Inhibition of Cholesterol and Fatty Acid Biosynthesis in Liver Enzymes and Chicken Hepatocytes by Polar Fractions of Garlic[1,2]

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ABSTRACT

Different concentrations of polar fractions, methanol-soluble (MESF), or water-soluble (WASF), of 1-8% equivalent to fresh garlic paste were added to yellow corn-soybean based diets and fed to 5-week-old male broiler chickens for 3 weeks to measure the inhibition of hepatic β-hydroxy-β-methylglutaryl coenzyme A (HMG-CoA) reductase, cholesterol 7α-hydroxylase (7α-hydroxy) and fatty acid synthetase (FAS). Dose-related decreases in the activities of these enzymes were obtained. Decreases in serum total cholesterol and in low density lipoprotein (LDL) levels were also observed. There was no effect on the level of cholesterol in high density lipoprotein (HDL). The most effective dose for these decreases was found 0.54% (MESF) and 1.2% (WASF) equivalent to 6% of the fresh garlic. The inhibition of HMG-CoA reductase and FAS by 25-300 μg of MESF or WASF for 15 min was tested in vitro, in male and female chicken hepatocytes. Inhibitions of activity were dose-dependent and the degree of inhibition increased with duration of incubation (150 μg of MESF or WASF 5 to 60 min). Dietary supplementation of odorless WASF of garlic was found to be very effective in lowering the total and LDL cholesterol levels compared to control chickens.

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INTRODUCTION

Although the rate of mortality has been considerably reduced, heart disease still remains as the leading cause of death in America. Most of the studies in this area are preventive in nature and focused on lowering plasma cholesterol. The role of nutritional factors such as the type of carbohydrate and dietary fiber in changing plasma cholesterol concentrations has been reported by a number of investigators (1-9).

The hypocholesterolemic, hypoglycemic and antibacterial properties of garlic oil and nonfibrous substances present in garlic bulb have been reported (10-15). Most studies reported in the literature have described the effects of garlic or its fractions on lowering the total cholesterol and lipids in serum and liver only after feeding cholesterol or fat to the experimental animals (11, 12). We have recently found that diets supplemented with different garlic fractions, particularly with polar solubles, decreased not only cholesterol and fatty acid biosynthesis, and serum total cholesterol but also lowered the cholesterol levels in low density lipoproteins (LDL) without affecting the high density lipoproteins (HDL) in chickens (16).

The inhibition of lipid metabolism and significant lowering of serum cholesterol and chol-LDL levels in chickens by polar fractions of garlic prompted us to determine the effective level of these fractions for the inhibition of lipid metabolism in male broiler chickens. We also report the inhibition of cholesterogenesis and lipogenesis by these fractions of garlic using isolated hepatocytes of male and female chickens.

MATERIALS AND METHODS

Experimental materials were purchased from the following sources: acetyl-CoA, malonyl-CoA, RS-mevalonic acid, glucose-6-phosphate, dithiothreitol, $NADP^+$, NADPH, glucose-6-phosphate dehydrogenase, cysteamine, Tween-80, triethanolamine hydrochloride, sodium malate, coenzyme A, malate dehydrogenase, nicotinamide, and DL-3-hydryoxy-3-methylglutaryl-CoA (Sigma Chemical Co., St. Louis, MO); cholesterol (Aldrich Chemical Co., Milwaukee, WI) was recrystallized twice in glacial acetic acid; 7α-hydroxycholesterol (5-cholesten-3β,7α-diol) and 7α-ketocholesterol (5-cholesten-3β-ol-7-one) (Steraloids, Inc., Wilton, NH); EDTA (Fisher Scientific Co., Itasca, IL); bovine serum albumin (Nutritional Biochemicals Corporation, Cleveland, OH); and DL-3-hydroxy-3-

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methyl-[3-^{14}C] glutaryl-CoA (sp act 26.3 mCi/mmol), [4-^{14}C] cholesterol (sp act 50-60 mCi/mmol) and Aquasol (scintillation solution) (New England Nuclear, Boston, MA). Fresh garlic (*Allium sativum*) and the other diet components were obtained in Madison, WI. All other chemicals were of analytical grade.

Animals and Diets

Five-week-old broiler male or female chickens, weighing ca. 800-900 g were obtained from a commercial source (Madison, WI). The birds were maintained on a corn-soybean based diet as a control and the addition of methanol- (MESF) or water-soluble fractions (WASF) of garlic in the control diet served as experimental diets.

Procedure for the Extraction of MESF and WASF of Garlic

The clean garlic cloves (600 g) were homogenized into a paste using a Waring blender. The paste was extracted with petroleum ether (600 ml) 3 times to remove fatty acids and terpenes. The residue was freeze-dried and lyophilized. The resulting powder (315 g) was extracted successively with methanol and water 3 times each, using 600 ml of solvent each time. The methanol-soluble extracts were combined and concentrated under vacuum at 60 C, yielding 55.3 g of semisolid material. The water-soluble combine extracts were lyophilized, resulted in 171.5 g of powder. These materials were stored at 4 C. The required amount of each fraction for each diet (equivalent to 1, 2, 4, 6 and 8% of the fresh garlic paste) was taken up either in a minimum volume of the methanol solvent (20 ml for 10 kg feed) or WASF powder was mixed thoroughly with the corn-soybean based diet (Table 1). The MESF-based diets were allowed to evaporate overnight in a pan (air-dried under the hood). These diets were fed to different groups of chickens. The WASF and MESF were also tested in vitro in chicken hepatocytes.

Experiment I: Effect of Different Concentrations of MESF or WASF of Garlic on Hepatic Enzyme Activities of Cholesterol and Serum Lipids in 8-week-old Male Broiler Chickens

Forty eight 5-week-old male broiler chickens were divided at random into 6 groups, housed in wire bottom cages. Eight chickens fed corn-soybean diet served as the control group; other groups of 8 were fed different levels of MESF or WASF of garlic, corresponding to 1, 2, 4, 6 and 8% of fresh garlic as shown in Table 1. The diets (Table 1) and water were provided ad libitum for 3 weeks with the photoperiod of 12 hr. At the end of the feeding period, the birds were killed and samples of blood and liver were removed, washed, held on ice, weighed and then prepared for the analysis as described previously (16).

Experiment II: Effect of Different Concentrations of MESF and WASF of Garlic on the Enzymic Activities of HMG-CoA Reductase and FAS in Isolated Hepatocytes of Chickens

Eight-week-old male or female chickens were fed corn-soybean diet. They were fasted (48 hr) and refed (72 hr) so that the enzymic activities were measured at the high points of activity, prior to the preparation of liver perfusion. During fasting and refeeding period, 12 hr of photoperiod was employed.

Preparation of Isolated Hepatocytes of Chicken for *in vitro* Assays

The recirculating perfusion buffer system was similar to that described by Zahlten and Stratman (17), which gave good yields of viable cells (2-4$\times$ 10^8 cells per liver) in the present experiment; the cell viability was determined by the dye exclusion method (0.004% erythrosin B) which showed 72-78% viable cells.

Calcium-free perfusate buffer. Krebs improved Ringer I (K-RI) buffer was prepared from the following solutions: 80 ml 0.154 M NaCl, 4 ml 0.154 M KCl, 3 ml H_2O, 1 ml 0.154 M KH_2PO_4, 1 ml 0.154 M $MgSO_4 7H_2O$, 21 ml 1.3% $NaHCO_3$, 4 ml 0.16 M Na pyruvate, 7 ml 0.1 M Na fumarate, 4 ml 0.16 M Na L-glutamate, and 5 ml 0.3 M glucose.

Calcium-free incubation buffer. Krebs-Heneleit (KH) buffer had the following composition: 100 ml 0.154 M NaCl, 4 ml 0.154 M KCl, 3 ml H_2O, 1 ml 0.154 M KH_2PO_4, 1 ml 0.154 M $MgSO_4 7H_2O$, 21 ml 1.3% $NaHCO_3$ and 1.5% gelatin.

Experimental Procedure

In the preparation of birds for liver perfusion, bile duct canulation was omitted, and perfusion of the liver was made through the portal vein. The bird was anesthesized with 50 mg/kg sodium pentobarbital injected intravenously in the wing vein, placed on its back on a support rack and secured in place with tape across each limb with the head slanted down. The abdominal skin was incised lengthwise using scissors and the skin was peeled from the muscle to each side. A midline incision was then made through the muscular layer up to the point where the diaphragm begins. The exposed muscles and organs were swabbed with saline solution. The intestine was displaced to the right. During the rest of the procedure, the liver was bathed with perfusion buffer and kept at 42 C.

The bird was then heparinized and a loose tie was placed around the inferior vena cava above where the artery branches off to the kidney. The splenic vein was tightened with a knot, the thoracic cavity

TABLE 1

Percent Composition of Chicken's Diets and Body Weight of 8-Week-Old Male Broiler Chickens

Ingredients (corn-soybean base)	Diets[a]		Body weight (g)			Gain in weight (%)	Feed consumption (kg)
	Corn (%)	MESF[b] (%)	WASF[b] (%)	Initial[c]	Final[d]		
Corn (9.5% control)	70.00	-	-	810 ± 102[e]	1710 ± 169[e]	111	12.96
Corn + MESF or WASF[b]	70.00	0.09 (1.0%)[f]	0.20 (1.0%)[f]	888 ± 117	1766 ± 141	99	12.90
Corn + MESF or WASF[b]	70.00	0.18 (2.0%)	0.40 (2.0%)	860 ± 112	1716 ± 161	100	12.94
Corn + MESF or WASF[b]	70.00	0.36 (4.0%)	0.80 (4.0%)	895 ± 121	1759 ± 142	96[g]	12.92
Corn + MESF or WASF[b]	70.00	0.54 (6.0%)	1.20 (6.0%)	892 ± 123	1739 ± 146	95[g]	12.91
Corn + MESF or WASF[b]	70.00	0.72 (8.0%)	1.60 (8.0%)	848 ± 111	1645 ± 132	94[g]	12.92

[a]Each diet also contains soybean meal-44% protein (23.0%); alfalfa meal-17% protein (2.0%); meat and bone meal (2.0%); dicalcium phosphate (1.0%); calcium carbonate (1.0%); iodized salt (0.5%); vitamin and mineral mixture (0.5%); vitamin and mineral mixture contains/kg feed: vitamin A 3000 IU, vitamin D_3 500 ICU, riboflavin 2.5 mg; calcium-pantothenate 3.0 mg, vitamin B_{12} 0.005 mg; zinc sulfate ($ZnSO_4$) 70 mg and manganese dioxide (MnO_2) 65 mg; grit (5.0%) was also incorporated at the expense of each diet.

[b]MESF and WASF = Methanol- and water-soluble fractions of garlic were added to the corn-soybean based diets. Out of the 380 g of clean garlic cloves; 1.4 g petroleum ether-soluble fraction, 34.2 g methanol-soluble fraction, 79.3 g water-soluble fraction, 12.1 g residue were obtained.

[c]Weight of five-week-old male chickens.

[d]Weight of eight-week-old male chickens. These weights were obtained with WASF of garlic. Similar gain in weights were obtained with MESF.

[e]Mean ± SD, N = 8 chickens per group.

[f]Percentages of respective amounts equivalent to fresh garlic paste are in parentheses.

[g]Significantly different from control at $P < 0.05$.

was opened to expose the superior vena cava and a loose tie was placed around it. The buffer pump was started slowly so that calcium-free K-RI buffer which was equilibrated to 42 C and gassed constantly by 95% O_2/5% CO_2 was just slightly dripping out of the syringe. The inferior vena cava was cut well below the first loose tie to allow blood to escape, a hole was made in the ventricle of the heart and a needle inserted through the heart into the superior vena cava and the second loose tie was tightened. The first loose tie was then tightened to make a closed circuit.

This perfusion was conducted for 10 min without added collagenase; then 40 mg collagenase (the best results were obtained by using the preparation made by Worthington; dissolved in 10 ml 0.154 M NaCl) was added and perfusion was continued for 7 min. After digestion, the liver was removed and transferred into a plastic beaker containing 50 ml perfusion buffer at room temperature. The liver was minced with scissors and the crude suspension gassed for 2 min with 95% O_2/5% CO_2 and filtered through one layer of cheesecloth into a second plastic beaker. The crude cell suspension containing hepatocytes and nonparenchymal cells was transferred to a centrifuge tube.

The cells were counted in a Neuhauer hemocytometer (total number of viable cells $37\text{-}40 \times 10^6$) and protein was estimated by the Biuret method (total protein 200-225 mg). The total volume was adjusted according to the number of viable cells ($5\text{-}6 \times 10^6$) or protein concentration (30 mg/0.9 ml) used per incubation. These cells were incubated with MESF or WASF of garlic (dissolved in saline solution) in a final volume of 1 ml at 42 C for 15 min. MESF was dissolved with 10 μg Tween 80 per assay. After incubation, the assay mixture was centrifuged at 5000 × g for 2 min at 4 C and the supernatant discarded. Homogenizing buffer (0.4 ml) was added, the cells were homogenized and processed to obtain the cytosolic and microsomal fractions.

Preparation of Tissues for Analyses

Homogenates of the liver and the sedimented hepatocytes from in vitro assays were prepared in 0.1 M potassium phosphate buffer, pH 7.4 containing 4 mM $MgCl_2$, 1 mM EDTA and 2 mM dithiothreitol. Livers were chopped and suspended in the buffer (1:2, w/v). Homogenization was done with a Polytron homogenizer. The 100,000 × g supernates (cytosolic fraction) and the microsomal fractions were stored at −20 C until they were assayed for enzymatic activities (18,19). Protein concentrations were estimated by a modification of the Biuret method using the bovine serum albumin as a standard (20).

Enzyme Assays and Estimation of Cholesterol in Serum

Assays for HMG-CoA reductase (EC 1.1.1.34), cholesterol 7α-hydroxylase (EC 1.14) and fatty acid synthetase were carried out as reported previously (18,19).

Serum cholesterol concentrations were estimated using Worthington "Cholesterol Reagent" set obtained from Worthington Diagnostics Division of Millipore Corporation, Freehold, NJ.

LDL and very low density lipoproteins (VLDL) were isolated from the serum (100 μl) by precipitation with a mixture of phosphotungstic acid 9.7 mM (10 μl) + $MgCl_2$ 0.4 M (10 μl). After standing for 5 min at room temperature, the mixtures were centrifuged at 2000 × g for 10 min, the supernatant was removed and was used to determine the level of cholesterol in HDL. The precipitate was dissolved in 0.1 M sodium citrate buffer (100 μl) and the level of cholesterol (LDL + VLDL) was determined using the above method.

Expression of Data and Statistical Methods

Enzyme data are presented as specific activities (units/mg cytosolic or microsomal protein/min). Statistical comparison of results was performed by a one-way analysis of variance (21).

RESULTS AND DISCUSSION

In those parts of the world where unrefined cereals and vegetable products form the major part of the diet, the incidence of cardiovascular disease is much lower than that found in the American population. Garlic has been credited since the days of ancient Rome with special health benefits (10-12), when used in trace amounts in food preparations. A number of investigators report that consumption of garlic reduces serum cholesterol levels in cholesterol- or lard-fed rats and rabbits (10-15,22). The significant decreases in the activities of HMG-CoA reductase, 7α-hydroxy, and FAS by feeding MESF or WASF of garlic at a 5% level in low cholesterol chicken diets (16), prompted us to determine the effective levels of these fractions in chickens. Male/female broiler chickens were used in the current studies due to their higher rate of feed efficiency conversion to muscle and lower rate of catabolism compared to those of White-Leghorn chickens (16).

Chickens were fed a normal corn-soybean diet consisting of corn (70.0%) and soybean (23.0%) as the major source of protein (Table 1). This diet was supplemented with MESF or WASF methanol or garlic equivalent to 1.0, 2.0, 4.0, 6.0 and 8.0% of fresh garlic paste. Weight gain and feed consumption are shown in Table 1. Weight gain was suppressed with increasing concentrations of the garlic fractions. Feed consumption by all experimental groups was slightly lower than that of the control group. The maximum suppression of weight gain (−8%) was found with 0.72% of MESF and 1.60% WASF equivalent to 8.0% of fresh garlic. Birds which were fed higher doses of these fractions (10, 15, 20%) did not show a further decrease in weight gain (unpublished results), and exhibited no visible evidence of any abnormal change in any organ upon sacrifice.

A dose-related decrease in activity was observed for each of the rate-limiting enzymes for the synthesis (HMG-CoA reductase) and the degradation (7α-hydroxy) of cholesterol and for fatty acid synthetase over the range of the concentrations of these fractions (Table 2). Values ranged from 14% to 42%, 11% to 36% and 3% to 54%, respectively, compared to the control. These results may reflect only in vivo response to the lowering of the substrate pool in liver effected by the inhibition of the biosynthetic activities of both cholesterogenesis and lipogenesis in liver.

These effects were accompanied by significant decreases in serum cholesterol levels (−18% to −25%), compared to control (Table 2). The levels of chol-HDL were not changed but significant decreases were found in chol-LDL levels with WASF of garlic (−32% to −37%) compared to the control. These values are also reflected in the ratios of total-cholesterol to chol-LDL 2.28 vs 2.99 (24% decrease) and chol-LDL to chol-HDL, 0.89 vs 1.39 (36% decrease), compared to control (Table 2). Similar inhibitions of these parameters were obtained with MESF of garlic. The most effective dose of MESF and WASF for these inhibitions was found to be equivalent to 6.0% fresh garlic paste.

The relationship between chol-HDL, chol-LDL levels and the risk of coronary heart disease is now well established, whereas it is not so between dietary practices and cholesterol levels in the serum lipoprotein fractions. The protective effect of HDL is suggested to lie in its role in the removal of cholesterol from arterial tissue (23,24). A decrease in the serum cholesterol level caused by antihypercholesterolemic agents is usually accompanied by the reduction of serum chol-LDL (25). The significant decrease in the chol-LDL affected by the addition of WASF of garlic to a corn-soybean based diet suggests that these materials might have a similar or closely related mode of action.

The effects of MESF and WASF of garlic on lipid metabolism were tested in isolated hepatocytes of female or male broiler chickens, which were incubated with 25-300 μg of each of the fractions for 15 min. Dose-related decreases in the activities of HMG-CoA reductase and FAS were obtained with increasing concentration of these fractions (Table 3) or time of incubation using 100 μg, 5-60 min, (Table 4) in these hepatocytes. The maximum inhibition of these enzymes occurred within 20 min, in incubation containing 100 μg of each of these fractions (Table 4). Slightly lower activities of these two enzymes were found when incubated with MESF compared to WASF (Tables 3 and 4), which is due to the presence of Tween-80 for dispersing MESF in the incubation. Moreover, the activities of these enzymes were also slightly higher in hepatocytes prepared from female broiler chickens (Table 3) than male chickens (Table 4).

TABLE 2

Effect of Different Concentrations of Methanol- and Water-Soluble Fractions of Garlic on Hepatic Enzyme Activities of Cholesterol and Serum Lipids in 8-Week-Old Male Broiler Chickens[a]

WASF of garlic[a]				Concentration (serum mg/100 ml)		
Concentration (%)	HMG-CoA reductase[b]	Cholesterol 7α-hydroxylase[c]	Fatty acid synthetase[d]	Total cholesterol	Chol-HDL	Chol-LDL
Corn (control)	332±24[f] (100)[e]	2.8±0.1[f] (100)[e]	126± 4[f] (100)[e]	165±14[f] (100)[e]	55.2±3.7[f] (100)[e]	76.8±7[f] (100)[f]
Corn+0.20	290±23[f] (86)	2.5±0.2[f] (89)	122± 4[f] (97)	136±10[g] (82)	53.6±4.1[f] (97)	52.5±5[g] (68)
Corn+0.40	280±25[f] (84)	2.4±0.1[f] (86)	118± 6[fg] (94)	131± 8[g] (79)	54.1±4.3[f] (98)	50.2±6[g] (65)
Corn+0.80	200±20[g] (60)	2.3±0.1[f] (82)	117±10[fg] (93)	130±10[g] (79)	53.2±4.2[f] (96)	48.3±5[g] (63)
Corn+1.20	200±20[g] (60)	1.9±0.1[g] (68)	112± 6[g] (89)	126± 8[g] (76)	53.5±4.5[f] (92)	47.6±4[g] (62)
Corn+1.60	190±20[g] (58)	1.8±0.2[g] (64)	96± 3[h] (76)	123± 6[g] (75)	53.9±5.0[f] (98)	48.1±6[g] (63)

[a]Feeding period was 3 weeks; time of killing was 0800; data expressed as mean±SD: N=8 chickens per group; HMG-CoA reductase=β-hydroxy-β-methylglutaryl-CoA reductase. WASF=water-soluble fractions of garlic. Amount is equivalent to 1, 2, 4, 6, 8% of fresh garlic paste. Similar data was obtained with MESF=methanol-soluble fractions of garlic.
[b]pmol of mevalonic acid synthesized/min/mg of microsomal fraction.
[c]pmol of [^{14}C]cholesterol into [^{14}C]7α-hydroxycholesterol/min/mg of microsomal fraction.
[d]nmol of NADPH oxidized/min/mg of cytosolic fraction.
[e]Percentage of respective control activity data are in parentheses.
[f-h]Values not sharing a common superscript letter are different at $p<0.05$.

The present data confirmed the in vivo studies by these fractions of garlic and the inhibition of lipid biosynthesis was independent of age and sex of the birds.

The present studies revealed the inhibition of cholesterol and fatty acid biosynthesis in vivo and in vitro by methanol- and water-soluble fractions of garlic, followed by the significant lowering of serum cholesterol and chol-LDL levels in chickens. Further studies in relation to the effects of these fractions of garlic, and their components on lipid metabolism are in progress.

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TABLE 3

Effect of Different Concentrations of Methanol- and Water-Soluble Fractions of Garlic on the Enzymic Activities of β-Hydroxy-β-Methylglutaryl-CoA Reductase and Fatty Acid Synthetase in Isolated Hepatocytes of Female Chickens[a]

Methanol- or water-soluble fractions of garlic	β-Hydroxy-β-methylglutaryl-CoA reductase[b]		Fatty acid synthetase[c]	
Concentration (μg)	Methanol-soluble fraction	Water-soluble fraction	Methanol-soluble fraction	Water-soluble fraction
0.0	22.5 (100)[d]	24.3 (100)[d]	69.9 (100)[d]	77.8 (100)[d]
25.0	15.3 (68)	14.5 (60)	68.8 (98)	75.2 (97)
50.0	14.7 (65)	14.0 (58)	65.2 (93)	71.3 (92)
75.0	11.2 (50)	10.2 (42)	59.7 (85)	65.4 (84)
100.0	10.4 (46)	9.5 (39)	53.8 (77)	61.2 (79)
200.0	9.7 (43)	9.0 (37)	46.7 (67)	54.3 (70)
300.0	8.6 (38)	9.1 (37)	44.4 (64)	52.2 (67)

[a]Eight-week-old female chickens were fed standard corn-soybean diets. They were fasted for 48 hr and refed 72 hr prior to the preparation of liver perfusion. Incubation period was 15 min. Values represent means of replicate within incubation set.
[b]pmol of mevalonic acid synthesized/min/mg of microsomal fraction.
[c]nmol of NADPH oxidized/min/mg of cytosolic fraction.
[d]Percentage of respective control activity data are in parentheses.

TABLE 4

Effect of Length of Incubation with Methanol- and Water-Soluble Fractions of Garlic on the Enzyme Activities of β-Hydroxy-β-Methylglutaryl-CoA Reductase and Fatty Acid Synthetase in Isolated Hepatocytes of Male Chickens[a]

Incubation time (min)	β-Hydroxy-β-methylglutaryl-CoA reductase[b]		Fatty acid synthetase[c]	
	Methanol-soluble fraction	Water-soluble fraction	Methanol-soluble fraction	Water-soluble fraction
0	15.5 (100)[d]	18.5 (100)[d]	52.0 (100)[d]	58.5 (100)[d]
5	13.0 (84)	14.5 (78)	46.0 (88)	52.5 (90)
10	11.5 (74)	11.0 (59)	44.0 (85)	46.0 (79)
15	9.5 (61)	10.0 (54)	40.5 (78)	43.5 (74)
20	8.5 (55)	9.5 (51)	38.4 (73)	41.0 (70)
40	8.0 (52)	9.0 (49)	37.0 (71)	41.0 (70)
60	8.0 (52)	9.0 (49)	35.5 (68)	37.0 (63)

[a]Eight-week-old male chickens were fed standard corn-soybean diets. They were fasted for 48 hr and refed 72 hr prior to the preparation of liver perfusion. Each incubation contains 100 μg of methanol- or water-soluble fractions of garlic; value represents means of replicate within incubation set.

[b]pmol of mevalonic acid synthesized/min/mg of microsomal fraction.

[c]nmol of NADPH oxidized/min/mg of cytosolic fraction.

[d]Percentage of respective control activity data are in parentheses.

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