Evaluation of Biological Activities of Triterpenoid Saponins from *Maesa lanceolata*†

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From a bioassay-guided fractionation procedure using Herpes simplex virus type 1 as the target model, a virucidal saponin mixture (maesasaponin mixture B) was isolated from the MeOH extract of leaves of *Maesa lanceolata*. The maesasaponin mixture B consisted of six homologous oleanane-type triterpenoid saponins **1**−**6**, identified by ^{1}H, ^{13}C, and 2D NMR spectroscopy and mass spectrometry. The maesasaponin mixture B (**1**−**6**) showed several biological activities expected for saponins. It exhibited a moderate virucidal activity against enveloped viruses. The maesasaponin mixture B (**1**−**6**) was tested for further properties. The saponin mixture was found to be highly hemolytic and molluscicidal: it hemolyzed 50% of human erythrocytes (1% suspension) at a concentration estimated at 1.6 μg/mL and exerted against *Biomphalaria glabrata* snails a severe toxic effect with LD_{95} and LD_{50} values of 4.1 and 2.3 ppm, respectively. In addition, the maesasaponin mixture B (**1**−**6**) showed moderate fungistatic and antimutagenic properties. The evaluation of these diverse activities is described.

Maesa lanceolata Forsskal var. *golungensis* Welw. (Myrsinaceae) is a shrub or small tree growing in many African countries including Rwanda,[2,3] where traditional healers include it in medicinal preparations used to treat a wide range of diseases.[4] This species is also used to prevent cholera in East African folk medicine.[5] Two other *Maesa* species, *M. chisia* and *M. indica*, have been reported to exhibit antiviral activity against Ranikhet disease and vaccinia viruses.[6]

In a preliminary antimicrobial and antiviral screening, the MeOH extract of *M. lanceolata* leaves exhibited virucidal effect (500 μg/mL) against enveloped viruses, in particular, Herpes simplex, vesicular stomatitis, semliki forest, and measles viruses. The MeOH extract showed no antibacterial activity. A bioassay-guided fractionation procedure, using Herpes simplex virus type 1 (HSV-1) as the target model, led to the isolation of a moderately virucidal saponin mixture, maesasaponin mixture B. This saponin mixture consisted of six homologous oleanane-type triterpenoid saponins **1**−**6** (Figure 1), identified by ^{1}H, ^{13}C, and 2D NMR spectroscopy and mass spectrometry.[1] The maesasaponin mixture B (**1**−**6**) was tested further for its virucidal and true antiviral activities against enveloped viruses including Herpes simplex virus types 1 and 2 (HSV-1 and -2), measles virus strain Edmonston A (MV-EA), semliki forest virus A7 (SF-A7), and vesicular stomatitis virus T2 (VSV-T2). It was also evaluated against two nonenveloped viruses, poliovirus type 1 (Polio-1) and coxsackie virus B2 (Cox-B2), and its cytotoxicity on Vero cell monolayers was also determined. In addition, the maesasaponin mixture B (**1**−**6**) isolated from *M. lanceolata* was compared to common disinfectants for its virucidal activity.

Saponins have been reported to possess antifungal properties.[7,8] The magnitude of the hemolytic activity of saponins has often been linked with the antifungal and antiinflammatory activities of the saponins.[9] In recent years, naturally occurring compounds including saponins have been reported to inhibit the effect of diverse environmental mutagens and carcinogens.[10,11]

Since the discovery of highly potent saponins in the berries of *Phytolacca dodecandra*, naturally occurring molluscicides have been receiving considerable attention.[12] Molluscicidal saponins have been isolated from many other promising plants.[7] Molluscicidal saponins are often hemolytic.[13] The wide range of reported saponin biological activities prompted us to design a study on in vitro antifungal, antimutagenic, hemolytic, and molluscicidal activities of maesasaponin mixture B (**1**-**6**), in addition to its antiviral and cytotoxic effects. The observed activities were compared, where possible, to that obtained with reference saponins.

Results and Discussion

As observed with the crude MeOH extract of the ground leaves of *M. lanceolata*, the isolated saponin mixture did not exert any inhibition on the replication of enveloped and nonenveloped viruses at subtoxic concentrations. β-Escin and saikosaponins A and D used as reference saponins also showed no inhibition on viral replication. Using the MTT method,[14] a 50% cytotoxic dose of maesasaponin mixture B (**1**−**6**) on Vero cell cultures was estimated at 17.4 μg/mL. This value was consistent with results obtained by direct microscopic examination of saponin-treated Vero monolayers.

The inactivation exhibited extracellularly against enveloped viruses by the maesasaponin mixture B (**1**−**6**) was evaluated at 25 °C for incubation periods ranging

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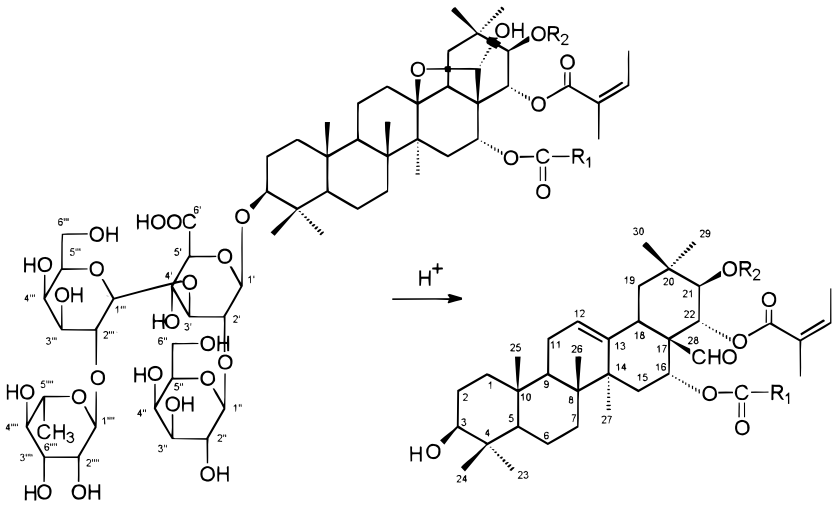

Maesasaponin mixture B	R_1	R_2	Amount (%)	Aglycon
1	C_2H_5	H	15	**1a**
2	C_3H_7	H	15	**2a**
3	C_4H_7	H	8	**3a**
4	C_4H_9	H	12	**4a**
5	C_2H_5	CH_3CO	31	**5a**
6	C_3H_7	CH_3CO	19	**6a**

Figure 1. Chemical structure of the constituents of the maesasaponin mixture B (**1**−**6**) and their aglycons (**1a**−**6a**) and the relative composition of **1**−**6**.

from 5 to 60 min. The virucidal saikosaponin D, whose extracellular virucidal activity was reported by Ushio and Abe,[15] was used as reference saponin. Saponins, as many other virucidal products, usually show such a high cytotoxicity that it is difficult to determine precisely the extent of viral infectivity reduction at cytotoxic concentrations. Therefore, ultracentrifugation and ultrafiltration techniques were used in order to separate the residual virus from toxic saponins. The former technique was applied to HSV (types 1 and 2) and VSV-T2, while the latter was used for MV-EA. The observed virucidal effect was dependent upon the test saponin concentration and the length of incubation periods. Concentrations of the maesasaponin mixture B (**1**−**6**) up to 50 μg/mL were practically inactive for incubation periods ranging from 5 to 60 min. The saponin mixture (**1**−**6**) reduced HSV-1 infectivity by 2.5 log at 100 μg/mL and after 30 min of incubation. For the same incubation period, the maesasaponin mixture B (**1**−**6**) inactivated more than 99.9% (3 log) of Herpes simplex virus (both types) at 250 μg/mL. MV-EA seemed to be as sensitive as HSV. The maesasaponin mixture B (**1**−**6**) was active extracellularly against VSV-T2 at concentrations equal to or higher than 250 μg/mL, when incubated at least 60 min. Even the highest concentration tested (1 mg/mL) showed no virucidal effect against VSV-T2 for shorter incubation periods. The aglycon mixture (**1a**−**6a**) resulting from the acidic hydrolysis of maesasaponin mixture B (**1**−**6**) was not active at all.

To check the possible influence of maesasaponin mixture B (**1**−**6**) cytotoxicity on the observed viral infectivity reduction, a subtoxic dose (5 μg/mL) was incubated with Vero cell cultures for 4 and 24 h prior to the virucidal test. The virucidal activity remained the same. Nevertheless, incubation for 24 h increased the cytotoxicity showed by the saponin mixture (**1**−**6**) against Vero cells by a factor of 2. The maesasaponin mixture B (**1**−**6**) interfered with viral infectivity by direct virus inactivation. Obviously, the maesasaponin mixture B (**1**−**6**) showed lower virucidal activity than expected, compared to that obtained with the MeOH extract. Disinfectants also impair viral infectivity by a direct virus inactivation.[16] Therefore, the virucidal activity of common disinfectants such as chloramine T, cetrimonium bromide, and phenylmercury borate was compared to that of the maesasaponin mixture B (**1**−**6**). The two groups of compounds showed equivalent virucidal activity against enveloped viruses (Table 1).

Table 1. Virucidal Activity of Some Common Disinfectants Compared to Maesasaponin Mixture B (1−6) from *Maesa lanceolata*[a]

	HSV-1[b]		polio-1[c]
disinfectant[d]	5 min (25 °C)	60 min (37 °C)	5 min (25 °C)
chloramine T	0.05	0.025	0.05
phenylmercury borate	0.025	0.01	>0.1
cetrimonium bromide	0.05	0.005	>5
maesasaponin mixture B (**1**−**6**)	>0.05	0.005	>0.1

[a] The activity corresponds to the reduction of viral infectivity by a factor $\geq 10^3$. [b] HSV-1: Herpes simplex virus type 1. [c] Polio-1: poliovirus type 1. [d] Active concentrations are expressed in % w/v.

The fungistatic activity expressed as minimum inhibitory concentration (MIC) of the test mixture was assayed using an agar dilution method, while the fungicidal effect was evaluated by the broth dilution method after incubation of microorganism with test product. The maesasaponin mixture B (**1**−**6**) inhibited the growth of *Epidermophyton floccosum*, *Microides interdigitalis* (= *Trichophyton mentagrophytes* var. *in-*

Table 2. Mutagenicity of Maesasaponin Mixture B (1−6) for *Salmonella typhimurium* Strains TA 98 and TA 100[a]

	TA 98		TA 100	
concn (nmol/plate)	−S9[b]	+S9[b]	−S9[b]	−S9[b]
100	10 ± 4[c]	16 ± 4[c]	98 ± 13[c]	124 ± 14[c]
50	13 ± 1[c]	20 ± 4[c]	80 ± 4[c]	133 ± 9[c]
0 (=SR)[d]	11 ± 3	17 ± 7	90 ± 21	126 ± 15

[a] The assay was performed in triplicate; mean values ± standard deviations are given. [b] ±S9: test with or without activation by microsomal fraction. [c] Not significantly different from spontaneous revertants at $p < 0.05$ (Student *t*-test). [d] SR: number of spontaneous revertants.

terdigitale), and *Trichophyton rubrum* at 50 μg/mL. *Candida albicans* and *Microsporum canis* were inhibited at 100 μg/mL, whereas the development of *Microsporum langeroni* was impaired at 250 μg/mL. *Aspergillus fumigatus* and *Aspergillus niger* were not sensitive at all test concentrations. No fungicidal activity against target microorganisms was demonstrated at concentrations up to 1 mg/mL. The fungistatic activity exhibited by the maesasaponin mixture B (**1**−**6**) was low. The maesasaponin B aglycon mixture (**1a**−**6a**) showed no antifungal activity. Miconazole and β-escin were used as reference antifungals. β-Escin was fungistatic only against *C. albicans*, *E. floccosum*, and *T. rubrum* at 250, 50, and 100 μg/mL, respectively. Miconazole was obviously much more active (MIC values were 12.5 and ≤3.13 μg/mL for *C. albicans* and dermatophyte species, respectively).

Mutagenic and antimutagenic assays were carried out according to Maron and Ames.[17] The antimutagenicity of the maesasaponin mixture B (**1**−**6**) was evaluated in *Salmonella typhimurium* strains TA 98 and TA 100 against chemical mutagens requiring metabolic activation (benzo[*a*]pyrene and 2-aminofluorene) or the direct-acting mutagen 4-nitroquinoline *N*-oxide. As shown in Table 2, the mean number of revertants observed in the presence of the maesasaponin mixture B (**1**−**6**) was not significantly different from that of spontaneous revertants noted in controls. The saponin concentrations tested for antimutagenic activity ranged from 0 to 250 nmol/plate. Saponins were found to inhibit in a dose-related manner the mutagenicity of 2-aminofluorene (2-AF) in *S. typhimurium* strain TA 98, that of 4-nitroquinoline *N*-oxide (4-NQNO) in *S. typhimurium* strain TA 100, and that of benzo[*a*]pyrene (BaP) in both test strains. This was corroborated by correlation coefficients (0.914−0.999) calculated from the dose−response curves. At concentrations equal to or higher than 100 nmol/plate, the maesasaponin mixture B (**1**−**6**) was active against the mutagenicity induced by BaP in *S. typhimurium* TA 98 (45−59% inhibition) and in *S. typhimurium* strain TA 100 (43−52% inhibition). The maesasaponin mixture B (**1**−**6**) was weakly active (30% inhibition) against 2-AF mutagenicity at the highest concentration tested and practically inactive against 4-NQNO mutagenicity. At 250 nmol/plate, β-escin exerted 40 and 48% inhibition against BaP in strains TA 98 and TA 100, respectively. At the highest concentration tested, α-hederin weakly inhibited the mutagenicity of BaP and 2-AF in *S. typhimurium* TA 98 (37 and 40% inhibition, respectively) and proved active (45%) against BaP in *S. typhimurium* TA 100. Saponin 50% inhibiting doses inferred from corresponding dose−response curves are compared in Table 3. The present results are in agreement with those reported on α-hederin.[18]

The hemolytic activity of the maesasaponin mixture B (**1**−**6**) was assessed on human erythrocytes (1% suspension in phosphate buffer saline, pH 7.4) using a method described earlier.[19] The maesasaponin mixture B (**1**−**6**) from *M. lanceolata* exhibited high hemolytic activity: the 50% hemolyzing concentration (HC_{50}) was estimated at 1.6 μg/mL. To have a clear idea of the extent of the observed hemolytic activity, the same experiments were performed with β-escin, whose hemolytic index was previously evaluated.[20] The HC_{50} of β-escin was 3.7 μg/mL. The maesasaponin B aglycon mixture (**1a**−**6a**) was practically inactive.

The maesasaponin mixture B (**1**−**6**) was assayed against the aquatic snails *Biomphalaria glabrata* for molluscicidal activity according to WHO specifications.[21] The saponin mixture showed a severe toxic effect: at active concentrations, snails were retracted in their shells, imobilized, and discolored. Using the probit method, the 95% and 50% lethal doses (LD_{95} and LD_{50}) of the maesasaponin mixture B (**1**−**6**) were estimated at 4.1 μg/mL and 2.3 μg/mL, respectively, with a 95% confidence interval of 2.7−6.1 μg/mL and 2.1−2.6 μg/mL. These results were comparable to that of known natural molluscicides.[7]

For its virucidal activity, the maesasaponin mixture B (**1**−**6**) possibly interacts with the viral envelope components leading to destruction of the envelope.[15,22] In the same way, the observed fungistatic, hemolytic, and molluscicidal activities would be due to the interaction of the maesasaponin mixture B (**1**−**6**) with cell membrane components. It has been suggested that the complexation of biological membrane cholesterol is likely to account for the fungistatic, hemolytic, and molluscicidal activities of saponins.[23,24] The mechanism of action of saponins related to membrane sterols is consistent with the lack of activity of the maesasaponin mixture B (**1**-**6**) on *Emericella* (*Aspergillus*) spp. that contain only traces of sterols. Saponins have been reported to produce a marked decrease of microsomal enzyme activities;[25] this property seems to justify the observed antimutagenic activity of the maesasaponin mixture B (**1**−**6**) against chemical mutagens requiring metabolic activation.

The complexity of the maesasaponin mixture B (**1**−**6**) makes the interpretation of the structure−activity relationship of its constituents quite difficult. Nevertheless, conclusions could be drawn tentatively from their common structural features. All the constituents of the bioactive maesasaponin mixture B (**1**−**6**) bear a 3β-*O*-tetrasaccharidic chain and a 13β,28-oxido group. Upon acidic hydrolysis, the oligosaccharide chain in the constituents of the maesasaponin mixture B (**1**−**6**) was cleaved, while the 13β,28-oxido group was transformed in a 28-aldehyde group and a 12,13-double bond.[1] The resulting aglycon mixture (**1a**−**6a**) did not show any of the tested activities including the hemolytic, fungistatic, and virucidal activities. Compared with the literature,[7,8,26−28] these observations suggest that the oligosaccharide in position C-3 and the 13β,28-oxido group are essential for the biological properties of the maesasaponin mixture B (**1**−**6**). The ester groups at

Table 3. Antimutagenic Activity of Triterpenoid Saponins

saponin	50% inhibitory concentration (nmol/plate)[a]			
	2-AF[b] (TA 98)[e]	4-NQNO[c] (TA 100)[e]	BaP[d] (TA 98)[e]	BaP[d] (TA 100)[e]
α-hederin	259.0	473.0	337.0	300.0
β-escin	240.0	540.0	323.7	256.6
maesasaponin mixture B (**1**−**6**)	447.4	550.0	174.0	228.4

[a] The 50% inhibitory concentrations (nmol/plate) were inferred from corresponding dose−response curves. [b] 2-AF: 2-aminofluorene. [c] 4-NQNO: 4-nitroquinoline *N*-oxide. [d] BaP: benzo[*a*]pyrene. [e] *Salmonella typhimurium* strains TA 98 and TA 100.

C-16, C-21, and C-22 seem to have no influence on the virucidal, hemolytic, and fungistatic activities exhibited by the maesasaponin mixture B (**1**−**6**). Polarity in ring A of pentacyclic triterpenes and relative hydrophobicity in the proximity of rings D/E are essential for saponin biological activities.[7,20] Therefore, the weakness of some activities showed by the maesasaponin mixture B (**1**−**6**) could be explained by the polarity in the proximity of rings D and E in the constuents of the saponin mixture.

The biological activities of *β*-escin were comparable to that showed by the maesasaponin mixture B (**1**−**6**). Although *β*-escin possesses no 13*β*,28-oxido group, it contains a 3*β*-*O*-saccharide chain and additional groups such as 23-OH and 16α-OH, which have been reported to contribute to the biological activities of saponins.[7]

The toxicity of saponins restrict their applications as therapeutic agents. Nevertheless, some applications could be considered for the maesasaponin mixture B (**1**−**6**) from *M. lanceolata*. Besides the high molluscicidal activity exhibited by its constituents, *M. lanceolata* fulfills most of the prerequisites for a viable candidate plant molluscicide as specified by Marston and Hostettmann.[29] Although the maesasaponin mixture B (**1**−**6**) showed moderate virucidal activities, it is expected to enhance the virucidal effect of disinfectants as do surface-active agents.[30]

Experimental Section

Plant Material and Purification of Saponin Mixtures. The purification of the maesasaponin mixture B (**1**−**6**) from the MeOH extract of the dried and ground leaves (1 kg) of *M. lanceolata* (Myrsinaceae) was described previously.[1] The maesasaponin mixture B (**1**−**6**) consisted of six homologous oleanane-type triterpenoid saponins (Figure 1), identified by ^{1}H, ^{13}C, and 2D NMR spectroscopy and mass spectrometry.[1] The maesasaponin B aglycon mixture (**1a**−**6a**) was obtained upon acidic hydrolysis of the maesasaponin mixture B (**1**−**6**).

Test Compounds. *β*-Escin, α-hederin, and saikosaponins A and B were purchased from Carl Roth and Co., Karlsruhe, Germany. Miconazole was obtained from Cilag, Brussels, Belgium. Chloramine T and cetrimonium bromide were obtained from Federa, Brussels, Belgium, and phenylmercury borate was obtained from Zyma, Brussels, Belgium. Benzo[*a*]pyrene and 2-aminofluorene were purchased from Aldrich, Bornem, Belgium, and 4-nitroquinoline *N*-oxide was obtained from Janssen Chimica, Beerse, Belgium.

Antiviral and Cytotoxicity Assays. The battery of antiviral screens was designed to cover as many as possible diverse virus groups in their morphology, replication mechanism, and pathological importance. Herpes simplex virus types 1 and 2 (HSV-1 and -2) were clinical isolates purified and identified by Dr. S. Pattyn, Laboratory for Microbiology, University of Antwerp, Antwerp, Belgium. Poliovirus type 1 strain 1A/S3 (Polio-1), coxsackie virus B2 (Cox-B2), and measles virus strain Edmonston A (MV-EA) were obtained from NIH, Bethesda, MD. Semliki forest virus A7 (SF-A7) was supplied by Dr. C. J. Bradish, Microbiology Research Establishment, Porton Down, Salisbury, England. Vesicular stomatitis virus T2 (VSV-T2) was obtained from Dr. Van Der Groen, Laboratory for Bacteriology and Virology, Institute of Tropical Medicine, Antwerp, Belgium. The viruses were propagated by infection of Vero cell monolayers at 37 °C. Supernatants obtained after infected cell freezing and thawing were divided and stored at −70 °C as stock viruses. The virus titer was estimated from the cytopathic effect (CPE) caused to Vero cell cultures using the end-point titration technique (EPTT)[31] and was expressed as 50% tissue culture doses ($TCID_{50}$) per mL. The virus titers were $10^{6.5}$, $10^{5.5}$, $10^{5.9}$, $10^{7.5}$, $10^{4.5}$, $10^{7.3}$, and $10^{6.7}$ $TCID_{50}$/mL for HSV-1, HSV-2, VSV-T2, SF-A7, MV-EA, Polio-1, and Cox-B2, respectively.

The antiviral activity and the cytotoxicity of the maesasaponin mixture B (**1**−**6**) were evaluated as described previously.[22] Saponin cytotoxicity was further evaluated by an optimized MTT-assay.[14] Confluent monolayers of Vero cells were incubated at 37 °C and for 4 days with dilutions of test compound in maintenance medium. The compound-containing medium was then removed and replaced with 0.1 mg of MTT (20 *μ*L of 5 mg/mL in maintenance medium); the cultures were incubated for a further 2 h. The MTT formazan crystals obtained were dissolved in 150 *μ*L of 2-propanol, and the absorbance was measured at 570 nm with a multiscan spectrophotometer (ELISA reader; MULTISCAN MCC/340, Labsystems, Helsinki, Finland). Controls of untreated cells and media without cells were run in parallel.

Virucidal Assays. The virucidal activity of the maesasaponin mixture B (**1**−**6**) was determined acccording to the methods described previously.[22,32] The mixtures were incubated at 25 °C for 5, 10, 15, 30, or 60 min. The cytotoxic components were separated from the residual virus using ultracentrifugation or ultrafiltration. The former technique was applied to HSV (types 1 and 2) and VSV-T2, whereas the latter was used for MV-EA.

Antifungal Assays. The fungal battery was composed of *C. albicans* ATCC 10231 and isolates of *A. fumigatus* RV 67686, *A. niger* RV 67644, *E. floccosum* RV 71625, *M. canis* RV 66973, *M. langeroni* RV 71268, *M. interdigitalis* RV 66466 (= *T. mentagrophytes* var. *interdigitale*), and *T. rubrum* RV 58125, provided by Dr.

Ch. De Vroey, Laboratory for Mycology, Institute of Tropical Medicine, Antwerp, Belgium. Filamentous fungi were grown on diluted Sabouraud agar[33] slants at room temperature for 15 days. Spores and mycelial fragments were collected in distilled water (10 mL) and then homogenized by ultrasonication using a titanium probe sonicator (Analis, Namur, Belgium) at low power and 10 μm amplitude. Yeasts were grown on Sabouraud streak plates at room temperature for 48 h. Blastospore suspensions from 1 day old subcultures were then prepared in distilled water after centrifugation at 3000 rpm for 5 min and washing. The titers of fungal suspension were determined by an endpoint dilution method. Dilutions of the maesasaponin mixture B (**1**−**6**) and the maesasaponin B aglycon mixture (**1a**−**6a**) comprising 50, 100, 200, 500, and 1000 μg/mL were prepared in distilled water. β-Escin and miconazole were used as standard antifungals.

In fungistatic assays, aliquots of drug dilutions were thoroughly mixed with an equal volume of molten double concentrated Sabouraud; the mixtures (0.2 mL) were each poured into a 96-well microtiter plate and allowed to cool. The wells were inoculated with 0.1 mL of 10^3 or 10^4 cfu/mL fungal suspension. Control experiments without test compounds were run in parallel. The plates were incubated at room temperature for 15 days, and the MIC value was recorded as the lowest saponin concentration at which no visible fungus development was observed.

In fungicidal assays, a preincubated drug dilution (0.5 mL) was thoroughly mixed with an equal preincubated volume (0.5 mL) of fungal suspension and incubated at 25 °C for 15 min. Fungi controls were run in parallel. To stop a possible residual antifungal effect, the mixture was diluted 100-fold in ice-cold liquid Sabouraud and then serially diluted 10-fold in the same medium to determine the endpoint dilution. Aliquots of the serial dilutions (0.2 mL) were subcultured in 96-well microtiter plates and incubated at room temperature. Survivors in saponin−fungus mixtures were recorded by light microscopy after 2 days or macroscopically after 7 and 15 days and compared to controls.

Antimutagenic Assays. Mutagenesis assays were carried out according to Maron and Ames.[17] Benzo[*a*]-pyrene, 2-aminofluorene, and 4-nitroquinoline *N*-oxide were used as standard mutagens. *S. typhymurium* strains TA 98 and TA 100 were obtained from R. Vrijsen, Free University of Brussels (VUB), Brussels, Belgium. The procedure followed in the antimutagenic test was described previously.[34] The data were analyzed for statistical significance using the Student *t*-test. A $p < 0.05$ was considered to be statistically significant. For interpretation of the results, the following qualitative ranking for antimutagenicity was used: 0−20%, not active; 20−40%, weakly active; 40−60%, active; more than 60%, strongly active.[35]

Hemolysis Assays. The hemolytic activity of maesasaponin mixture B (**1**−**6**), maesasaponin B aglycon mixture (**1a**−**6a**), and β-escin was assessed using a previously described method[19] that was modified slightly. A 1% human erythrocyte suspension in phosphate buffer saline (PBS, pH 7.4) was incubated with saponin or sapogenin dilutions in PBS at 37 °C for 30 min. The mixtures were centrifuged (2500 rpm), the supernatants were properly diluted, and the hemoglobin (Hb) content was then measured at 414 nm by a scanning multiwell spectrophotometer (MULTISCAN MCC/340). Blank tests with saponin dilutions (without erythrocytes), erythrocytes (without saponin dilutions), and buffer (without erythrocytes and saponin dilutions) were run in parallel. The results were compared to a positive control sample containing erythrocytes in distilled water. The 50% hemolyzing concentrations (HC_{50}) were inferred from hemoglobin absorbance vs saponin concentration curves.

Molluscicidal Assays. The evaluation of the molluscicidal activity was based on WHO specifications.[21] Uniform *Biomphalaria glabrata* snails (6−8 mm) were incubated at 25 °C for 24 h with saponin dilutions. Dead snails were counted and removed, and the survivors were maintained in fresh desionized water for further 24 h. A new count of possible additional dead snails was then made. The LD_{95} and LD_{50} values were estimated using the probit method.

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