

Diallyl Polysulfides from *Allium sativum* as Immunomodulators, Hepatoprotectors, and Antimycobacterial Agents

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ABSTRACT *Mycobacterium tuberculosis* remains one of the world's deadliest killers, with an annual death rate of ~1.5 million. The medicinal effects of garlic have been well documented, and natural products have been shown to have anti-mycobacterial activity. The current study evaluated the efficacy of six *Allium sativum* L. polysulfide mixtures as anti-mycobacterial agents together with their cytotoxic, immunomodulatory, and hepatoprotective activities. The microtitre PrestoBlue assay was used to determine the minimum inhibitory concentrations (MIC). Cytotoxicity was evaluated by using peripheral blood mononuclear cells (PBMC). Excreted cytokine levels were determined by utilizing an enzyme-linked immunosorbent assay (ELISA), by exposing isolated PBMCs to varying concentrations of polysulfide mixtures. Human C3A liver cells were utilized in the hepatoprotective study, to assess the protective effect against the toxicity induced by acetaminophen. Samples with higher amounts of diallyl trisulfide (Sample G4) showed the highest antimycobacterial activity, exhibiting an MIC of 2.5 µg/mL against *M. tuberculosis* H37Rv. Five samples showed moderate toxicity in PBMC, with G1 showing no toxicity. The selective index of G4 was the highest, with a selectivity index close to one. Two samples, G3 and G6 containing higher amounts of diallyl tetrasulfide and lower amounts of diallyl trisulfide, showed >50% hepatoprotection. This is comparable to a hepatoprotective agent, Silymarin, which showed a hepatoprotective effect of 30% at the tested concentration. Diallyl tetrasulfide showed significant antimycobacterial activity. A combination of higher diallyl tetrasulfide and lower diallyl trisulfide was indicative of hepatoprotective activity.

KEYWORDS: *Allium sativum*; antimycobacterial; diallyl tetrasulfide; hepatoprotective; immune stimulant; polysulfides, tuberculosis

INTRODUCTION

Tuberculosis is a worldwide problem and an epidemic in most low-income countries. Mycobacteria are showing resistance in many cases, and patients, especially in developing countries, do not comply with the current regimen, thus spreading the infection and increasing resistance.

Allium sativum L., commonly known as garlic, belongs to the Alliaceae family. Garlic has a very high nutritional value, and the *Allium* species are one of the world's oldest cultivated plants. *A. sativum* extracts are known to have many biological activities. These include antibacterial, antiviral, antifungal, and anti-protozoan properties, as well as beneficial effects on the cardiovascular and immune systems.¹ Garlic extracts have been shown to inhibit the growth of not only susceptible but also resistant strains of *Mycobacterium tuberculosis*, showing activities between 1 and 3 mg/mL.² Alliinase, present in the garlic cell vacuoles, enzymatically converts alliin to allicin when the cells are crushed. Allicin quickly degrades to form polysulfides.

These garlic polysulfide oils are the active oils that contribute toward the aforementioned biological properties. Diallyl disulfide is an efficient agent for detoxification. It significantly upregulates the glutathione-S-transferase gene and increases the production of the enzyme.^{3,4}

It is also known that plants have various positive effects when used as medicines, and one of these effects is hepatoprotective activity. *A. sativum* extracts have previously been tested for *in vivo* hepatoprotective activity on isoniazid-induced hepatotoxicity in mice and have been established to act as hepatoprotectants, reducing the toxic markers associated with liver damage.⁵

In this study, six polysulfide oil mixtures containing various concentrations of diallyl polysulfides have been evaluated for their antimycobacterial, cytotoxic, immunomodulatory, and hepatoprotective abilities.

MATERIALS AND METHODS

Sample preparation

Diallyl disulfide (Sample code G1 [Fig.1]) was purified from polysulfide synthetic oil mixture (G6 [Fig.2]) by distillation. Garlic oils: Naturex (G2), Treatt (G3), Octavus (G4), and Stringer (G5) were obtained commercially and are garlic oils containing mixtures of diallyl mono to hexasulfide in slightly varying proportions (Table 1).

Microorganism culturing

The microorganism, maintained in complete 7H9 broth (2% Glycerol, 10% OADC and 2% PANTA), was supplied by the national health laboratories at the University of Pretoria. The *M. tuberculosis* H37Rv (ATCC 27264) strain was used in the study. Bacteria inoculum was cultured on LJ media as well as in 7H9 broth that was supplemented with 10% OADC, 2% Glycerol, and 2% PANTA (antibiotics to prevent the growth of other micro-organisms), for 2 to 3 weeks at 37°C until the logarithmic growth phase was reached. From the 3-week-old active culture, a McFarland standard # 1 was prepared in 7H9 broth and diluted 100 times to reach a colony-forming unit (CFU) of 3×10^6 cfu/mL.

Microtitre PrestoBlue assay

The samples were dissolved in 100% dimethyl sulfoxide (DMSO) and maintained at room temperature for at least 1 h. The samples were further diluted to their final concentration with fresh culture broth. The highest concentration of DMSO was 2.5%. DMSO solvent, bacteria, and media controls were included in the assay. Two positive controls, isoniazid (INH) and ethambutol (EMB), were included. Stock solutions of INH and EMB were prepared in sterile distilled water. The stock solutions were diluted in complete 7H9 broth to four times the maximum desired final testing concentrations before the addition to the 96-well microplates. The method was adapted from Franzblau et al., as described by Lall et al.^{6,7} The samples were assayed in triplicate. All tests were carried out in sterile, flat-bottomed 96-well microplates. Perimeter wells were filled with sterile distilled water to prevent or limit evaporation of the media during incubation. To the remaining test wells, 100 μ L of the freshly prepared 7H9 medium, supplemented with 2% glycerol, 10% OADC, and 2% PANTA, was added. The working compound mixtures (100 μ L) were added to the first well in each row, from which twofold serial dilutions were made in 7H9 broth. The final concentrations of these preparations ranged from 0.781 to 400 μ g/mL for the G1–G6 samples, 0.125–4.0 μ g/mL for INH, and 0.3125–10 μ g/mL for EMB. One hundred microliters of the prepared *M. tuberculosis*

culture inoculum (3×10^6 cfu/mL) was added to each well, yielding a final volume of 200 μ L. A drug-free inoculum control was also included in the experiment as well as a medium, drug, and solvent control. The plates were sealed with parafilm and incubated for 7–10 days at 37°C on a rotation table to limit the formation of bacteria biofilm complexes. After 10 days of incubation, 40 μ L of a 1:1 alamar blue:10% Tween 80 or 40 μ L of PrestoBlue:10% Tween 80 (1:1) solution was added to one growth control well. Tween 80 was used to limit the clumping effect as this has been shown to inhibit biofilm production. The plates were resealed and incubated for 24 h at 37°C. Once the color change was observed, the viability reagent mixture was added to all the remaining wells. The plates were resealed and incubated for an additional 18–24 h. A blue color indicated no growth, and a pink color indicated growth of the bacteria. The minimum inhibitory concentrations (MIC) were described as the lowest concentrations where no color change from blue to pink occurred. All the experiments were done in a Biosafety level 2 laboratory.

Peripheral blood mononuclear cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by using the Histopaque®-1077 method. The blood samples were collected from a healthy volunteer in EDTA tubes, transferred into a 50 mL falcon tube, and diluted 1:1 with incomplete Roswell Park Memorial Institute (RPMI) medium. Half the volume of the blood was determined, and that amount of Histopaque-1077 was added to a fresh, sterile 50 mL falcon tube. The diluted blood was layered on top of the Histopaque-1077 and centrifuged at 400 g for 30 min. The cloudy layer of PBMCs was carefully removed by using a pipette and transferred into a new 50 mL tube. The removed cells were washed with incomplete RPMI media and centrifuged again at 300 g for 10 min. The supernatant was removed, and ~5 mL of ACK buffer (0.15 M NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA at a pH of 7.2) was added to lyse the contaminating erythrocytes. The ACK was deactivated by the addition of 15–20 mL of incomplete RPMI and centrifuged at 200 g for 10 min at room temperature. The supernatant was removed, and the cells were re-suspended in 5 mL complete RPMI (RPMI +10% fetal bovine serum [FBS]) and counted by using a hemocytometer.

Cytotoxicity assay

A series of twofold dilutions (200–0.78 μ g/mL) of the test samples G1–G6 were prepared in DMSO, and the cytotoxicity was measured by the PrestoBlue resazurin-based method.⁶ The dilutions were added to the inner wells of the microtiter plate and incubated for 72 h. The outer wells

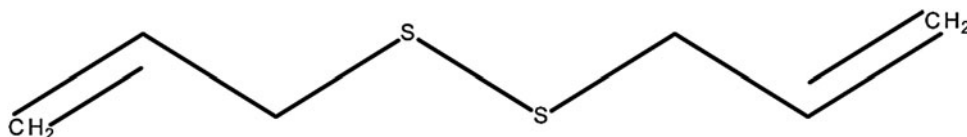


FIG. 1. Chemical structure of diallyl disulfide (Sample G1).

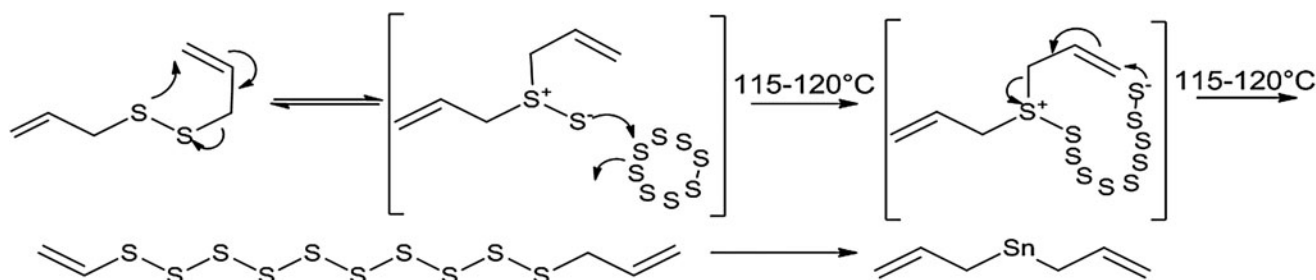


FIG. 2. The proposed reaction mechanism of synthesized oil containing different diallyl polysulfides, diallyl monosulfide to diallyl hexasulfide.

contained 200 μL of sterile distilled water to limit evaporation. After 72 h, 20 μL of PrestoBlue reagent was added to the wells and the plates were incubated for 1–2 h. After the incubation, the absorbance of the color complex was spectrophotometrically quantified by using an enzyme-linked immunosorbent assay (ELISA) plate reader (PowerWave XS, Bio-Tek) at 570 nm with a reference wavelength of 600 nm. DMSO (0.04%) served as a solvent control, and doxorubicin served as a positive control. GraphPad Prism software (4.03) was utilized to statistically analyze and determine the 50% inhibitory concentration (IC_{50}) values by using analysis of variance (ANOVA).

Immunomodulatory activity cell treatment

PBMCs were isolated as described earlier. The cells were counted, and 1000 μL of a 100,000 cells/mL suspension was plated in a 24-well plate. The cells were treated with 1000 μL of the compounds that were prepared at $\frac{1}{2} \text{IC}_{50}$ as determined by the cytotoxicity assay. A media control, solvent control, and positive control containing Phytohaemagglutinin (PHA) at 2 $\mu\text{g}/\text{mL}$ were used as the various controls. A viability test was conducted, after incubation, to determine the viability of the cells when supernatants were collected.

Immune modulatory assay

All the treated cells were incubated for 18–24 h at 37°C with 5% CO_2 . The plates were removed, sealed with parafilm, and centrifuged for 5 min at 800 g . Supernatants were collected in 150 μL aliquots and used directly or stored at

–72°C. The BD OptEIA™ Human IL12 (p40) ELISA set kit and Reagent set B were used (Cat nos. 555171 and 550534). The 96-well ELISA plates were coated with the primary capture antibody, according to the lot-specific recommendations, 24 h in advance. The plates were blotted dry and blocked by using 200 μL of assay diluent. Plates were incubated for 1 h and washed again as mentioned earlier. The standards and samples were prepared as described in the kit brochure, and 100 μL of each supernatant sample and standard (in triplicate) was added to the plates and incubated at room temperature for 2 h. After sample/standard incubation, the plates were washed five times and 100 μL of working detector was added to each well. The plates were sealed and incubated for another hour, followed by aspiration and seven washes. One hundred microliters of the substrate solution [1:1 Substrate A (hydrogen peroxide in buffer solution): Substrate B (3, 3', 5,5' Tetramethylbenzidine)] was added to each well and incubated for 30 min at room temperature. After 30 min, 50 μL of stop solution (1 M phosphoric acid) was added; the absorbance was read at 450 nm (primary); and 570 nm was used as the reference wavelength.

C3A cell culture

C3A liver cells (ATCC HB-8065) are a derivative of HepG2 human liver cells. The cells were cultured in 10% FBS in Eagle's minimum essential medium. After the cells formed a confluent layer, they were washed, trypsinized, and counted and 100 μL of cells were seeded at a concentration of 10,000 cells/well in a 96-well plate.

TABLE 1. GARLIC POLYSULFIDE MIXTURES WITH VARYING PERCENTAGES OF DIFFERENT DIALLYL POLYSULFIDES

Garlic oils		Naturex (G2)	Treatt (G3)	Octavus (G4)	Stringer (G5)	Synthetic oil (G6)
Diallyl monosulfide		7	7	<5	5	<5
Diallyl disulfide		37	24	15	28	5
Diallyl trisulfide		35	49	60	54	20
Diallyl tetrasulfide		15	16	20	12	45
Diallyl pentasulfide		4	2	<5	1	20
Diallyl hexasulfide		2	1	<5	1	5

In vitro hepatoprotective assay

Samples were tested at $\frac{1}{2}$ IC₅₀ (a concentration half of that of the 50% inhibition concentration) and $\frac{1}{4}$ IC₅₀ (a concentration quarter of that of the 50% inhibition concentration) and were prepared in the appropriate media (10% FBS, Eagle's Minimum Essential Medium [EMEM]). The concentrations were selected based on the IC₅₀ values to test at-concentration ranges that will not affect the viability of the cells but still have a therapeutic effect. From the prepared concentrations, 50 μ L was added to the already seeded plates. The toxic inducer used in the assay was Acetaminophen (obtained from Sigma-Aldrich cat. no. 1001325327) at a concentration of 2 mM. Acetaminophen was dissolved in the appropriate media at a concentration of 8 mM, and 50 μ L was added to each well. A positive control, Silymarin (obtained from Sigma-Aldrich cat. no. S0292), was used to assess the hepatoprotective effect. Silymarin was prepared in the appropriate medium at a concentration of 400 μ g/mL. Fifty microliters were added in triplicate to the plated cells. The test concentration of Silymarin was 100 μ g/mL. Cultured C3A cells were counted, adjusted and 100 μ L of cells were plated as stated earlier. Fifty microliters of the prepared samples were added to each well in triplicate. Fifty microliters of the toxic inducer, acetaminophen, was added to all the wells and the plates were incubated at 37°C, 5% CO₂ for 3 h. After incubation, 20 μ L of PrestoBlue was added to all the wells and incubated for another 0.5–1 h. The fluorescence was measured at 535–560/590–615 nm, and the protective effect was determined by comparing the samples with the untreated cells and the cells treated with acetaminophen only.

RESULTS AND DISCUSSION

Antimycobacterial activity

Good growth of *M. tuberculosis* (H37Rv) was observed in the 7H9 broth media within 4–6 weeks. Four diallyl poly-

sulfide mixtures out of six samples inhibited the growth of *M. tuberculosis* at a concentration of 20 μ g/mL or less (Table 2).

The best antimycobacterial sample was found to be G4 with an MIC of 2.5 μ g/mL. This is comparable to the MIC of some existing TB drugs, such as ethambutol with an MIC of 2–8 μ g/mL.⁷ Polysulfide oils extracted from Alliaceae plants showed a significant decrease in gram-positive and gram-negative bacteria numbers in previous studies.⁸ In another study done by Münchberg *et al.*, in 2007, it was also observed that diallyl disulfide (G1) showed the lowest antimycobacterial activity.⁹ Diallyl disulfide, in contrast to monosulfides, can form thiols (RSH) and act as oxidants. The higher activity observed in sample G4 indicates that the chemistry and biological activity involved in trisulfides are more complex than its disulfide counterparts. The higher activity may be linked to the formation of perthiols (RSSH) in tri-, tetra-, penta-, and hexasulfides. Trisulfides are also the most stable of the longer chained diallyl sulfides. The chemical mechanism and ability of the thiols and perthiols to act as redox- and metal-binding agents explains the higher activity in some of the samples. Synthetic garlic oil, sample G6, contains the lowest amount of diallyl trisulfide, indicating the importance of the perthiols in antimycobacterial activity. The higher concentration of diallyl tetrasulfide in sample G6 did not increase the activity to the same range as the samples containing higher amounts of diallyl trisulfide. The lipophilicity of the polysulfides can influence the activity observed. In a study conducted by Barry *et al.*, in 2000, they determined that the optimum LogP values for antimycobacterial properties are between 1.3 and 4.1. The LogP values of the polysulfides increase with an increase in sulfides, which might explain the decrease in activity with higher amounts of larger polysulfides.¹⁰

Cytotoxicity

Cytotoxic evaluation of the compounds was done on isolated PBMC (Table 2). The selectivity index of a sample

TABLE 2. MINIMAL INHIBITORY CONCENTRATIONS ON *MYCOBACTERIUM TUBERCULOSIS* H37RV, CYTOTOXIC ACTIVITY AND INTERLEUKIN 12 PRODUCTION ON ISOLATED PERIPHERAL BLOOD MONONUCLEAR CELLS, OF DIALLYL POLYSULFIDES AND REFERENCE ANTIBIOTICS

Sample	MIC ^a (μ g/mL)	Cytotoxicity			Cytokine production	
		IC ₅₀ ^b (μ g/mL)	\pm SD	SI (PBMC) ^c	% Viability	IL 12 pg/mL
G1	1000	37.97	11.33	0.04	76.37	75.23
G2	6.25	4.27	0.33	0.68	86.26	83.25
G3	6.25	6.03	0.17	0.96	94.36	50.33
G4	2.5	0.29	0.25	0.12	88.27	71.2
G5	20	1.39	0.13	0.07	90.14	31.36
G6	50	1.8	0.16	0.04	89.37	68.52
Isoniazid ^d	0.25	—	—	—	—	—
Ethambutol ^d	1.25	—	—	—	—	—
ActinomycinD ^d	—	0.038	—	—	—	—
Phytohaemagglutinin ^d	—	—	—	—	100	103.6

^aMinimum inhibitory concentration on *M. tuberculosis* H37Rv.

^bFifty percent inhibitory concentration on PBMCs.

^cSelectivity index on PBMCs = IC₅₀ (PBMC)/MIC.

^dReference antibiotics, isoniazid and ethambutol (antimycobacterial), actinomycinD (cytotoxicity), and phytohemagglutinin (immunomodulation), not tested. MIC, minimum inhibitory concentrations; SD, standard deviation.

is the representation of the selectivity of the samples for antibacterial activity over toxicity. The selectivity index was determined according to formula 1.

The IC_{50} values of the tested samples G1 to G6 ranged from 0.2 to 37.97 $\mu\text{g/mL}$. The selectivity indexes of the samples were found to be quite low. Polysulfides have been found to be multifaceted cytotoxins. The mechanism of toxicity ranges over many biological functions and processes. Sulfides, especially tri- and tetrasulfides, act as thiolation agents, reacting with protein thiols, including cysteine residues within proteins, resulting in mixed disulfides at the protein site.⁹ This modification to mixed disulfides can, in some instances, disturb protein function, altering cellular responses and can lead to cell death, as in the case with diallyl trisulfide, which thiolates β -tubulin and reduces its biological function.¹¹ Thiolation of a trisulfide also results in a mixed disulfide and a persulfide (RSSH). The latter product is believed to be the actual active agent of polysulfides. It is important to note that cells with sufficient glutathione concentrations can reverse this toxic effect.⁹ The toxic mechanism can not only be attributed to thiol-polysulfide reactions, as the biological chemistry of polysulfides is much more complex. Studies and *ab initio* calculations have indicated the nonlinearity of longer chain polysulfides, with structures showing folded and even helical arrangements.¹² These longer chain polysulfides (in higher concentrations) may act in hydrophobic interactions, disrupting cell walls and binding to hydrophobic pockets within proteins, resulting in the unfolding of the protein structure.⁹

Immunomodulatory activity

The excreted levels of Interleukin 12 (IL12) were evaluated in PBMC (Table 2). IL12 is one of the first cytokines produced by macrophages at the start of a mycobacterial infection and is important for the containment of the bacterial

cells. PBMC contain monocytes that can be stimulated to differentiate into macrophages. The production of IL12 from PBMC, after the stimulation with diallyl polysulfides, was measured to determine the baseline effect before an infection. Cell viabilities evaluated after treatment were higher than 70% for all the tested sample wells. Samples G1 to G6 caused a decrease in IL 12 cytokine production at the tested concentrations ($1/2 IC_{50}$) when compared with the positive control PHA 2 $\mu\text{g/mL}$. In a study done by Hodge *et al.* in 2002, it was found that garlic extracts decreased inflammatory cytokines, including IL12, in whole blood and PBMC samples.¹³ Lectins from aged garlic have shown immunomodulatory activity, but isolated compounds and polysulfide immunomodulatory mechanisms are understudied.¹⁴ Sample G2 containing relatively the same amount of diallyl di- and trisulfide showed the highest amount of IL12 produced, indicating that the formation of thiols and perthiols also play a role in the immune response. The second highest amount of IL12 was produced through G1 (pure diallyl disulfide), and higher amounts of trisulfide in the polysulfide mixture (Sample G3) were associated with decreased production of IL12, demonstrating the importance of diallyl disulfide for IL12 production.

Hepatoprotective activity

The hepatoprotective effect of the polysulfide mixtures was determined by comparing the viability of the samples with the viability of the toxicity induced (0% protected) and the untreated cells (100% protected) (Fig. 3). A significant decrease in cell viability was observed on treatment of C3A cells with acetaminophen (2 mM). Sample G6, which contained increased amounts of diallyl pentasulfide, exhibited an $\sim 60\%$ protection at the tested concentration toward cell toxicity induced by acetaminophen. This is indicative of the not before seen importance of a pentasulfide for biological activity. Pentasulfides would also produce perthiols in the cell, similar

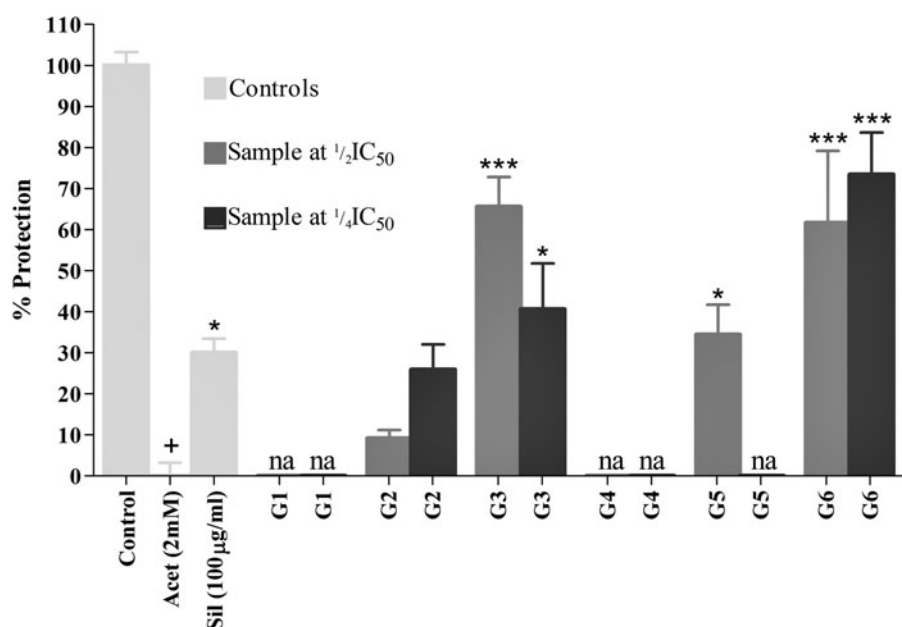


FIG. 3. Hepatoprotective effect of diallyl polysulfide mixtures on acetaminophen toxicity-induced C3A cells. Data are expressed as mean% protection \pm SD with respect to the toxic inducer acetaminophen, $n=3$; Silymarine (Sil [100 $\mu\text{g/mL}$]); +acetaminophen (Acet [2 mM])(0% Protection); * $P < .05$, when compared with the control group (+); *** $P < .001$, when compared with the control group (+); na, not active; SD, standard deviation.

to the other longer chained polysulfides. The positive control Silymarin had a protective effect of 30%. Previous studies indicated that garlic homogenates showed hepatoprotective activity in mice treated with Isoniazid.¹⁵ Diallyl sulfides were also reported to have a protective effect, *in vivo*, on carbon tetrachloride-induced liver injury in mice.¹⁶ Thus, it was expected for the polysulfide mixtures (G1 to G6) to show a hepatoprotective effect against acetaminophen toxicity. In a work done by Tsai *et al.*, it was established that diallyl disulfides and trisulfides increased the gene expression of the pi class of glutathione S-transferase (GST).⁴ This enzyme is directly involved in the detoxification of xenobiotics and, thus, would exhibit a hepatoprotective effect in the case of liver damage.

Several polysulfide mixtures showed good antimycobacterial activity. Sample G4 showed an MIC of 2.5 µg/mL, and this can be attributed to the increased concentrations of diallyl tri- and tetrasulfide and the associated lipophilicity of these. Increased amounts of diallyl tetrasulfide in the polysulfide mixtures had a decreased biological activity when compared with the trisulfides. This is potentially due to the chemistry involved in the cell, with the formation of thiols (RSH) by disulfides and perthiols (RSSH and RSSH) by longer chained polysulfides (RS_xR, x>2). The absorbance of these polysulfides and the associated lipophilicity can influence activity. The logP value of thiols (RSH) is lower than that of perthiols (RSSH), which is indicative of a more readily absorbed compound. This explains the increase in activity with lower polysulfides. The samples with good antimycobacterial activities showed relative toxicity in PBMC. The samples were further tested for their hepatoprotective activity to substantiate their potential as antimycobacterial agents. Out of the six garlic polysulfide mixtures, G6 was the only sample to have significant hepatoprotective activity, probably due to the higher amount of diallyl tetra- and/or pentasulfide in the mixture. The main biological activities are related to the lower length polysulfides (diallyl tri- and tetrasulfide), and their associated lipophilicity and absorbance and increased sulfide numbers did not increase activity.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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