

Antioxidant activity of fish sauces including puffer (*Lagocephalus wheeleri*) fish sauce measured by the oxygen radical absorbance capacity method

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Abstract. Fish sauces are fermented seasonings traditionally used throughout Asia, including Japan. Here, we report on the antioxidant activity of 30 fish sauces, among them a puffer fish sauce developed specifically for this study. To determine the antioxidant activity (i.e., the peroxyl radical elimination capacity) of the fish sauces, the oxygen radical absorbance capacity (ORAC) was measured. ORAC values ranged between 10^4 μmol (flatfish sauce 1) and 10^3 μmol (sandfish sauce) trolox equivalent (TE)/100 ml of fish sauce. Hydroxyl radical scavenging activity (IC_{50}) was measured using electron spin resonance. IC_{50} values ranged between 0.081% (puffer fish sauce) and 0.653% (sardine fish sauce 7). Puffer fish sauce had a high ORAC value (8,365 $\mu\text{mol TE}/100$ ml) and the highest hydroxyl radical scavenging activity (0.081). The relationship between the ORAC and IC_{50} values of the 30 fish sauces was determined to be intermediate ($r = -0.521$, $p = 0.01$).

Introduction

Active oxygen species generated in the body cause damage to DNA and the lipid membrane structure of cells, and have been implicated in the processes of aging and cancer development (1). It is therefore important to eliminate excess active oxygen within the body. Antioxidants absorbed from food have been

shown to be effective scavengers of active oxygen (2). Our research group has previously reported on the peroxyl and hydroxyl radical scavenging activities of fish sauce (3,4), sea urchin gonads (5), gelatin gel food 'Nikogori' made from fish meat (6-9), dried bonito stock (Katsuo-dashi) (10), buckwheat (11,12) and soy sauces (13,14) measured by chemiluminescence and electron spin resonance (ESR) spectroscopy. We have also demonstrated the DNA protective activities of fish sauces, including tiger puffer (*Takifugu rubripes*) fish sauce, against hydroxyl radicals using an apurinic/apyrimidinic (AP) site assay (15).

Fish sauces are traditional seasonings used in Asian, including Japanese, cuisine, and have been studied in various countries, including countries outside of Asia (16-18). The measurement of oxygen radical absorbance capacity (ORAC) using fluorescence intensity for the determination of antioxidant activity (in the form of peroxyl radical elimination capacity) has been approved by the USDA (United States Department of Agriculture). Since Cao *et al* first developed the method in 1993 (19), various studies have applied the ORAC assay in both the US (20-26) and Japan (27-31). Though Yamada *et al* reported the ORAC value of dried bonito stock (Katsuo-dashi) (32,33), no studies have determined the ORAC value of fish sauce.

Here, we report on the antioxidant activity (the peroxyl radical elimination capacity) of 30 fish sauces, among them a puffer fish sauce developed specifically for this study, by comparing the ORAC values of the sauces to their hydroxyl radical scavenging activities as measured by ESR spectrometry. The puffer fish sauce was compared to previously experimentally developed and commercially available fish sauces.

Materials and methods

Fish sauce samples. The fish sauces used in the study are listed in Table I. The antioxidant activity of the unnumbered fish sauces was reported in our previous study (4). The puffer

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Table I. List of the examined fish sauces.

| No. | Type of sauce | Original name | Ingredients besides salt | Country of production |
|-----|--|---------------|---|-----------------------|
| 1 | Squid fish sauce 4 | Ishiri 3 | Squid | Japan |
| 2 | Squid fish sauce 5 | | Squid, soybean, wheat and koji mold | Japan |
| 3 | Squid fish sauce 6 | Ishiri 4 | Squid | Japan |
| 4 | Sardine fish sauce 6 | | Sardine, soybean and barley | Japan |
| 5 | Sardine fish sauce 7 | Shottsuru 3 | Sardine and seaweed | Japan |
| 6 | Sardine fish sauce 8 | Ishiru 2 | Sardine and sugar | Japan |
| 7 | Anchovy fish sauce 1 | Nam plaa 3 | Anchovy and sugar | Thailand |
| 8 | Anchovy fish sauce 2 | | Anchovy | Japan |
| 9 | Anchovy fish sauce 3 ⁴ (formerly fish sauce 4) | Nam plaa 2 | Anchovy and sugar | Thailand |
| 10 | Anchovy fish sauce 4 | Nam plaa 4 | Anchovy and sugar | P.R. China |
| 11 | Anchovy fish sauce 5 | | Anchovy, soybean, wheat and koji mold | Japan |
| 12 | Anchovy fish sauce 6 | | Anchovy, soybean, wheat and koji mold | Japan |
| 13 | Salmon fish sauce 4 | | Salmon, soybean, wheat and koji mold | Japan |
| 14 | Sea-bream fish sauce 1 | | Sea-bream and rice | Japan |
| 15 | Sea-bream fish sauce 2 | | Sea-bream and chicken egg white | Japan |
| 16 | Flatfish fish sauce 1 | | Flatfish, soybean, wheat and koji mold | Japan |
| 17 | Flatfish fish sauce 2 | | Internal organs of flatfish, soybean, wheat and koji mold | Japan |
| 18 | Puffer fish sauce | | Puffer, soybean, wheat and koji mold | Japan |
| 19 | Ayu fish sauce | | Ayu | Japan |
| 20 | Cod fish sauce | | Cod, soybean, wheat and koji mold | Japan |
| 21 | Sandfish fish sauce ⁴ | Shottsuru 1 | Sandfish | Japan |
| 22 | Sand lance fish sauce ⁴ | Ikanago shoyu | Sand lance | Japan |
| 23 | North Pacific lightfish fish sauce | | North Pacific lightfish and rice | Japan |
| 24 | Fish sauce 2 ⁴ | Yuiru | Fish | P.R. China |
| 25 | Fish sauce 4 | Nam plaa 5 | Fish | Thailand |
| 26 | Fish sauce 5 | | Fish and alcohol | Japan |
| 27 | Oyster sauce 2 | | Oyster, sugar and amino acid | Japan |
| 28 | Shrimp sauce 2 | | Shrimp, amino acid and alcohol | Japan |
| 29 | Sea urchin gonad fish sauce | | Sea urchin gonad, sugar and onion | Japan |
| 30 | Walleye pollack roe fish sauce | | Walleye pollack roe, amino acid and alcohol | Japan |

fish sauce was developed at our laboratory using the flesh, skin and bones (but not the internal organs, which contain the poison tetrodotoxin) from the white chestnut puffer fish (*Lagocephalus wheeleri* Abe, Tabeta et Kitahama). The puffer fish materials were combined with soybean, wheat, soy sauce koji mold (*Aspergillus sojae*), NaCl and water, then fermented for approximately 1 year at room temperature. For the experiments, the supernatant from the original fermented mash was used after sterilization (15).

Chemicals. 2,2'-Azobis (2-amidinopropane) dihydrochloride (AAPH) and dipotassium hydrogenphosphate (K_2HPO_4) were obtained from Wako Pure Chemical, Ltd. (Osaka, Japan). Potassium dihydrogenphosphate (KH_2PO_4) was obtained from Kanto Chemical Co., Inc. (Tokyo, Japan) and hydrogen peroxide (H_2O_2) from Santoku Chemical Co., Ltd. (Tokyo, Japan). Fluorescein sodium salt and iron (II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) were obtained from Sigma-Aldrich Japan (Tokyo, Japan). A spin trapping reagent, 5,5-dimethyl-1-pyrroline

N-oxide (DMPO), as well as 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan).

Sample preparation. Phosphate buffer was used as the assay (control) buffer and was prepared by combining 75 mM K_2HPO_4 and 75 mM KH_2PO_4 for a final volume of 75 μ mol, adjusted to pH 7.0. AAPH reagent was dissolved in the buffer at a concentration of 31.7 mM. Fluorescein working solution was prepared at a concentration of 94.4 nM by dissolving fluorescein sodium salt in the buffer. Trolox standard solutions were prepared at concentrations of 100, 50, 25, 12.5 and 6.25 μ M by dissolving trolox in the buffer.

Measurement of oxygen radical absorbance capacity. The ORAC value was obtained by measuring the peroxyl radical elimination capacity of peroxyl radicals generated by AAPH reagent, and by measuring the time lapse degradation of fluorescein (i.e., the rate of decrease in the intensity of

fluorescence) (21). The ORAC assay was performed on a 96-well multilabel microplate reader (Mithras LB940; Berthold Technologies GmbH & Co. KG) as described by Huang *et al* (22,23,25). In brief, 20 μ l each of sample buffer (obtained by appropriate dilution with assay buffer), various concentrations of trolox standard solution (for construction of a standard curve) or blank buffer (as a control) were placed in the individual wells of a 96-well transparent microplate (Sanplatec Corp., Osaka, Japan). Fluorescein working solution (200 μ l) was added and the wells were agitated at 37°C for 10 min. Subsequently, 75 μ l of AAPH solution was added to each of the wells to initiate the reaction. The total volume of each reaction solution was 295 μ l. The fluorescence intensity [485 nm (excitation)/535 nm (emission)] was then measured every 2 min over 90 min at pH 7.4 and 37°C. As the reaction progressed, fluorescein was consumed and the fluorescence intensity decreased. The inhibition of fluorescence decay was taken to indicate the presence of an antioxidant.

Typical ORAC assay kinetic curves in the presence of various concentrations of trolox are shown in Fig. 1. ORAC values were determined:

The area under the kinetic curve (AUC) of the standards and samples was calculated as follows:

$$\text{AUC} = (0.5 + f_{10 \text{ min}}/f_{8 \text{ min}} + f_{12 \text{ min}}/f_{8 \text{ min}} + f_{14 \text{ min}}/f_{8 \text{ min}} + f_{90 \text{ min}}/f_{8 \text{ min}}) \times 2$$

where $f_{x \text{ min}}$ = fluorescence reading at cycle x min (23).

The standard regression line was obtained by plotting the trolox concentrations against the net $\text{AUC}_{\text{trolox}}$ of each concentration:

$$\text{Net AUC}_{\text{trolox}} = \text{AUC}_{\text{trolox}} - \text{AUC}_{\text{control}}$$

$$\text{Net AUC}_{\text{sample}} = \text{AUC}_{\text{sample}} - \text{AUC}_{\text{control}}$$

where $\text{AUC}_{\text{trolox}}$ = AUC in the presence of trolox; $\text{AUC}_{\text{control}}$ = AUC with blank control; $\text{AUC}_{\text{sample}}$, AUC with sample buffer. The horizontal axis was the net $\text{AUC}_{\text{trolox}}$; the vertical axis, the concentration of trolox.

The equation $Y = ax + b$ was derived from the above data, and the values for a and b were obtained.

The final ORAC values of the samples were calculated using the equation:

$$\text{ORAC value } (\mu\text{mol trolox equivalent}/100 \text{ ml}) = [a \times (\text{net AUC}_{\text{sample}})] \times 100/[\text{sample}]$$

where [sample] = the diluted concentration ratio of the sample.

Data were analyzed using Microsoft Excel.

Electron spin resonance analysis. ESR was conducted as previously described (5,10-12,14,15). Briefly, hydroxyl radical generation was first examined by the DMPO method and iron (II) sulfate with or without the fish sauce samples. Next, the addition of 8.8 mM H_2O_2 (50 μ l) to the reaction mixture (320 μ l) was used to initiate Fenton's reaction as depicted in the chemical equation: $\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \cdot\text{OH} + \text{OH}^-$. After 1 min of hydroxyl radical generation, spin adduct DMPO-OH \cdot was measured using the ESR spectrometer (JES-FR30; JEOL

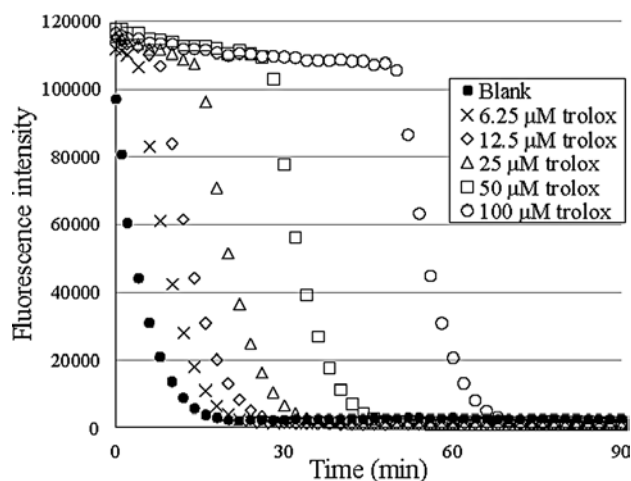


Figure 1. Typical fluorescence decay curves induced by AAPH in the presence of different concentrations of trolox.

Ltd., Tokyo, Japan). ESR measurement conditions were: output, 4 mW (9.4 GHz); magnetic field, 342.790 \pm 5 mT; modulation amplitude, 0.079 mT; time constant, 0.1 sec; sweeping time, 1 min; amplification ratio, 32-125.

Calculation of the IC_{50} of hydroxyl radical scavenging. IC_{50} values were defined as the concentration of each fish sauce that reduced the control peak height ratio of ESR (generation of hydroxyl radical) by half. The antioxidative value was calculated using the formula: $(\log \text{Io/I}) \times 100$, where Io = ESR peak height ratio as the control, and I = ESR peak height ratio as the samples. Thus, the IC_{50} value was the concentration of samples at $\text{Io/I} = 1/2$, calculated from the antioxidant results of ESR obtained in the experiments (5,10-12,14,15).

Results

Oxygen radical absorbance capacity values. As shown in Fig. 2, ORAC values ranged between 9,664 and 1,001 μ mol trolox equivalent (TE)/100 ml of flatfish sauce 1 or sandfish sauce, respectively. The flatfish, squid, puffer, ayu, salmon and cod fish sauces had high ORAC values (>6,000 μ mol TE/100 ml). The ORAC value of puffer fish sauce was the fourth highest value at 8,365 μ mol TE/100 ml. Sauces containing soybean, wheat and koji mold tended to have higher ORAC values, while the sardine, anchovy, sandfish and sea-bream fish sauces had lower ORAC values (i.e., weak antioxidant activity) ranging between 1,000 and 5,500 μ mol TE/100 ml. Sardine fish sauce 7, 8 and sandfish sauce had the lowest ORAC values, of <2,000. A similar trend was reported in a previous study (4), in which squid fish sauces had high antioxidant activity and sardine fish sauces had weak antioxidant activity, determined using chemiluminescence. The concurrence of these results is not unexpected, since both studies measured peroxyl radical elimination capacity or scavenging activity. The average ORAC value of the 30 types of fish sauce was 5,060 μ mol TE/100 ml. The ORAC value of soy sauce (light color soy sauce) was 4,944 μ mol TE/100 ml. This value was similar to the average ORAC value of the fish sauces (unpublished data).

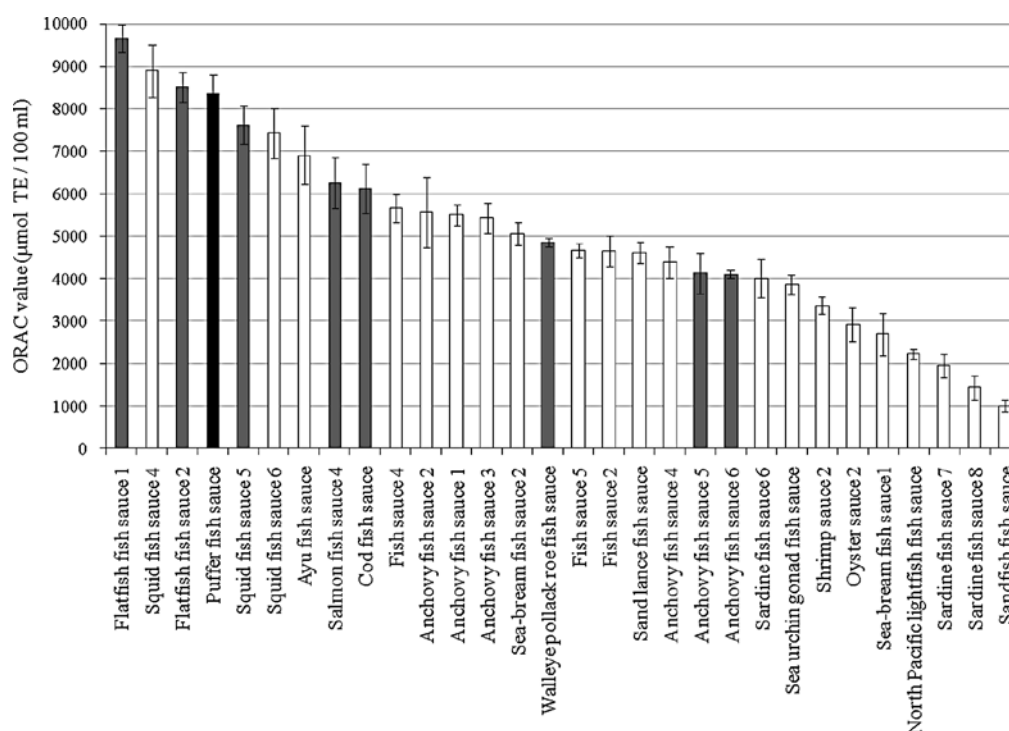


Figure 2. ORAC values (μmol trolox equivalent/100 ml) representing the peroxy radical elimination capacity of the 30 types of fish sauce. The black bar represents the puffer fish sauce, and the gray bars represent the fish sauces that include soybean, wheat and koji mold ($n=3$).

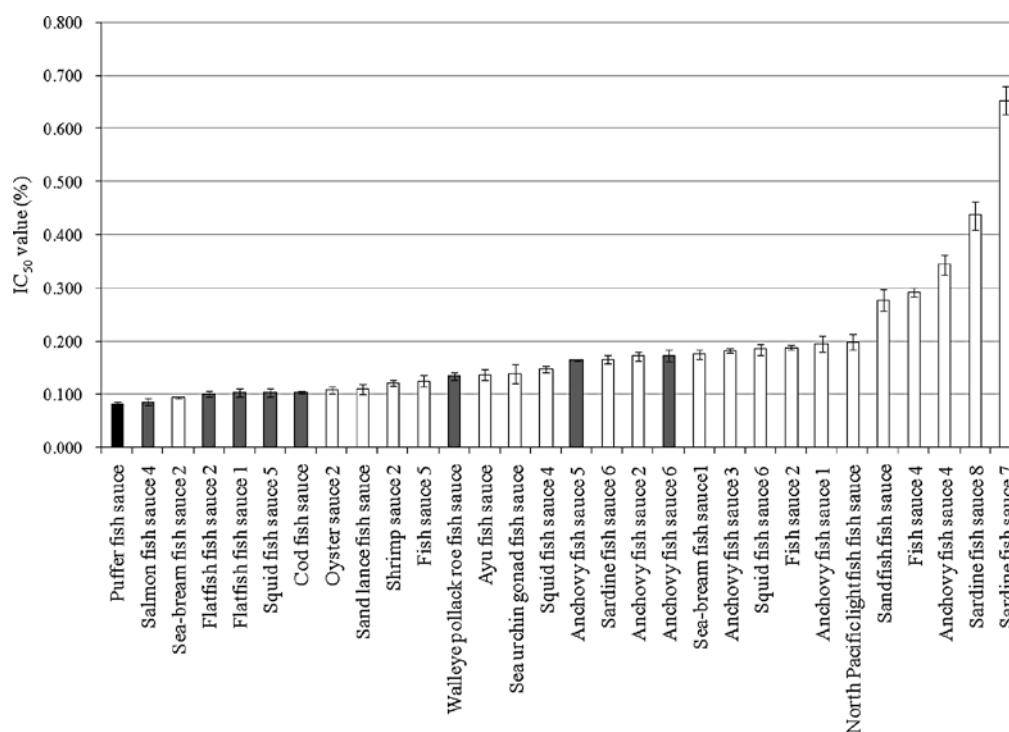


Figure 3. IC₅₀ values (%) representing the hydroxyl radical scavenging activities of the 30 types of fish sauce. The black bar represents the puffer fish sauce, and the gray bars represent the fish sauces that include soybean, wheat and koji mold ($n=3$).

IC₅₀ values from electron spin resonance spectrometry. The IC₅₀ value was estimated from the results of ESR as the hydroxyl radical scavenging activity. The order of strength of hydroxyl radical scavenging activity is shown in Fig. 3; the lower the height of the bar, the stronger the radical scavenging

activity. Based on the data, the IC₅₀ values (%) ranged between 0.081 (puffer fish sauce) and 0.653 (sardine fish sauce 7). The puffer fish sauce made using white chestnut puffer showed the highest hydroxyl radical scavenging activity, though previously we reported the IC₅₀ of puffer fish sauce made with tiger

puffer (*Takifugu rubripes* Temminck et Schlegel) to be 0.20% (15). The flatfish, puffer, salmon and cod fish sauces had high hydroxyl radical scavenging activities, with IC_{50} values $<0.105\%$. Fish sauces with soybean, wheat and koji mold as ingredients tended to have higher hydroxyl radical scavenging activities. By contrast, the IC_{50} values of the squid fish sauces ranged between 0.104 and 0.185%, though they had high antioxidant activities based on the ORAC values. Sardine and sandfish sauces had weak hydroxyl radical scavenging activities as well as low ORAC values.

Relationship between ORAC and IC_{50} values. ORAC values represented peroxy radical elimination capacity and IC_{50} values represented hydroxyl radical scavenging activities. The relationship between the two was analyzed using the correlation coefficient (r), line regression and coefficient of determination (R^2) (Fig. 4). The regression line equation was $y = -9712.3x + 6836.4$, the correlation coefficient was -0.521 and the coefficient of determination was 0.272. Therefore, the correlation was found to be intermediate at $p=0.01$.

Discussion

We previously demonstrated, using chemiluminescence, ESR and an apurinic/apyrimidinic site assay, that fish sauces commonly used for seasoning foods in Asia have high antioxidant activities, (3,4,15). The data from these previous studies and the current study reveal that the sauces with the weakest antioxidant activity are fish sauce 1, determined using chemiluminescence (0.246%) (4), sandfish sauce, determined by measuring ORAC (1,001 $\mu\text{mol TE}/100\text{ ml}$), and sardine fish sauce 7, determined using ESR (0.653%). When sardine fish sauce 7 was diluted 153-fold, the IC_{50} value was 0.653%, half (50%) of the scavenging activity of the generated hydroxyl radicals. According to the USDA databases (34), the ORAC value of sandfish sauce (1,001 $\mu\text{mol TE}/100\text{ ml}$) was higher than the values of apple juice (408 $\mu\text{mol TE}/100\text{ ml}$), pear juice (704), orange juice (726), white grape juice (793), lime juice (823), canned tomato juice (486), canned vegetable juice (548), apple vinegar (270) and red wine vinegar (410). Therefore, even fish sauces with weak antioxidative activity had higher antioxidant activity than fruit and vegetative juices.

Puffer fish sauce had a high ORAC value (8,365 $\mu\text{mol TE}/100\text{ ml}$) and the lowest IC_{50} value (0.081%) – in other words, the highest hydroxyl radical scavenging activity, and highest ratio of DNA protection against hydroxyl radicals, of 68.9% (15). Thus, the puffer fish sauce showed high antioxidant activity against peroxy and hydroxyl radicals from the viewpoint of radical elimination capacity or scavenging activity and DNA protection. This is not generally characteristic of fish sauce, based on the correlation coefficient data as seen in Fig. 4. We speculate that some peptides, including oligopeptides, may contribute to the high antioxidant activity of the puffer fish sauce (unpublished data). Furthermore, puffer fish sauce and fish sauces containing soybean, wheat and koji mold tended to have higher ORAC values and lower ESR IC_{50} values. As previously suggested by Ando *et al*, aminocarbonyl reaction products, i.e., melanoidin produced during fermentation from soybean, wheat and koji mold, contribute to the high antioxidant activities in such sauces (13).

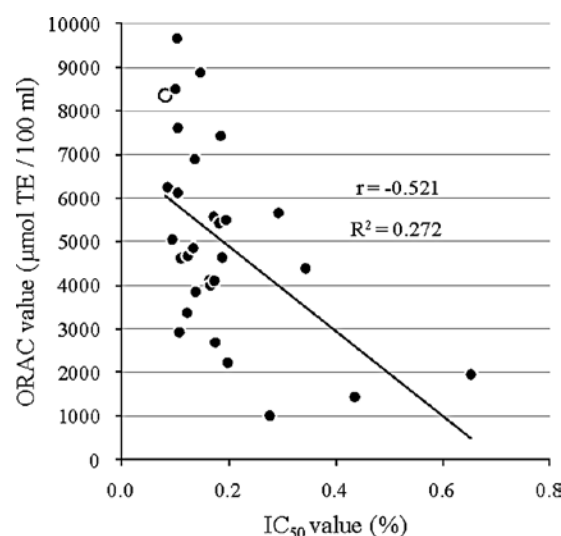


Figure 4. Relationship between ORAC and IC_{50} values of the 30 types of fish sauce. The open circle represents the puffer fish sauce.

Hernández-Ledesma *et al* reported on the angiotensin-converting enzyme (ACE)-inhibitory activity and ORAC value of β -lactoglobulin-derived peptides (35). It is known that the activity of ACE induces the onset of disease hypertension. This report by Hernández-Ledesma *et al* is notable as it extends the ORAC research field. As the data in this study represent hydrophilic-ORAC values, in the future it is essential to measure the lipophilic ORAC and the total ORAC values as well.

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