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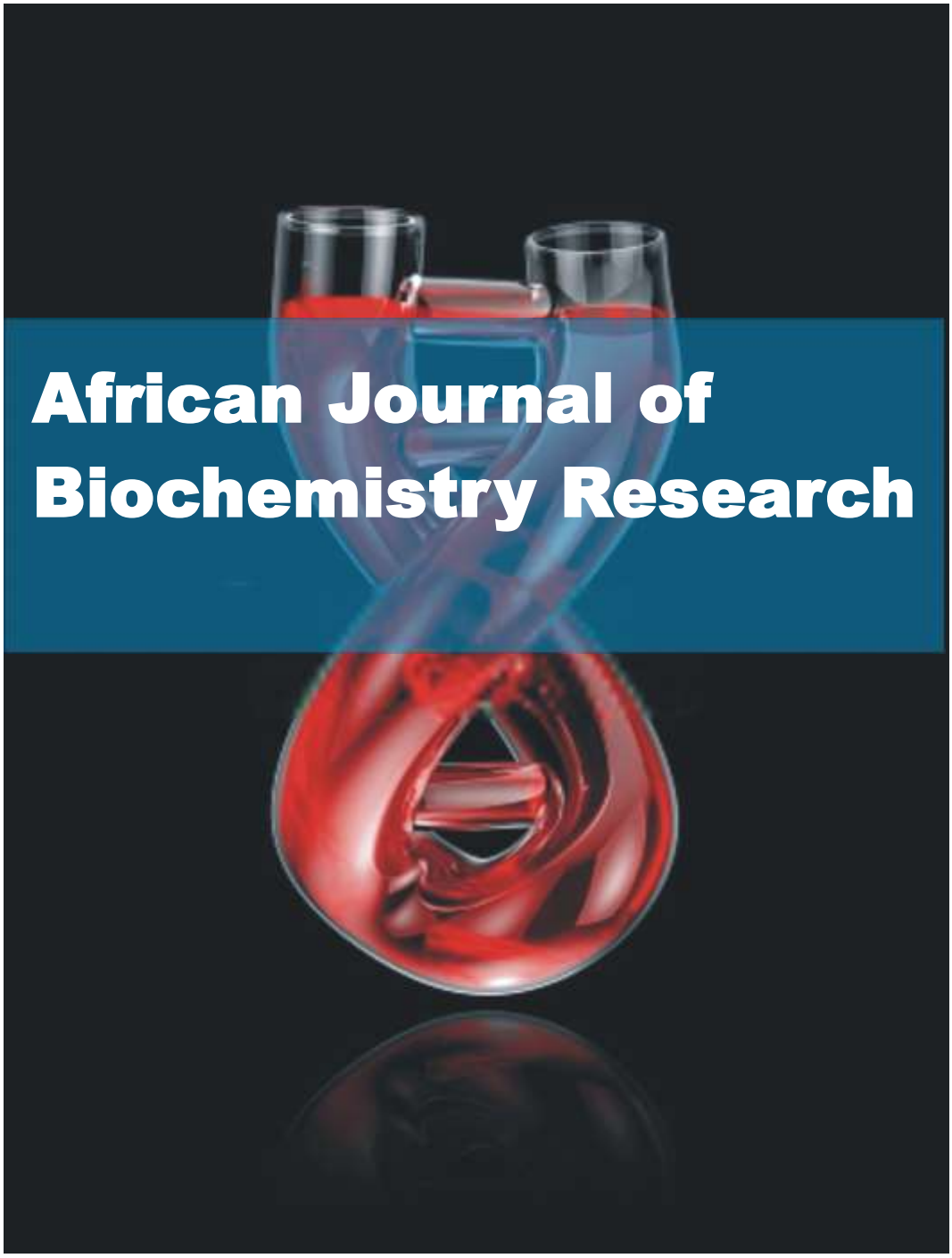

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Full Length Research Paper

Effects of garden egg, carrot and oat-supplements on biochemical parameters in cadmium exposed rats

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Cadmium (Cd) toxicity is influenced by dietary components, such as fiber and minerals. Garden egg, carrot and oat are rich sources of fiber. Thus, this study examines the effect of *Solanum melongena* (garden egg), *Daucus carota* (carrot) and oat-supplements on selected biochemical parameters in the plasma and tissues of cadmium-exposed rats. Twenty-five healthy male Wistar rats (140±50 g) were distributed into five treatment groups in which rats in group one were not exposed to cadmium, and served as control while rats in group two were exposed to cadmium only in addition to their normal diet. Cadmium was administered by gastric intubation at a dose of 5 mg Cd/kg body weight as $CdCl_2$. H_2O was given three times a week for six weeks. Rats in Groups 3 to 5 were treated similarly with cadmium, but with their normal diet supplemented with 5% garden egg, carrot and oat, respectively. A significant (P<0.05) increase was observed in alanine aminotransferase (ALT)/aspartate aminotransferase (AST) activity in the plasma/kidney of rats exposed to Cd, while a significant (P<0.05) decrease was observed in liver ALT/AST activity. Likewise, the levels of liver, kidney and intestine alkaline phosphatase (ALP), superoxide dismutase (SOD) and lipid peroxidation were increased compared to the control. Conversely, feeding with garden egg, carrot and oat significantly (P<0.05) reversed these effects of cadmium, compared to rats maintained on cadmium only. The results suggest that garden egg, carrot and oat contain bioactive/antioxidant properties which help in ameliorating cadmium toxicity.

Key words: Cadmium, lipid peroxidation, dietary fiber, carrot.

INTRODUCTION

Cadmium (Cd) is an abundant and ubiquitously distributed heavy toxic metal that is widely used in modern industries (Novelli et al., 2000). It is of great commercial importance due to its agricultural and industrial use (WHO, 2000; Jarup, 2003), but is a serious environmental and industrial pollutant because it easily contaminates soil, plants, air and water (Ognjanovic et al., 2010). Cd has a long biological half-life, contaminates water and food and accumulates in human tissues, especially the liver and kidney, causing damage (WHO,

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2000). It is not biodegradable and thus the risk of human exposure is constantly increased as it enters the food chain (Agency for Toxic Substances and Disease Registry (ATSDR), 2008).

Exposure to cadmium through food sources, especially leafy vegetables is also common and it is the main route of exposure for the non-smoking and non-occupationally exposed population (Kierstin, 2003). Cadmium toxicity results from its promotion of oxidative damage by increasing the cellular concentration of reactive oxygen species (ROS) and by reducing the cellular antioxidant capacity (Corticeiro et al., 2006). During exposure, cadmium accumulates predominantly in the liver, kidneys, reproductive organs and tissues (Godt et al., 2006; Takamure et al., 2006) and this is due to the liver and kidney being the most susceptible organs (Asagba, 2009). Increase in aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities in the blood is a manifestation of Cadmium-induced damage of the liver (Kowalczyk et al., 2003). At the cellular level, cadmium induces oxidative stress, cell proliferation, and apoptosis (Turner and Lysiak, 2008; Tremellen, 2008).

Efforts to ameliorate cadmium (Cd) absorption and toxicity are thus of great importance since exposure to Cd due to environmental contamination is still inevitable in many populations (Callegaro et al., 2010). Ishihara et al*.* (2001) reported that nutritional deficiencies are believed to have aggravated the concurrent liver, kidney and bone disorders seen in "Itai-Itai" disease patients. Recent studies have shown a correlation between cadmium related kidney and bone diseases and nutritional deficiencies, such as proteins, trace elements and antioxidants (Asagba et al*.,* 2004; Lawal and Ellis, 2011; Ferramola et al., 2012). This, according to Asagba et al*.* (2004), indicates that nutritional status greatly influences the metabolic fate and toxicity of cadmium. Liu et al. (2004) reported that diets containing high fiber content decrease absorption of cadmium and Asagba and Eriyamremu (2007) reported that the uptake, distribution and toxicity of cadmium are influenced by the type or composition of the diet of a population.

Fruits and vegetables have long been recognized as very useful in controlling and modulating various functions in the body to maintain normal state of health and reduce the risk of diseases. This is because they contain phytochemicals that help in the prevention of diseases (Prior, 2003) and fiber, which pushes food through the digestive system, absorbing water and easing defecation.

Fibers act by changing the nature of the contents of the gastrointestinal tract and influencing how other nutrients and chemicals are absorbed (Eastwood and Kritchevsky, 2005). Carrot, garden egg and oat are good sources of crude fiber (Ejoh et al., 1996), but few studies have evaluated their effects on Cd toxicity. The present study was aimed at investigating the effects of carrots, garden egg and oat- on some biochemical parameters in the plasma and tissues of cadmium exposed rats.

MATERIALS AND METHODS

Chemicals

The reagents used in this study were of analytical grade from the British Drug House.

Plant materials

Garden egg, carrot and oat were purchased from Effurun market, Effurun, Delta State, Nigeria. Garden egg and carrot were grated and sun-dried to constant weight, while the oat was mashed with hands. Experimental diets were then prepared by supplementing grower's mash with 5% of the processed garden egg, carrot and oat.

Experimental animals

Twenty five healthy male Wistar rats with an average weight of 140±5 g were used for this study. They were obtained from the animal house, Faculty of Basic Medical Sciences, Delta State University, Abraka, and housed in standard cages. They were allowed for two weeks to acclimatize to laboratory condition before the commencement of the experiment. The animals were treated in accordance with internationally accepted standards and protocols for animal care.

Experimental design

The rats were distributed into five treatment groups with five rats in each group. Rats in group one were not exposed to cadmium and served as control. Rats in group two were exposed to cadmium only in addition to their normal diet. Cadmium was administered by gastric intubation at a dose of 5 mg Cd/kg body weight as $CdCl_2$. H_20 three times a week for six weeks. Rats in Groups 3 to 5 were treated similarly with cadmium, but with their normal diet supplemented with 5% garden egg, carrot and oat, respectively. At the end of the treatment period, the rats were weighed and sacrificed by cervical dislocation.

Collection and treatment of samples

The abdominal and thoracic region of each rat was opened with the aid of dissecting kit to expose the kidney, heart, intestines, etc. Blood was collected from the heart by means of 5 ml hypodermic syringe and needle into a heparinised tube; it was centrifuged at 3,000 *g* for 10 min, and the plasma was obtained for biochemical assay. The liver, kidney and intestine of each rat were excised and weighed, and 10% homogenates were prepared under cold conditions with phosphate buffer (pH 7.4). The homogenates were centrifuged at 5000 g for 10 min and the supernatants were carefully separated from the residue and used for biochemical assays.

Biochemical analysis

The activities of ALT and AST in the plasma and tissues were assayed by the method of Reitman and Frankel (1957). The activity of the aminotransferases is expressed in units/ml. Assay for superoxide dismutase (SOD) was done by the method of Misra and

Table 1. Effect of garden egg, carrot and oat on body weight gain and organ body/weight ratio of cadmium exposed rats.

Parameter	Group 1 (Ctrl+ND)	Group 2 (Cd + ND)	Group 3 (Cd+ + ND + GE) (%)	Group 4 (Cd+ ND +C) (%)	Group 5 (Cd+ ND +O)
Body weight gain (g)	152.5±2.5^{a}	116.3±2.39^{b}	137.5±4.77^{c} (18.2)	135.0±2.89^{c} (16.1)	126.7±3.39^{d} (8.9)
Liver/body weight ratio($\times10^{-2}$)	2.61±0.124^{a}	1.51±0.037^{b}	2.02±0.044^{c}	2.21±0.123^{c}	2.09±0.126^{c}
Kidney/ Body($\times10^{-3}$)	2.17±0.124^{a}	1.51±0.037^{b}	1.69±0.15^{c} (27.1)	1.66±0.035^{c} (24.8)	1.75±0.12^{c} (31.6)

ND = Normal Diet; +Cd = + Cadmium; +Cd + GE = +cadmium + garden egg; +Cd + C = +cadmium + carrot; +Cd + O = + cadmium + Oat. Values are mean ± SD. Values on the same row with different superscript differ significantly ($p< 0.05$) from each other.

Table 2. Effect of garden egg, carrot and oat on SOD activity and lipid peroxidation levels in the liver, kidney and intestine of Cd exposed rats.

Parameter	Group 1 (Ctrl+ND)	Group 2 (Cd + ND)	Group 3 (Cd+ + ND + GE) (%)	Group 4 (Cd+ ND +C)	Group 5 (Cd+ ND +O)
SOD (units/g tissue)					
Liver (Unit/g tissue)	84.4± 3.3^{a}	37.0± 10.7^{b}	75.9± 2.5^{c} (105.1)	58.4±6.9^{c} (57.9)	67.5± 2.6^{d} (82.4)
Kidney (Unit/g tissue)	79.1± 8.0^{a}	52.2± 4.4^{b}	73.2±5.0^{a} (40.2%)	66.5±3.3^{c} (27.4)	54.9±2.0^{b} (5.2)
Intestine (Unit/g tissue)	32.6±2.8^{a}	19.2±1.4^{b}	20.3±2.1^{b} (5.7)	24.6±2.4^{b} (28.1)	22.9±2.0^{b} (19.3)
Lipid peroxidation (µmole MDA/g tissue)					
Liver (µmole MDA/g tissue)	139.0±6.11^{a}	201.1±3.55^{b}	162.3±8.53^{c} (19.3)	158.7±7.55^{c} (21.1)	161.4±2.72^{c} (19.7)
Kidney (µmole MDA/g tissue)	81.92±8.02^{a}	140.28±2.2^{b}	128.78±3.87^{b} (8.2)	114.8±2.40^{c} (18.2)	124.3±3.24^{b} (11.4)
Intestine (µmole MDA/g tissue)	168.0±1.58^{a}	183.0±1.52^{b}	175.8±1.60^{a} (3.9)	171.0±4.02^{a} (6.6)	175.0±1.50^{a} (4.4)

ND = Normal Diet; +Cd = + Cadmium; +Cd + GE = +cadmium + garden egg; +Cd + C = +cadmium + carrot; +Cd + O = + cadmium + Oat . Values are mean ± SD. Values on the same row with different superscript differ significantly ($p< 0.05$) from each other.

Fridovich (1972) based on the inhibitory effect of SOD on the initial rate of epinephrine auto-oxidation. The activity of SOD is expressed in unit/g tissue. One unit is the amount of the enzyme necessary to cause 50% inhibition of the oxidation of epinephrine to adenochrome for 1 min. The activity of alkaline phosphate (ALP) in the tissues was determined by the method of Annino and Giese (1976). The activity of the enzyme is expressed in units/g tissue. One unit is the amount of the enzyme that forms one micromole of p-nitrophenol per minute. The level of thiobarbituric acid reactive substances (TBARS) which is an index of lipid peroxidation was determined by the method of Gutteridge and Wilkins (1982). Values of TBAS are reported in terms of malondialdehyde (MDA) and expressed as µmole MDA/g tissue. The amount of MDA in the samples was quantified using a molar extinction coefficient of 1.56×10^{5} m/cm.

Data analysis

The values are reported as Mean ± SEM. The mean values between the groups were compared by using analysis of variance (ANOVA) and least significance test (LSD) procedure using the statistical package for the social sciences software (SPSS). The results were considered significant at $P<0.05$ level.

RESULTS

The effect of garden egg, carrot and oat on body weight gain and organ/body weight ratio of rats exposed to cadmium is presented in Table 1. The body weight gain of rats exposed to cadmium (Cd) significantly ($p< 0.05$) decreased in comparison to the control. However, this parameter significantly ($p< 0.05$) increased on feeding the Cd exposed rats with diet supplemented with garden eggs, carrots and oats in relation to rats fed on Cd+ Normal Diet only (control, not included). The above observation was the same trend for organ/ body weight ratio. Consequently, the study indicates that feeding of garden egg, carrots and oats to rats can reduce the effect of cadmium on body weight gain and organ/ body weight ratio of the rats.

The effect of garden eggs, carrot and oat on levels of SOD and lipid peroxidation in the liver, kidney and intestine of rats exposed to cadmium are presented in Table 2. The liver SOD level of rats administered Cd significantly ($p <0.05$) decreased compared to the control. However, feeding cadmium exposed rats with garden egg, carrot and oat increased the SOD level significantly ($p<0.05$) in relation to rats maintained on Cd and normal diet only. The garden egg, carrot, and oat caused 105.1, 57.9 and 82.4%, increase respectively. Like in the liver, the kidney SOD level of rats exposed to Cd decreased significantly ($p<0.05$) in relation to the control.

Conversely, this enzyme increased significantly ($p<0.05$) on feeding cadmium exposed rats with garden

Table 3. Effect of garden egg, carrot and oat on alkaline phosphate activity in the liver kidney and intestine of Cd exposed rats.

Parameter	Group 1 (Ctrl+ND)	Group 2 (Cd + ND)	Group 3 (Cd+ + ND + GE) (%)	Group 4 (Cd+ ND +C) (%)	Group 5 (Cd+ ND +O) (%)
Liver (unit/g tissue)	98.8±5.3[a]	88.1±4.4[b]	91.6±3.0[b] (4.0)	94.4±2.8[a] (7.2)	89.0±5.3[b] (1.0)
Kidney (unit/zg tissue)	47.4±1.8[a]	48.2±3.8[a]	47.2±0.9[a] (2.1)	49.0±5.7[a] (1.7)	47.5±0.9[a] (1.5)
Intestine (unit/g tissue)	9.9±1.3[a]	5.8±0.5[b]	7.8±0.6[a] (34.5)	8.8±0.03[a] (51.7)	8.8±0.03[a] (43.1)

ND = Normal Diet; +Cd = + Cadmium; +Cd + GE = +cadmium + garden egg; +Cd + C = +cadmium + carrot; +Cd + O = + cadmium + Oat . Values are mean ± SD. Values on the same row with different superscript differ significantly (p< 0.05) from each other.

egg, carrots, and oat in relation to rats maintained on Cd and normal diet only. Garden egg, carrots and oat caused 40.2, 27.4 and 5.2%, increase respectively. Also, like in the liver and kidney, the intestine SOD level of cadmium exposed rats decreased significantly (p<0.05) in relation to the control. However, unlike the liver and the kidney, the SOD level of intestine of cadmium exposed rats fed with garden egg, carrots and oat was not significantly different in relation to rats maintained on Cd and normal diet only. Thus, the study indicates that feeding of rats with garden egg, carrot and oat ameliorated effect of cadmium on SOD activities in both liver and kidney, but not in intestine.

The effect of garden egg, carrot and oat on the level of lipid peroxidation in the liver, kidney and intestine of rats administered cadmium is presented in Table 2. The liver lipid peroxidation of rats exposed to Cd increased significantly (p<0.05) compared to the control. This however decreased significantly (p<0.05) on feeding the Cd exposed rats with garden egg, carrots and oat by percentage of 19.3, 21.1 and 19.7, respectively, in relation to the rats exposed to Cd only. Similarly, the kidney lipid peroxidation level of rats administered Cd increased significantly (p<0.05) in comparison to the control.

However, when the Cd exposed rats were fed garden egg, carrot and oat, there was an insignificant decrease (for garden egg and oat) and a significant (P<0.05) decrease (for carrot) in the level of lipid peroxidation compared to rats maintained on Cd only. The percentage decrease in kidney lipid peroxidation was 8.2, 18.2 and 11.4% of garden egg, carrot and oats, respectively. Like the liver and kidney, the intestine LPO level of rats administered Cd increased significantly in relation to the control, but insignificantly decreased on feeding with garden egg, carrot and oat in relation to the rats maintained on Cd only. The decrease caused by garden egg, carrot and oat was 3.9, 6.6 and 4.4%, respectively. The study thus indicates that garden egg, carrot and oat have the ability to reduce the effect of cadmium on tissue membrane lipid peroxidation.

Table 3 represents the effects of garden egg, carrot and oat on ALP activity in the liver, kidney and intestine of cadmium exposed rats. In the liver, the alkaline phosphates activity of Cd exposed rats was significantly (p<0.05) reduced compared to the control. However, feeding Cd exposed rats with garden egg, carrot and oat had no significant effect (except with carrot which was observed to have a significant increase) on ALP activity in relation to rats maintained on Cd only. The Kidney ALP activity of rats treated with Cd was not significantly different from control. The activity of the enzyme remained comparable to the control even after the feeding of carrot, garden egg and oat to cadmium treated rats. The intestine ALP activity of Cd exposed rats was observed to be significantly reduced in relation to control. On the other hand, the activity of the enzyme increased significantly (p<0.05) on feeding with garden egg, carrot and oat by 34. 5, 5.7 and 43.1%, respectively in relation to rats maintained on Cd only.

Table 4 shows the effect of garden egg, carrot and oat on ALT and AST activities in the liver, plasma and kidney of rats administered cadmium. The kidney ALT activity of Cd exposed rats was significantly (p<0.05) increased in relation to control. This was reversed when garden eggs, carrot and oat were added to the diet of the exposed rats as the ALT activity was reduced significantly (p<0.05) in comparison with those maintained on Cd only. The garden egg, carrot and oat caused 48.9, 13.8 and 8.8%, decrease respectively. The ALT activity of the liver of Cd exposed rats decreased significantly (p<0.05) in relation to control. However, with garden egg, carrots and oat supplements in the diet of the Cd exposed rats, there was significant (p<0.05) increase in the ALT activity in relation to rats maintained on Cd only. The increase caused by garden egg, carrot and oat was to the tune of 166.7, 162.5 and 170.8%, respectively.

The plasma of rats administered cadmium had a significantly (p<0.05) increased ALT activity in comparison to the control. When fed with garden egg, carrot and oat, the activity of plasma ALT of Cd exposed rats was grossly reversed significantly (p<0.05) in relation to those maintained on Cd only. Garden egg, carrot and oat reduced the effect of Cd on plasma ALT activity by 23.8%, 20.5% and 21.9%, respectively. The study therefore shows that supplementing diet of rats with garden egg, carrot and oat reversed the cadmium decrease of liver ALT activity and Cd induced increase of both kidney and plasma ALT activity.

The kidney AST activity of Cd exposed rats significantly

Table 4. Effect of garden egg, carrot and oat on ALT and AST activities in the kidney, liver and plasma of Cd exposed rats.

Parameter	Group 1 (Ctrl+ND)	Group 2 (Cd + ND)	Group 3 (Cd+ + ND + GE) (%)	Group 4 (Cd+ ND +C)	Group 5 (Cd+ ND+O)
ALT (units/ml)					
Kidney U/ml	476.6±35.1[a]	913.33±25.16[b]	466.66±24.43[c] (48.9)	787.50±27.58[d] (13.8)	833.33±49.32[d] (8.8)
Liver U/ml	920.00±103.27[a]	160.00±65.31[b]	426.66±46.18[c] (166.7)	420.00±76.57[c] (162.5)	433.33±76.57[d] (170.8)
Plasma U/ml	437.50±47.87[a]	750.00±91.28[b]	571.25±56.62[c] (23.8)	596.25±18.87[c] (20.5)	586.00±35.04[c] (21.9)
AST (units/ml)					
Kidney U/ml	150.00±14.49[a]	550.00±130.00[b]	433.33±24.94[c] (21.2)	490.00±110.00[d] (10.9)	433.33±9.42[d] (21.2)
Liver U/ml	840.00±138.56[a]	285.20±7.88[b]	348.80±28.48[c] (22.3)	348.00±28.60[c] (22.0)	358.60±50.96[c] (25.7)
Plasma U/ml	440.00±43.58[a]	768.75±51.47[b]	626.25±36.37[c] (18.5)	697.50±53.77[d] (9.3)	628.30±32.53[c] (18.3)

ND = Normal Diet; +Cd = + Cadmium; +Cd + GE = +cadmium + garden egg; +Cd + C = +cadmium + carrot; +Cd + O = + cadmium + Oat . Values are mean ± SD. Values on the same row with different superscript differ significantly ($p< 0.05$) from each other.

($p<0.05$) increased in relation to control. Conversely, the activity of the enzyme was significantly reduced by 21.2, 10.9 and 21.2% relative to those maintained on Cd only when Cd exposed rats were fed with garden egg, carrots and oat supplemented diets, respectively. The liver AST activity was significantly ($p<0.05$) reduced in rats administered Cd compared to the control. However, on feeding with garden egg, carrot and oat, there was a significant ($p<0.05$) increase in the liver AST activity in relation to rats maintained on Cd only. The increase caused by garden egg, carrot and oat was 22.3, 22.0 and 25.7%, respectively. The plasma AST activity of Cd treated rats was significantly ($p<0.05$) increased in relation to the control. However, when fed with garden egg, carrot and oat, there was a significant reduction of AST activity compared to rats maintained on Cd only by 18.5, 9.3 and 18.3%, respectively. Thus, the study indicates that feeding of garden egg, carrot and oat ameliorates the effects of cadmium on plasma and tissue AST activity.

DISCUSSION

An attempt was made in this study to examine the effect of garden egg, carrot and oat on some biochemical parameters in the organs and plasma of Cd-exposed rats. The significant decrease ($P<0.05$) of body weight gain of Cd exposed rats when compared to control is consistent with previous studies (Eriyamremu et al., 2005; Asagba and Eriyamremu, 2007).

The significant difference ($P<0.05$) noticed in the weight gain and organ/body weight ratio in the Cd exposed rats (compared to the control) is an indication of Cd toxicity which is also consistent with previous reports (Asagba et al., 2004; Asagba and Eriyamremu, 2007; Eriyamremu et al., 2005). It has been established that weight loss caused by cadmium can be linked to its influence on nutrient digestion and availability (Eriyamremu et al., 2005). The reversal of the effect of Cd on body weight gain and organ/body weight ratio by garden egg, carrot and oat suggests strongly that these plants have bioactive components that may be protective in Cd toxicity.

The result of the present study indicates that the administration of Cd induces peroxidative injury in the liver, kidney and intestine of rats, which is mediated by the inhibition of SOD activity and increase in the level of lipid peroxidation (Table 2). This is consistent with findings of previous studies (Asagba et al., 2004; 2006; Asagba and Eriyamremu, 2007; Ding et al., 2013, Nazima et al., 2015; Ayala et al., 2014; Akomolafe et al., 2016, Oyinloye et al., 2016). One of the mechanisms attributed for the inhibition of SOD by Cd is the displacement of essential metals such as copper and zinc from the enzyme (SOD) and the inhibition of SOD activity might lead to oxidative stress (Timbrell, 1995). Under the condition of oxidative stress, lipid peroxidation occurs as evidenced by the production of malondialdehyde (MDA), which can be used as an index of per oxidative injury *in vivo* and susceptibility of tissues to oxidative stress. In addition, Arroyo et al. (2012) showed that Cd competes with essential metals such as zinc, selenium, copper and calcium and thus interfere with various cellular processes such as metal membrane transport and energy metabolism.

The plasma, liver and kidney were assayed for amino transferees (ALT/AST) activities, which are the indices of tissues (particularly liver) damage (WHO, 2000).

According to reports, exposure to Cd may lead to kidney and liver damage (Alfvén et al., 2002; Asagba et al., 2002, 2006). Thus, the activity of plasma ALT/AST is expected to increase in Cd exposed rats and decrease correspondingly in the liver as damage to this organ may lead to leakage of these enzymes into the plasma (Lee et al., 2009; Kowalczyk et al., 2003; Shati, 2011). The result of the study confirmed this for plasma and liver ALT/ AST activities (Table 4). However, it is noteworthy that the

kidney ALT/AST activity of Cd exposed rats was higher compared to control (Table 4).

Reports show that amino transferees are involved in amino acid metabolism (Kowalezyk et al., 2003). Thus, the observed increase in the activity of kidney amino transferees may be an indication of increased metabolism of amino acid occasioned by Cd stress in this organ. Cd contamination has severally been shown to induce oxidative stress, which leads to the progression of severe pathological conditions after prolonged retention in tissues (Baba et al., 2013; Matović et al., 2011). Many authors have shown that Cd metabolism in the body promotes the generation of reactive oxygen species (ROS) such as superoxide ion, hydroxyl radicals and hydrogen peroxide (Baba et al., 2013; El-Refaiy and Eissa, 2013).

The toxicity of Cd can also be accounted for by the reduction in the activity of alkaline phosphatase (ALP) in the liver, kidney and intestine of Cd exposed rats as recorded in this study. Cadmium, being a non-redox metal, is capable of indirectly eliciting oxidative damage to the liver by depleting cellular antioxidant levels especially glutathione as well as depleting protein-bound sulfhydryl groups (Wang et al., 2015). According to Timbrell (1995), the inhibition of enzymes such as ALP by Cd may be by the displacement of essential co-factors in the active site of the enzyme by Cd and also by binding of Cd to SH groups which are essential for the activity of enzyme.

Furthermore, the inclusion of garden egg, carrot and oat to the diet of Cd exposed rats ameliorated the effect of Cd on the above parameters in the indicated organs. This shows that these plants may be effective against Cd induced stress. This may be because they contain antioxidants and high fiber content, which has been shown to terminate formation of free radicals (Sarkar et al., 1995; Anderson et al., 2009; Noda et al., 2001) and Chelates metals (Asagba et al., 2004) in biological systems, respectively. Reports show that these plants contain antioxidants such as Vitamin A, C, and E, Caffeic acid, chlorogenic acid, amongst others and are rich in crude fiber, which have been shown to reduce Cd uptake (Jung et al., 2011; Bliss and Elstein, 2004; Berglund et al., 1994; Asagba et al., 2004).

Comparatively, no consistent trend is noticeable for the effect of these plants on Cd induced toxicity in rats. It can therefore be assumed that any of these plants is equally effective in ameliorating Cd toxicity in rats.

Conclusion

The results of the presents study indicate that cadmium caused an increase in the level of lipid peroxidation and the activity of plasma and kidney ALT/AST and a decrease in the activity of liver ALT/AST. Likewise, the activity of ALP and SOD in the liver, kidney and intestine of rats was also decreased. But feeding with garden egg, carrot and oat reversed these effects of cadmium, except for the effect on intestine SOD activity. These results indicate that these plants are protected from Cd toxicity.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Effect of organic acids on the alteration of black rice anthocyanins by *Enterobacter aerogenes* NBRC 13534

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In a previous study, it was reported that *Enterobacter aerogenes* and four organic acids are involved in the alteration of black rice anthocyanins. To investigate the factors involved in altering the coloration of black rice anthocyanins, organic acids (caffeic acid, ferulic acid, malonic acid, and *p*-hydroxybenzoic acid) were individually added to potato dextrose agar medium containing black rice anthocyanins and cultured with *E. aerogenes* NBRC 13534 at 37°C for five days. As a result, the color change of black rice anthocyanins was found in the medium supplemented with caffeic acid or ferulic acid among the four organic acids. HPLC analysis of anthocyanins in each medium revealed three new peaks not found in the original black rice anthocyanins in the sample supplemented with caffeic acid. Also, two new peaks were observed in the medium supplemented with ferulic acid.

Key words: Anthocyanin, anthocyanin modification, black rice, *Enterobacter aerogenes*, microorganism, color, pigment.

INTRODUCTION

Anthocyanins are a group of flavonoid-based pigments that impart various colors such as red, blue, and purple to flowers and fruits (Adachi and Yoshitama, 2004). The structure of anthocyanins is based on various combinations of anthocyanidin, sugar, and organic acid. Six types of anthocyanidins are predominantly found in nature: cyanidin, delphinidin, malvidin, pelargonidin, petunidin and peonidin (Zhao et al., 2014). As the sugar component, D-glucose, D-galactose, L-rhamnose, D-xylose, D-arabinose, and the like are often bound to anthocyanidins by a β-glycosidic bond. In addition, acylated anthocyanins are characterized by ester linkage of the organic acid to the sugar moiety and exhibit increased stability of color quality (Terahara, 1993). In contrast, anthocyanidins are characterized by low color stability, fading rapidly (Tsuda, 2012). Organic acids that bind to anthocyanins are largely divided into aromatic organic acids and aliphatic organic acids. Aromatics include hydroxycinnamic acids such as *p*-coumaric acid, caffeic acid, ferulic acid, and sinapinic acid, as well as hydroxybenzoic acids such as *p*-hydroxybenzoic acid and gallic acid. In addition to malonic acid and acetic acid, aliphatic organic acids include oxalic acid, succinic acid, and malic acid. Anthocyanins also undergo structural

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changes in response to pH, temperature, and the presence of enzymes. As a result of these properties, differences in components and environmental factors result in changes in the color quality of various plants (Ohba et al., 2000). Although anthocyanins are easily discolored and tend to fade, they are considered to be safer than artificial coloring compounds and are often used for coloring processed foods (Anna et al., 2014). Further, it has recently been reported that anthocyanins exhibit antioxidant activity (Tsuda, 2012). Thus, from the viewpoint of safety, anthocyanins are attractive compounds as colorants.

However, since anthocyanins are natural plant pigments, a multi-step process, including crop production, extraction, filtration, and purification, is necessary (Adachi and Yoshitama, 2004). Yields are also dependent on factors such as weather during crop cultivation (Aung et al., 2014). Therefore, in vitro approaches to the production of anthocyanins, where weather is not a factor, are being studied. Gregorio et al. (2017) reported that ethephon increased the content of anthocyanins in black carrot roots. However, despite advances in anthocyanin production in vitro, yields have not yet reached levels required commercially. To overcome this limitation, we speculated that the identification of microorganisms capable of modifying anthocyanins could lead to the rapid and inexpensive production of novel anthocyanins for use in foods. Anthocyanins are plant pigments. So, plant enzymes are usually used for structural modification of anthocyanins. However, this paper is an attempt to modify the structure of anthocyanin using microbial enzymes. Microbial growth and enzyme production is faster than that of plant. Therefore, microbial enzymes are very useful to convert from black rice anthocyanin to another anthocyanin as compared with that of plants. In addition, the main anthocyanin of black rice anthocyanin is Cyanidinn-3-glucoside, which is a very simple structure. Therefore, we believe it is easy to convert to various structures. We previously screened and identified microorganisms that could potentially modify the structure of anthocyanins (Saigusa et al., 2014).

The screened microorganisms were confirmed to have high homology with *E. aerogenes* NBRC 13534. *E. aerogenes* are known to exist in the soil or in the human intestinal tract. In addition, it has been reported that anthocyanin cleaves glucoside bonds and decomposes anthocyanidin heterocycle by intestinal microorganisms (Aura et al., 2005; Cheng et al., 2016; Mueller et al., 2017). From this, it was suggested that anthocyanin was degraded by *E. aerogenes*, and a change in anthocyanin occurred due to the binding of a new organic acid or sugar to the degraded anthocyanidin. In this study, *E. aerogenes* NBRC 13534 was used to investigate the effects of four organic acids (caffeic acid, ferulic acid, *p*-hydroxybenzoic acid and malonic acid) on alterations in black rice anthocyanins.

MATERIALS AND METHODS

Caffeic acid, ferulic acid, *p*-hydroxybenzoic acid, and malonic acid were purchased from Nacalai Tesque Co., Ltd. (Kyoto, Japan). Potato Dextrose Agar (PDA) medium was purchased from Nissui Co., Ltd. (Tokyo, Japan). All other reagents were of the highest grade available. Black rice (*Oryza sativa* var. *Indica* cv. *Shiun*) was purchased from Kajiwara Beikoku Co., Ltd. (Kyoto, Japan). *E. aerogenes* NBRC 13534 was purchased from NITE Bioresource Center (Chiba, Japan) as the microbial strain.

Incubation of *E. aerogenes* NBRC 13534

In this experiment, PDA medium containing anthocyanin extracted from black rice and organic acid was used to confirm the influence of organic acid on color quality change of black rice anthocyanin by *E. aerogenes* NBRC 13534. Black rice bran contains a lot of cyanidin - 3 - glucoside which is one kind of anthocyanin. (Nakagawa and Maeda, 2017). To separate the rice bran, unpolished black rice was subjected to flour mill "Hikiko" (Tokyo Unicom, Tokyo, Japan). It is necessary to always keep the pH acidic for extraction of pigment (Adachi and Yoshitama, 2004). So, to extract the anthocyanins, 25 g of bran was added to 250 mL of McIlvaine buffer (pH 4) and the resulting mixture was left in the dark at 15°C for 12 h. After filtering with gauze, the sample was centrifuged (10 min, 1309.23 ×g) and the supernatant was filtered using No. 1 filter paper (ADVANTEC Toyo Co. Ltd., Tokyo, Japan) to obtain the black rice anthocyanins. We prepared six different media. At first, PDA medium supplemented with 100 mL of black rice extract and 0.01 g of four organic acids (caffeic acid, ferulic acid, *p*-hydroxybenzoic acid, malonic acid) was prepared. Then, PDA medium containing 100 mL of black rice extract and 0.01 g of each organic acid was also prepared, respectively. As a control experiment, a medium supplemented with no organic acid was prepared. Each medium was sterilized in boiling water for 5 min. In order to maintain the anthocyanin structure, sterilization of the medium by autoclaving was avoided. After cooling, 20 mL of the medium was aseptically dispensed into a petri dish. After that, *E. aerogenes* NBRC 13534 was inoculated on the medium using a platinum loop and incubated at 37°C for 5 days. Changes in the color of the medium were assessed.

Absorption spectra

First, we extracted the anthocyanin from the medium. 15% acetic acid was used for anthocyanin extraction. The medium showing color changes was collected and added to a 15% acetic acid solution. After 12 h at 5 °C, anthocyanin was extracted from the medium. After that filtration of anthocyanin extract was performed using No. 1 filter paper (ADVANTEC Toyo Co. Ltd., Japan). Then, absorbance spectra of the anthocyanin extract were analyzed using a U-3010 spectrophotometer (Hitachi, Tokyo, Japan).

Anthocyanin analysis

Anthocyanins were analyzed using a high performance liquid chromatograph (HPLC) (Shimadzu, Kyoto, Japan) equipped with a CTO-10AC column oven and SPD-10AV UV-VIS detector. For analytical HPLC, an ODS-3 column (4.6 id×250 mm, GL Sciences, Inc. Tokyo, Japan) with solvents A (H_3PO_4:H_2O = 15:985) and B (H_3PO_4:AcOH:CH_3CN:H_2O = 15:200:250:535) under gradient conditions at a flow rate of 1 mL/min. Anthocyanin analysis was carried out using the following gradient conditions: 0-100 min, linear gradient from 35 to 55% of solvent B in solvent A; 100-140 min, linear gradient from 55 to 65% of solvent B in solvent A. Detection

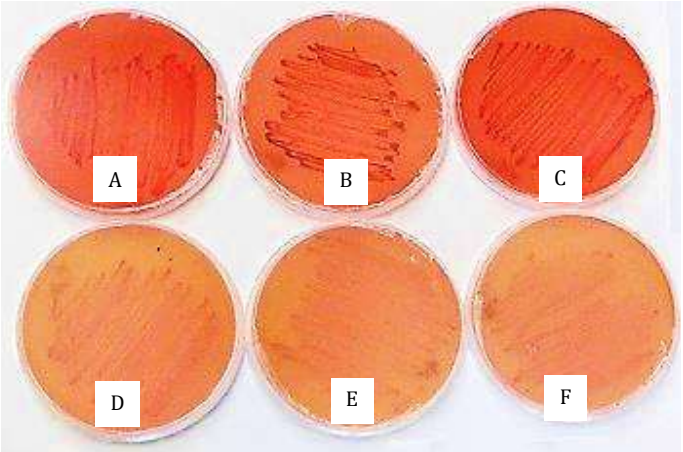

Figure 1. Effect of organic acids on alterations of black rice anthocyanins by *E. aerogenes*. (*E. aerogenes* NBRC 13534 was incubated at 37°C for 5 days in PDA medium supplemented with various organic acids) (A: All four added organic acids; B: ferulic acid; C: caffeic acid; D: without organic acids; E: *p*-hydroxybenzoic acid; F: malonic acid).

was performed at 520 nm. The column temperature was 35°C. The anthocyanin extract was diluted 2-fold with 15% acetic acid solution, then diluted 2-fold with solvent (solvent A: B = 65: 35) and filtered through disposable membrane filter DISMIC-13 HP (0.2 µm, ADVANTEC). The filtrate was measured by HPLC.

RESULTS AND DISCUSSION

Color changes were observed in PDA medium to which all four organic acids were added and in PDA to which caffeic acid or ferulic acid was added. These medium changed to deep red. It has been reported that the color quality of anthocyanins is increased by mixing caffeic acid with ferulic acid and gallic acid with anthocyanin (Qian et al., 2017). However, in a previous study report, color change was observed by culturing *E. aerogenes* in a medium containing phenolic compounds (caffeic acid and ferulic acid), but no color change was observed in the uncultured medium (Saigusa et al., 2014). From this, it was suggested that not only caffeic acid and ferulic acid but also *E. aerogenes* are important factors for changing the color quality of black rice anthocyanin. There was no color change in the PDA medium without organic acids or in that supplemented with malonic acid or *p*-hydroxybenzoic acid (Figure 1). The maximum absorption value of the PDA medium supplemented with all four organic acids was shifted from 520 nm, which is the maximum absorption value of black rice anthocyanins, to 500 nm. In addition, caffeic acid was shifted to 500 nm and ferulic acid to 510 nm. However, the PDA medium without organic acids and that containing malonic acid or *p*-hydroxybenzoic acid did not show changes in absorption (Figure 2). HPLC analysis revealed that the PDA medium to which all four organic acids were added showed four peaks not observed in black rice anthocyanins.

Three new peaks were observed in caffeic acid, while ferulic acid showed two new peaks. However, no new peaks were observed for PDA medium without organic acids or that containing malonic acid or *p*-hydroxybenzoic acid (Figure 3). From these results, it was confirmed that caffeic acid and ferulic acid are involved in the color changes of black rice anthocyanins. From our previous studies (Saigusa et al., 2014), among the four new peaks (peak 1-4) in a medium supplemented with four organic acids, it was confirmed that the two pigments corresponding to peaks 1 and 4 exhibit absorption maxima at 500 nm and peaks 2 and 3 have absorption maxima at 520 nm. From these results, it was suggested that a new peak may be involved in shifting to 500 nm in the medium supplemented with caffeic acid and in shifting to 510 nm in the medium supplemented with ferulic acid. Furthermore, since the structure of these two organic acids are similar, it was suggested that there is an important relationship between the change of color and the structure of the organic acid (Figure 4).

Since anthocyanin is a plant pigment, cultivation of plants is necessary to apply this pigment to foods. However, if anthocyanins can be synthesized *in vitro* freely, cultivation of plants for obtaining of anthocyanin becomes unnecessary, and it may be possible to obtain the necessary kinds of anthocyanins when necessary. Therefore, we tried modification and synthesis test of black rice anthocyanin using microbial enzyme. By incubation of microorganisms, microbial enzymes can be obtained much faster than plant enzymes. In the paper on the synthesis of anthocyanins, mainly plant enzymes are used (Yonekura et al., 2012). There is no paper on

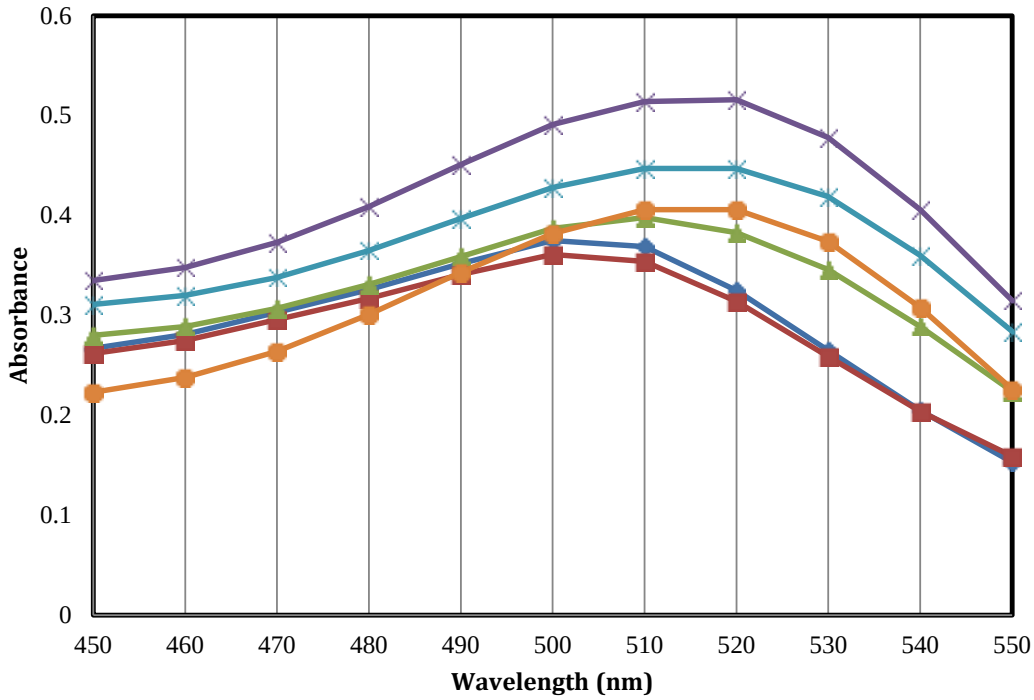

Figure 2. Absorbance spectrum of discolored black rice anthocyanin. Changes in maximal absorption value of black rice anthocyanins in due to *E. aerogenes* and organic acids. Incubate at 37°C for 5 days (◆- four organic acid, ■- caffeic acid, ▲- ferulic acid, ×- *p*-hydroxybenzoic acid, ✳- malonic acid, ●- no organic acids).

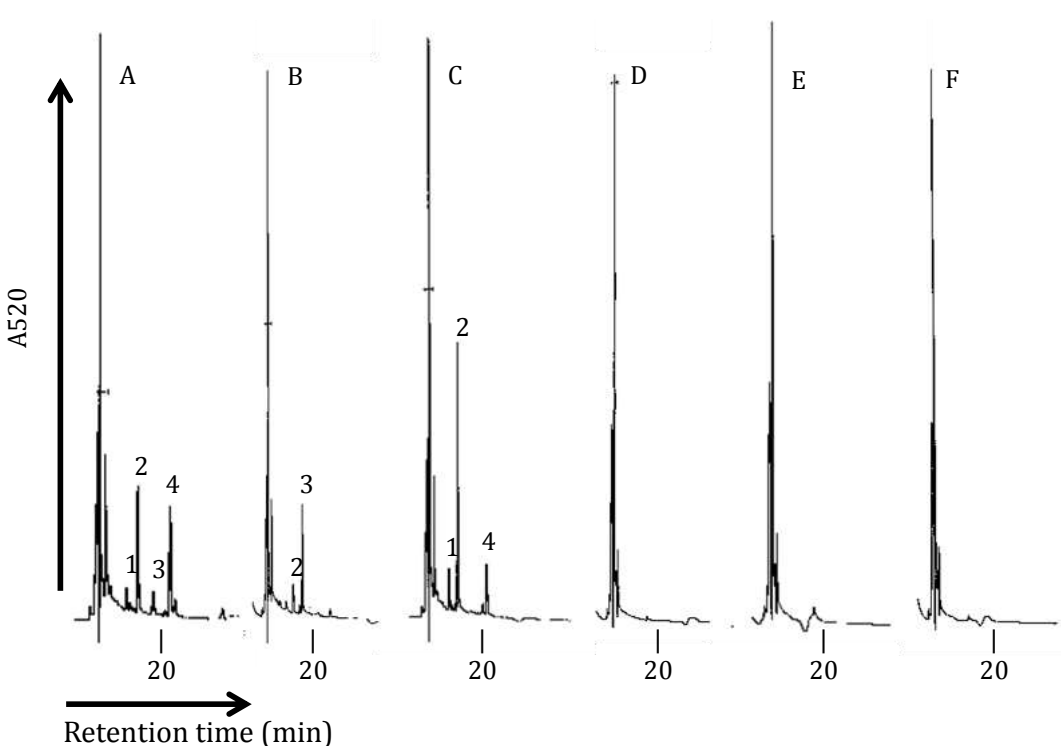

Figure 3. HPLC chromatograms of PDA medium with or without organic acids (A: four organic acids; B: ferulic acid; C: caffeic acid; D: *p*-hydroxybenzoic acid; E: malonic acid; F: no organic acids; Detection: 520 nm).

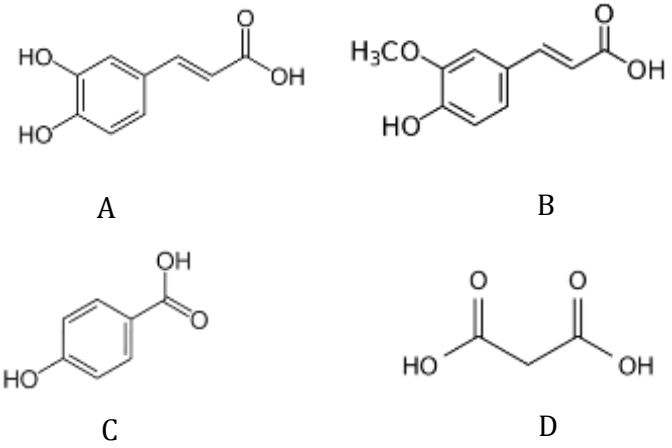

Figure 4. Structure of organic acid (A- caffeic acid; B- ferulic acid; C- *p*-hydroxybenzoic acid; D- malonic acid.

synthesis or modification by microbial enzymes. For that reason, we believe this paper is a very rare information. The main component of the black rice anthocyanin is cyanidin-3-glucoside (Terahara et al., 1994), which is a very simple structure. Also, as mentioned earlier, decomposition of anthocyanins by intestinal microorganisms has been reported (Aura et al., 2005; Cheng et al., 2016; Mueller et al., 2017). Also, improvement in color quality and stability of anthocyanins by organic acids has also been reported (Qian et al., 2017) which suggests that intestinal microorganisms other than *E. aerogenes* may also alter the structure of anthocyanins. For these reasons, depending on the combination of microbial enzyme and organic acid, various new anthocyanins may be born. However, we just got the possibility to produce anthocyanin *in vitro* from the result of this experiment. In the future, structural analysis of newly created peaks is necessary. The combination of various anthocyanins, organic acids and microbial enzymes must necessarily be further investigated. It will also be essential to establish an anthocyanin synthesis technology by microbial enzymes in the future.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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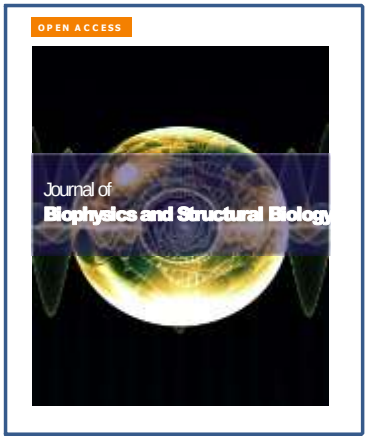

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