), while the DPPH inhibition showed a significant difference ($p < 0.05$) (C). Labels 1–6 refer to the band numbers after fractionation via preparative TLC. Both ABTS (B) and DPPH (D) inhibitions were significantly ($p < 0.05$) affected by the bands. Different superscript letters indicate significant differences at 95 % level of confidence. Values are expressed as mean ± SD.*

The highest inhibition for both assays was displayed by Band 1, where the solubility was closer to methanol. The inhibition decreased as the band numbers increased up to Band 6, where the solubility was closer to ethyl acetate (Fig. 3B and D). These findings suggested that the antioxidant properties of fermented pea extracts were majorly contributed by the compounds soluble in methanol instead of ethyl acetate. According to Magro and de Casto [54], the selection of solvents determined the extraction of bioactive compounds. Proper solvents can enhance the extraction process, as indicated by an increase in extraction yield and antioxidant activity.

Protic solvents (e.g., water, methanol, and ethanol) facilitate the extraction of compounds with higher free radical scavenging activity in comparison to aprotic solvents (e.g., n-dichloromethane, ethyl acetate, and acetone).

Phenols are the most common antioxidants found in nature [55]; therefore, the bands were subjected to TPC and TFC analyses. The bands displayed higher TPC and TFC compared to those of methanol extracts, regardless of the fermentation cultures (Fig. 4B and D). Although the TPC and TFC were not significantly ($p > 0.05$) affected by either the fermentation cultures (Fig. 4A and C) or the bands (Fig. 4B and D), the highest TPC and TFC were noticeable. The highest TPC was displayed by the most polar band (i.e., Band 1), while the highest TFC was exhibited by the most nonpolar band (i.e., Band 6).

The pattern of the bands for cytotoxic activity is displayed in Fig. 5B. The statistical analysis revealed no significant differences ($p > 0.05$) in fermentation cultures (Fig. 5A). However, the effect of the bands was significant ($p < 0.05$) (Fig. 5B). A correlation test was conducted to gain a better understanding of the relationship between the TPC or TFC and the bioactivities of the bands, assessing whether the absolute value of the correlation was strong (0.70—1.00), moderate (0.40—0.69), or weak (0.00—0.39) [56]. The results showed that TFC demonstrated a strong positive correlation with cytotoxicity ($R = 0.722$) (Table 3). Its correlation with either ABTS or DPPH inhibition was strong and negative. Meanwhile, TPC displayed a strong correlation ($R = 0.916$) only with DPPH inhibition. Moreover, TPC correlated moderately with both ABTS inhibition and cytotoxicity. This implies that less polar flavonoid compounds in the upper band induced the cytotoxicity of fermented peas. Meanwhile, polar phenolic compounds other than flavonoids, such

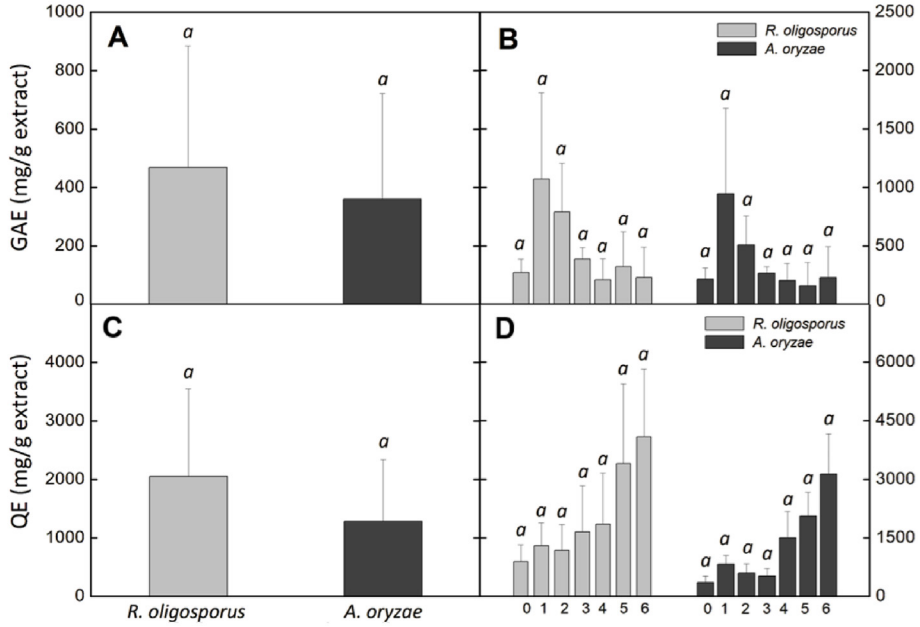


Fig. 4. TPC (B) and TFC (D) of methanol extracts of fermented peas after elution with a mixture of methanol and ethyl acetate at the ratio of 1:1. The extracts were prepared from peas fermented with either R. oligosporus *or* A. oryzae*. Both TPC (A) and TFC (C) were not significantly ($p > 0.05$) affected by the fermentation cultures. Label 0 refers to extracts prior to fractionation. Labels 1–6 refer to the band numbers after fractionation using preparative TLC. Both TPC (B) and TFC (D) were not significantly ($p > 0.05$) affected by the bands. Different superscript letters indicate significant differences at 95 % level of confidence. Values are expressed as mean ± SD.*

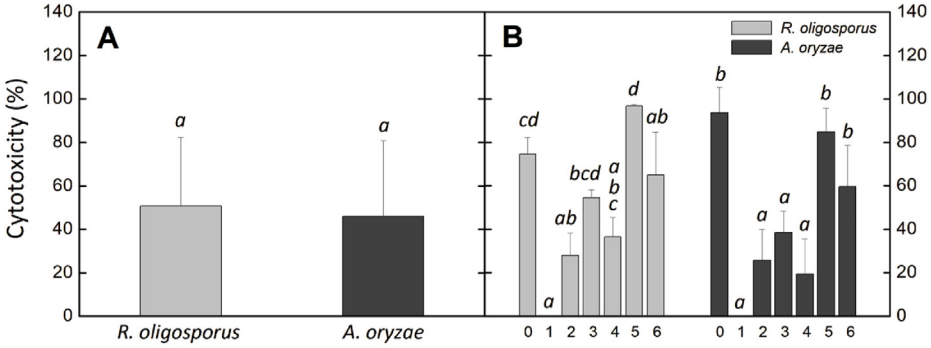


Fig. 5. Cytotoxicity (B) of methanol extracts of fermented peas after elution with a mixture of methanol and ethyl acetate at the ratio of 1:1. The extracts were prepared from peas fermented with either R. oligosporus *or* A. oryzae*. Cytotoxicity was not significantly ($p > 0.05$) affected by the fermentation cultures. Label 0 refers to extracts prior to fractionation and tested at 420 μg/mL. Labels 1–6 refer to the band numbers after fractionation using preparative TLC and tested at 420 μg/mL. Cytotoxicity was significantly ($p < 0.05$) affected by the bands. Values are expressed as mean ± SD.*

Table 3. Spearman correlation (R) among TPC, TFC, and bioactivities of fractionated fermented pea methanol extracts. Fermentation was carried out using either R. oligosporus *or* A. oryzae*, while extraction was performed using methanol. Fractionation was conducted on TLC plates using a mixture of methanol and ethyl acetate (ratio of 1:1) as the mobile phase.*

Assays	ABTS Inhibition	DPPH Inhibition	Cytotoxicity
TPC	0.608	0.916	−0.529
TFC	−0.790	−0.741	0.722

as gallic acid, which were concentrated in the lower bands, were most likely responsible for the antioxidant activity.

Flavonoids exhibit cytotoxicity against MCF-7 breast cancer cells through multiple mechanisms, including apoptosis induction, cell cycle arrest, oxidative stress modulation, oestrogen receptor inhibition, and suppression of key oncogenic pathways [57,58].

4. Conclusions

Peas subjected to 24-h fermentation with both *R. oligosporus* and *A. oryzae* exhibited different volatile profiles compared to unfermented peas, as well as improved antioxidant properties. This enhancement was evidenced by the lower IC_{50} values of fermented pea methanol extracts, as determined by the ABTS assay, in comparison to that of unfermented peas ($IC_{50} > 500$). Further investigation involving fractionation revealed that the compounds collected from the bottom bands of the TLC plates possibly contributed to the antioxidant properties of fermented peas. These compounds were more soluble in protic solvents and displayed high antioxidant activity and TPC. In contrast, the compounds collected from the upper bands of the TLC plates were more soluble in aprotic solvents and showed high TFC and cytotoxicity against MCF-7 breast cancer cells. A strong correlation was observed between TFC and bioactivities (i.e., ABTS inhibition, DPPH inhibition, and cytotoxicity), with an absolute value of R greater than 0.7. In contrast, TPC showed a stronger correlation with DPPH inhibition ($R = 0.916$) but a moderate correlation with the two other bioactivities. This finding is important in the development of pea-based functional ingredients, functional foods, or nutraceuticals, such as antioxidant and cancer prevention supplements. In order to support these future possible applications, the isolation and characterisation of bioactive compounds are required, as well as conducting in vivo bioactivity studies.

Ethics information

This research did not have any ethical concerns because it involved neither human participants nor animals tests.

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Conflicts of interest

The authors declare that they have no competing interests.

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