Relative Toxicity of Cottonseed Gossypol Enantiomers in Broilers

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Abstract: In cottonseed meals gossypol exist as a mixture of (+)- and (-)-enantiomers. Because of limited data on the effects of specific gossypol enantiomers on poultry and variation in (-)-gossypol in seed of Upland [34 to 47% (-)-gossypol] and Pima cotton cultivars [52 to 57% (-)-gossypol], the relative toxicity of these enantiomers to broilers was evaluated. Seeds of three cotton cultivars differing in the proportion of (-)-gossypol (35.2, 52.2 and 66.3 %) were included in three different diets at levels to give a final concentration of 400 mg/kg of total gossypol [259 and 141; 161 and 209; 135 and 265 mg/kg of (+)- and (-)-enantiomers respectively]. A total of 72 One-Day-Old, male broilers were placed in 12 Petersime battery brooder pens at 6 birds per pen and randomly assigned to the three cottonseed meat diets and a soybean meal based control. All diets were formulated to fulfill the NRC (1994) suggested requirements for essential amino acids and poultry metabolizable energy for starting broiler chickens. Gossypol enantiomers were determined in plasma, liver, heart, kidney, breast muscle and pancreas collected at 21 days of age. BW was significantly depressed as the relative dietary concentration of the (-)-enantiomer increased ($P<0.01$). Relative liver weight significantly increased with the use of all three cottonseeds. Body weights were negatively associated with the concentration of (-)-gossypol in plasma, liver, heart, kidney and muscle. These results clearly indicate the toxic effects of gossypol in broilers are due primarily to the (-)-enantiomer.

Keywords: Broilers, cottonseed, gossypol, enantiomers, plasma.

INTRODUCTION

Gossypol is a pigment produced by plants belonging to the genera *Gossypium* (cotton) of the family *Malvaceae* (Heinstein 1977) [1]. It has two naphthalene rings (with identical constituents), and restricted rotation about the bond connecting the rings, forming two identical structures that have no plane of symmetry and differing optical properties [(+)- and (-)-enantiomers] (Huang 1987) [2]. Gossypol content and the proportion of (+)- and (-)-enantiomers in cottonseed are under genetic control, and vary within and between species of cotton (Cass 1991, Percy 1996) [3,4]. The seed of Upland cotton (*G. hirsutum*), which represents about 98% of the cotton grown in the United States, has a total gossypol content ranging from 0.50 to 0.76% with (-)-gossypol of 33.8 to 43.2% (Calhoun 1995c) [5]. Seed of Pima cotton (*G. barbadense*) grown in the United States has a gossypol content of 0.67 to 1.25%, with the (-)-enantiomer ranging from 35.2 to 55.2% (Stipanovic 2009) [6]. Distribution of gossypol enantiomers in cottonseed meal reflects the enantiomer distribution in the cottonseed.

Gossypol has toxic and antifertility effects on animals and humans. These effects are related to the species of animal, source of gossypol, level and duration of consumption, composition of the diet, age of the animal and stress (Phelps 1966, Berardi 1980, Nomeir 1985, Calhoun 1991) [7-10].

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Gossypol absorption from the gastrointestinal tract, tissue distribution and rate of elimination from the body vary between species (Lyman 1969, Chen 1987, Othman 1988, Garlich 1989, Dowd 1995) [11-15] Lyman 1969 [11], using ^{14}C labeled gossypol, established that gossypol is absorbed from the gastrointestinal tract of chickens. Dowd 1995 [15], using lambs, reported a slow absorption of gossypol. Chen 1987 [12], observed that both enantiomers are poorly absorbed from the gastrointestinal tract of rats; however, (-)-gossypol was absorbed at a faster rate than (+)-gossypol.

The liver of the animals that have been fed cottonseed have the highest concentration of total gossypol in the body (Lyman 1969, Tang 1980, Morgan 1988, Kim 1996, Lordelo 2005) [11,16-19], whereas blood, heart, pancreas, muscle, kidney, spleen and lung all have lower concen-trations (Chen 1987, Calhoun l995a) [12,20]. Dowd 1995 [15] reported in lamb the proportions of the (+)- and (-)-enantiomers are specific for each tissue. Concentrations of (+)- and (-)-gossypol in plasma, liver, kidney and muscle of chicken increase linearly as the level of free gossypol increases in the diets (Gamboa 2001) [21]. Lordelo (2005) [19] reported higher accumulation levels of (+)-gossypol to (-)-gossypol in the liver, heart, kidney, spleen, testes and muscle of chicken. Liver of lambs had the highest proportion of (+)-gossypol, whereas the heart had the highest proportion of the (-)-enantiomer.

Roehm 1967 [22] indicated that the presence of the highly reactive carbonyl and nucleophylic groups allows for a wide array of reactions such that gossypol in animal tissues is always bound to cell components. This reactivity and the

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diversity in types of bonds formed, as well as the slow release of (+)- and (-)-gossypol bound to proteins most probably affects the metabolism rate and elimination time of the enantiomers of gossypol from the body (Roehm 1967, Othman 1988) [13,22]. There is evidence that (+)-gossypol is cleared at a slower rate than (-)-gossypol (Chen 1987, Zhou 1987) [12,23]. Due to the higher rate of (-)-gossypol clearance, Chen 1987 [12] suggested metabolites of (-)-gossypol might be responsible for its toxic effects.

Different sources of gossypol have been shown to have different levels of availability. Using free gossypol from cottonseed meats (decorticated seed), intact cottonseed pigment glands and ruptured cottonseed pigment glands. Heywang 1966 [24] found differences in toxicity suggesting differences in gossypol availability in chickens. Heywang 1955 [16] reported that free gossypol from raw cottonseed was more toxic than free gossypol from hydraulic processed cottonseed meal. Knabe 1995 [25] using broilers found that gossypol from cottonseed meats was more available than gossypol from processed cottonseed meal regardless of the type of process. Free gossypol from hydrolyzed cottonseed soap stock is not as toxic as it is in cottonseed meal (Curtin 1956, Lipstein 1964) [27,28]. Bailey 2000 [29] showed that broilers fed moco crushed cottonseed containing a relatively high (+)- to (-)- gossypol enantiomer ratio performed better than broilers receiving commercial crushed cottonseed with a lower (+)- to (-)- gossypol enantiomer ratio. Differences in cottonseed processing can affect free gossypol concentrations as well as availability (Gamboa 2001) [21]. In broilers, body weight gains were lower in birds fed either enantiomer. However, (-)-gossypol was more detrimental to the growth than (+)-gossypol (Lordelo 2005) [19]. Santos 2005 [30] reported that animal response to processing of cottonseed tended to differ according to form of cottonseed. They also showed that feeding crushed pima cottonseed meat, increased gossypol availability versus whole pima seed in Holstein steers. Because of limited data on the effects of specific gossypol enantiomers on poultry and variation in the concentrations of (-)-gossypol in seed of *G. barbadense* cotton, an experiment was designed to provide information on the relative toxicity of (+)- and (-)-gossypol to broiler chickens.

MATERIALS AND METHODS

Seeds of two *G. barbadense* accessions (P-469 and P-470) and a *G. barbadense* cultivar (1935-95) were used in this study. Total gossypol and percentage (-)-gossypol were 13.1 mg/gm and 35.3 (P-469); 11.6 mg/gm and 52.2 (1935-95); 11.9mg/gm and 66.3 (P-470), respectively, for the three sources of cottonseed. Seeds were ground to pass a 6 mm screen and used in three different diets at levels of 3.05, 3.45 and 3.63 %, respectively, to give concentrations of 259 and 141, 161 and 209, and 135 and 265 mg/kg of the (+)- and (-)-enantiomers respectively. All diets contained 400 mg/kg of total gossypol (Table **1**). All of the gossypol present in unprocessed cottonseed is considered to be unbound and in the free state.

One-Day-Old, Argo male broiler chicks (n=72) were placed in 12 Petersime battery brooder pens (6 birds/pen)

Table 1. Gossypol Content of Cottonseed and Diets (as Fed Basis)

Identification	Gossypol in Seed			Gossypol in Diet[3]		
	Total[1]	(+)[2]	(-)[2]	Total	(+)	(-)
	____% of total ____			__________mg/kg________		
P-469	1.31	64.7	35.3	400	258.8	141.2
1935-95	1.16	47.8	52.2	400	191.2	208.8
P-470	1.19	33.7	66.3	400	134.8	265.2

[1]Total gossypol was determined by the Official Methods of the American Oil Chemist Society (AOCS, 1985).
[2](+)- and (-)-gossypol were determined by high performance liquid chromatography as described by Hron *et al.* (1995).
[3] Concentrations of total and the (+)- and (-)-enantiomers of gossypol in diets.

and three pens were randomly assigned to each of the cottonseed diets and a soybean meal based control diet. All diets were formulated to meet the National Research Council's (NRC, 1994) [31] requirements for essential amino acids and poultry metabolizable energy for starting broiler chickens (Table **2**). The chicks were housed in an environmentally regulated room which provided 23 h light and 1 h dark. Diets and water were provided *ad libitum*. Birds were observed twice daily to assess general condition and mortality. Body weights (BW) were obtained at day one and weekly thereafter. Feed intakes were also recorded weekly. Feed to gain ratios were calculated based on this information.

At 21 days of age, blood samples were obtained by heart puncture (1.5 ml/bird) from all birds using 10 ml vacuum tubes containing sodium heparin as an anticoagulant. The contents were mixed by repeated, gentle inversion. Tubes were kept on ice and protected from light until plasma was separated by centrifugation. Plasma was stored at -20^{o}C until used for gossypol analysis. Birds were then immediately killed by cervical dislocation and samples of liver, kidney, heart, pancreas and breast muscle were collected and stored at -20^{o}C until analyzed for gossypol.

Total gossypol in cottonseed was determined by the official method of the American Oil Chemists Society (AOCS, 1985) [32]. The proportions of the (+)- and (-)-enantiomers in seed were determined by high performance liquid chromatography (HPLC) after precolumn derivatization with a chiral amine [(-)-2-amino-1-propanol] (Hron *et al*., 1995) [33]. Total, and (+)- and (-)-gossypol in lyophilized plasma and tissues were determined by HPLC as described by Kim 1995 [34].

Data Analysis

The experimental design was a completely random arrangement of four treatments with three replications. The General Linear Models Procedure of the Statistical Analysis System (SAS) [35] was used to analyze the data. A means separation test (Duncan's Multiple Range Test) was used to test for differences between means when the F test for treatments was significant ($P < 0.05$). Correlation and regression analyses were used to test the associations between gossypol intakes and performance criteria and between gossypol intakes and plasma and tissue concentrations of

Table 2. Percentage Composition and Calculated Nutritional Values for Starter Diets

Ingredients	Control	P-469[1]	1935-95[2]	P-470[3]
Corn	50.56	48.67	48.01	48.15
Soybean meal (48% CP)	37.86	35.58	35.73	36.71
Cottonseed	-	3.05	3.45	3.36
Fat (animal & vegetable blend)	6.92	7.38	7.50	7.47
Calcium carbonate	1.70	1.72	1.72	1.72
Mono-dicalcium phosphate[4]	1.66	1.61	1.60	1.60
Salt	0.43	0.43	0.43	0.43
DL-methionine (98%)	0.19	0.19	0.19	0.19
Vitamin premix[5]	0.25	0.25	0.25	0.25
Mineral premix[6]	0.05	0.05	0.05	0.05
Coban 60®[7]	0.075	0.075	0.075	0.075
Calculated Nutrient Content				
Crude protein, %	23	23	23	23
ME, kcal/kg	3200	3200	3200	3200
Calcium, %	1.00	1.00	1.00	1.00
Phosphorus (available), %	0.45	0.45	0.45	0.45
Lysine, %	1.10	1.10	1.10	1.10
Methionine, %	0.50	0.50	0.50	0.50
Methionine + cystine, %	0.90	0.90	0.90	0.90

[1] P-469 = 259:141, Dietary concentrations of (+)- and (-)- enantiomers (mg/kg) respectively.
[2] 1935-95 = 191:209, Dietary concentrations of (+)- and (-)- enantiomers (mg/kg) respectively.
[3] P-470 = 135:265, Dietary concentrations of (+)- and (-)- enantiomers (mg/kg) respectively.
[4] Each kg provided 15,555 mg of iron.
[5] Supplied per kilogram of diet: Vitamin A acetate, 11,023 IU; cholecalciferol, 3858 IU; vitamin E, 46 IU; menadione (menadione sodium bisulfite complex), 1.47 mg; thiamine (thiamine mononitrate) 2.94 mg; riboflavin, 5.85 mg; niacin, 45.93 mg; choline (choline chloride), 477.67 mg; pantothenic acid (calcium d-pantothenate), 20.21 mg; pyridoxin (pyridoxine hydrochloride), 7.17 mg; vitamin B_{12}, 16.5 µg; d-biotin, 0.55 mg; folic acid, 1.75 mg; antioxidant, 55 mg.
[6] Trace mineral premix provided the following per kilogram of diet: manganese (sulfate or oxide), 68 mg; zinc (oxide), 55 mg; copper (sulfate or oxide), 4.4 mg; iron (ferrous sulfate), 26.4 mg; iodine (calcium iodate), 1.1 mg; selenium (sodium selenite), 0.1 mg.
[7] Each pound contains 60 g monensin as monensin sodium.

gossypol. Since plasma and tissues of birds fed the control diet contained no gossypol, only the gossypol data for the birds fed the three cottonseed treatments were included in the correlation and regression analysis.

RESULTS

None of the birds used in this experiment became sick or died. The only overt signs of gossypol toxicity observed during this study were reductions in feed intake and body weight gains. A depression in feed consumption was observed during the second week in birds consuming the diet containing cottonseed variety P-470, ($P < 0.05$) but the difference was not significant (Table **3**). During the third week, birds consuming the two highest concentrations of dietary (-)-gossypol consumed significantly less feed ($P < 0.05$) than the control birds or those fed the lowest level of (-)-gossypol (Table **3**). Overall, for the 21-day period, there was a negative correlation between the dietary level of (-)-gossypol and feed intake.

During the first week there was no significant effect of diet on body weight. However, by the end of the second week, birds fed the highest level of (-)-gossypol weighed less than either the control birds or those receiving the other two cottonseed treatments. At the end of the third week, body weights were reduced as the level of (-)-gossypol increased in the diet (Table **3**).

Relative liver weight (liver weight expressed as a percentage of body weight) was greater in birds fed the diets containing cottonseed. There were not significant correlations for (-)-gossypol consumption and relative liver weights, however, there was a significant correlation between (+)-gossypol consumption and relative liver weight ($r = 0.77$, $P < 0.01$). Compared with the controls, relative heart weight was less in birds fed the diet containing cottonseed variety P-469 ($P < 0.05$) (Table **3**). Relative heart weight was not correlated with intakes of either the (+) or (-) enantiomers of gossypol, but it was correlated with total gossypol consumption ($r = 0.69$, $P < 0.04$).

Concentrations of total gossypol decreased linearly in plasma, liver, kidney and muscle as the consumption of total gossypol decreased (Table **4**). Total gossypol levels in the heart were not correlated with cumulative gossypol consumption. Liver had the highest concentration of total

Table 3. Weekly Feed Consumption and Body Weight of Broilers Fed Diets Containing 400 mg/kg of Total Gossypol, Provided by Cottonseed with Different Proportions of (+)- and (-)-Gossypol, from 1 to 21 d

Week		Cottonseed Variety			PSEM
	Control[1]	P-469[2]	1935-95[3]	P-470[4]	
	Feed Consumption (g/bird)				
1	90.4	109.6	108.8	99.4	5.1
2	284.0	288.5	307.2	241.1	15.3
3	525.4[a]	472.2[ab]	431.8[b]	323.9[c]	25.4
Cumulative	899.8[a]	870.3[a]	846.8[a]	664.4[b]	37.6
	Body Weight (g/bird)				
1	125.1[b]	143.1[a]	133.6[ab]	126.8[b]	4.0
2	332.4[a]	348.1[a]	328.4[a]	271.1[b]	11.5
3	718.6[a]	654.3[b]	595.0[c]	460.8[b]	16.7
	Relative Organ Weights (%)[5]				
Liver	2.74[c]	3.22[a]	2.98[b]	2.95[b]	0.07
Heart	0.65[a]	0.60[ab]	0.64[a]	0.57[b]	0.02

[1]Corn soy diet with no cottonseed
[2] P-469 = 259:141, Dietary concentrations of (+)- and (-)- enantiomers (mg/kg) respectively.
[3]1935-95 = 191:209, Dietary concentrations of (+)- and (-)- enantiomers (mg/kg) respectively.
[4] P-470 = 135:265, Dietary concentrations of (+)- and (-)- enantiomers (mg/kg) respectively.
[5]Grams of organ per 100g of BW.
[a-c] Means within a row without a common superscript differ ($P < 0.05$).

Table 4. Concentrations of Gossypol in the Plasma, Liver, Heart, Kidney and Muscles of Broilers Fed Diets Containing 400 mg/kg of Total Gossypol, Provided by Cottonseed with Different Proportions of (+)- and (-)-Gossypol, from 1 to 21 d

	Cottonseed Variety			
	P-469[1]	1935-95[2]	P-470[3]	PSEM
Plasma (μg/ml)				
(+) enantiomer	32.8[a]	22.3[b]	15.0[c]	0.9
(-) enantiomer	7.5[c]	9.6[b]	12.3[a]	0.5
Liver (μg/g DM)				
(+) enantiomer	1013.7[a]	797.5[b]	544.3[c]	56.3
(-) enantiomer	92.1[c]	159.5[b]	227.6[a]	10.9
Heart (μg/g DM)				
(+) enantiomer	55.9[a]	44.5[ab]	28.9[b]	5.5
(-) enantiomer	14.6[b]	21.5[b]	33.8[a]	2.6
Kidney (μg/g DM)				
(+) enantiomer	192.7[a]	110.6[b]	73.2[b]	11.3
(-) enantiomer	30.0[b]	36.8[ab]	46.7[a]	3.5
Muscle (μg/g DM)				
(+) enantiomer	20.4[a]	14.1[b]	8.5[c]	0.5
(-) enantiomer	3.2[b]	4.4[ab]	5.5[a]	0.4

[1] P-469 = 259:141, Dietary concentrations of (+)- and (-)- enantiomers (mg/kg) respectively.
[2]1935-95 = 191:209, Dietary concentrations of (+)- and (-)- enantiomers (mg/kg) respectively.
[3] P-470 = 135:265, Dietary concentrations of (+)- and (-)- enantiomers (mg/kg) respectively.
[a-c] Means within a row without a common superscript differ ($P < 0.05$).

gossypol. Concentrations of the (+)- and (-)-enantiomers of gossypol in plasma, liver, kidney and muscle reflected consumption of the individual (+)- and (-)-enantiomers of gossypol. The concentrations of (-)-gossypol in the heart were not correlated with the cumulative consumption of (-)-gossypol.

Proportions of (+)- and (-)-gossypol in the diet were positively correlated with proportions of these enantiomers in plasma. There was a high degree of association between plasma total gossypol and liver, kidney and muscle total gossypol; whereas, plasma total gossypol was not significantly correlated with heart total gossypol. The correlations for plasma levels of (-)-gossypol and tissue concentrations of (-)-gossypol were lower than the correlation for the (+)-enantiomer. The coefficients were $r = 0.90$ ($P < 0.0009$), $r = 0.83$ ($P < 0.0053$), $r = 0.73$ ($P < 0.0257$), and $r = 0.71$ ($P < 0.03$) for liver, heart, kidney and muscle, respectively.

DISCUSSION

Animal research in which the pure enantiomers of gossypol have been used, suggested that toxic effects of gossypol are mainly related to concentration of the (-)-enantiomer of gossypol (Calhoun 1995b) [36]. The results are in agreement with reports by Heywang 1966 and Knabe 1995 [24,26] who indicated that gossypol from cottonseed meats was much more available than gossypol from cottonseed meals. Results of the present study indicate the primary toxic effect of dietary (-)-gossypol is depressed feed consumption. There were no significant differences in feed to gain ratio. Depression in feed consumption resulted in low BW gains as well as low consumption of gossypol, which in turn resulted in a lower concentration of tissue gossypol.

Lordelo 2005 [19] reported that total feed consumption was reduced only in broilers consuming (-)-gossypol, but BW gains were lower for birds fed either enantiomer. Wang 1987 [37] reported a significant depression in BW gain after administration of (-)-gossypol to rats. These authors did not indicate whether the reduced BW gain was the result of depressed feed consumption or inefficiency in feed utilization. Lordelo 2007 [38] reported that in laying hens and broiler breeder hens ingestion of (+)-gossypol has a greater effect on egg yolk discoloration than the consumption of (-)-gossypol. They also reported that addition of (+)-gossypol to the diet reduced egg production.

In this study relative liver weight increased as dietary gossypol increased regardless of the enantiomer ratio or cumulative consumption. Lordelo 2005 [19] showed that relative liver weight increased in chicks fed the highest concentration of (+)-gossypol and decreased in chicks fed the highest level of (-)-gossypol. Relative heart weight on the other hand was significantly lower than the control in broilers receiving the high (-)-gossypol treatment (265 mg/kg).

The liver and kidney had the highest concentrations of total gossypol regardless of the dietary enantiomer ratio. Similar results were previously reported for chickens (Lyman 1969, Knabe 1995) [11,26], trout (Roehm 1967) [22] and lambs (Kim 1996) [18]. Tissue deposition of total gossypol and gossypol enantiomers is specifically related to individual organs. On the other hand, the concentration of total gossypol in heart tissue was independent of gossypol consumption. Concentrations and proportions of gossypol enantiomers in plasma, liver, and muscle linearly increased as gossypol consumption increased. Similar results were previously reported for broiler chickens by Gamboa 2001 [21]. But Lordelo 2005 [19] showed that except for the liver, tissue concentrations of either enantiomer did not increase from 21 d to 42 d of age and as dietary concentrations increased, concentrations of (-)- and (+)-gossypol at 42 d of age increased linearly in the heart, kidney, muscle and plasma; however, in the liver, the concentration increased quadratically. Information from a previous experiment in which gossypol was furnished from 8 different cottonseed meals indicated that concentration of total, (+) and (-)-gossypol in plasma, liver, kidney and muscle were linearly related to dietary gossypol.

Concentrations of (+) or (-)-gossypol in liver tissue as a function of cumulative consumption of gossypol enantiomers were higher in liver (3.48:1.51 μg/1 mg; (+)-:(-)-gossypol), followed by plasma (0.127 and 0.054 μg/1 mg;(+)-:(-)-gossypol), and muscle (0.085 and 0.028 μg/1mg; (+)-:(-)-gossypol). In all cases, tissue response to dietary (+)-gossypol was higher than the response to dietary (-) gossypol. Similar results were also reported by Lordelo 2005 [19] in broilers. All tissues responded to changes in dietary ratios of (+) and (-) gossypol. For all experimental treatments liver had the highest proportion of (+)-gossypol followed by kidney, plasma, muscle and heart. Lordelo 2005 [19] reported that liver had the highest accumulation of total gossypol followed by bile, spleen, kidney, testes, heart, plasma and muscle. Chen 1987 [12] reported a greater accumulation of (+)-gossypol due to its longer half life. Yu 1987 [39] also reported that (-)-gossypol has a low rate of binding to proteins making it more suitable for degradation. Due to the high rate of (-)-gossypol clearance, Chen 1987 [12] suggested that metabolite products of (-)-gossypol degradation might be responsible for its toxic effects.

Plasma concentrations of total gossypol and gossypol enantiomers clearly represent dietary concentrations and distribution of gossypol enantiomers. Plasma gossypol analysis may be valuable for establishing safe levels of dietary gossypol enantiomers as previously suggested by Calhoun 1995b, Calk 1992 and Knabe 1995 [26,36,40].

In cottonseed and cottonseed meals gossypol exist as a mixture of (+)- and (-)-enantiomers. Data from this experiment clearly show that (-)-gossypol is significantly more toxic than (+)- gossypol. These results are consistent with the research conducted by Lordelo 2005 [19] whose results also indicated that both gossypol enantiomers were toxic to broilers but (-)-gossypol was more harmful to efficient broiler production than (+)-gossypol. Variation in (-)-gossypol in seed from Upland [34 to 47% (-) gossypol] and Pima cotton cultivars [52 to 57% (-)-gossypol] require that the specific enantiomer concentrations be considered when assessing gossypol toxicity. In this study we were able to correlate dietary and tissue concentrations of (-)-gossypol to depressed performance leading us to suggest that cottonseed meals containing predominantly concentration of (+)-gossypol are desirable for broiler nutrition. The toxicity of methyl ethers of gossypol have not been determined in this study, but the levels and ratios of enantiomers may be important.

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Received: November 09, 2009 Revised: December 11, 2009 Accepted: March 3, 2010