Antioxidative Effect of Coconut (*Cocos nucifera L.*) Water Extract on TBARS Value in Liver of Rats Fed Fish Oil Diet

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ABSTRACT

*Ethanolic extract of the coconut water showed antioxidant activity in the **in vitro** assay using an emulsified-aqueous system containing linoleic acid and β-carotene. To evaluate the possible antioxidative effect **in vivo**, a study with rats fed fish oil diet,was performed. Oral administration of the coconut water extract (CWE, 340mg/mL) did not influence food intake, daily body weight gain nor average liver weight of rats. It was investigated that the increase of liver TBARS value of rats fed fish oil diet was significantly (P<0,01) suppressed by oral administration 0,8ml of the coconut water extract (CWE, 340mg/mL)/rat twice per day during 14 days of experimental period. Oral administration of the coconut water extract also tended to suppress the decreasing effect of fish oil diet on liver ascorbic acid. Liver total glutathione of rats fed fish oil diet was significantly lower than that of rats fed AIN-93G standard diet, but oral administration of the coconut water did not significantly change liver glutathione. SOD activity in liver homogenate of rats fed fish oil diet and orally administrated by coconut water extract was significantly higher than that of those orally administrated by water. The result showed that the antioxidative properties of the coconut water extract have been demonstrated by in vivo assay using rats fed fish oil diet.*

INTRODUCTION

Coconut water, that is the liquid endosperm found in the fruit of coconut (*Cocos nucifera* L.), is well consumed by the peoples in the coconut producing countries as a natural refreshing drink (Rosario & Rubico, 1979; Jayalekshmy *et al.*, 1986; Pue *et al.*, 1992). The water from the young fruit is regarded as a delicious and nutritious drink. It has been reported that the water from unripe coconut is sterile, pyrogenic free and there was no evidence of an *in vitro* or *in vivo* hemolysis of canine and human blood (Fernandez, 1988; Brito & Dreiss, 1944[ab], Eiseman 1954). The green coconut water has been used for intravenous purposes in pediatric practice (Mojumdar, 1951), a readily available source of potassium for the cholera patients (Carpenter *et al.*, 1964), and for home rehydration in children with mild gastroenteritis (Adam & Bratt, 1992). However, on the nutritional point of view the young coconut water has received a little attention.

An investigation in our laboratory (Santoso, 1996) showed that the coconut water or its ethanolic extract exhibited an obvious antioxidant activity in the assay using an emulsified-aqueous system containing linoleic acid and β-carotene according to Miller method (Miller, 1971). The coconut water or its extract also exhibited a radical scavenging activity in the assay using superoxide dismutase which produces superoxide radicals.

The targets of antioxidant application are shifting from food ingredients to medical or nutritional

purposes because some human diseases including aging and cancer are now ascribed to oxidation of cellular components (Nakayama *et al.*, 1994). Recently, radical scavengers have attracted special concern because they can protect the human body from free radical attack which may cause many diseases including aging and cancer. It has been reported that numerous pathological conditions are produced by reactive oxygen species including superoxide, hydroxyl radical and hydrogen peroxide. And there are some indications that not only endogenous antioxidants but also dietary antioxidants may be effective protection from peroxidative damage in living systems (Namiki *et al.*, 1993).

It has been well established that fish oil diet has some beneficial effects such as in lowering plasma tryglycerides, cholesterol, incidence of atherosclerosis and cardiovascular, however, due to high content of n-3 polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (20:5 n-3, EPA) and docosahexaenoic acid (22:6 n-3, DHA) fish oil diet can potentiate the susceptibility of cellular membranes to lipid peroxidation (Hue *et al.*, 1989; Nardini *et al.*, 1995; Meydani *et al.*, 1991, Herold & Kinsella, 1986; Harris, 1989). A high dietary fish oil might overwhelm the normal antioxidant systems. The effect of dietary fish oil on increasing lipid peroxidation in rat organ has been proven with higher content of thiobarbituric acid rective substances (TBARS) in liver of rats fed with higher content of fish oil (Kobatake *et al.*, 1983; Saito & Nakatsugawa, 1994).

In order to evaluate the possible antioxidative effect of the coconut water extract *in vivo*, a study with rats fed with fish oil diet was conducted. The antioxidative status was studied by measuring the levels of lipid peroxidation product (TBARS) and antioxidant defense system (ascorbic acid, glutathione, superoxide dismutase and glutathione peroxidase activity) in liver of rats after 14 days of experimental period.

MATERIALS AND METHODS

Materials

The young coconut fruits originated from the Philippines were purchased from a fruit shop in Tokyo, Japan.

Preparation of coconut water extract

Preparation of the coconut water extract (CWE) principally was as follows. A 1000ml of the coconut water from freshly split fruits was freeze-dried. The freeze-dried material was then extracted with approximately 500ml of 99.5% ethanol (Wako Pure Chem. Indust., Ltd.) by stirring for 15 minutes at room temperature. After filtration (Whatman No. 2), the filtrate was evaporated until all ethanol evaporated, and the residue (34g) was then filled up to 100ml with water.

Evaluation of antioxidant activity

Antioxidant activity of the coconut water ethanolic extract (CWE) was evaluated by the method of Miller (Miller, 1971). This procedure is based on minimizing β-carotene loss in the coupled oxidation of linoleic acid and β-carotene in an emulsified-aqueous system.

A 2.0mg of crystalline β-carotene (Wako Pure Chem. Indust., Ltd.) was dissolved in 10mL chloroform. A 1.0mL of this solution was pipetted into a round-bottomed flask which contained 20mg purified linoleic acid (Wako Pure Chem. Indust., Ltd.) of Tween 40 (polyoxyethylene sorbitan monopalmitate). After removal of the chloroform on a rotary evaporator, 50mL of oxygenated deionized water was added, and then immediately stir vigorously. A 5.0mL aliquot of this emulsion was then pipetted into each of a series of test tubes which contains 0.2mL of antioxidant sample in ethanol. The control (blank) test contained only ethanol, and 100 μg/mL of BHA (butylated hydroxyanisole) or 100 μg/mL of BHT (butylated hydroxytoluene) was used as a reference antioxidant. The mixture in the test tubes was incubated at 50°C and read its absorbance at 470nm on a spectrophotometer (UV-160 SHIMADZU UV-Visible Recording Spectrophotometer) at time intervals.

Animals and diet

Male Wistar rats five weeks old were purchased from Tokyo Animal Experimental Co., Ltd. (Tokyo). After one week of adaptation, the rats were divided into four groups and used for this experiment, the schematic of experimental design is shown in Table 1. Groups FOW and FOC (eight rats/group) would be fed fish oil diet that the composition basically

followed that of the standard diet of AIN-93G (Reeves *et al.*, 1993) by replacing soybean oil with fish oil and by omitting TBHQ, groups AINW and AINC would be fed AIN-93G standard diet. The composition of the diets was shown in Table 2. Fatty acid composition of fish oil (San Omega EPA-18, Nihon Yushi Co. Ltd.) and soybean oil (Wako Pure Chem. Indust., Ltd.) used for this experiment are shown in Table 3 as determined by gas chromatography after methylation with BF_3 in methanol according to MetCalfe (1961). The rats were maintained individually in cages in a room with temperature of 22 ± 1°C and a humidity of 55 ± 5% with a 12 diurnal system. Feed and drinking water were provided *ad libitum*. Body weight of rats and food intake were monitored daily. The rats of groups FOC and AINC were orally administrated by 0.8ml of CWE/rat twice per day, those of group FOW, and AINW were by water in the same amount as a control treatment. Oral administration was done using a syringe started at 10.00a.m. and 5.00p.m. daily. The rats were sacrificed after 14 days of experimental period.

Table 1. **Design of animal experiment**

Group (No.of rat*)	Treatment
FOW (8)	Fish oil diet + Water
FOC (8)	Fish oil diet + CWE**
AINW (5)	AIN-93G diet + water
AINC (5)	AIN-93G diet + CWE

* Male Wister rats, six weeks old, were maintained under experimental condition for 14 days.
** CWE, coconut water extract (340 mg/ml). Water or CWE was orally administrated 0.8 ml/rat twice per day started at 10.00 a.m. and 5.00 p.m.

Table 2. **Diet composition**

Ingredient	AIN-93G#	Fish Oil Diet
	g/kg diet	
Cornstarch	397.486	397.500
Casein (≥85% protein)	200.000	200.000
Dextrinized cornstarch (90-94% tetrasaccharides)	132.000	132.000
Sucrose	100.000	100.000
Soybean oil*	70.000	–
Fish oil**	–	70.000
Fiber	50.000	50.000
Mineral mix (AIN-95G-MX)	35.000	35.000
Vitamin mix (AIN-93G-VX)	10.000	10.000
L-Cystine	3.000	3.000
Choline bitartrate (41.1% choline)	2.500	2.500
Tert-butylhydroquinone	0.014	–

Reeves *et al. (1993). J. Nutr.* **123:** 1939-1951
* Wako Pure Chem. Indust., Ltd.
**San Omega EPA-18, Nihon Yushi Co.Ltd.

At the end of experiment the rats were anaesthetized under Nembutal (70 μl/100g body weight of rat). After opening of the abdominal cavity the livers were perfused with 0.9% NaCl solution *via* the portal vein, and the livers were removed for analyses of thiobarbituric acid reactive substances, total ascorbic acid, glutathione, SOD and glutathione peroxidase activity.

Analysis of thiobarbituric acid reactive substances:

Thiobarbituric acid reactive substances (TBARS) in liver was determined by the standard procedure of Ohkawa *et al.* (1979). A 1.0g (wet basis) of liver tissue was homogenized with 9.0ml of 1.15% KCl solution in a Teflon Potter-Elvehjem homogenizer. A 0.2ml of liver homogenate was added 0.2ml of 8.1% sodium dodecyl sulfate, 1.5ml of 20% acetic acid solution adjusted to pH 3.5 with NaOH, and 1.5ml of 0.8% aqueous solution of TBA (Wako Pure Chem. Indust. Ltd.). The mixture was made up to 4.0ml with water, and then heated at 95°C for 60minutes. After cooling in a tap water, 1.0ml of water and 5.0ml of the mixture of n-butanol and pyridine (15:1, V/V) were added and shaken vigorously. After centrifugation at 4000rpm for 10minutes, the organic layer was taken and its absorbance at 532nm was measured on a spectrophotometer. 1,1,3,3-tetramethoxypropane (Wako Pure Chem. Indust., Ltd.) was used as a reference standard, and TBARS value was expressed as nmol MDA/g liver tissue.

Ascorbic acid determination

Total ascorbic acid in liver tissue was determined by hydrazine method according to Baba *et al* (1992). An appropriate amount of liver tissue with 10% of trichloroacetic acid was homogenized, and the homogenate was then centrifuged at 3500rpm for

5minutes. A 0.5ml of supernatant was added 1-2 drops of 2% 2,6-dichlorophenolindophenol, 0.5ml of 2% thiourea and 0.25ml of 2% 2,4-dinitrophenylhydrazine. The mixture was incubated at 50°C for 1.5 hour and then added 1.25ml of 85% H_2SO_4. After standing for 30minutes, the absorbance at 520nm was read on a spectrophotometer. L-ascorbic acid (Wako Pure Chem. Indust., Ltd.) was used as a standard.

Glutathione determination

Liver glutathione (GSH) was determined by DTNB method (Ellman, 1959; Beutler *et al.*, 1963; Hafeman *et al.*, 1974). An appropriate amount of liver homogenate was treated with a precipitating solution of metaphosphoric acid solution. Precipitating solution was: 1.67 g of glacial metaphosphoric acid (a mixture of HPO_3 and $NaPO_3$), 0.2g of disodium ethylenediamine tetraacetic acid (EDTA) and 30g of sodium chloride per 100mL of distilled water. DTNB reagent was: 40mg of 5,5' dithiobis (2-nitrobenzoic acid) (Wako Pure Chem. Indust., Ltd.) in 100mL of 1% trisodium citrate solution.

A 2.0mL of sample (a precipitated liver homogenate) with 2.0mL of 0.4mM Na_2HPO_4 was mixed with 1.0mL of DTNB reagent. After mixing, absorbance 412nm was recorded on a spectrophotometer (UV-160 SHIMADZU UV-Visible Recording Spectrophotometer) against glutathione (Wako Pure Chem. Indust., Ltd.) solution standards. Liver glutathione was expressed in μg/g tissue.

Superoxide dismutase activity

Superoxide dismutase (SOD) activity of liver homogenate was determined after the method of Elstner & Heupel (1976) and Oyanagui (1984). Into a 0.1ml of liver homogenate in a test tube was added 0.20ml of H_2O; 0.20ml of 0.1M phosphate borax buffer pH 8.2; 0.1ml of 10mM hydroxylamonium and 0.5mM xanthine (Wako Pure Chem. Indust., Ltd.) solution. This mixture was preincubated at 37°C for 10minutes, and then added 0.20ml of 0.005unit/ml xanthine oxidase (EC 1.2.3.2; Wako Pure Chem. Indust., Ltd.) solution. After incubation at 37°C for 30minutes, into the mixture was added 2.0mL of 30 μM N-1-naphtylethylenediamine-sulfanilic acid solution. After standing for 30minutes, the absorbance at 550nm was read on a spectrophotometer (UV-160 SHIMADZU UV-Visible Recording Spectrophotometer). Protein in liver homogenate was determined by LOWRY method (Lowry *et al.*, 1951). SOD activity was described as the amount (mg) enzyme that yielded 50% inhibition of nitrite formation from hydroxylamine under experimental condition, and it was expressed as nitrite unit (NU).

Glutathione peroxidase activity

Glutathione peroxidase (GSHPx) activity in liver homogenate was determined by the method of Noguchi *et al.* (1973). One milliliter of 0.4M sodium phosphate pH 7.0 containing 0.4mM EDTA, 1 mL of 2.0mM reduced glutathione (GSH) and 0.5mL of 0.01M sodium azide was mixed and kept in ice water. A 1.5mL of liver homogenate was added, after 5minutes preincubation at 37°C, 1 mL of 4.17mM H_2O_2 was added and mixed quickly. Immediately after mixing, duplicate 1 mL aliquots of the mixture were taken for a zero time control, transferred into 4mL of metaphosphoric acid solution, and measured the GSH. After 9 minutes of incubation, the amount of remaining GSH was determined. The activity was calculated as the difference of in the amount of GSH and expressed as unit per milligram protein. One unit equals the disappearance of one percent of the substrate (GSH) per minute. Protein concentration was determined by LOWRY method (Lowry *et al.*, 1951).

Statistical analysis

Data are presented as mean ± SD. Data were analyzed by one way analysis of variance (ANOVA), and least significant differences with Student's t-test was used for multiple comparison. Differences with $P<0.05$ were considered statistically different.

RESULTS AND DISCUSSION

Antioxidant activity of CWE *in vitro*

Figure 1 shows antioxidant activity of the coconut water extract (CWE, 340mg/mL) along with that of BHA (100 μg/mL) and BHT (100 μg/mL) as evaluated by the Miller method (Miller, 1971). In the emulsified system containing linoleic acid and β-carotene CWE exhibited a remarkable high antioxidant activity compared to that of BHA or BHT.

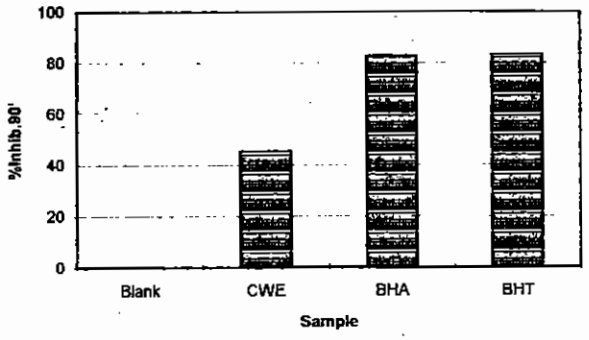

Figure.1. **Antioxidant activity (as % inhib. abs. 470nm at 90mnt) of the coconut water extract (CWE, 340mg/mL); BHA, 100 μg/mL and BHT, 100 μg/mL.**

CWE was found to contain 2.30 μg/mL of ascorbic acid, and on this assay the antioxidant activity of CWE was higher than that of 2.30 μg/mL ascorbic acid, this indicated that CWE contains natural antioxidant(s) other than ascorbic acid. The bioctive substances occuring in the coconut water extract seemed to delay the propagation rate of oxidation of linoleic acid and β-carotene when the system was incubated at 50°C. In the previous experiment, CWE also exhibited a superoxide scavenging activity in the *in vitro* assay using xanthine/xanthine oxidase system that produces superoxide radicals (Santoso, 1996), thus CWE also acted as a secondary antioxidant. It has also been reported that heating at boiling water temperature for ten minutes did not alter the radical scanvenging activity of the extract, this indicated that the coconut water contains antioxidants other than vitamin nor enzymatic antioxidant. The identification of the antioxidant substances naturally occuring in the coconut water is now in progress.

Food intake, body weight gain and liver weight

Average food intake, daily body weight gain and liver weight of rats are shown in Table 3. Daily food intake of rats fed fish oil diet was significantly lower than that of those fed AIN-93G standard diet. Feeding fish oil diet showed to decrease the daily food intake of rats, this result was in agreement with the study of Saito & Nakatsugawa (1994). Oral administration of 0.8ml CWE (340mg/mL)/rat twice per day did not change the daily food intake of rats fed fish oil diet nor that of those fed the standard diet. There was no significant differences in the daily body weight gain and in liver weight between rats fed fish oil diet and those fed the standard diet (Table 4). Oral administration 0.8mL CWE/rat twice per day did not influence the daily body weight gain nor average liver weight of rats fed fish oil diet and of those fed AIN-93G standard diet. During experimental period all rats were visually in a normal condition, there was no rat with diarrhea phenomenon although the fecal appearrance of rats fed fish oil diet showed whitish. Oral admintration of CWE did not affect the fecal appearrance of the experimental rats. The coconut water has been used as a natural drink for centuries and there is no report on the toxicological effects of the water has been published.

Table 3. **Food intake, body weight gain and liver weight of rats***

	Food intake	Body weight gain	Liver weight	
Group	(g/day)	(g/day)	(g)	(% body weight)
FOW	24.14 ± 3.65**	9.86 ± 1.40[a]	17.97 ± 0.85[a]	5.51 ± 0.86[a]
FOC	24.30 ± 0.65[a]	8.71 ± 0.94[a]	17.02 ± 1.05[a]	4.97 ± 0.40[a]
AINW	26.27 ± 1.32[b]	9.45 ± 2.22[a]	18.31 ± 2.51[a]	5.15 ± 0.46[a]
AINC	26.31 ± 1.42[b]	9.33 ± 1.83[a]	18.66 ± 3.92[a]	5.20 ± 0.68[a]

* Rats of group FOW and FOC were fed fish oil diet, those of group AINW and AINC were AIN-93G standard diet for 14 days. Group FOC and AINC were orally administrated by 0.8ml CWE/rat twice per day, group FOW and AINW were by water at the same amount.

** Each value represents mean ± SD, means within the same column without a common superscript letter are significantly different at $P < 0.05$.

Antioxidative effect of CWE on liver TBARS value

Fish oil diet showed to increase lipid peroxidation in liver as determined by a higher value of TBARS (Fig. 2A and B), this result is in agreement with some studies that due to rich in n-3 polyunsaturated fatty acids such as eicosapentaenoic acid (20:5 n-3, EPA) and docosahexaenoic acid (22:6 n-3, DHA) fish oil can potentiate the susceptibility of lipid peroxidation in cellular membranes (Saito & Nakatsugawa, 1993; Simopoulus, 1991; Hue *et al.*, 1989). Saito & Nakatsugawa (1993) reported that a significant increase was observed on liver TBARS of rats fed diet containing 15% fish oil, however, in this study

the diet containing 7% fish oil has been effective to increase the liver TBARS value of rats. This might be possible due to the high degree of peroxidation of the dietary fish oil as indicated by high POV and TBARS value (Table 4).

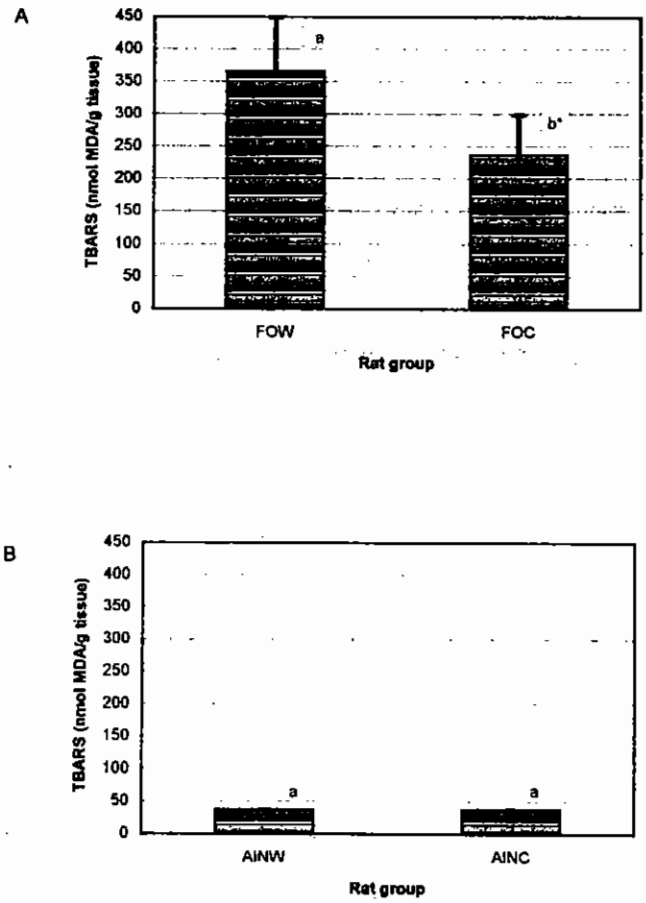

Figure. 2. Liver TBARS of rats. A) Group FOC and FOW were fed fish oil diet, B) group AINC and AINW were fed AIN-93G standard diet for 14 days.
Group FOC and AINC were orally administrated by 0.8ml CWE/ rat twice per day, FOW and AINW were by water at the same amount as a control treatment.
* Significantly different at P<0.01.

Table 4. **Fatty acid composition (g/100g)* and degree of peroxidation of dietary lipids**

Fatty acid	Fish oil#	Soybean oil##
12;0	2.99	0.00
14;0	6.97	0.08
16;0	18.78	11.24
16;1	8.42	0.12
16;4 n-3	1.13	0.00
17;0	1.64	0.00
18;0 iso	0.00	0.00
18;0	7.68	2.34
18;1	10.72	18.89
18;2 n-6	3.01	59.63
18;3 n-6	0.00	7.69
18;4 n-3	3.54	0.00
20;0	0.00	0.00
20;1	3.20	0.00
20;2 n-6	0.00	0.00
20;3 n-6	0.00	0.00
20;4 n-6	1.30	0.00
20;4 n-3	3.33	0.00
20;5 n-3	16.89	0.00
22;1	1.19	0.00
22;5 n-3	1.64	0.00
22;6 n-3	7.57	0.00
SFA	38.06	13.67
MUFA	23.54	19.01
PUFA	38.40	67.32
M/S	0.61	0.28
P/S	1.01	4.93
n-6 P	4.31	67.32
n-3 P	**34.09**	**0.00**
n-3/n-6	7.92	0.00
Degree of peroxidation		
POV *(meq kg)*	163	5.50
TBARS *(μ moleMDA/g*	616	0.22

*Determined by gas chromatography.
#San Omega EPA 18, Nihon Yushi Co., Ltd.
##Wako Pure Chem. Indust., Ltd.

Oral administration of 0.8mL CWE/rat twice per day significantly (P<0.05) suppressed the increase in liver TBARS of rats fed fish oil diet after 14 days of experimental period. The result (Table 5) also showed that in the absence of lipid peroxidation inducer, CWE did not cause a substantial increase nor decrease in

liver peroxidation level. CWE seems to assure a better protection against oxidative risk induced by fish oil diet. On the liver antioxidant defense systems, there were no significant differences on ascorbic acid, glutathione (GSH) nor glutathione peroxidase (GSHPx) in liver of rats orally administrated by CWE and those orally administrated by water. A significant increase was observed on SOD activity in liver of rats orally administrated by CWE for 14 days of experimental period. The higher activity of liver SOD caused by oral administration of CWE might be, in part, one of the factors of the suppressing effect on liver TBARS value of rats fish oil diet. Otherwise, the antioxidant substances in CWE directly reacted with lipid radicals to convert them to more stable products (Larson, 1988). CWE is a crude extract containing substances with rather diverse activities, and therefore the antioxidant activity can not be onsidered as a single activity. Further study using an isolated antioxidant from the coconut water is inevitable to elucidate the mechanism of this antioxidative effect.

Table 5. **Liver ascorbic acid, glutathione (GSH), superoxide dismutase (SOD) and glutathionine peroxidase (GSHPx)**

Group	Ascorbic acid (μg/g tissue)	GSH (μmol/g tissue)	SOD act (Nitrite unit)	GSHPx (unit*/g)
FOW	152.14 ± 29.39[a]	11.95 ± 3.67[a]	37.08 ± 7.83[a]	33.39 ± 4.52[a]
FOC	185.82 ± 35.58[ab]	11.19 ± 4.78[a]	52.85 ±10.67[b]	32.81 ± 7.02[a]
AINW	255.44 ±28.04[c]	22.78 ± 3.28[b]	39.02 ± 4.86[a]	35.85 ± 6.37[a]
AINC	214.73 ±41.11[bc]	21.98 ± 2.28[b]	34.70 ± 2.20[a]	35.55 ± 2.91[a]

*Rats of group FOW and FOC were fed fish oil diet, those of group AINW and
AINC were AIN-93G standard diet for 14 days. Group FOC and AINC were orally administrated by 0.8ml CWE/rat twice per day, group FOW and AINW were by water at the same amount.

**Each value represents mean ± SD, means within the same column without a common superscript letter are significantly different at P<0.05.

*One unit equals the disapperance of one percent of glutathione per minute.

The young coconut water is commonly consumed as natural drink in the growing areas. In the natural state coconut water or meat is sterile (Fernandez, 1988) and the use of coconut as food is notable for its reported lack of antinutritional factors (Padua-Resurreccion & Banzon, 1979). The above results showed that the coconut water contained antioxidant substances that its efficacy has been demonstrated by *in vivo* assay using rats fed fish oil diet. The result may encourage us to become more aware of the possibility that the coconut water can be used as a part of functional drink.

CONCLUSION

In conclusion, it was found that oral administration of ethanolic extract of the coconut water did not affect the performance nor the growth of rats fed normal diet and of those fed fish oil diet. Oral administration 0.8mL of the coconut water extract (340mg/mL) per rat per day for 14 days was effective to suppress the increase in liver TBARS of rats fed fish oil diet. However, in the absence of lipid peroxidation inducer, the coconut water did not cause a substantial increase nor decrease in liver peroxidation level. Further study with isolated substances from the coconut water is necessary to elucidate the mechanism of the antioxidant activity.

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