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Molluscicidal Saponins from *Sapindus mukorossi*, Inhibitory Agents of Golden Apple Snails, *Pomacea canaliculata*

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Extracts of soapnut, *Sapindus mukorossi* Gaertn. (Sapindaceae) showed molluscicidal effects against the golden apple snail, *Pomacea canaliculata* Lamarck. (Ampullariidae) with LC_{50} values of 85, 22, and 17 ppm after treating 24, 48, and 72 h, respectively. Bioassay-directed fractionation of *S. mukorossi* resulted in the isolation of one new hederagenin-based acetylated saponin, hederagenin 3-*O*-(2,4-*O*-di-acetyl-α-L-arabinopyranoside)-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside (**1**), along with six known hederagenin saponins, hederagenin 3-*O*-(3,4-*O*-di-acetyl-α-L-arabinopyranoside)-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside (**2**), hederagenin 3-*O*-(3-*O*-acetyl-β-D-xylopyranosyl)-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside (**3**), hederagenin 3-*O*-(4-*O*-acetyl-β-D-xylopyranosyl)-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside (**4**), hederagenin 3-*O*-(3,4-*O*-di-acetyl-β-D-xylopyranosyl)-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside (**5**), hederagenin 3-*O*-β-D-xylopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside (**6**), and hederagenin 3-*O*-α-L-arabinopyranoside (**7**). The bioassay data revealed that **1**−**7** were molluscicidal, causing 70−100% mortality at 10 ppm against the golden apple snail.

KEYWORDS: ***Sapindus mukorossi*; Sapindaceae; molluscicidal effects; *Pomacea canaliculata*; hederagenin saponins**

INTRODUCTION

Golden apple snails (*Pomacea canaliculata* Lamarck. (Ampullariidae)), which were introduced into Taiwan from Argentina in 1979, have become major pests of rice and other aquatic crops throughout the whole island (*1*). These snails are also spread out and distributed in Philippines, Malaysia, Thailand, Indonesia, China, and the other Asian countries (*2*). Concern arose when the snails became a pest, causing serious damage to paddy rice and the other vegetables in Asia (*3*, *4*). It has been reported that golden apple snails cause an agricultural harvest loss of ca. $3 million dollars per year in Taiwan (*1*). In addition, the snails may also serve as a vector for diseases and parasites, as it may play potential role as an intermediate host for *Angiostrongylus cantonensis* (*5*). The snails can be controlled by chemical pesticides such as niclosamide and metaldehyde (*6*). However, synthetic molluscicides are expensive and generally toxic to other organisms and may produce damage to the environment (*7*). Natural plant molluscicides are gradually gaining attention, because they are considered ecologically more appropriate for snail control. In searching for suitable molluscicidal agents from natural sources, we found that the extracts of the soapnut, *Sapindus mukorossi* Gaertn. (Sapindaceae) exhibited the molluscicidal effects against golden apple snails. The pericarp of *S. mukorossi* has been traditionally used as an expectorant, as well as a source of natural surfactants. This genus has been reported to contain water-soluble mondesmosidic saponins (*8*−*10*). These saponins were known to possess molluscicidal activity and are present in various plants (*11*−*12*). However, there is no report concerning the isolation of natural products employed to kill golden apple snails. Bioassay-directed isolation and characterization of a new acetylated triterpene saponin, which named hederagenin 3-*O*-(2,4-*O*-di-acetyl-α-L-arabinopyranoside)-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside (**1**), together with six known triterpenoidal hederagenin saponin (**2**−**7**) from *S. mokorossi* (*13*−*16*). Bioassay data revealed that all of the isolated saponins (**1**−**7**) exhibited molluscicidal effects against *P. canaliculata*.

MATERIALS AND METHODS

General Experimental Procedures. Infrared (IR) spectra were measured on a Mattson Genesis II spectrophotometer (Thermo Nicolet, Madison, Wisconsin) using a KBr matrix. FABMS data were performed on a JEOL SX-102A instrument (JEOL USA, Inc., Peabody, Mas-

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sachusetts). High-resolution FABMS were measured on a Finnigan/Thermo Quest MAT mass spectrometer (Scientific Instrument Services, Inc., Ringoes, New Jersey). GC-MS were measured on a Finnigan/Thermo Trace GC. Optical rotations were recorded on a JASCO p-1020 polarimeter (JASCO International Co., Tokyo, Japan) at λ 589 nm. NMR spectra were performed on Varian NMR spectrometers (Unity Plus 500 and 400 MHz) (Varian Inc., Palo Alto, California.) using CD_3OD and C_5D_5N as solvent for measurement. Diaion HP-20 (Mitsubishi Chemical Co., Tokyo, Japan) and silica gel (Merck 70-230 mesh and 230-400 mesh) was used for column chromatography, and precoated silica gel (Merck 60 F-254) plates were used for TLC. The spots on TLC were detected by spraying with 50% H_2SO_4 and then heating on a hot plate. HPLC separations were performed on a Hitachi-7100 series apparatus with a Bischoff refractive index detector (Bischoff Chromatography, Leonberg, Germany), equipped with a 250 × 10-mm preparative Cosmosil 5SL-II column.

Plant Material. The powder of pericarp of *S. mukorossi* (soapnut) was purchased from Dharani Forestry and Orchards Limited administrative office in Guntur, India.

Extraction and Isolation. The powder of pericarp of *S. mukorossi* (soapnut) (35.5 g) was homogeneously suspended in MeOH for 72 h, filtered, and then was concentrated under reduced pressure to yield a MeOH extract. Preliminary purification of the MeOH extract (28.0 g) was made by using column chromatography over a 23 × 4.5-cm highly Diaion HP-20 porous polymer resin column, eluting with H_2O, 20, 40, 60, 80, and 100% aq. MeOH, successively. The bioactive fraction (100% MeOH) was further chromatographed on a 24 × 3.0-cm Diaion HP-20 column and eluted with 40, 60, 80, and 100% Me_2CO, successively. The bioactive fraction (40% Me_2CO) was concentrated (4.4 g) and then chromatographed on a 23 × 2.5-cm silica gel column with $CHCl_3$/MeOH, 21:1→2:1 to give five fractions (fractions 1−5). Fractions 4 and 5 were further purified by HPLC on a 250 × 10-mm silica gel column at a flow rate of 2.2 mL/min. Compound **2** (30.5 mg) was purified from fraction 4 by eluting with $CHCl_3$/MeOH (12:1), while compound **3** (50.2 mg) and compound **4** (30.3 mg) were isolated from fraction 5 by eluting with $CHCl_3$/MeOH (16:1). Fraction 6 was further purified by HPLC at a flow rate of 2.3 mL/min with $CHCl_3$/MeOH (12:1) to afford compound **6** (12.8 mg). The 60% acetone fraction (9.2 g) was chromatographed on Sephadex LH-20 with 100% MeOH to give 3 fractions (1′-3′). Fraction 1′ was chromatographed on silica gel with $CHCl_3$/MeOH, 10:1→1:1 to give five fractions (1′-1 to 1′-5). Fraction 1′-3 was further purified by HPLC with $CHCl_3$/MeOH (16:1) to afford compound **5** (30.4 mg). Fraction 1′-4 was chromatographed on silica gel in $CHCl_3$/Me_2CO/MeOH, 7:0.5:0.5→1:2:2 to give five fractions (1′-4-1 to 1′-4-5). Fraction 1′-4−2 was purified by HPLC at 2.5 mL/min with $CHCl_3$/MeOH (18:1) to obtain compound **7** (20.2 mg). Fraction 1′-4-3 was further purified by HPLC with $CHCl_3$/MeOH (12:1) to afford compound **1** (12.8 mg).

Hederagenin 3-*O*-(2,4-*O*-Di-acetyl-α-L-arabinopyranoside)-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside (1). White amorphous powder. $[\alpha]^{24}_D$ +30° (*c* 0.2, MeOH). IR ν_{max} (KBr): 3450, 2940, 1728, 1690 cm^{-1}. F ABMS *m*/*z*: 989 $[M+Na]^+$. HRFABMS *m*/*z*: 989.5095 (calcd 989.5086, $C_{50}H_{78}O_{18}$+ Na). ^1H NMR (400 MHz, CD_3OD): δ 3.63 (1H, m, H-3), 5.25 (1H, m, H-12), 2.83 (1H, d, J = 3.2, 13.6, H-18), 3.34, 3.51 (2H, m, H-23), 0.70 (3H, s, H-24), 0.97 (3H, s, H-25), 0.81 (3H, s, H-26), 1.71 (3H, s, H-27), 0.91 (3H, s, H-29), 0.94 (3H, s, H-30), 4.53 (1H, d, J = 6.0, Ara H-1), 3.69 (1H, br s, Ara H-2), 3.68 (1H, m, Ara H-3), 3.75 (1H, m, Ara H-4), 3.50 (1H, m, Ara H-5a), 3.85 (1H, m, Ara H-5b), 5.17 (1H, d, J = 1.2, Rha H-1), 4.06 (1H, dd, J = 1.6, 2.4, Rha H-2), 3.81 (1H, dd, J = 2.4, 9.2, Rha H-3), 3.47 (1H, t, J = 9.6, Rha H-4), 3.75 (1H, m, Rha H-5), 1.23 (1H, d, J = 6.0, Rha H-6), 4.70 (1H, d, J = 7.0, outer Ara H-1), 5.01 (1H, t, J = 7.2, outer Ara H-2), 3.90 (1H, dd, J = 3.6, 9.2, outer Ara H-3), 5.05 (1H, m, outer Ara H-4), 3.79 (1H, m, outer Ara H-5a), 3.96 (1H, dd, J = 2.4, 12.8, outer Ara H-5b), 2.12 (3H, s, OAc), 2.17 (3H, s, OAc). ^{13}C NMR (100 MHz, CD_3OD): 39.6 (C-1), 26.5 (C-2), 82.4 (C-3), 43.9 (C-4), 48.2 (C-5), 18.8 (C-6), 33.4 (C-7), 40.5 (C-8), 49.6 (C-9), 37.6 (C-10), 24.5 (C-11), 123.6 (C-12), 145.3 (C-13), 42.9 (C-14), 28.8 (C-15), 23.9 (C-16), 47.6 (C-17), 42.7 (C-18), 47.2 (C-19), 31.5 (C-20), 34.8 (C-21), 33.8 (C-22), 64.1 (C-23), 13.7 (C-24), 16.3 (C-25), 17.7 (C-26), 26.4 (C-27), 181.8 (C-28), 33.5 (C-29), 24.0 (C-30), 104.3 (Ara C-1), 76.4 (Ara C-2), 73.8 (Ara C-3), 69.3 (Ara C-4), 65.0 (Ara C-5), 101.3 (Rha C-1), 71.6 (Rha C-2), 81.3 (Rha C-3), 72.6 (Rha C-4), 70.2 (Rha C-5), 18.8 (Rha C-6), 103.9 (outer Ara C-1), 73.8 (outer Ara C-2), 70.1 (outer Ara C-3), 72.3 (outer Ara C-4), 64.1 (outer Ara C-5), 20.9, 21.1, 172.3, 172.5 (OAc × 2).

Hederagenin 3-*O*-(3,4-*O*-Di-acetyl-α-L-arabinopyranoside-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside (2). White amorphous powder, $[\alpha]^{24}_D$ +60.0° (*c* 0.2, MeOH). IR ν_{max} (KBr) 3450, 2942, 1730 cm^{-1}. FABMS *m*/*z*: 989 $[M + Na]^+$, molecular formula $C_{50}H_{78}O_{18}$, (identical with the literature (*13*)).

Hederagenin 3-*O*-(3-*O*-Acetyl-β-D-xylopyranosyl)−(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside (3). White amorphous powder, $[\alpha]^{24}_D$ +11.0° (*c* 0.2, MeOH). IR ν_{max} (KBr) 3401, 2930, 1727, 1693 cm^{-1}. FABMS *m*/*z*: 947 $[M + Na]^+$, molecular formula $C_{48}H_{76}O_{17}$, (identical with the literature (*14*)).

Hederagenin 3-*O*-(4-*O*-Acetyl-β-D-xylopyranosyl)−(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside (4). White amorphous powder, $[\alpha]^{24}_D$ +40° (*c* 0.18, MeOH). IR ν_{max} (KBr) 3416, 2942, 1725, 1694 cm^{-1}. FABMS *m*/*z*: 947 $[M + Na]^+$, molecular formula $C_{48}H_{76}O_{17}$, (identical with the literature (*15*)).

Hederagenin 3-*O*-(3,4-*O*-di-Acetyl-β-D-xylopyranosyl)−(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside (5). White amorphous powder, $[\alpha]^{24}_D$ +39° (*c* 0.2, MeOH). IR ν_{max} (KBr) 3397, 2939, 1694 cm^{-1}. FABMS *m*/*z*: 989 $[M + Na]^+$, molecular formula $C_{50}H_{78}O_{18}$, (identical with the literature (*15*)).

Hederagenin 3-*O*-β-D-Xylopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside (6). White amorphous powder, $[\alpha]^{24}_D$ +6.0° (*c* 0.2, MeOH), IR ν_{max} (KBr) 3398, 2938, 1690 cm^{-1}. FABMS *m*/*z*: 889 $[M + Na]^+$, molecular formula $C_{46}H_{74}O_{15}$, (identical with the literature (*14*)).

Hederagenin 3-*O*-α-L-Arabinopyranoside (7). White amorphous powder, $[\alpha]^{24}_D$ + 10.0° (*c* 0.2, MeOH), FABMS *m*/*z*: 627 $[M + Na]^+$, molecular formula $C_{35}H_{56}O_8$, (identical with the literature (*16*)).

Acid Hydrolysis of 1. Compound **1** (21 mg) was treated with 2 N methanolic HCl (2 mL) under reflux at 90 °C for 3 h. The mixture was extracted with CH_2Cl_2. The organic layer was evaporated to dryness to give hederagenin. The aqueous hydrolysate was neutralized with Na_2CO_3 and filtered. The dried filtrate was acetylated with pyridine−Ac_2O. GC-MS analysis showed peracetylrhamnose and peracetylarabinose (1:2), in comparison with reference compounds.

Molluscicidal Assay. Molluscicidal activity was tested against the golden apple snail, *P. canaliculata*, which was collected in June 2001 from streams and farms in Ping-Tung County, Taiwan. Before testing, adult (large snails of 20−25 mm in diameter used for field and soapnut crude extracts bioassay) and young snails (small snails of 6−8 mm in diameter used for soapnut fraction bioassay) were acclimatized to laboratory conditions for one week, by feeding them the leaves of sweet potato. Niclosamide 70% wettable powder (WP) (Dupont Co., Taipei, Taiwan) and metaldehyde 80% WP (Sinon Co., Taichung, Taiwan) were used as the control chemicals. Six different concentrations for each compound were prepared and three replicates (10 snails for each) were kept in a 1-L glass jar containing 300 mL of solution, according to the protocols of the World Health Organization (*17*). Snails were submerged in the respective test compound solution in a jar covered with cloth netting to prevent them from escaping. Control snails were treated with water. The snails were fed for 24, 48, and 72 h and checked for dead snails by probing with a stainless steel needle to detect their response (*17*). The molluscicidal effect was also compared by spraying soapnut extract powder (4 ppm), niclosamide (0.8 ppm), and metaldehyde (20 ppm), as well as water alone as a blank, in a young rice field. The dimension of the experiment at field was 3 m × 3 m covered with water about 5 cm in depth per treatment. Twenty snails per unit were placed into respective tested fields with three replications, and the number of dead snails was calculated after 72 h.

Statistical Procedure. Percentage mortality was corrected by Abbott's formula (*18*). Lethal concentration for 50% (LC_{50}) values for each treatment was obtained by Probit analysis, using the computer software of Finney (*19*).

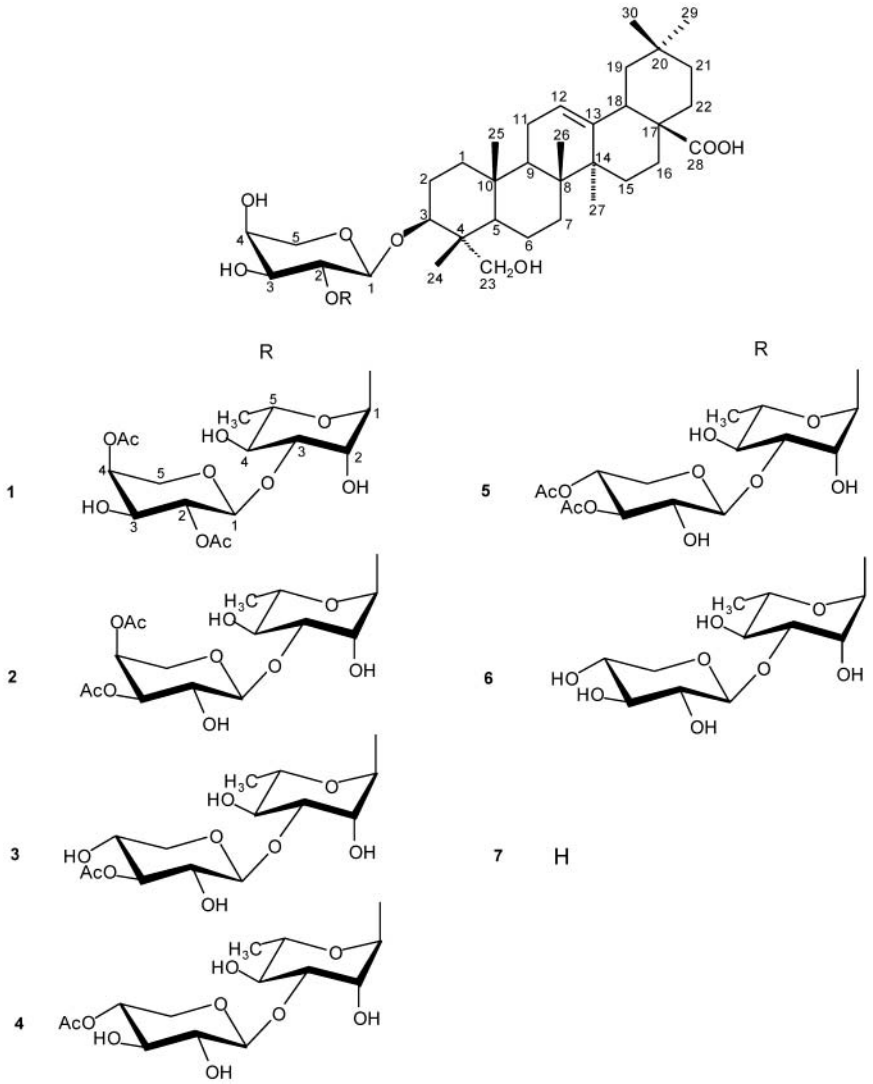

Figure 1. Saponins isolated from *S. mukorossi.*

Table 1. Molluscicidal Activity of the Saponin Extracts and Niclosamide 70% WP against the *Pomacea canaliculata* by Submersion Method

sample	exposure time (h)	LC_{50}[a] (ppm)
saponin extracts	24	85
saponin extracts	48	22
saponin extracts	72	17
niclosamide	24	0.2
niclosamide	48	AD[b]

[a] LC_{50} is the lethal concentration (ppm) that kills 50% of the snails. [b] The snails exposed to niclosamide were all dead after 48 h.

RESULTS AND DISCUSSION

The preliminary bioassay revealed that the soapnut exhibited molluscicidal effects against the golden apple snails in a jar with LC_{50} values of 85, 22, and 17 ppm after treating 24, 48 and 72 h, respectively (**Table 1**). The soapnut showed the time dependent molluscicidal property. For the security limitation, the usage of two commercialized pesticides, nicolsamide (70% WP) and metaldehyde (80% WP), were expressed as 0.4 and 4.55 kg/ha (0.8 and 20 ppm for 9 m^2), respectively, in accordance with the official guideline *Plant protection manual* edited by Council of Agriculture, Executive Yuan, R. O. C. (*20*). Different from the field assay, the laboratory assay for the soapnut extracts (4 ppm) revealed that its optimum concentration equals one-fifth those of noclosamide. Compared with niclosamide (0.8 ppm) and metaldehyde (20 ppm), which had 85 and 40% mortality, respectively, the soapnut extracts (4 ppm) displayed 62% mortality to golden apple snails in a rice field (**Table 2**). These promising results prompted us to further purify bioactive constituents from the plant. Thus, the MeOH extract of soapnut was repeatedly chromatographed on columns of Diaion HP-20 eluting with H_2O−MeOH by a successive

Table 2. Molluscicidal Activity of the Saponin Extracts, Metaldehyde, and Niclosamide against *P. canaliculata* in Paddy Field after 72 h Treatment

	number of dead snails				
compound	I	II	III	means ± S.D.[a]	mortality (%)
niclosamide (70% WP)	19/20	16/20	16/20	17.00 ± 1.73 d	85
metaldehyde (80% WP)	8/20	7/20	9/20	8.00 ± 1.00 c	40
soapnut extracts powder	11/20	12/20	14/20	12.33 ± 1.53 b	62
control (H2O)	0/20	0/20	0/20	0	0

[a] Means in a column followed by the same letter are not significantly different by Duncan's multiple range tests at the 5% level.

gradient system. All the eluting fractions were monitored for molluscicidal activity to furnish the bioactive fractions. The most active fraction (100% MeOH) caused 100% mortality at 50 ppm, which was further purified by HPLC to yield compounds **1**−**7** (**Figure 1**).

The high-resolution FABMS of compound **1** showed the molecular formula to be $C_{50}H_{78}O_{18}$. Its IR spectrum showed absorptions at 1690 (C═O of COOH) and 3450 (OH) cm^{-1}. The ^{13}C NMR spectrum suggested that **1** had a triterpene aglycone and three sugar moieties. In addition to the signals of sugar moieties, the presence of six tertiary methyls, eleven methylenes, five methines, and seven quaternary carbon signals in the ^1H-, ^{13}C NMR and DEPT spectra indicated that the aglycone was a hederagenin-type triterpenoid (*10*). Compound **1** possessed a single olefinic proton at δ_H 5.25 (1H, br s, H-12) and two olefinic carbons at δ_C 123.6 (C-12) and 145.3 (C-13). In addition, hydroxyl-methylene protons (H-23a,b) at δ_H 3.34 and 3.51, one methylene carbon at δ_C 64.1 (C-23), and one carbonyl carbon at δ_C 181.8 (C-28) were observed. The chemical shifts of δ_C 82.4 and δ_H 3.63 predicted that one of the sugar moieties was attached to C-3 of the aglycone. Together with NOESY data, the correlation of Hax-3 with H-23a,b and H-5 indicated that the hydroxyl at C-3 should have a β-configuration.

Acid hydrolysis of **1** afforded the aglycon hederagenin, which was identified by comparing its ^1H- and ^{13}C NMR data with published values (*16*). The final products, L-arabinose and L-rhamnose were also identified by GC-MS analysis.

From the HMQC spectrum, three anomeric protons at δ 4.53 (1H, d, $J = 6.0$ Hz, α-arabinopyranosyl), 5.17 (1H, d, $J = 1.2$ Hz, α-rhamnopyranosyl), and 4.70 (1H, d, $J = 7.0$ Hz, α-arabinopyranosyl) were correlated with ^{13}C NMR signals at δ 104.3, 101.3, and 103.9, respectively, that supported the presence of three sugar residues. Most of the protons and carbons of each sugar unit were assigned on the basis of extensive NMR experiments such as ^1H COSY, TOCSY, HMQC, and HMBC. The TOCSY spectrum of **1** showed that the proton at δ 4.53 (Ara-H1) was coupled to the signals at δ 3.69 (Ara-H2), 3.68 (Ara-H3), 3.75 (Ara-H4), 3.50 (Ara-H5a), and 3.85 (Ara-H5b). Furthermore, the protons at δ 5.17 (Rha-H1) and δ 4.70 (Ara-H1) were correlated, sequentially, with the other protons of rhammose and arabinose, respectively.

Two proton signals at δ 2.12 and 2.17, together with carbon signals at δ 21.1, 20.9, 172.3, and 172.5 indicated the presence of two acetoxyl groups. The HMBC spectrum further confirmed two acetyl groups from the correlations between δ_H 5.01 (Ara-2) and δ_C 172.5 and δ_H 5.05 (Ara-4) and δ_C 172.3. Thus, the structure of **1** was elucidated as hederagenin 3-*O*-(2,4-*O*-diacetyl-α-L-arabinopyranoside)−(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside.

Compounds **2**−**7** were identified as known saponins by analyses of their spectroscopic data (FAB-MS, ^1H and ^{13}C NMR, and 2D spectra) and comparison with literature values (*13*−*16*).

Compounds **1**−**7** were subjected to molluscicidal assay. The bioassay data showed that **2**−**5** had 100% mortality, **1** and **6** had 90% mortality, and **7** had 70% mortality, at a concentration of 10 ppm against *P. canaliculata* by submersion method after 24 h treatment. These results, showed that both of the isolated saponins and the crude soapnut extract possess molluscicidal activity against the apple snails. Furthermore, the activities of hederagenins with three sugar moieties (**1**−**6**) are better than those of triterpene with one sugar (**7**) are noted. In conclusion, further studies should be undertaken to develop a natural molluscicide, not only pure saponins but also the crude extract of *S. mukorossi*.

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