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Bioactivities of Gossypol, 6-Methoxygossypol, and 6,6′-Dimethoxygossypol

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6-Methoxygossypol and 6,6′-dimethoxygossypol were recently isolated from the root tissue of cotton plants. Together with gossypol, these natural products were investigated for a wide range of bioactivities. Antioxidant effects, the potential to prevent DNA damage, anticancer activity, and antitrypanosomal effects were studied. The 6-methoxygossypol generally exhibited equal bioactivities to the 6,6′-dimethoxygossypol, but gossypol showed greater activities than both methylated derivatives in scavenging free radicals, reducing ferric ions, and preventing UV-induced DNA damage. All three compounds inhibited the growth of cervical (SiHa), breast (MCF-7), and colon (Caco-2) cancer cells. At ≥5 ppm of test concentrations, 6,6′-dimethoxygossypol showed a stronger ability than gossypol to inhibit the growth of *Trypanosoma brucei* cells.

KEYWORDS: Cottonseed; gossypol; gossypol derivative; antioxidant; anticancer; trypanosome

INTRODUCTION

Gossypol is a polyphenolic compound of cotton plants, and this secondary metabolite appears to inhibit insect predation (*1*) and microbial growth (*2*). This compound initially garnered considerable attention because of its contraceptive effects (*3, 4*), and recently it was found to possess a wide spectrum of interesting biological activities, particularly its capability to inhibit the growth of several types of human cancer cells. Breast, colon, prostate, and leukemia cells are all sensitive to the presence of gossypol (*5–8*). In addition, the compound is reported to have antiviral (*9, 10*), antiamoebic (*11*), and antiprotozoan effects (*12–14*). However, its toxicity at higher concentrations limits the use and marketability of cottonseed and cottonseed products in some animal feeding applications.

6-Methoxygossypol and 6,6′-dimethoxygossypol (**Figure 1**), have been found in some varieties of cotton (*15, 16*). Although considerable work on the activities of gossypol has been conducted, few reports are available on the biological activities of the gossypol derivatives. In the initial report on the identification of these compounds, mention was made that both compounds exhibited antimicrobial activity, but few details were provided (*15*). Another report on chemically synthesized methylated gossypol indicated that the derivative had limited activity as a male contraceptive (*17*). However, no other studies have been reported because these gossypol derivatives are not commercially available.

Both compounds were until recently isolated from the root bark of *Gossypium barbadense* (Sea Island cotton) by preparative chromatography (*18*). In this report, we compare and discuss the activities of gossypol, 6-methoxygossypol, and 6,6′-dimethoxygossypol in several bioassays. The tested bioassays included free radical scavenging activity, reducing power, DNA damage prevention capability, and cancer and trypanosomal cell growth inhibition.

MATERIALS AND METHODS

Materials. Roswell Park Memorial Institute 1640 (RPMI 1640), gossypol acetic acid, 2,2′-diphenyl-1-picrylhydrazyl (DPPH), and butylated hydroxytoluene (BHT) were purchased from Sigma Chemical Co. (St. Louis, MO). Tissue culture plates were purchased from Costar Corp. (Cambridge, MA). Heat-inactivated fetal bovine serum, fetal

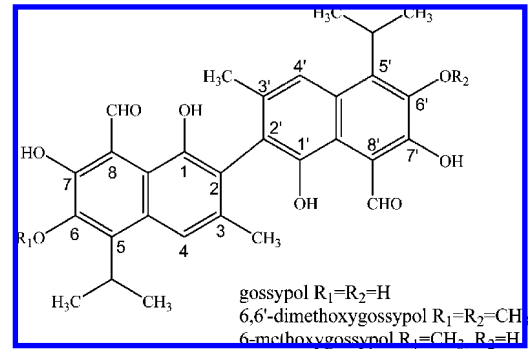

Figure 1. Structures of gossypol, 6-methoxygossypol, and 6,6′-dimethoxygossypol.

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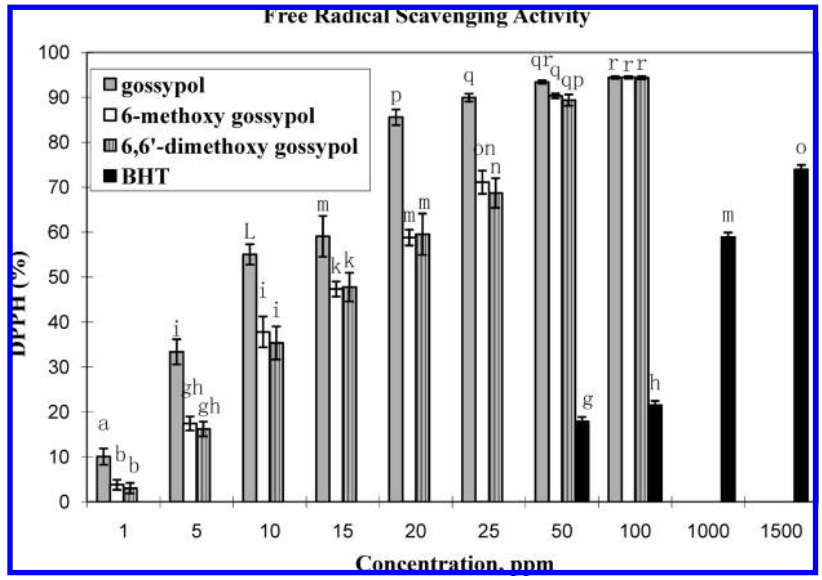

Figure 2. DPPH free radical scavenging activity of gossypol, 6-methoxygossypol, and 6,6′-dimethoxygossypol. Error bars represent the standard deviation of triplicate experiments, and different letters represent significant difference at $P < 0.05$.

bovine serum, and newborn calf serum were purchased from Hyclone Laboratories, Inc. (Logan, UT). Dichloromethane (DCM), acetone, and trichloroacetic acid (TCA) were from Fisher Scientific (Suwanee, GA), and potassium ferricyanide was from J. T. Baker Chemical Co. (Phillipsburg, NJ). 6-Methoxygossypol and 6,6′-dimethoxygossypol were isolated as previously reported (*18*). Both methoxy gossypol derivatives were 1:1 molar solvates of acetic acid. Like gossypol acetic acid (1:1), both methylated gossypols are racemic with 1/1 of (+)-gossypol/(−)-gossypol ratio (unreported data).

Antioxidant Activity. DPPH free radical scavenging and reducing power were used to evaluate antioxidant activities

Free radical scavenging activity was determined by the method of Yamaguchi, et al. (*19*) with slight modification. Solutions of each gossypol compound in DCM were prepared at concentrations from 1 to 100 ppm. An aliquot of 0.4 mL of the solution was mixed with 0.4 mL of 0.25 mM solution of DPPH in DCM. The solution was shaken vigorously and then incubated in the dark at room temperature for 30 min. UV absorption was measured at 517 nm. Less absorption at 517 nm indicates higher free radical scavenging activity which was calculated as

$$\text{scavenging activity (\%)} = \left(1 - \frac{\text{absorbance of sample at 517 nm}}{\text{absorbance of control at 517 nm}}\right) \times 100 \quad (1)$$

BHT at concentrations of 100, 1000, and 1500 ppm in DCM was included as a comparative standard.

Reducing power was determined according to the method of Chung, et al. (*20*). Acetone solutions of each gossypol compound were prepared to yield test concentrations of 1, 10, 100, and 125 ppm. A 0.5-mL aliquot of each acetone solution was mixed with 1 mL of a 1% potassium ferricyanide [$K_3Fe(CN)_6$] solution and was incubated at 50 °C for 20 min. One milliliter of 10% TCA was then added into the mixture, and the solution was centrifuged at 3000 rpm for 10 min. The upper layer (1 mL) was recovered and mixed with 1 mL of distilled water and 0.2 mL of a 0.1% solution of $FeCl_3$. Solution absorption was measured at 700 nm. Higher absorbance indicates greater reducing power (*20*).

DNA Strand Breakage. DNA strand scission was performed as described by Keum et al. (*21*), with minor modifications. Plasmid DNA was prepared and purified from *Escherichia coli* as described by Sambrook and Russell (*22*). The reaction mixture (15 μL) contained 10 mM Tris-HCl, 1 mM EDTA buffer (pH 8.0), 0.04 M H_2O_2 with 1 μL of plasmid DNA. The test compounds were dissolved in DMSO at defined concentrations and were added prior to H_2O_2 addition. Hydroxyl radicals were generated by irradiation of the reaction mixture in a 1.5 mL centrifuge tube at a distance of 30 cm with a 12 W UV lamp. After incubation at room temperature for 30 min, the reaction was terminated by adding a loading buffer (0.25% bromophenol blue tracking dye and 40% sucrose), and the DNA was analyzed by electrophoresis on 1% agarose gels. Bands were visualized by staining with ethidium bromide and were photographed on a transiluminator (Bio-Rad Laboratories).

Anticancer Activities. MCF-7 (human breast), Caco-2 (human colon), and SiHa (cervical) cancer cell lines were purchased from the American Type Culture Collection (ATCC) (Rockville, MD). MCF-7 and SiHa cells were cultured in RPMI-1640 with 10% newborn calf serum (*23*). Caco-2 cells were cultured in RPMI with 10% fetal bovine serum instead of the newborn calf serum. All cell lines were incubated at 5% CO_2 and 90−100% relative humidity at 37 °C. Medium renewal was carried out 2−3 times per week, and cells were subcultured when they achieved 80−90% confluence.

Prior to chemical treatment, 10^4 cells/well were seeded into a 96-well tissue culture plate and were allowed to attach for 24 h. The cells were then treated with a defined concentration of the test compound dissolved in DMSO, which was limited to a 2% concentration in each well. As negative controls, cells were treated with DMSO only. After a 24-h incubation period, cell proliferation was determined with the CellTiter 96 aqueous nonradioactivity cell proliferation assay (Promega, Madison, WI). Results were recorded on a universal EL800 Bio-Tek microplate reader at 490 nm.

Antitrypanosomal Activities. *Trypanosoma brucei* bloodstream form parasites (90−13, provided by Rockefeller University) were grown in HMI-9 medium supplemented with 10% heat-inactivated fetal bovine serum and cultured as previously reported (*24*). Parasites were seeded into the 96-well tissue culture plates (10^4 cells/well/200 μL) and treated with gossypol or methoxygossypol derivatives dissolved in DMSO. After 24-h incubation at 37 °C under 5% CO_2, trypanosomes were counted on a Becton Dickinson FACScan flow cytometer. The percentage of survived cells was determined by comparing the treated cell counts with those from wells treated only with DMSO.

Statistical Analysis. Each experiment was conducted at least three times. All data were subjected to the statistical analyses by using SAS program to examine the least significant difference (LSD) at the 95% confidence level.

RESULTS

Free Radical Scavenging Activity. The concentrations of gossypol, 6-methoxygossypol, and 6,6′-dimethoxygossypol needed to scavenge 50% of the free radicals in the test system were 8.2, 16.4, and 16.8 ppm, respectively (**Figure 2**). 6-Methoxy-

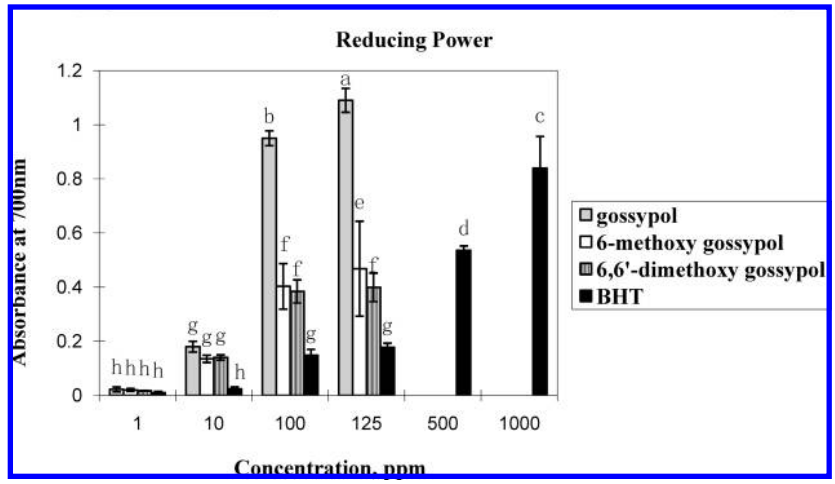

Figure 3. Reducing power of gossypol, 6-methoxygossypol, and 6,6′-dimethoxygossypol. Higher absorbance indicates greater reducing power. Error bars represent the standard deviation of triplicate experiments, and different letters represent significant difference at $P < 0.05$.

gossypol exhibited a similar free radical scavenging activity as 6,6′-dimethoxygossypol, while gossypol possessed a stronger radical scavenging activity than either methylated derivative.

Reducing Power. Gossypol, 6-methoxygossypol, and 6,6′-dimethoxygossypol all reduced ferric ions to ferrous ions in a dose-dependent manner (**Figure 3**). Because of gossypol's solubility limitation, the highest concentration of the test compounds was 125 ppm. Like the DPPH assay, gossypol showed greater reducing power and higher efficiency than 6-methoxygossypol or 6,6′-dimethoxygossypol. All three test compounds showed much stronger reducing power than BHT. 6,6′-Dimethoxygossypol exhibited the same reducing power at a 10 ppm concentration as BHT at a 100 ppm concentration. Regardless of the negative influence of the methylation, the study demonstrated that gossypol and its two methoxy derivatives had substantial antioxidant activity compared with compounds currently in use.

Assay of DNA Damage. Hydroxyl radicals are known to cause DNA strand scission (*25*), and these effects can be observed by monitoring the banding patterns of electrophoretically separated DNA plasmids, i.e., observing a reduction of the major supercoiled band and the formation of bands corresponding to nicked-circles and linearized plasmids or polymerized DNA. When exposed to oxidative conditions in the presence of gossypol or 6-methoxygossypol, plasmid DNA showed much less damage than did the control case without one of the test compounds (**Figure 4**), an effect that appeared to be dose-dependent. At the 800 μM concentration (~400 ppm), gossypol and 6-methoxygossypol appeared to protect against any DNA damage. 6,6′-Dimethoxygossypol was not as effective as other two chemicals, so significant damage was observed at 8 or 800 μM concentrations (**Figure 4**).

Anticancer Activity. Gossypol, 6-methoxygossypol, and 6,6′-dimethoxygossypol exhibited similar dose-dependent growth inhibition against cervical, breast, and colon cancer cells (**Figure 5A−C**). There were no significant differences in activity between the tested gossypol forms against each cell line ($P > 0.05$), except at the 10 ppm concentration. At this level, 6-methoxygossypol and 6,6′-dimethoxygossypol showed higher anticancer activity than gossypol. The IC_{50} values are around 10 ppm for 6-methoxygossypol and 6,6′-dimethoxygossypol, but a much higher IC_{50} value (>10 ppm) for gossypol was observed in the presence of serum proteins.

Antitrypanosomal Activity. Gossypol and gossypol derivatives essentially stopped *T. brucei* cell growth at concentrations

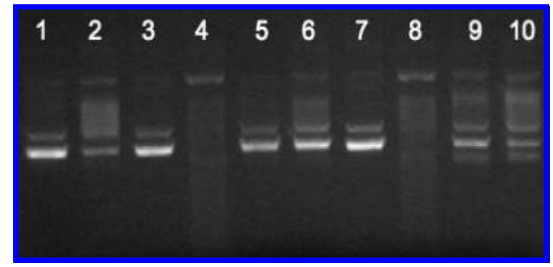

Figure 4. Protection effect of gossypol and methoxygossypol on DNA strand scission induced by H_2O_2 and UV: lane 1, 800 μM gossypol + UV + H_2O_2; lane 2, 8 μM gossypol + UV + H_2O_2; lane 3, control DNA; lane 4, control DNA + UV + H_2O_2; lane 5, 800 μM 6-methoxygossypol + UV + H_2O_2; lane 6, 8 μM 6-methoxygossypol + UV + H_2O_2; lane 7, control DNA; lane 8, control DNA + UV + H_2O_2; lane 9, 800 μM 6,6′-dimethoxygossypol + UV + H_2O_2; lane 10, 8 μM 6,6′-dimethoxygossypol + UV + H_2O_2.

greater than or equal to 10 ppm (**Figure 6**). The IC_{50} values are 7.83, 3.98, and 3.21 ppm for gossypol, 6-methoxygossypol, and 6,6′-dimethoxygossypol, respectively. At the 5 ppm concentration, 6,6′-dimethoxygossypol exhibited stronger activity than either gossypol or 6-methoxygossypol. At the 10 ppm concentration, both 6-methoxygossypol and 6,6′-dimethoxygossypol showed stronger activity than gossypol. At this concentration, both methoxy derivatives essentially stopped cell growth.

DISCUSSION

Gossypol has long been known to be toxic to animals. This property has limited the use of cottonseed as ruminant diets. Meanwhile, the compound has also been found to possess some special biological activities. It has been reported that the compound had anticancer (*26, 27*), insect inhibitory effect (*1*), antimicrobial activity (*2*), antiprotozoan effect (*12–14*), and antioxidant activity (*28*). Recently, considerable work has focused on the gossypol's ability to inhibit the growth of cancer cells (*6, 8, 29–31*).

Although gossypol's biological activities have been widely investigated, the structural and conditional factors that influence the activities are still unclear. For example, there is a significant difference in activity of the optical forms of gossypol; e.g., the (−)-gossypol enantiomer is more active than the (+)-gossypol enantiomer (*27, 30*). Such observation suggests that the molecular functional group responsible for activity extends over

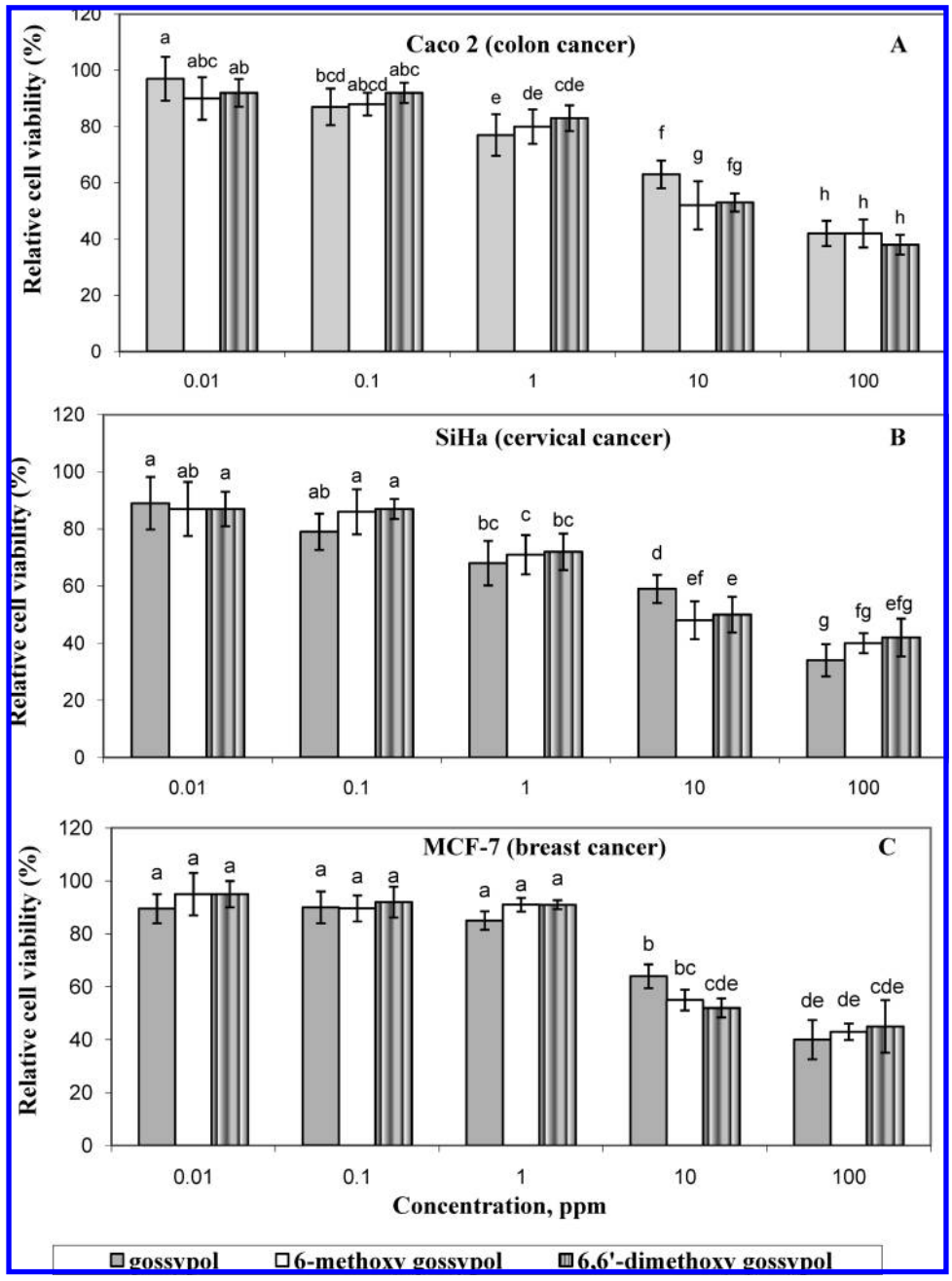

Figure 5. Growth inhibition of three cancer cell lines (human breast MCF-7, human colon Caco-2, and cervical SiHa) incubated with gossypol, 6-methoxygossypol, and 6,6′-dimethoxygossypol. Error bars represent standard deviations of four experiments, and different letters represent significant difference at $P > 0.05$.

the binaphthalene bridge bond and that the particular orientation of the substituent of each naphthalene ring also affects the activity. In addition, some researchers have noted that gossypol's activity is markedly reduced in the presence of protein. It has been suggested that gossypol's aldehydic groups can readily react with the primary amines of protein and/or other amino groups to form Schiff's bases, which significantly reduces bioactivities (*32*). Because of these influences, gossypol's therapeutic effectiveness often appears to require a higher concentration but with an increasing risk of toxicity. Consequently, efforts have been made to synthesize gossypol derivatives to improve activity and/or lower toxicity. Unfortunately, many of these efforts have been piece-meal, either in terms of the numbers and types of compounds synthesized or the array of bioactivities studied. Few convincing conclusions have been drawn at present regarding what features of the gossypol molecule are important for activity. Nevertheless, derivatives retaining the bioactivity with less toxicity have been reported. For example, Radloff and co-workers (*9*) showed that modification of the compound's aldehydic groups lowered its toxicity to the host Vero cells but did not abolish the compound's antiviral (HSV-II) activity. Other reports (*33, 34*) also confirmed that such modification could decrease the compound's toxicity while retaining gossypol's antiviral bioactivity.

In this work, 6-methoxygossypol and 6,6′-dimethoxygossypol were found to have many of the activities of gossypol. Regarding gossypol's antioxidant effects, methylation of even one of the phenolic hydroxyl groups has significantly reduced the activity, which indicates that the hydroxyl group of gossypol is critical for quenching free radicals. Similarly, replacement of hydroxyl groups with methoxy groups in modified chalcones has also been noted to affect free radical scavenging ability, though the

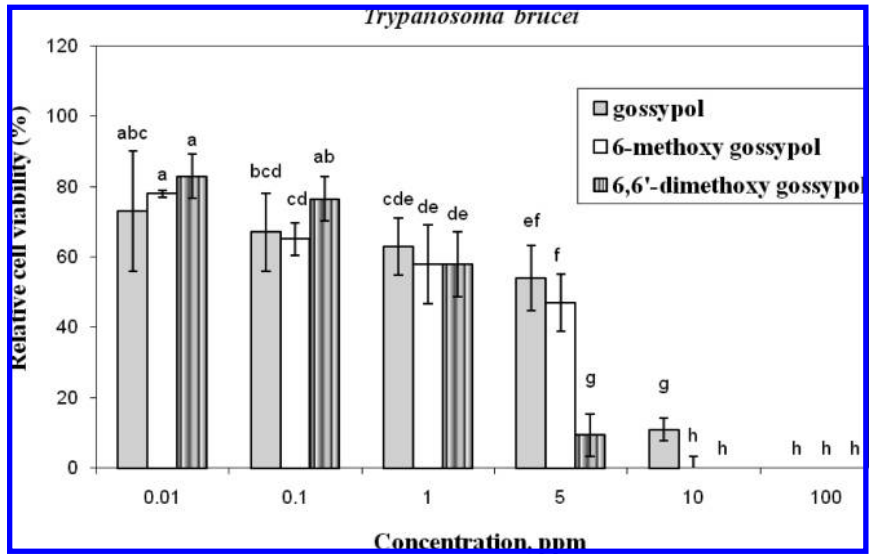

Figure 6. Growth inhibition of trypanosomes incubated with gossypol, 6-methoxygossypol, and 6,6′-dimethoxygossypol. Error bars represent standard deviations of four experiments, and different letters represent significant difference at $P < 0.05$.

methylation may improve or worsen the free radical scavenging ability, depending on the methylation pattern on the chalcone (*35*). Still, all three gossypol forms had a significantly greater free radical scavenging effect ($p < 0.05$) and a greater ability to reduce ferric ions (**Figures 2** and **3**) than BHT. The relative capability of gossypol and its related methylated derivatives to prevent the DNA damage was consistent with the compound's antioxidant effects. This suggests that gossypol's protection of DNA may occur, at least in part, by quenching free radicals, alleviating the oxidative stress that causes DNA damage.

Regardless of the toxicity of gossypol and its methylated derivatives, their antioxidant property may have industrial applications and food or medicinal applications, because the protective effects were found to occur at concentrations much lower than those imposed on gossypol in food products, i.e., FDA stipulates that cottonseed protein use must contain less than 450 ppm free gossypol (*36*) and the Protein Advisory Group of the United Nations Food and Agriculture and World Health Organizations (FAO/WHO) specifies limits of 600 ppm free gossypol and 12 000 ppm total gossypol in food products (*37*).

Methylation of the hydroxyls at the 6- and 6′-positions did not diminish gossypol's anticancer activity in all three cell lines tested. In cancer cells, gossypol is believed to arrest cell growth at the G0/G1 phase (*38*) and induce cell apoptosis (*8*) by regulating the cell cycle, antiapoptosis, and pro-apoptosis proteins and related enzymes. Yet, the details of gossypol effects toward cancer cells are an active research area that is in its infancy. In this study, a wide range dosages of gossypol compounds was tested, and the growth inhibition of the cancer cells by gossypol and its derivatives might occur because of their toxicity at high concentrations. Cell death maybe due to either apoptosis or necrosis, depending on the dosage. The mechanisms of cell death of gossypol compounds are being investigated. Neverthless, that the methoxygossypol derivatives have comparable or better activity suggests that the phenolic hydroxyl groups at the 6- and 6′-positions are not critical for this activity.

Few drugs are available for the treatment of trypanosomal infections that cause significant mortality in man and livestock in Africa. Gossypol is reported to inhibit trypanosomes (*12, 14, 39*). Montamat and co-workers (*14*) reported that a 5-min exposure to 100 μM gossypol (~50 ppm) immobilizes cultures of *Trypanosoma cruzi*.

Blanco et al. (*12*) reported that a 30-min exposure to 25 μM gossypol (~12 ppm) immobilizes and alters the cell morphology of *T. cruzi*. More recently, Kaminsky and Zweygarth (*39*) reported that, for three separate *T. brucei* strains (including one drug resistant strain), the IC_{50} value for a 24-h gossypol exposure was >10 ppm. Our study showed a similar level of gossypol's antitrypanosomal activity with an IC_{50} value of 7.8 ppm after 24-h exposure. Moreover, both 6-methoxygossypol (IC_{50} value, 3.98 ppm) and 6,6′-dimethoxygossypol (IC_{50} value, 3.21 ppm) more effectively inhibited growth than gossypol. In *T. cruzi*, gossypol has been reported (*13, 14*) to inhibit some oxidoreductases, such as α-hydroxyacid and malate dehydrogenases; NAD-linked enzymes; and, of glutamate dehydrogenase, malic enzyme and glucose-6-phosphate dehydrogenase and NADP-dependent enzymes.

All of the above-mentioned protozoan experiments included serum in the media. Hence, attenuation of gossypol's effect against trypanosomes is possible because of gossypol−protein interactions. Unfortunately, there have been no protozoan-based experiments with aldehyde-modified gossypol compounds. As the methoxygossypol derivatives were more active than gossypol, the combination of methylation and changes at the aldehyde position may yield stronger inhibition. This has prompted us to extend our investigation on further chemical modifications of the gossypol structure that may enhance its bioactivities.

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