DOI:10.1021/jf902439m

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Screening for Feeding Deterrent and Insect Growth Regulatory Activity of Triterpenic Saponins from *Diploknema butyracea* and *Sapindus mukorossi*

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Antifeeding and insect growth regulatory effects of saponins and its hydrolyzed products from *Diploknema butyracea* and *Sapindus mukorossi* on the insect pest *Spodoptera litura* (F.) were investigated in the laboratory. *D. butyracea* saponins as well as their hydrolyzed prosapogenins were found to be better biologically active in controlling pests. A concentration of 1200 and 3400 mg L^{-1} alkaline and acid hydrolyzed *D. butyracea* saponins exhibited significant antifeeding and toxic effects to third instar larvae when compared to the emulsified water as control. The *n*-BuOH extract after prep-HPLC separation provided two saponins from the *D. butyracea* saponin mixture: 3-*O*-[β-D-glucopyarnosyl-β-D-glucopyranosyl]−16-α-hydroxyprotobassic acid-28-*O*-[ara-glc-xyl]-ara (**MI-I**) and 3-*O*-β-D-glucopyranosyl-glucopyranosyl-glucopyranosyl-16-α-hydroxyprotobassic acid-28-*O*-[ara-xyl-ara]-apiose (**MI-III**). The single saponin extracted from the *S. mukorossi* saponin mixture was 3-*O*-[β-D-xyl-(OAc)·β-D-arabinopyranosyl·β-D-rhamnopyranosyl] hederagenin-28-*O*-[β-D-glc·β-D-glc·β-D-rhamnopyranosyl] ester (**SM-I**). Five days after saponin treatment on larvae, the growth index (GI_{50}) was reduced from 0.92% to 1520 ppm in alkaline hydrolyzed *D. butyracea* saponins. Upon hydrolysis, growth regulatory activity was improved in *S. mukorossi* saponin, whereas very little difference was found in antifeedant activity. Hydrophile−lipophile balance is important for the proper functioning of saponin/prosapogenin/sapogenin, which could be achieved by manipulating the sugar molecule in the triterpenic skeleton.

KEYWORDS: Saponin; IGR activity; feeding deterrent; *Diploknema butyracea*; *Sapindus mukorossi*

INTRODUCTION

New plant protection chemicals derived from natural sources are considered to be more environmentally benign than synthetic pesticides (*1*). Plants produce a variety of compounds that provide certain protection against insect attack (*2*−*5*). Many of these compounds affect feeding behavior as well as growth of insects. Saponins also have been suggested as possible chemical defensive agents of plants against generalist herbivores (*6*−*8*).

Out of several agriculturally important insects, *Spodoptera litura* is an economically important polyphagous pest in India, China, and Japan. It is causing considerable economic loss to many vegetable and field crops. It is a polyphagous insect present in high numbers in tropical countries even during rainy seasons with at least 48 families (*9*, *10*).

Saponins are widely distributed secondary plant metabolites and are found among almost 100 plant families (*11*, *12*). Saponins are a class of natural products that are surface-active sterol or triterpene glycosides. Triterpenoid saponins consist of a triterpenoid aglycone composed of a C30 pentacyclic structure. Triterpenic saponins may be monodesmosidic or bidesmosidic, based on the attachment of sugar either in C-3-OH or C-28-COOH or both. Saponins are biological detergents because of glycolysation of the hydrophobic aglycone, and when agitated in water, they produce copious foam. Saponins have also been characterized by their hemolytic index and formation of precipitates with cholesterol in alcohol.

Monodesmosidic triterpenoid saponins from *Barbarea vulgaris* evidenced antifeedant activity against third instar larvae of the diamondback moth, *Plutella xylostella* (*13*). *Sapindus mukorossi* and *Murraya koenigii* extracts (0.5%) have been found to effectively control the mustard aphid when evaluated for their antifeedant/insecticidal properties (*14*). *Sapindus trifoliatus* was found moderately active when ether extract was tested at up to 1000 ppm for antifeedant activity against fourth instar larvae of the noctuid *Spodoptera litura*. Triterpenic saponins were shown to reduce larval growth and cause mortality in the flower beetle, *Tenebrio molitor* (*15*), European grape moth, *Lobesia botrana* (*16*), European corn borer, *Ostrinia nubialis* (*17*), army worm, *Spodoptera littoralis* (*18*), and a number of other insects.

Though, *Diploknema butyracea* J. F. Gmel (Family: sapotaceae) and *Sapindus mukorossi* GAERTN (Family: sapindeae) are known to contain triterpenic saponin in their seed and fruit pericarp,

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 Published on Web 12/11/2009

respectively, information is limited on their pesticidal properties (*19*−*21*, *33*) . *Diploknema butyracea* is a medium to large size deciduous tree found in central and northern India and Malaysia. The tree bears ovoid shaped berries up to 5 cm long that are greenish turning reddish yellow or orange when ripe: seeds 1−4, brown colored, ovoid shaped, 2.5−3.75 cm long. Fruits ripen in May through June. *Sapindus mukorossi* is a handsome deciduous tree of the Indian subcontinent. It is known as a soap-nut tree. It flowers during summer, and the fruit appears in July and August and ripens by November to December. Fruits are solitary globose, i.e., round nuts, of 2−2.5 cm diameter, fleshy, saponaceous, and yellowish brown in color. The seed is enclosed in a black, smooth, and hard globose endocarp. The fruit is collected during winter months for seed and or sale in the market as a soap nut. In general, *D. butyracea* and *S. mukorossi* contain 2.5 and 2.6% saponins. Crystalline saponins isolated from *Sapindus mukorossi* fruit were Sapinoside—A and B were characterized as 3 (α-L-arabinopyranosyl-2-α-L-rhamnopyranoside) and 3 (α-L-rabinopyranosyl-2-α-L-rhamnopyranosyl-3-β-D-xylopyranoside) of hederagenin, respectively (*19*). From pericarp of *Sapindus mukurossi*, a number of monodesmosidic and bisdesmosidic saponins were also reported. The bisdesmosidic saponins were named as mukuroz-saponin X, mukuroz-saponin Y_1, and mukuroz-saponin Y_2 (*20*).

Lalitha and Venkataraman (*21*) reported the antifungal activity and mode of action of saponins from *Madhuca butyracea* Macb. (*Diploknema butyracea*) against four different fungi. *Sapindus mukorossi* and *Murraya koenigii* extracts (0.5%) have been found to effectively control the mustard aphid, *Lipaphis erysimi*, when evaluated for their antifeedant/insecticidal properties (*14*). Petroleum ether extracts of ritha seeds were tested at up to 1000 ppm for antifeedant activity against fourth-instar larvae of the noctuid *Spodoptera litura* and found that *Sapindus trifoliatus* was moderately active. The activity was correlated with the percentage content of linoleic and oleic acid in the seed oil (*22*).

The objective of the investigation was to evaluate the saponins for insecticidal activity for use as environmentally benign pesticides.

MATERIALS AND METHODS

General Experimental Procedure. All chemicals and reagents were procured from Merck India, Ltd. Double distilled water was used throughout the analysis. Separation of saponins was performed on a Waters HPLC system, fitted with a 600 series pump, Rheodyne injector, 996 PDA detector at 206 and 213 nm for *S. mukorossi* and *D. butyracea*, respectively. Purification of individual saponins was performed on a Prep-HPLC instrument (MERCK-KNAUER) fitted with a Wellchrom preparative HPLC pump K-1800 and RP-18 column (Lichrospher, 250 × 25 mm; 10 μm) and self-packed RP-18 column (250 × 50 mm; 15−25 μm) and an automatic fraction collector. ^{1}H NMR spectra of compounds were recorded on a Bruker (300 MHz) spectrometer. Deuterio-methanol (CD_3OD) was used as solvent, and tetramethyl silane (TMS) was used as standard. Electrospray mass spectroscopy was carried out on AB/MDS Sciex-API 2000 triple quadrupole mass spectrometer using TurboSpray source.

Plant Material. *Sapindus mukorossi* fruits were procured from Palampur, Himachal Pradesh. *Diploknema butyracea* seeds were procured from Bihar.

Insect Rearing. Insect cultures of *Spodoptera litura* (F.) (Noctuidae: Lepidoptera) were maintained in the laboratory on castor leaves (*Ricinus communis* L.). Rearing conditions were a 12 h photo regime at 28 ± 1 °C and 60% relative humidity. Insect cultures were continuously refreshed with new one, found in the vicinity of the research farm of the Indian Agricultural Research Institute, New Delhi, India.

Extraction of Saponins. *D. butyracea* seeds and *S. mukorossi* fruit pericarp were used for the extraction of saponin (*33*). Ground seeds/pericarp (1 kg) placed in a conical flask containing hexane (2 L) were soaked overnight and agitated the next day with a mechanical stirrer for 45 min. The material was filtered through a buchner funnel in vacuo, and the resulting cake was again extracted twice with 1 L of hexane to ensure complete removal of oil/fat. The deoiled seed cake was then extracted in the same manner with methanol (3 × 1 L), and the combined methanol extract after filtration was concentrated in vacuo at 45 °C to viscous syrup. The extract was partitioned between water and *n*-butanol to remove water-soluble free sugars. The combined butanol extract was then concentrated to viscous liquid at < 70 °C in vacuo. The viscous concentrate was dissolved in a minimum quantity of methanol (25 mL) and precipitated with a large excess of acetone to obtain a saponin mixture. The yield of saponin was ≈12.5 and 20.5 g from *D. butyracea* and *S. mukorossi*.

Preparation of Prosapogenin and Sapogenin. Both the saponin mixtures in methanol were refluxed with 1.5 M HCl−MeOH for 8 h. After hydrolysis was complete, the reaction mixture was diluted with water and extracted with chloroform or dichloromethane (3 × 50 mL). While prosapogenin was detected in the organic phase, sugars were found in aqueous hydrolysate.

Both *D. butyracea* and *S. mukorossi* saponin (2 g) were dissolved in methanol and refluxed with 20% aqueous KOH (1:1) for 4 h. The solution was cooled at room temperature and diluted with water. After neutralizing the solution with 5% H_2SO_4, partially hydrolyzed saponins were separated out. The prosapogenin (~600 mg) was filtered through a buchner funnel in vacuo and repeatedly washed with water to remove the free sugars.

Separation of Saponins. To find out the number of saponins in both the saponin mixtures, analytical reverse-phase high-performance liquid chromatography (HPLC) was run. HPLC was performed on a Waters HPLC system fitted with LiChrosphere R 100 RP-18e column (5 μm) procured from Merck KgaA, Darmstadt, Germany, or on Novapack phenyl 16R, 4 μm particle size, 3.9 × 150 mm ID cartridge column containing dimethylphenyl propyl silyl bonded amorphous silica. The waters HPLC system equipped with a 600 series pump, Rheodyne injector, and a 996 PDA detector at 206 and 213 nm for *D. butyracea* and *S. mukorossi* saponins, respectively. A 20 μL volume of sample was injected each time via a Rheodyne injector (20 μL loop) for a run time of 15 min. The samples were filtered through a 0.25 μm Millipore filter before injection. Peaks were detected at the corresponding λ_{max}. The retention time (R_t) for each compound was measured. Methanol:water (60:40 v/v) at a flow rate of 0.4 mL min^{-1} with pressure 1750 psi was chosen for optimum separation of *D. butyracea* saponin constituents, whereas acetonitrile:water (47:53 v/v) at a flow rate of 0.4 mL min^{-1} with pressure 1200−1250 psi has been chosen for optimum separation of the *S. mukorossi* saponin constituents.

Chromatographic Conditions. Methanol:water (60:40 v/v) at a flow rate of 0.4 mL min^{-1} with pressure 1750 psi was chosen for optimum separation of *D. butyracea* saponin constituents, whereas acetonitrile: water (47:53 v/v) at a flow rate of 0.4 mL min^{-1} with pressure 1200−1250 psi has been chosen for optimum separation of the *S. mukorossi* saponin constituents.

Characterization of *Diploknema* Saponins. *Diploknema Saponin **MI-I***. The first saponin isolated by repeated silica gel column chromatography of the crude saponin concentrate was obtained as amorphous white powder. It was designated as **MI-I** (mp 235−238 °C, R_f= 0.65 in $CHCl_3$:MeOH:H_2O (65:35:10).

^{1}H NMR (CD_3OD) δ: 0.901, 0.939, 1.059, 1.13, 1.164, and 1.226 (3H each, s, H-29, H-30, H-27, H-26, H-24, and H-25, respectively), 1.247 (2H, H-5), 1.270, 1.613 (2H, H-15), 1.306 (2-H, H-7), 1.610 (1H, H-9), 1.722, 1.761 (1H, H-11, or H-22), 3.41 (1H, s, H-3), 3.371 (2H, H-23), 3.56 (1H, m, H-18), 4.502 (1H, d, H-2), 5.092 (1H, br s, H-16), 5.127 (1H, br s, H-6), 5.347 (1H, glc anomeric), 5.630 (1H, br s, H-12). Signal pattern of protons attached to glycone moiety at δ 3.4−4.82 was unclear.

ES-MS: *m*/*z* 1241 $[M+H]^+$, *m*/*z* 1223 $[M-18]^+$, *m*/*z* 1205.9 $[M-18-18]^+$, *m*/*z* 1073 $[M-18-150]^+$, *m*/*z* 1059 $[M-182]^+$, *m*/*z* 927.7 $[M-182-132]^+$, *m*/*z* 795.8 $[M-182-132-132]^+$.

*Diploknema Saponin **MI-III***. ^{1}H NMR (CD_3OD) δ: 0.903, 0.940, 1.060, 1.133, 1.224, 1.310 (3H each, s, H-29, H-30, H-27, H-26, H-24, and H-25, respectively), 1.182, 1.760 (2H, H-2), 1.203 (1H, H-5), 1.248, 1.613 (2H, m, H-7), 1.273, 1.294, 1.724 (1H, m, H-9), 1.761 (2H, H-22), 3.34, 3.75 (2H, H-23), 3.564 (1H, m, H-18), 3.713 (1H, m, H-3), 4.524 (1H, d, H-2), 3.487, 3.520, 3.770, 3.826, 3.837, 3.858, 3.915, 4.420, 4.460, 4.540

(anomeric and glycon moiety protons), 5.088 (1H, br s, H-16), 5.150 (1H, br s, H-6), 5.349 (glc H-1), 5.629 (1H, br s, H-12).

ES-MS: m/z 1535.9 $[M]^+$, m/z 1518 $[M-18]^+$, m/z 1403 $[M-132]^+$, m/z 1373 $[M-132-30]^+$, m/z 1373 $[M-162]^+$, m/z 1355 $[M-162-18]^+$, m/z 1241 $[M-132-162]^+$, m/z 1241 $[M-162-132]^+$.

Sapindus mukorossi Saponin, ***SM-I.*** 3-*O*-[β-D-xyl(OAc)·β-D-arabinopyranosyl·β-D-rhamnopyranosyl] hederagenin-28-*O*[β-D-glc·β-D-glc·β-D-rhamnopyranosyl] ester.

^{1}H NMR (CD_3OD) δ: 0.701, 0.815, 902, 0.936, 0.972, and 1.173 (3H each, s, H-29, H-30, H-26, H-27, H-24, and H-25, respectively), 3.299 (1H, m, H-3), 3.520 (2H, m, H-23), 5.215 (1H, d, H-12). Other NMR peaks appearing in the region 3.4−5.0 corresponded to anomeric and other protons of the glycone moiety.

ES-MS: m/z 1417 $[M+Na]^+$, m/z 942.7 $[M^+-$ (rha+ara+xyl-OAc, 452 amu)], m/z 925 $[M^+-$ (glc+glc-A+ara, 490 amu], m/z 1374 [1417 − $COCH_3$, m/z 793 [925-ara], m/z 782 [942 − glc], (m/z 414, 386.7, 369.7) derived from the glc-glc-A-ara fragment. m/z 223, 248, 205, and 187 (RetroDiels−Alder fragments)

Bioassay of Saponins against *Spodoptera litura*. The test compounds (100 mg) were weighed accurately in a 5 mL volumetric flask and dissolved into 0.5 mL of distilled acetone. The volume was then made to 5 mL with acetone to obtain 2% stock solution. From these stock solutions, different concentrations (1.0, 0.5, 0.25, 0.1, 0.07, 0.05, 0.01, 0.007, and 0.005%) were prepared separately by serial dilution with 0.5% emulsified water, which in turn was prepared by dissolving 5 mL of Tween 80 emulsifier in 1 L of distilled water.

Antifeedant activity of saponins was evaluated against *Spodoptera litura* larvae using no choice bioassay method. A leaf-dipping method was used to evaluate the activity of the test samples. Castor leaves were washed with 70% double distilled alcohol and air-dried for 15 min before dipping into the required amount of plant product. The leaf disk (6.5 cm) of castor was used for evaluating feeding deterrent activity of the samples against *S. litura*. Ten leaf disks per dose were separately dipped in each test solution for 30 s. Solvents were evaporated, and the larvae were transferred individually on treated and control (disks treated with solvent, emulsified water only) leaf disks placed in Petri plates. Treated leaves were fed to third-instar larvae of *S. litura*. Five replications were used per dose for the test. To calculate the larvae antifeeding activity, the percentage of leaf damage was estimated after 48 h of treatment by taking observations using graph papers. Experiments were maintained at 28 ± 1 °C and 65 ± 5% relative humidity. Corrected feeding inhibition (%) was calculated by the following formula:

$$\text{corrected feeding inhibition (\%)} = [(C-T)/(C+T)] \times 100$$

where T=Consumption of leaf in treatment and C=consumption of leaf in control.

For insect growth regulatory activity, third-instar larvae of *Spodoptera litura* weighing between 30 and 60 mg were treated with various concentrations of the test emulsions under Potter's direct spray tower at a pressure of 340 g cm^{-2}. The sprayed dishes were dried for five min under a fan after which the larvae were transferred to separate rearing bottles. The larvae, similarly sprayed with emulsified water, served as a control. Larval weight was taken at 3 and 7 days after treatment. Ten replications were used per dose for the test. The raw data on different parameters were subjected to angular transformation (arc sine percentage)$^{1/2}$ and analyzed statistically by complete randomized design. Analysis of variance was done, and means were separated by square difference, i.e., critical difference. Inhibition concentration (IC_{50}) was determined based on probit analysis. Percent reduction in larval weight gain over control (% growth inhibition) was calculated as

$$[(\text{weight gain in control} - \text{weight gain in treatment})/\text{weight gain in control}] \times 100$$

Data Analysis. Antifeedancy index (AI_{50}) values were calculated by using a Basic LD_{50} program version 1.1 as described by Trevors (*23*). The raw data on different parameters were subjected to angular transformation (arc sine percentage) and analyzed by complete randomized design. Analysis of variance was done, and means were separated by critical difference (CD) using the statistical package for social sciences (SPSS, version 10).

RESULTS

The deoiled/defatted plant material (*D. butyracea* seed cake or *S. mukorossi* fruit pericarp) was extracted with methanol, and the residue was partitioned with water:*n*-butanol. Then the concentrated organic phase was precipitated in a large excess of acetone to yield saponin powder concentrate. An improved LC method has been developed to estimate triterpenic saponin content by reverse HPLC using a photodiode array detector. The method was chosen because of the ready solubility of triterpenic saponins in polar solvents. On the basis of an initial screen of various proportions of methanol:water and acetonitrile:water solvent systems, methanol:water (60:40 v/v) at a flow rate of 0.4 mL min^{-1} with pressure 1750 psi and λ_{max} of 213 nm was chosen for optimum separation of *D. butyracea* saponin constituents. Three saponins eluted at 16.4, 20.6, and 21.9 min. The *Sapindus mukorossi* saponin concentrate was comprised of a single major saponin (>80%), and the rest were detected as minor constituents. The retention time of the major *S. mukorossi* saponin under these LC conditions is 13.04 min.

Triterpenic saponins after alkaline hydrolysis using 10% KOH to yield a partial hydrolytic product in which the glycon moiety attached to C-3-OH functions remain intact, while that attached to C-28-COOH functions was hydrolyzed while the other prosapogenin obtained after acidic hydrolysis had partially hydrolyzed glycon moieties at both C-3-OH and C-28 carboxylic acid functions.

The initial leaf disk no choice bioassay assessed how effectively the saponin deterred insect feeding. As extract concentration increased, deterrency indices also increased, in a dose-dependent manner (**Table 1**). Leaf damage on castor treated with 1% saponin was significantly different from the other four concentrations ($P < 0.05$). At the end of the experiment, *S. litura* larvae consumed about 95% of control leaves, but about 30−40% of castor leaves were treated with 1% saponin. Therefore, we conclude that saponin is an antifeedant to *S. litura* larvae, but larvae still consume a quite large amount of treated leaves.

Antifeedant activity of *D. butyracea* and *S. mukorossi* saponins was in a similar range with shade better in *D. butyracea*. The activity ranged between 39.7 and 51.8 and 32.8 and 48.2% in *D. butyracea* and *S. mukorossi*, respectively. The activity was enhanced when the saponin was subjected to hydrolysis in the case of *D. butyracea*, whereas enhancement was minimum in the case of *S. mukorossi* saponin. Alkaline hydrolyzed saponin was found to be more active than the acid hydrolyzed one. This is reflected in antifeedant index (AI_{50}) (**Table 2**). Hydrolyzed saponins were more active than the parent saponins in the case of *D. butyracea*. AI_{50} was reduced from 1.39% to 1200 and 3400 mg L^{-1} in alkaline and acid hydrolyzed *D. butyracea* saponins, respectively. In the case of hydrolyzed *S. mukorossi* saponin, activity was far less than *D. butyracea* saponins. Both the sapogenins had the same activity as their saponin.

Saponins lowered food consumption and reduced larval growth rate, which was measured in larval weight (**Table 1** and **3**). A pronounced decrease of the weight increments occurred with acid and alkaline hydrolyzed saponins. In the case of *D. butyracea*, both the hydrolyzed saponins were found more active than their parent molecule, which is depicted in the GI_{50} data (**Table 4**). IGR activity of *D. butyracea* saponins was 39.6%, whereas it was 83.7 and 62.2% in alkaline and acid hydrolyzed saponins, respectively, at 1% concentration. Further removal of sugars yielded less in activity, which was depicted in GI_{50} of sapogenin. Upon hydrolysis of *S. mukorossi* saponin, enhancement in IGR activity was not as significant as that of *D. butyracea*. In this case, GI_{50} was reduced from 1.39% to

Table 1. Antifeedant and Insect Growth Regulatory (IGR) (%) Activity of *Diploknema butyracea* Saponins and Their Derivatives against *S. litura*[a]

conc.	1.0	0.5	0.25	0.1	0.05	CD	1.0	0.5	0.25	0.1	0.05	CD
comp.						(5%)						(5%)
			Antifeedant						IGR			
saponin	51.8	44.3	43.3	40.3	39.7	8.54	39.6	38.6	25.0	20.3	15.1	5.98
	(*46.0*)	(*41.7*)	(*41.1*)	(*39.4*)	(*39.8*)		(*39.0*)	(*38.4*)	(*30.0*)	(*26.7*)	(*22.8*)	
alkaline hydrolyzed saponin	81.8	77.1	56.7	47.6	36.4	10.67	83.7	66.3	57.2	52.5	26.1	7.65
	(*64.9*)	(*61.4*)	(*48.9*)	(*43.6*)	(37.0)		(*66.4*)	(*54.6*)	(*52.2*)	(*45.5*)	(*30.7*)	
acid hydrolyzed saponin	64.5	56.7	38.1	35.5	26.4	8.28	62.2	61.6	56.1	41.0	38.3	5.81
	(*53.5*)	(*48.9*)	(*39.6*)	(*36.5*)	(*30.9*)		(*52.1*)	(*51.7*)	(*48.5*)	(*39.8*)	(*38.2*)	
sapogenin (protobassic acid)	39.6	27.1	25.9	11.1	10.3	5.86	41.9	38.6	26.1	21.4	15.1	5.01
	(*39.0*)	(*31.4*)	(*30.6*)	(*19.4*)	(*18.6*)		(*40.4*)	(*38.4*)	(*30.7*)	(*27.6*)	(*22.8*)	

[a] arc sin transformed data (within parentheses).

Table 2. AI_{50} of Saponins and Its Hydrolyzed Products of *D. butyracea* and *S. mukorossi*

compounds	AI_{50} (%)	$\chi 2$ exp (3 d.f)	fiducial limit
Diploknema butyracea			
saponin	1.39	0.81	0.14−14.24
alkaline hydrolyzed prosapogenin	0.12	3.14	0.10−0.18
acid hydrolyzed prosapogenin	0.34	2.69	0.24−0.483
sapogenin (protobassic acid)	1.31	1.68	0.70−0.244
Sapindus mukorossi			
saponin	1.40	0.08	0.27−0.715
alkaline hydrolyzed prosapogenin	1.18	1.27	0.59−0.237
acid hydrolyzed prosapogenin	0.91	0.14	0.18−0.461
sapogenin (hederagenin)	1.49	0.37	0.29−0.759

5500 and 5100 mg L^{-1} in alkaline and acid hydrolyzed saponin, respectively.

Our results suggest that partially hydrolyzed saponins are a feeding deterrent and also regulate insect growth to *S. litura* larvae. After feeding for 5 day on saponin treated castor leaves, larval growth was reduced depending upon the concentration as compared to control. Saponin feeding also extended the length of time to pupal ecdysis. While the controls took 15.1 days from the start of the second larval instar to the pupal ecdysis, insects surviving the feeding with 1% saponins needed 18.4 days.

DISCUSSION

Structure Elucidation. The ^{1}H NMR spectrum of **MI-I** showed the existence of six singlet peaks at δ 0.91, 0.94, 1.06, 1.13, 1.16, and 1.23 corresponding to six methyl groups at H-29, H-30, H-27, H-26, H-24, and H-25 positions of the aglycone moiety. A broad singlet at δ 5.63 corresponded to the olefinic proton located at the 12th position of the aglycone moiety. Similarly, proton(s) located at carbons adjacent to the hydroxyl functions such as H-3 and H-23 were located at δ 3.41 (m) and 3.71 (br, s).

The mass spectrum displayed a quasi-molecular ion peak $[M + H]^+$ at m/z 1241, which corresponded to its molecular formula $C_{57}H_{92}O_{29}$ (**Figure 1**). It further showed two characteristic peaks at m/z 1223 and 1205 emanating as a result of the successive loss of two neutral water (18 amu) molecules from the parent protonated molecular ion. Further, two fragment ions of lower intensity, detected at m/z 1060 and 1074, were attributed to the loss of the glucose moiety (m/z 180 amu) and arabinose moiety (m/z 150 amu), respectively, from the parent ion (m/z 1241) and its dehydrated fragment ion (m/z 1223), respectively. The preferential loss of glucose and pentose units such as arabinose suggest that these monosaccharide units are located at the outer periphery of the glycone moiety of the saponin molecule. Further, these monosaccharides (glucose and arabinose) were also detected in the initial phase of Kiliani hydrolysis of **MI-I** saponin. In addition, characteristic fragment ions at m/z 927.7 and 795.8 were postulated to arise as a result of the successive loss of the second and third pentose moiety (150 amu), which may consist of either arabinose and/or xylose. On the basis of spectral studies, the structure of *Diploknema butyracea* saponin (**MI-I**) was tentatively assigned as 3-*O*-[β-D-glucopyarnosyl-β-D-glucopyranosyl]−16-α-hydroxyprotobassic acid-28-*O*-[ara-glc-xyl]-ara (**Figure 1**).

On the basis of ^{1}H NMR spectral data, the more polar *Diploknema* saponin (**MI-III**) was tentatively identified as bidesmoside of 16-hydroxyprotobassic acid. It showed the presence of six methyl signals at δ 0.90, 0.94, 1.06, 1.13, 1.224, and 1.31 ascribable to H-29, H-30, H-27, H-26, H-24, and H-25 protons, respectively. The ^{1}H NMR spectrum also showed signals at δ 4.52, 3.71, 5.088, and 5.15 corresponding to H-2, H-3, H-6, and H-16 protons on the carbons bearing hydroxyl functions. The NMR spectrum also confirmed the existence of the olefinic proton attributable to the H-12 proton at δ 5.63 and two hydroxymethyl protons at δ 3.34 and 3.75 located at the C-23 position of the aglycone moiety. The remaining anomeric and other protons at δ 3.49, 3.52, 3.77, 3.83, 3.84, 3.86, 3.92, 4.42, 4.46, and 4.54 located on the two glycone moieties attached to C-3 or C-28 positions of the aglycone nucleus could not be assigned to their respective positions.

The mass spectrum of more polar *Diploknema* saponin (**MI-III**) showed a molecular ion peak at m/z 1535.9 corresponding to its molecular formula $C_{68}H_{111}O_{38}$ (**Figure 1**). The characteristic fragment ions detected at m/z 1403 and 1373 were attributed to the respective cleavage of pentose such as xylose, arabinose, apiose (132 amu) and hexose such as glucose (162 amu) from the parent ion indicating that one molecule each of glucose and apiose were present in the outer periphery of the two sugar chains attached to the saponin nucleus. Another mass ion peak located at m/z 1241 probably originated as a result of the loss of the pentose unit from the parent ion m/z 1373 or hexose unit (glucose) from the fragment ion 1373. Two other fragment ions detected at m/z 1518 and 1355 were caused by the loss of one water molecule (18 amu) from the molecular ion m/z 1535.9 and the fragment ion m/z 1373, respectively. On the basis of spectral studies, the structure of **MI-III** was tentatively assigned as 3-*O*-β-D-glucopyranosyl-glucopyranosyl-glucopyranosyl-16-α-hydroxyprotobassic acid-28-*O*-[ara-xyl-ara]-apiose (**Figure 1**).

Earlier reports found two major saponins named Mi-saponin A and Mi-saponin B from seed kernels of *Madhuca longifolia*, and the saponins were elucidated as 3-*O*-β-D-glucopyranosyl−28-*O*-[α-L-rhamnopyranosyl (1-3)-β-D-xylopyranosyl (1-4)-α-L-rhamnopyranosyl (1-2)-α-L-arabinopyranosyl]-protobassic acid

Table 3. Antifeedant and Insect Growth Regulatory (IGR) (%) Activity of *Sapindus mukorossi* Saponins and Their Derivatives Against *S. litura*[a]

conc.	1.0	0.5	0.25	0.1	0.05	CD	1.0	0.5	0.25	0.1	0.05	CD
comp.						(5%)						(5%)
	Antifeedant						IGR					
saponin	48.2	45.6	40.5	38.4	32.8	8.57	42.2	31.3	28.1	20.0	18.6	15.87
	(44.0)	(42.5)	(39.5)	(38.3)	(34.9)		(40.4)	(38.8)	(31.8)	(26.4)	(25.5)	
alkaline hydrolyzed saponin	50.7	41.6	29.5	22.3	20.1	11.53	56.4	53.5	37.8	36.9	30.0	9.16
	(45.4)	(40.1)	(32.9)	(28.1)	(26.6)		(48.6)	(47.0)	(37.9)	(37.4)	(33.1)	
acid hydrolyzed saponin	51.2	47.2	44.1	40.7	38.7	9.89	69.5	63.5	57.6	54.5	54.5	8.86
	(45.7)	(43.4)	(41.6)	(39.6)	(38.4)		(56.5)	(52.9)	(49.4)	(47.6)	(47.6)	
sapogenin (protobassic acid)	48.0	45.2	39.2	35.4	33.1	4.15	44.3	38.5	32.5	24.4	18.5	7.40
	(43.9)	(42.3)	(38.8)	(36.5)	(35.1)		(41.7)	(38.4)	(34.7)	(29.5)	(25.5)	

[a] arc sin transformed data (within parentheses).

Table 4. GI_{50} of Saponins and Its Hydrolyzed Products of *D. butyracea* and *S. mukorossi*

compounds	GI_{50} (%)	$\chi 2$ exp (3 d.f)	fiducial limit
Diploknema butyracea			
saponin	0.916	0.898	0.582−1.59
alkaline hydrolyzed prosapogenin	0.152	6.85	0.117−0.198
acid hydrolyzed prosapogenin	0.192	1.56	0.118−0.313
sapogenin (protobassic acid)	2.00	1.04	0.790−5.07
Sapindus mukorossi			
saponin	1.40	0.532	0.265−7.23
alkaline hydrolyzed prosapogenin	1.18	2.52	0.309−0.988
acid hydrolyzed prosapogenin	0.91	1.65	0.30−0.85
sapogenin (hederagenin)	1.49	0.202	0.66−4.34

and 3-*O*-*β*-D-glucopyranosyl−28-*O*-{3-*O*-*β*-D-apio-D-furanosyl-4-*O*-[α-L-rhamnopyranosyl(1-3)-*β*-D-xylopyranosyl]-α-L-rhamnopyranosyl(1-2)-α-L-arabinopyranosyl]-protobassic acid (*33*), whereas Nigam et al. (*34*) reported two major saponins as 3-*O*-*β*-D-glucopyranosyl protobassic acid−28-*O*-*β*-D-apio-D-furanosyl (1−3)-*β*-D xylopyranosyl (1-4)-α-L-rhamnopyranosyl (1-2)-α-L-arabinopyranoside and 3-*O*-*β*-D glucopyranosyl 16-α-hydroxy protobassic acid−28-*O*-*β*-D-apio-D-furanosyl (1-3)-*β*-D xylopyranosyl (1-4)-α-L-rhamnopyranosyl (1−2)-α-L-arabinopyranoside.

In our investigation, we found **MI-I** as 3-*O*-[*β*-D-glucopyarnosyl-*β*-D-glucopyranosyl]−16-α-hydroxyprotobassic acid-28-*O*-[ara-glc-xyl]-ara and **MI-III** as 3-*O*-*β*-D-glucopyranosyl-glucopyranosyl-glucopyranosyl-16-α-hydroxyprotobassic acid-28-*O*-[ara-xyl-ara]-apiose. In our study, we found hydroxyprotobassic acid as sapogenin, whereas Kitagawa et al. (*33*) reported protobassic acid as sapogenin. Furthermore, Nigam et al. (*34*) reported both as sapogenin.

Sapindus saponin on acidic and alkaline hydrolysis yielded the sapogenin which was identified as hederagenin by comparison of its ^{1}H NMR and mass spectrum data with literature values (*20*). On hydrolysis with mineral acid, it was completely hydrolyzed and yielded free monosaccharides identified as glucose, arabinose, xylose, and rhamnose, as evident by comparison with authentic samples (TLC, PC). Different partial hydrolysis products inferred that *Sapindus* saponin is bisdesmosidic with two-sugar moieties attached at the C-3 hydroxyl and C-28 carboxyl functions. The constitution of the sugar moiety attached at the C-3 hydroxyl appears to be the same as reported earlier (*20*). The sugar moiety located at the C-28 position was probably comprised of glucose, acid, and arabinose. The prosapogenin, resulting from alkaline hydrolysis of the saponin, furnished only three sugars, namely, arabinose, rhamnose, and xylose. Besides the characteristic ^{1}H NMR peaks corresponding to six methyl singlets (0.70, 0.82, 0.90, 0.94, 0.97, and 1.17), multiplets at δ 3.3 (1H, H-3) and 3.52 (2H, H-23) corresponding to protons adjacent to hydroxyl functions, and an olefinic proton (H-12) at δ 5.22, typical of a hederagenin aglycone moiety, other peaks appearing in the region 3.4−5.0 corresponded to anomeric and other protons of the molecule. Instead of a molecular ion $[M]^+$ peak, it exhibited a metal adduct peak at m/z 1416.9 $[M + Na]^+$. Other prominent peaks emerging at m/z 925 are postulated to arise as a result of the C-28 ester cleavage. The fragment ion at m/z 490, 414, 386.7, and 369.7 originated from the C-28 glycone moiety comprised of glucose and the arabinose molecule. Similarly, another major fragment ion peak at m/z 942 originated as a result of the cleavage of the C-3 glycosidic linkage. Similarly, peaks at m/z 793 and 782 were attributed to the loss of glucose and arabinose moieties from fragment ions m/z 925 and 942, repectively. The structure of the saponin was established as the 3-*O*-[*β*-D-xyl(OAc)·*β*-D-arabinopyranosyl·*β*-D-rhamnopyranosyl] hederagenin-28-*O*[*β*-D-glc·*β*-D-glc·*β*-D-rhamnopyranosyl] ester (**Figure 1**).

From the pericarp of *S. mukurossi*, a number of monodesmosidic saponins were isolated, and three bisdesmosidic saponins were also reported. The structures of bisdesmosidic saponins were elucidated as the -sophorosyl ester of 3-*O*-α-L-rhamnopyranosyl (1-2)-α-L-arabinopyranosyl hederagenin (Mukuroz-saponin X), 3-*O*-*β*-D-xylopyranosyl (1-3)-α-L-rhamnopyranosyl (1-2)-α-L-arabinopyranosyl hederagenin (Mukuroz-saponin Y1), and 3-*O*-α-arabinopyranosyl (1−3)-α-L-rhamnopyranosyl 275 (1-2)-α-L-arabinopyranosyl hederagenin (Mukuroz-saponin Y2) (*20*). In our study, saponin isolated from *S. Mukorossi* was characterized as 3-*O*-[*β*-D-xyl(OAc)·*β*-D-arabinopyranosyl·*β*-D-rhamnopyranosyl] hederagenin-28-*O*[*β*-D-glc·*β*-D-glc·*β*-D-rhamnopyranosyl] ester. Our experiment included saponins, sapogenins, and partially hydrolyzed saponin with a similar carbon skeleton but differing in the attachment of the sugar molecule (**Figure 1**). The results revealed that the activity of saponin and its sapogenin was similar in both cases. Previous observations reported that insecticidal activity was due to triterpenoid aglycones (*18*, *35*).

Mechanism of Action in *Spodoptera litura*. Investigations concluded that saponins act as insect feeding deterrents (*13*, *18*, *24*−*27*). Food consumption was reduced in our experiments also due to antifeedant activity, and this was obviously the major cause of the diminished body growth (**Tables 1** and **3**). Inhibition of the digestive enzymes (*29*) and interference with the sterol metabolism (*30*, *31*) could be involved in the negative effects of saponins against *S. litura*. Negative effects of saponins on herbivore performance, e.g., reduction of larval growth and pupal mass, could be a consequence of shortening or suppressing the feeding process. Thus, the results presented here confirm that saponins are natural feeding barriers for phytophagous insects (*17*, *25*−*27*, *32*). Moreover, a

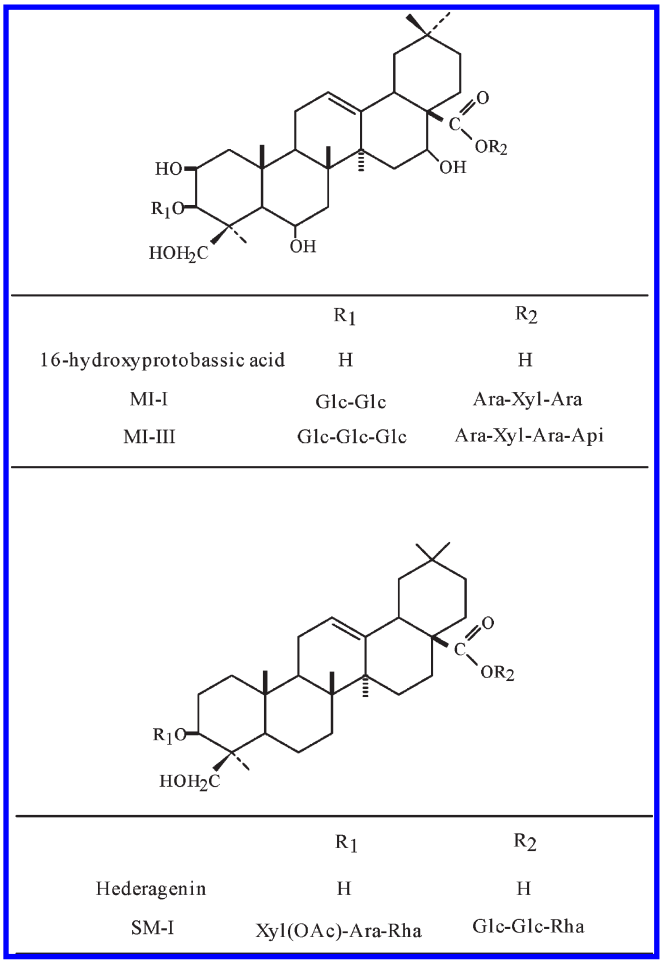

Figure 1. Structure of (**A**) *D. butyracea* and (**B**) *S. mukorossi* saponin and sapogenin.

similar reduction in insect food consumption by saponins has been reported by Adel et al. (*18*) and Golawska et al. (*28*).

Both the genins, protobassic acid and hederagenin, exhibit similar activities. The activity of glycosylated saponins depends on the sugar moiety (*18*). Thus, the result presented here confirms that selective attachment of the sugar molecule to the aglycone moiety resulted in higher insecticidal activity than its aglycone part. Glycosylated saponins exert insecticidal activity only when they are hydrolyzed by insect gut glycosidases and liberate an active aglycone (*18*). Complex glycosides containing arabinopyranyl apparently resist the action of gut glycosidases and are therefore inactive. On the other hand, glycosidation may render the apolar aglycones water-soluble and thereby facilitate their ingestion. It was thus inferred that for optimum activity an appropriate hydrophile−lipophile balance (HLB) would be necessary which can be provided by a shorter glycone moiety comprised of a lesser number of monosaccharide units.

Triterpenic saponins after alkaline hydrolysis yielded a molecule, in which the glycon moiety attached to C-3-OH functions remains intact, while that attached to C-28-COOH functions was hydrolyzed. The other prosapogenin obtained after acidic hydrolysis had partially hydrolyzed glycon moieties at both C-3-OH and C-28 carboxylic acid functions.

Unlike saponins, the partial hydrolytic products have a smaller number of sugar units attached to the sapogenin nucleus. While a lower level of growth inhibitory effect of saponins containing a larger number of sugar units can be attributed to a comparatively more polar nature of the saponin molecule, the lower level of activity of sapogenins (16-α-hydroxyprotobassic acid and hederagenin) is attributed to their nonpolar nature. It is likely that the saponin derivative exerts insecticidal activity, only when the hydrophile lipophile balance (HLB) is optimum for penetration inside the insect body. Thus, our results presented here confirm that monodesmosidic saponins were more active than bisdesmosidic (*36*).

The study indicated that the antifeedant activity of *D. butyracea* saponins increased with a decrease in the number of monosaccharide units in the glycon moiety attached to the prosapogenin molecule. Thus, monosaccharide with one sugar chain attached at the C-3 hydroxyl function was more active than bidesmoside with two sugar moieties at the C-3 hydroxyl and C-28 carboxylic functions. The corresponding products obtained after partial hydrolysis of *S. mukorossi* were just a shade better than the corresponding saponins, but the increase in the activity was not significant.

In conclusion, *D. butyracea* prosapogenin offers potential against *S. litura*, particularly the alkaline hydrolyzed prosapogenin, containing protobassic acid with sugars attached in C-3-OH. Further studies are in progress to evaluate the individual prosapogenins on larvicidal activity.

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Received for review July 15, 2009. Revised manuscript received November 21, 2009. Accepted November 21, 2009.