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Triterpenic saponins as regulator of plant growth

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Summary

To investigate the plant growth regulatory effect of saponins, two bisdesmosidic triterpenic saponins with hederagenin and 16-α-hydroxy protobassic acid as an aglycon were isolated from *Sapindus mukorossi* Gaertn (Family: Sapindeae) and *Diploknema butyracea* (Family: Sapotaceae). Two triterpenoids were applied on the rice and maize seeds for germination and growth regulation tests. We concluded the following. 16-α-hydroxy protobassic acid glycosides had a growth promoting activity in maize and rice. The effect was more prominent in maize than in rice. Whereas, hederagenin glycoside had a slight growth inhibitory effect on rice. The saponin showed growth promoting activity in maize below 250 μg ml^{-1}. In general, hederagenin glycoside showed root growth inhibiting effect in rice and 16-α-hydroxy protobassic acid glycosides had promotive effects on the entire plant of rice and maize at low concentration. These active compounds from *D. butyracea* could be further exploited as potential plant growth regulators in agricultural applications in the future.

Introduction

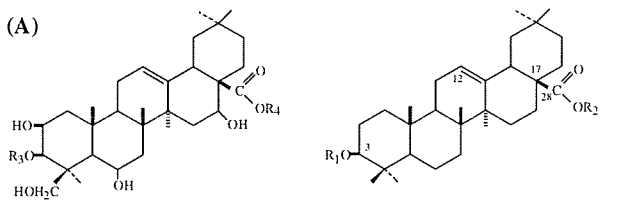
Saponin-triterpenoid glycosides are one of the most interesting groups of secondary plant metabolites. The saponins are a group of plant glycosides in which hydrophilic sugars are attached to a lipophilic steroid or triterpenoid moiety. The basic structural molecule that is found in largest number of plants is the pentacyclic triterpene of oleanane type. The most common triterpene of this type is oleanolic acid (Fig. 1), which occurs both in a free and more often as glycoside or glycoside ester. Its glycosides differ in the structure of carbohydrate moieties, containing sometimes up to 10 monosaccharide units, often in the form of branched chains. Some of them are mono- or bidesmosides, i.e. they contain one sugar chain usually present at C-3 hydroxyl group and the other attached to C-28 carboxyl group of oleanolic acid. Oleanolic acid and its derivatives were discovered in at least 120 species in the 6 subclasses of the Magnoliopsydia class (LIU, 1995) and in only one species of the Liliopsida class (*Crocus sativa*) (BISSET and WICHTL, 2001).

They are widely distributed in higher plants, and it is well known that they show various biological activities such as antimicrobial, antitermitic, molluscicidal, insect growth regulatory and anti-human immunodeficiency virus-1 (HIV-1) protease (HARBORNE et al., 1999; MARSTON and HOSETTMAN, 1987; SAHA et al., 2010). Recently, the relations between the chemical structures of saponins and their biological activities have been examined using isolated or synthesized saponins.

Saponins are usually found with some concentration in roots, foliage, fruit and seeds (HARBORNE, 1999; KITAGAWA et al., 1988). It has been reported that allelopathic compounds, which show stimulating or inhibiting activity for growth of other plant species, are exuded from seeds of various plant species into the environment (KUSHIMA et al., 1998). These facts prompted us to study the plant growth regulation effects of saponins. Limited reports are available on the plant growth regulation effects of saponin, whereas literature is available on the plant growth regulation effects of phenolic compounds. (OHARA and OHIRA, 2003; ISHII et al., 2007).

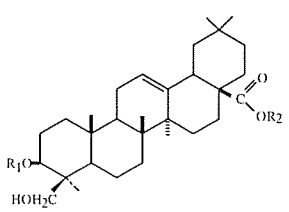
We isolated and characterized bisdesmosidic saponins with 16-α-hydroxyl protobassic acid and hederagenin as an aglycon, and these triterpenoid saponins were submitted to the germination and plant growth regulation assays using rice and maize seeds.

(A)



	R_1	R_2	R_3	R_4
Oleanolic acid	H	H		
16-hydroxyprotobassic acid			H	H
MI-I			Glc-Glc	Ara-Xyl-Ara
MI-III			Glc-Glc-Glc	Ara-Xyl-Ara-Api

(B)



	R_1	R_2
Hederagenin	H	H
SM-I	Xyl(OAc)-Ara-Rha	Glc-Glc-Rha

Fig. 1: Structure of (A) *D. indica* and (B) *S. mukorossi* saponin(s) and sapogenin

Materials and methods

Plant material

Sapindus mukorossi seeds were procured? from Palampur, Himachal Pradesh, India. *Diploknema butyracea* seeds were procured from Bihar, India.

Spectrometry

^{1}H-NMR spectra of compounds were recorded on a Brucker (300 MHz) spectrometer. Deuterio-methanol (CD_3OD) was used as solvent and tetramethyl silane (TMS) was used as standard. Electro-

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spray mass spectroscopy was carried out on AB/MDS Sciex-API 2000 triple quadrupole mass spectrometer using TurboSpray source.

Isolation of triterpenic saponins

Ground plant material (1kg; seed kernel of *D. butyracea* and fruit pericarp of *Sapindus mukorossi*) was defatted with hexane for complete removal of oil/fatty substances and the de-oiled material was then extracted with methanol (3 × 1 L). The combined methanol extract was concentrated under vacuum at 45 °C to viscous syrup and it was partitioned between water and n-butanol. The combined butanol extract was then concentrated to viscous liquid at < 70 °C under vacuum. The viscous concentrate then precipitated in acetone to obtain saponin mixture.

Separation of triterpenic saponins

To find out the number of saponins in both the saponin mixture, analytical reverse phase high performance liquid chromatography was run. HPLC was performed on Waters HPLC system fitted with LiChrosphere R 100 RP-18e column (5µm) procured from Merck KgaA, Darmstadt, Germany or on Novapack phenyl 16R, 4µm particle size, 3.9 mm × 150 mm ID cartridge column containing dimethylphenyl propyl silyl bonded amorphous silica. A 20µl volume of sample was injected via a Rheodyne injector (20µl loop) for a run time of 15 minutes. 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* saponins 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 butyracea saponin MI-I

^{1}H NMR (CD_3OD) δ: 0.901, 0.939, 1.059, 1.13, 1.164 and 1.226

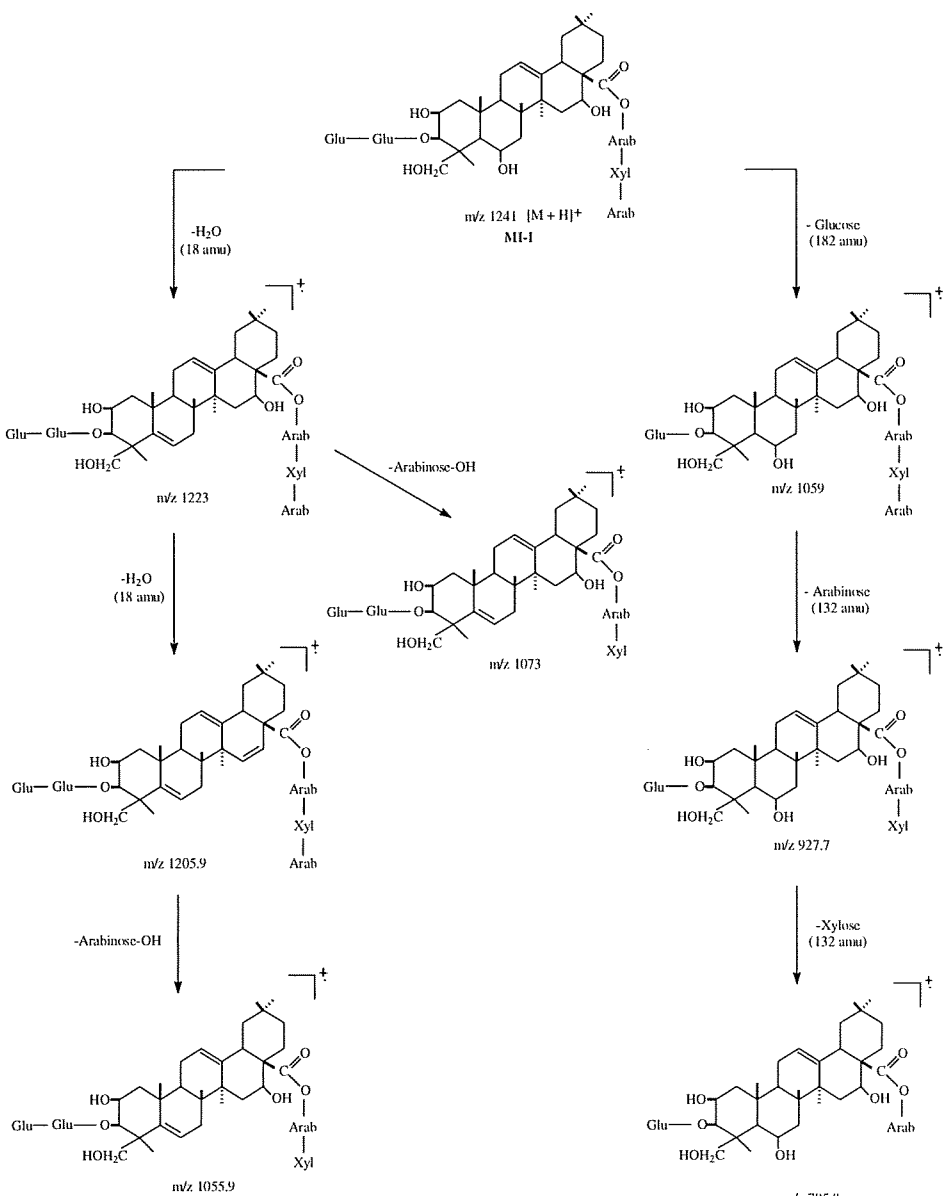

Fig. 2: Mass fragmentation pattern of *Diploknema butyracea* saponin MI-I

(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 $[M - 182-132-132]^+$.

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 (m.p. 235-238 °C, R_f 0.65 in $CHCl_3$: MeOH: H_2O (65:35:10).

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 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 $C_{57}H_{92}O_{29}$ (Fig. 1). The structure was confirmed by its mass fragmentation pattern (Fig. 2). On the basis of spectral studies, the structure of *Diploknema butyracea* saponin (MI-I) was assigned as 3-O- [β-D-glucopyranosyl-β-D-glucopyranosyl] – 16-α-hydroxyprotobassic acid-28-O-[arabinopyranosyl-glucopyranosyl-xylopyranosyl]-arabinopyranoside (Fig. 1).

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 $[M - 162-132]^+$.

On the basis of ^{1}H-NMR spectral data, the more polar *Diploknema* saponin (MI-III) was 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 olefinic proton attributable to H-12 proton at δ 5.63 and two hydroxymethyl protons at δ 3.34 and 3.75 located at C-23

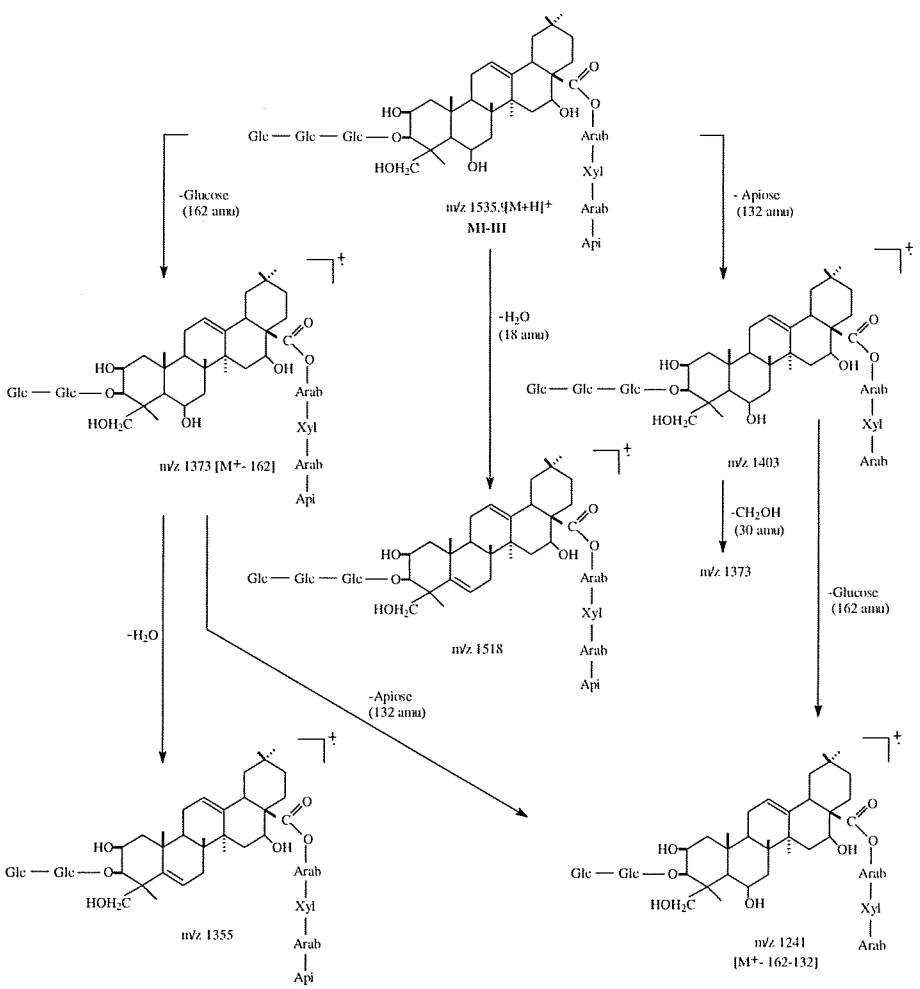

Fig. 3: Mass fragmentation pattern of *Diploknema butyracea* saponin MI-III

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 molecular ion peak at m/z 1535.9 corresponding to its molecular formula $C_{68}H_{111}O_{38}$ (Fig. 1). The characteristic fragmentation pattern confirmed the structure (Fig. 3). 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-[arabinopyranosyl-xylopyranosyl-arabinopyranosyl]-apiofuranoside (Fig. 1).

Sapindus mukorossi saponin, SM-I

^{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 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+rha, 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 glc-glc-rha fragment. M/z 223, 248, 205, and 187 (RetroDiels-Alder fragments).

Sapindus mukorossi 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. On hydrolysis with mineral acid it was completely hydrolysed 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 C-3 hydroxyl and C-28 carboxyl functions. The constitution of sugar moiety attached at C-3 hydroxyl appears to be the same as reported earlier (KIMATA et al., 1983). The sugar moiety located at C-28 position probably comprised of glucose, acid and arabinose.

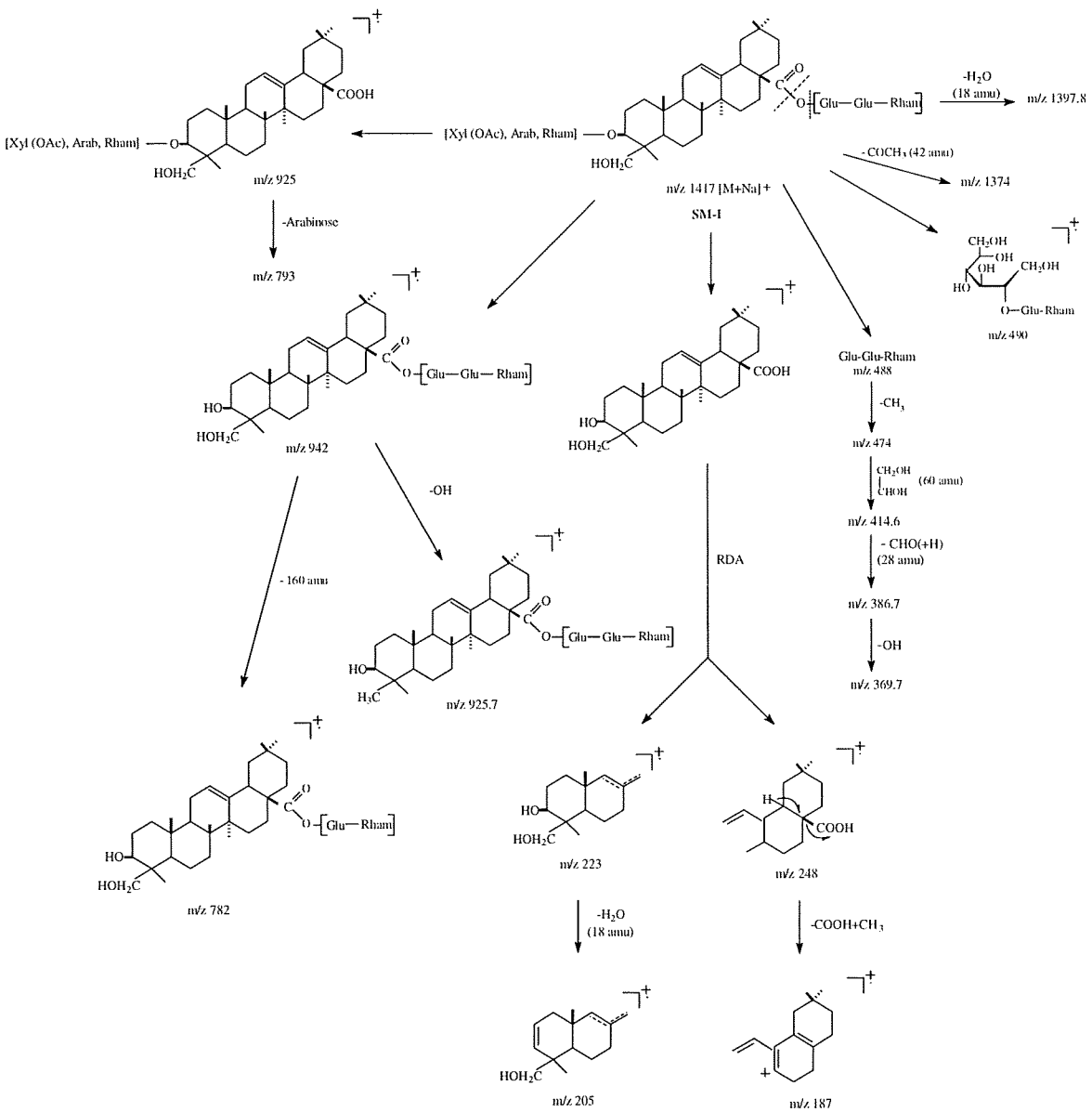

Fig. 4: Mass fragmentation pattern of *Sapindus mukorossi* saponin SM-I

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 moieties, 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 peaks emerged after fragmentation helped in structural confirmation (Fig. 4). The structure of the saponin was established as 3-O-[O-acetyl-β-D-xylopyranosyl-β-D-arabinopyranosyl-β-D-rhamnopyranosyl] hederagenin-28-O[β-D-glucopyranosyl-β-D-glucopyranosyl-β-D-rhamnopyranosyl] ester (Fig. 1).

Bioassay

Germination and initial growth tests were conducted using rice (*Oryza sativa*) (var. BTC) and maize (*Zea mays* L.) (var. Jaunpur local) as follows: nine seeds were placed on filter paper moistened with 2 ml of test solution in a Petri dish (15 mm × 90 mm diam.) and incubated at 25 °C for 7 days under light-dark cycles of 12:12 h. Petri dishes were sealed with Para film to ensure a closed-system model and to keep the environment moist. After 10 days of growth, the lengths of the shoots and roots were measured using a ruler, and then compared with those of the control.

The test saponins were dissolved in 0.2 % emulsified water. Control experiments were conducted in distilled water (without a test compound). The effects on shoot and root length were registered and expressed as a percentage of the control. All data are expressed as the mean. Statistical analysis of the bioassay data was performed by the DMRT/Student's t-test. The data were considered statistically significant when $P < 0.05$.

Results

Characterization of the active compounds

In our study, saponins are having C_{28} carboxylic groups with C_{12} olefinic double bond, in both the plants. Sapogenins were 16-α-hydroxy protobassic acid and hederagenin in *D. butyracea* and *S. mukorossi*, respectively. 16-α-hydroxy protobassic acid contains four more hydroxyl groups at C- 2,6,16, 24 positions of oleanane ring.

Saponin of *S. mukorossi* and *D. butyracea* contains one and two saponins, respectively. Single saponin of *S. mukorossi* was characterized as 3-O-[O-acetyl-β-D-xylopyranosyl-β-D-arabinopyranosyl-β-D-rhamnopyranosyl] hederagenin-28-O[β-D-glucopyranosyl-β-D-glucopyranosyl-β-D-rhamnopyranosyl] ester (SM-I). Two saponins of *D. butyracea* was characterized as 3-O-[β-D-glucopyarnosyl-β-D-glucopyranosyl]-16-α-hydroxyprotobassic acid-28-O-[arabinopyranosyl-glucopyranosyl-xylopyranosyl]-arabinopyranoside (MI-I) and 3-O-β-D-glucopyranosyl-glucopyranosyl-glucopyranosyl-16-α-hydroxyprotobassic acid-28-O-[arabinopyranosyl-xylopyranosyl-arabinopyranosyl]- apiofuranoside (MI-III).

As evident from their different molecular mass (MS), two saponins of *D. butyracea* reported herein, appear to be different from those namely Mi-saponin A and Mi-saponin B reported earlier from *Madhuca latifolia* (KITAGAWA et al., 1975), and butyroside A, B and other saponins reported from the seeds of *Madhuca butyracea* (NIGAM et al., 1992). While *M. latifolia* saponins are derived from the genin protobassic acid, those from *M. butyracea* are glycosides of 16-α-hydroxyprotobassic acid. Two *D. butyracea saponins* MI-I and MI-III also be the glycosides of 16-α-hydoxyprotobassic acid.

Biological activity of triterpenic saponins

Rice

To clarify the biological role of triterpenic saponins isolated from *D. butyracea* and *S. mukorossi*, and plant growth assay using rice and maize was conducted. Effect of *D. butyracea* and *S. mukorossi* saponins on the germination and growth of rice and maize seedlings is given in Tab. 1 and 2. Duncan's multiple range test (DMRT) was conducted to compare the values using SPSS software. Perusal of data revealed that growth (%) of rice coleoptile and root in rice seedlings increased with decrease in *Diploknema* saponin concentrates. At

Tab. 1: Germination and growth regulation effects of triterpenoid saponins on rice seeds.

Concentration	Per cent based on control		
(ppm)	Germination	Radicle	Hypocotyl
D. butyracea saponin			
2000	90	-17.6^a	0.9^a
1000	90	-15.4^a	1.1^a
500	100	-10.8^b	2.5^b
250	100	12.5^c	9.4^c
125	100	21.2^d	9.8^c
50	100	34.7^e	10.0^c
S. mukorossi saponin			
2000	90	-9.1^{bc}	8.9^a
1000	90	-7.5^{ab}	10.4^a
500	100	-5.5^a	38.7^d
250	100	-8.6^b	21.9^c
125	90	-9.8^c	17.5^b
50	90	-13.5^d	16.1^b

Values sharing the same letter are not significantly different (P<0.05)

Tab. 2: Germination and growth regulation effects of triterpenoid saponins on maize seeds.

Concentration	Per cent based on control		
(ppm)	Germination	Radicle	Hypocotyl
D. butyracea saponin			
2000	90	-28.1^a	66.8^a
1000	100	-17.6^b	67.9^a
500	100	30.2^c	76.9^b
250	100	129.8^f	93.8^c
125	100	111.8^e	115.4^d
50	100	101.5^d	131.0^e
S. mukorossi saponin			
2000	90	-58.2^a	30.2^a
1000	90	-10.4^b	45.1^b
500	100	35.2^e	74.1^c
250	100	33.3^{de}	122.8^d
125	100	24.1^d	78.0^e
50	90	18.1^c	70.6^c

Values sharing the same letter are not significantly different (P<0.05)

higher concentrations of 1000 ppm, the increase in rice coleoptile and root growth was 1.1 and -15.4% while at lowest concentrations of 50 ppm, coleoptile and root growth was found to be 10 and 34.7%. The application of *Sapindus* saponin on rice seed, however, gave somewhat different results. While at higher concentration of 1000 ppm, it inhibited 90% of the coleoptile growth and 7.5% of the root growth. Whereas, at lower concentrations, it recorded significant increase in coleoptile (shoot) growth (Tab. 1). The highest coleoptile growth (40%) recorded at 500 ppm decreased with further decrease in concentration. At 500 ppm concentration, root growth was stunted by only 5.6%, while at lower concentration of 50 ppm, the root growth was decreased to the extent of 13.8%. Thus at the optimum test concentrations of 500 ppm, *Diploknema* saponin recorded 39% increase in coleoptile growth, and marginal decrease 5.6% in the root length growth.

Maize

Effect of *Diploknema* and *Sapindus* saponins on growth of maize seedlings is given in Tab. 2. Perusal of data indicated that like in rice, the coleoptile length of maize seedlings after germination increased with decrease in application dose of *Diploknema* saponin concentrate. At higher concentration of 2000 ppm, it recorded 66.8% increase in shoot length, while at lowest concentration of 50 ppm it showed significantly higher increase (134%) in the shoot length. Roots on the other hand showed different pattern of growth. While at concentrations of 1000 ppm and higher, it recorded 12.2 to 29.8% decrease in root growth, the root length increased with decrease in concentration of the test compound until it reached 250 ppm when it showed maximum (130%) root growth. Decrease in test concentration level beyond 250 ppm led to decrease in the root growth.

Following treatment with *Sapindus* saponin concentrate, maize coleoptile length increased with decrease in concentration of the test saponin. At optimum concentration of 250 ppm, it recorded maximum shoot growth (123%). The shoot growth decreased with further decrease in test concentration. Similarly maize root growth was inhibited (58.2% and 10.4%) at higher concentration (2000 and 1000 ppm), and further decrease in concentration led in increase in the root growth. At lower concentrations, of 250, 125 and 50 ppm; 33.2, 24.1 and 18.1% increase in root length has observed (Tab. 2). Thus for optimum growth of maize seedling, 250 ppm concentration of *Sapindus* saponin was ideal for treating maize seeds before germination.

Discussion

The growth test with *O. sativa* and *Z. mays* revealed that both the saponins possessed some growth-regulating effect. Therefore we attempted to isolate and determine the structure of plant growth regulators and to evaluate their biological activities. Growth-promoting activity was shown by both the saponins on maize. Although both (*D. butyracea* and *S. mukorossi*) the group of saponins was previously isolated, no report documented its physiological activity. The comparative study of the plant growth-promoting activity of these saponins were investigated, and provided some information with structure-activity relationship.

There have been many investigations on structure-activity relations of the growth regulation effects of different glycosides. As for the growth inhibitory effects of phenolic glycosides, early works demonstrated that the glycosides were less effective than their aglycons (OHARA and OHIRA, 2003; STENLID, 1968). These studies suggest that the aglycon moiety plays a substantial role in the growth inhibitory activity of glycosides. That is, betulin glycosides showed stronger effects than betulin, and betulin glycosides with two to four glucose residues had the most inhibitory activity (OHARA and OHIRA, 2003). Study by ISHII et al. 2007 indicates that the glycoside moiety plays a minor role in the activity, and the ceramide moiety constitutes the essential part of the activity of plant growth rather than the glycoside moiety. Therefore, the inhibitory effects of triterpenoid saponins are thought to be attributed not to the property of their aglycons but the total structure is important for its activity. As chemical and physical properties of triterpenoid saponins vary with the sugar chain length, saponins with an adequate number of sugar residues would exhibit high activity.

Previous studies have reported that saponins possess various toxic properties (ANDERSSON et al., 1989; PROKOFEVA et al., 2003). HIRADATE et al. (1999) reported that a triterpenoid-type saponin, durantanin, isolated as an alleochemical from the leaves of *Duranta repens*, demonstrated plant-growth inhibitory activity against seedlings of *Brassica juncea*.

These results suggested that the saponins derived from *D. butyracea* and *S. mukorossi are* expected to be both of unique and practical. The results of the present study, which demonstrated the plant growth regulating activities of plant secondary metabolites from seed and fruit pericarp of two plants, suggest that these active natural products could be employed in ecofriendly agricultural applications of economy in consumption of chemical fertilizers and agrichemicals. However, on the basis of data presented here, it is difficult to explain the observed effects. Further investigation is required to fully elucidate the plant growth regulating activity of metabolites in maize.

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