Brazilian Journal of Poultry Science
Revista Brasileira de Ciência Avícola

ISSN 1516-635X Jan - Mar 2019 / v.21 / n.1 / 001-008

http://dx.doi.org/10.1590/1806-9061-2018-0791

Original Articles

Dietary Effects of Natural Polyphenol Antioxidant on Laying Performance and Egg Quality of Laying Hens Fed Diets with Oxidized Oil

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■Keywords

Laying hens, oxidized oil, natural antioxidants, vitamin E, egg quality.

Submitted: 20/June/2018
Approved: 17/November/2018

ABSTRACT

This study was conducted to investigate the effects of dietary oxidized oil and natural polyphenol antioxidants on laying performances and egg quality of laying hens. A total of two hundred, 61-week-old Lohmann Brown laying hens were divided into five groups, consisting in four replicates of 10 birds each. They were fed one of the five experimental diets (fresh oil only, oxidized oil without antioxidants, oxidized oil with vitamin E 200 ppm, oxidized oil with vitamin E 100 ppm + Cabanin CSD® 100 ppm, oxidized oil with Cabanin CSD® 500 ppm)respectively, for 6 weeks. There were no significant differences in laying performances and egg weight among the treatments. However, egg mass in group fed oxidized oil with vitamin E 100 ppm + Cabanin CSD® 100 ppmwas significantly higher than group fed oxidized oil only (p<0.05). Eggshell thickness and eggshell strength was not affected by feeding of oxidized oil and antioxidants. Haugh unit checked after storage of 14 d from the end date of the trial showed significant difference (p<0.05). Serum superoxide dismutase (SOD) like activity was significantly higher in antioxidant treated groups. The level of serum glutamic oxaloacetic transaminase (GOT) was significantly lower in groups fed vitamin E 100 ppm + Cabanin CSD® 100 ppm or Cabanin CSD® 500 ppm compared to groups fed oxidized oil without antioxidant. The number of total microbes, *lactobacilli*, and *Enterocossus faecium* showed significant difference favored to antioxidants groups. This study indicates that addition of oxidized oil to layer diet may have harmful effect on oxidative status and egg quality, but concurrent addition of vitamin E and natural polyphenol can alleviate the toxic effect of oxidized oil.

INTRODUCTION

Fat and oils are concentrated and relatively higher energy sources. These are routinely added to poultry diets to increase the energy density and to supply essential fatty acids. It is generally known that oils rich in polyunsaturated fatty acids (PUFA) have the advantage of digestion and absorption but are more prone to oxidation because of their double bonds, in particular, under the condition that heat is added (Urso & Clarkson, 2003). Heat is inevitably applied in processing for feed manufacture and in frying for meal preparation from oilseeds. During repeated heat application, oxidative and thermal effects result in the formation of potentially toxic compounds (Warnants *et al.*, 1996). Still controversial, direct products of fat oxidation including aldehydes, ketones and esters decreased available energy values of animal feeds (Shermer & Calabotta, 1985). Zhang *et al.* (2011) suggested that oxidation of fats induces an unfavorable odor, causing quality problems such as lowering the nutritional value of chicken meat.

The active antioxidants, vitamin E and selenium, have been shown to have a protective effect against oxidative changes (Meluzzi *et al.*, 2000). Supplementation of these antioxidants reduced the formation of malondialdehyde (MDA) in eggs enriched with n-3 PUFA (Ren *et al.,* 2013). The natural polyphenols also exhibited potent antioxidative and free-radical scavenging properties. Kang *et al.* (2010) suggested that blood total antioxidant status and intestinal superoxide dismutase (SOD) could be increased in laying hens fed natural antioxidant products. In previous studies, we found that Skullcap extract could be used as a valuable natural source for reducing lipid oxidation developed during storage (An *et al.*, 2010). If so, it is anticipated that natural polyphenols will reduce oxidative change under the harsh conditions that cause oxidation, such as the feeding oxidized oil. The objective of this study was to test the effects that vitamin E and natural polyphenol antioxidants have on laying performances, egg quality and oxidative status of laying hens fed diets containing oxidized oil.

MATERIALS AND METHODS

Test substrates

Cabanin CSD® were composed of grape pomace extract, sweet chestnut extract, black current extract and citrus extract and provided by DaehoCo. Ltd. To obtain oxidized oil, fresh food grade soybean oils were purchased from the supermarket. The oils were contacted with oxygen for 96 h using a bubble generator and then heated at a temperature of 130°C for 8 h and stored for the rest of the time at room temperature. This work lasted 4 days. As finally identified, the acid value of oxidized oil and the peroxide value (POV) were 2.15 mg of KOH/g and 98.5 meg/kg, respectively, and these values are similar to the heated soybean oil used in another study (Yue *et al.*, 2011). The oil purchased at the same time was refrigerated to avoid further oxidative changes. Fatty acid composition of fresh and oxidized soybean oils is presented in Table 1.

Animals, diets and management

The Institutional Animal Care and Use Committee at Konkuk University approved the techniques and procedures involved in the animal care and handling (KU15186). An experiment was conducted using two hundred, 61-week-old Lohmann Brown laying hens and lasted 6 wks. The hens were housed in a caged layer house of commercial design with water and feed provided *ad libitum*. They were randomly assigned into the 5 experimental groups with 4 replicates of 10 hens each and reared under routine conditions of temperature (25±3 °C), humidity (50%) and artificial lighting (15L/9D). Two hens at a time were put into one wire cage (35×40 cm). The hens were fed one of the five experimental diets which were PC, fresh soybean oil only; NC, oxidized soybean oil only; T1, oxidized oil with vitamin E 200 ppm; T2, oxidized oil with vitamin E 100 ppm + Cabanin CSD® (as natural polyphenol antioxidant) 100 ppm; T3, oxidized oil with Cabanin CSD® 500 ppm, respectively. The composition of the experimental diet was based on the requirements stipulated by NRC (1994) for Brown laying hens and the nutrition levels were shown in Table 2. Diets were freshly added every day. Feed intake of each replicate was recorded weekly.

Table 1 – Fatty acid compositions of fresh and oxidized soybean oils 1.

Fatty acids	Fresh soybean oil	Oxidized soybean oil
C14:0	0.00	0.28
C14:1ω5	0.00	0.00
C16:0	10.60	13.78
C16:1ω7	0.00	1.19
C18:0	4.26	5.32
C18:1ω9	26.46	24.35
C18:2ω6	52.12	49.57
C18:3ω3	5.99	4.22
C20:0	0.00	0.44
C20:1ω9	0.00	0.50
C20:2ω6	0.00	0.00
C20:3ω6	0.00	0.00
C20:4ω6	0.00	0.00
C20:5ω3	0.00	0.00
C22:0	0.57	0.00
C22:1ω9	0.00	0.00
C22:6ω3	0.00	0.00
C24:0	0.00	0.35
SFA	15.43	20.57
PUFA	58.11	53.79
MUFA	26.46	25.64
Total ω3	5.99	4.22
Total ω6	52.12	49.57
ω6/ ω3	8.70	11.75
SFA : MUFA : PUFA	1 : 1.71 : 3.76	1 : 1.25 : 2.61

[1] SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

Egg production

Egg production and egg weight were measured daily. Daily average of egg weight was determined, excluding abnormal eggs and recorded on replicate basis. Egg mass was calculated as the percentage of egg production multiplied by the egg weight. Feed consumption were measured at the beginning and the

Table 2 – Ingredients and composition of the basal diet (%, as-fed basis) 1,2.

Ingredients	
Yellow corn	46.49
Soybean meal	11.23
Soybean oil (fresh or oxidized form)	1.50
Corn dried distill'sgrains with dolubles	6.00
Rapeseed meal	3.00
Wheat bran	6.70
Lupin	7.00
Corn gluten meal	2.20
Tallow	0.60
Rice bran	2.90
Limestone, coarse	10.80
Mono dicalcium phosphate	0.60
Salt	0.20
L-Lys, 78%	0.22
DL-Met, 98%	0.08
Choline-Cl, 50%	0.08
Mineral premix	0.17
Vitamin premix	0.18
Sodium bicarbonate	0.05
Total	100.00
Calculated Analysis of basal diet	
CP, %	16.50
Crude fat, %	4.12
Crude fiber, %	3.59
Crude ash, %	13.95
Avail. P, %	0.45
Ca, %	4.18
TMEn, kcal/kg	2,750

[1] Mineral mixture provided following nutrients per kg of diet: Fe, 55mg; Zn, 88mg; Mn, 88mg; Cu, 5.5mg; I, 1.7mg; Se, 0.3mg;

[2] Vitamin mixture provided following nutrients per kg of diet: vitamin A, 8,666 IU; vitamin D_3, 2,666 IU; vitamin E, 20 IU; vitamin K_3, 2mg; vitamin B_1, 2mg; vitamin B_2, 4.6mg; vitamin B_6, 3.3mg; vitamin B_{12}, 0.013mg; biotin, 0.1mg; niacin, 33mg; pantothenic acid, 8mg; folic acid, 1mg.

end of the experimental period, and average daily feed intake, and egg production were adjusted for mortality.

Egg quality measurements

Egg qualities were determined at a 7 d interval. Five eggs from each replicate were collected, individually weighed and stored overnight at room temperature for subsequent measurements. The breaking strength of uncracked eggs was measured with an eggshell strength tester (FHK, Fujihara Ltd., Tokyo, Japan). Eggshell thickness without shell membrane was measured with a micrometer (Digimatic micrometer, Series 293-330, Mitutoyo, Japan). Albumen height was measured using Egg multi-tester (QCM+ Technical Services and Supplies Ltd., York, England). Haugh unit, along with albumen height and egg weight, was calculated as previously described (An *et al.*, 2010). Egg yolk color was measured by comparing to Roche yolk color fan (Hoffman-La Roche Ltd., Basel, Switzerland). The collected eggs were kept in storage temperature of 18ºC during 7 or 14 d to observe the change of Haugh unit.

Blood sampling and analysis

At the end of the experimental period, blood samples were collected from the jugular vein of 8 birds per each treatment. After centrifugation at 2,000 × *g* for 15 min the serum samples were stored at -60ºC until analysis. The activities of glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) in sera sampled were measured with colorimetric method using GOT-GPT assay kits (Asan Pharmaceutical Co., Korea) according to the manufacture's direction. For superoxide dismutase (SOD) like activity test, 2ml of Tris-HCl buffer (pH 8.5) and 0.1 ml of 5 mM pyrogallol were incubated with or without sera at 25ºC for 30 min. The reaction was stopped with 0.1 ml of 1 N HCl, which was followed by reading the absorbance at 405 nm against the blank (Marklind & Marklund, 1974).

Measurement of malondialdehyde

Some modified methods were used to determine thiobarbituric acid reactive substances (TBARs) values in egg yolk to evaluate lipid oxidation as described by Botsoglou *et al.* (1994). To study the oxidative stability of egg yolk, 8 eggs from each group was placed in a high temperature cabinet over 3 days of storage at 35ºC. A 1.5g egg yolk sample was homogenized with 5% aqueous trichloroacetic acid solution containing 0.8% butylated hydroxytoluene and then centrifuged for 3min at 3,000*g*. Following reaction with thiobarbituric acid reagent, MDA was directly quantified by third-derivative spectrophotometry against blank reaction mixture (Beckman DU-650, Beckman Counter, Inc.).

Cecal microbial population

Cecal contents of slaughtered birds were aseptically sampled and immediately chilled on ice for microbial test at the end of the experimental period. Immediately, digesta homogenates in PBS were serially diluted from 10^{-1} to 10^{-7} and subsequently plated onto duplicate selective agar media for enumeration of target bacterial strains. Total microbes, *coliforms*, lactic acid bacteria, and *Enterocossus faecium* were enumerated using plate count agar, MacConkey agar, and MRS agar, respectively. Each plate was incubated at 37°C, for 24 to 72 h anaerobically or aerobically. The number of colonies were then counted. Results obtained were presented as base-10 logarithm colony-forming units per gram of cecaldigesta.

Statistical analysis

The main effects between treated groups were subjected to ANOVA using the general linear models procedure of SAS (2002) and significant differences were determined using Duncan's multiple range test at the level of $p<0.05$. Differences between $0.05<p<0.10$ were discussed as a trend toward significance. Log transformation was performed for variables that was not normally distributed.

RESULTS

The levels of C16:0 and C18:0 of oxidized soybean oil were slightly higher as compared with fresh soybean oil. Oxidized soybean oil contained less unsaturated fatty acid, such as C18:1 ω9, C18:2 ω6 and C18:3 ω3. The rate of SFA:MUFA:PUFA of oxidized oil was numerically decreased through repetitive heat treatment (Table 1). Egg production and egg weight were not significantly affected by dietary treatments (Table 3). As compared with NC, hens fed oxidized oil with vitamin E 100 ppm + Cabanin CSD® 100 ppm showed a higher daily egg mass ($p<0.01$). No significant difference was found among groups in feed intake ($p<0.05$).

As shown in Table 4, Egg quality parameters did not show significant responses to the dietary treatments ($p<0.05$). Dietary antioxidant supplementation did not affect the Haugh unit of eggs stored for 1 d or 7 d. However, the Haugh unit of the group fed diet containing oxidized oil with Cabanin CSD®500 ppm was significantly higher($p<0.05$) than NC or PC over14 d of storage at 18ºC (Table 5).The Haugh unit of eggs stored for 14 d was the lowest numerically in NC group. In contrast, MDA contents of egg yolk tended to decreased in groups fed antioxidants ($p<0.10$). The serum SOD like activity in supplemental vitamin E 200

Table 3 – Dietary effects of dietary vitamin E and natural antioxidants on laying performances in laying hens fed oxidized oil 1, 2.

	PC	NC	T1	T2	T3	SEM	*p*-value
Egg production rate, %	73.5	71.7	73.1	76.2	75.1	1.33	0.144
Egg weight, g/egg	62.81	61.89	63.02	63.63	61.98	0.53	0.144
Egg mass	46.2^{ab}	44.4^{b}	46.1^{ab}	48.5^{a}	46.6^{ab}	0.69	0.004
Feed intake, g/day/bird	110.37	107.26	110.50	112.30	108.60	4.24	0.925

[1]PC, fresh soybean oil only; NC, oxidized soybean oil only, T1, NC + vitamin E 200 ppm; T2, NC + vitamin E 100 ppm + Cabanin CSD®100 ppm; T3, NC + Cabanin CSD®500 ppm; SEM, pooled standard error of the means.

[2]Data are mean of 4 replicates with 5 cages with 2 birds per cage.

[a,b]Values with different superscripts within a row differ significantly ($p<0.05$).

Table 4 – Dietary effects of dietary vitamin E and natural antioxidants on egg qualities in laying hens fed oxidized oil 1, 2.

	PC	NC	T1	T2	T3	SEM	*p*-value
Yolk color, R.C.F	6.72	6.89	6.70	6.75	6.75	0.09	0.612
Eggshell thickness, mm/100	34.63	34.17	34.44	34.08	34.56	0.33	0.711
Eggshell strength, kg/cm^2	2.56	2.41	2.36	2.33	2.42	0.07	0.280

[1]PC, fresh soybean oil only; NC, oxidized soybean oil only, T1, NC + vitamin E 200 ppm; T2, NC + vitamin E 100 ppm + Cabanin CSD®100 ppm; T3, NC + Cabanin CSD®500 ppm; SEM, pooled standard error of the means.

[2] Mean values from the overall experiment period.

Table 5 – Dietary effects of dietary vitamin E and natural antioxidants on the change of Haugh unit, yolk lipid peroxidation and blood profiles in laying hens fed oxidized oil 1, 2.

	PC	NC	T1	T2	T3	SEM	*p*-value
Haugh unit							
1d	76.17	76.49	73.99	75.26	78.23	3.62	0.944
7 d	62.82	63.24	66.68	66.85	67.83	2.86	0.635
14 d	44.24^{b}	42.40^{b}	47.33^{ab}	49.55^{ab}	53.30^{a}	2.41	0.016
MDA, μg/g yolk	0.039	0.058	0.041	0.032	0.035	0.007	0.097
SOD, IU/L	62.61^{a}	50.08^{b}	65.80^{a}	57.60^{ab}	65.31^{a}	3.69	0.024
GOT, IU/L	109.99^{ab}	125.53^{a}	116.31^{ab}	80.96^{b}	81.46^{b}	12.47	0.042
GPT, IU/L	31.42	29.03	24.62	37.75	29.90	2.84	0.056

[1]PC, fresh soybean oil only; NC, oxidized soybean oil only, T1, NC + vitamin E 200 ppm; T2, NC + vitamin E 100 ppm + Cabanin CSD®100 ppm; T3, NC + Cabanin CSD®500 ppm; MDA, malondialdehyde; SOD, superoxide dismutase; GOT, glutamic-oxaloacetic transaminase; GPT, glutamic-pyruvic transaminase; SEM, pooled standard error of the means.

[2]Haugh unit is expressed as means of 20 eggs and other data are expressed as means of 8 replicate per dietary group.

[a,b]Values with different superscripts within a row differ significantly ($p<0.05$).

ppm or Cabanin CSD®500 ppm diet fed hens were significantly higher (p<0.05) as compared with NC. In contrast, levels of serum GOT in group with vitamin E 100 ppm + Cabanin CSD®100 ppm or CSD®500 ppm were significantly lower (p<0.05) than that of NC. No significant differences in level of GPT were observed among all groups.

The effects of dietary oxidized oil and antioxidant on the profiles of cecal microflora are summarized in Table 6. Compared with PC, the total microbe significantly decreased (p<0.05) with dietary natural antioxidant supplementation. The number of *Lactobacilli* was significantly increased (p<0.05), with dietary natural antioxidant supplementation (group fed diet with vitamin E 100 ppm +Cabanin CSD®100 ppm or CSD®500 ppm). The number of coliform bacteria was not significantly affected by dietary treatments.

Table 6 – Dietary effects of dietary vitamin E and natural antioxidants on theprofiles of cecal microflora in laying hens fed oxidized oil 1, 2.

	PC	NC	T1	T2	T3	SEM	*p*-value
Total microbes, logcfu/g	5.47^{a}	5.13^{ab}	5.37^{a}	4.87^{b}	4.75^{b}	0.16	0.013
E. coli, logcfu/g	4.57	5.16	4.12	4.12	4.01	0.21	0.380
Lactobacilli, logcfu/g	5.31^{b}	5.25^{b}	5.27^{b}	5.79^{a}	6.03^{a}	0.11	<0.001
Eterococcusfaecium logcfu/g	5.07^{bc}	4.60^{c}	4.88^{bc}	5.67^{a}	5.15^{b}	0.15	<0.001

[1]PC, fresh soybean oil only; NC, oxidized soybean oil only, T1, NC + vitamin E 200 ppm; T2, NC + vitamin E 100 ppm + Cabanin CSD®100 ppm; T3, NC + Cabanin CSD®500 ppm; SEM, pooled standard error of the means.

[2] Data are expressed as means of 8 replicate per dietary group.

[a,b,c]Values with different superscripts within a row differ significantly (p<0.05).

DISCUSSION

It has been well known that the oxidized oil exerted nutritionally harmful effect that would have been added to animal feeds (Shermer & Calabotta, 1985). The harmful effects ranged from slight reduction of performance to fatal toxicity leading to death. Oxidative changes also affected the structure that constituted the oil. Oxidized soybean oil used in this study contained less monounsaturated and polyunsaturated fatty acids and more saturated fatty acids, this means that the nutritional quality was lowered by oxidation. Consistence result has been reported by Gulla & Waghray (2011), who observed a gradual increase in C16:0 and C18:0 and decreased in C18:1 ω9 and C18:2 ω6 when sesame oil was stored for 12 mo.

The objective of this study was to investigate whether the dietary oxidized oil have an adversary impact on laying performance and addition of vitamin E or natural antioxidant could alleviate the negative effect of oxidized oils. Unexpectedly, the egg production and feed intake were not affected by either supplemental oxidized oil or antioxidants. Instead, hens fed oxidized oil with vitamin E 100 ppm +Cabanin CSD® 100 ppm showed a higher daily egg mass (p<0.01) as compared with groups fed oxidized oil only. Yue *et al.* (2011) found no perceivable effect on feed intake and egg production and Lewis-McCrea & Lall (2007) did not also observe an effect on egg production by dietary oxidized oil. The inconsistence results have been reported by Cabel *et al.* (1988) and Sanchez-Muniz *et al.* (1998), who found a significant reduction in egg production and feed efficiency by dietary oxidized oil. Dietary natural antioxidant or vitamin E has been demonstrated to improve laying performance (Radwan Nadia *et al.*, 2008). Ajakaiye *et al.* (2011) reported that dietary addition of 150 ppm vitamin E could improve the egg weight and oxidative stability in laying hens raised at high temperature. These results indicate that the use of antioxidants may prevent deterioration of productivity under the condition where oxidative damage is expected.

We found that the Haugh unit was not affected during 1 d or 7 d of storage at 18°C, but decreased significantly over the following 14 d of storage in group fed oxidized oil only. The Haugh unit of layers fed oxidized oil with Cabanin CSD®500 ppm was significantly higher than NC group. The Haugh unit, an indicator of the most widely accepted measure of internal egg quality, tended to decrease according to the elapsed time of storage (Williams, 1992). It suggested that certain antioxidants such as vitamin C, vitamin E and selenium being beneficial to albumen quality by it's antioxidant property. Lim *et al.* (2006) reported that Haugh unit was linearly increased with increasing dietary garlic powder during storage and this was presumed to be due to the antioxidant properties of garlic powder. An *et al.* (2010), however, demonstrated that the Haugh units in the groups fed diets containing Skullcap extract tended to increase after 2 weeks of storage, but not significantly. These different responses

were partly attributed to differences in type of natural substances, storage conditions of eggs and oxidative status of laying hens. It is also suggested that dietary antioxidant nutrients and natural antioxidants are effective in improving the quality of eggs and meats during extended storage (Surai, 2000). When vitamin E was fed, the TBRA in eggs enriched with PUFAs was reduced at 40 d of storage (Cherian *et al.*, 1996). In previous study, we also observed that dietary use of Skullcap extract has been demonstrated to reduce lipid oxidation of egg yolks (An *et al.*, 2010). In this study, the levels of MDA in stored eggs were numerically decreased, but not significantly, by dietary antioxidant supplementation and this is probably because the storage period was relatively short.

Overall, serum SOD like activity and GOT levels were significantly affected by dietary treatments. The results of serum SOD like activity clearly showed that there were differences between groups of fresh oil and oxidized oils. Dietary supplementation of vitamin E 200 ppm or Cabanin CSD®500 ppm increased significantly the serum SOD like activity as compared with NC. This result is supported by a report that blood total antioxidant status and intestinal SOD could be increased in laying hens fed natural products, which has a high content of antioxidants (Kang *et al.*, 2010). The SOD is one of the most important antioxidant enzymes and plays a role in protecting cells from damage caused by reactive oxygen species (Marklind & Marklund, 1974). The serum SOD like activity results shown in this study suggest that oxidized oil decreases the antioxidant capacity, while vitamin E and Cabanin CSD® may improve the antioxidant status of laying hens by elevating the activity of antioxidant enzyme. Serum GOT is closely related liver and tissue damages in avian and are valuable for the determination of safety of new feedstuffs and feed additives (Diaz *et al.* 2003). In our current study, the level of serum GOT was the highest in group fed oxidized oil without antioxidants and this in an indication that oxidized oil may have a negative effect on liver function. Serum GOT also increased in rats fed diet containing autoxidized safflower oil (Nakamura *et al.*, 1973). In contrast, the levels of serum GOT were significantly lower in Cabanin CSD® fed groups compared to groups fed oxidized oil without antioxidant.

The number of *Lactobacilli* significantly increased ($p<0.05$), with dietary natural antioxidant supplementation. The observed beneficial effect of Cabanin CSD® agree with our previous report with cecal microbial population (An *et al.,* 2010). Jamroz *et al.* (2005) also reported a decrease in *E Coli* and an increase in *Lactobacillus pp.* of chicks fed plant extracts. On the other hand, Cross *et al.* (2007) observed that the profiles of gut microflora were not affected by feeding herbs or their associated essential oil. In this study, the natural antioxidant, Cabanin CSD® added to diets modulated the profiles of cecal microflora, reflecting a potential to alter gut micro ecology in laying hens.

The present study gives evidence that not only vitamin E but the natural antioxidant efficiently prevents lipid oxidation under the condition where oxidative damage is expected. There are demands for using natural products that can prevent lipid oxidation in lipid enriched animal foods, due to consumer preferences for natural substances and toxicological concerns of synthetic antioxidants (Miller *et al.*, 2005). The natural antioxidant, Cabanin CSD® can fully replace the antioxidant function of vitamin E and be used as a valuable source for reducing oxidative stress and maintaining liver function.

CONFLICTOF INTERESTS

We declare that we have no financial or personal relationships with other organizations that can inappropriately influence our work.

ACKNOWLEDGEMENTS

This paper was supported by the KU Research Professor Program of Konkuk University (314028-03-3-CG000).

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