Assay of snake venom phospholipase A_2 using scattering mode of a spectrofluorimeter

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When aggregated micelles of phospholipids are hydrolysed by phospholipase A_2 (PLA_2) in an aqueous dispersion, scattering from the solution is decreased. Hydrolysis of dimyristoyl phosphatidylcholine by PLA_2 from Russell's viper venom has been investigated using a spectrofluorimeter at 650 nm. The rate of decrease in scattering was linearly dependent with venom concentration, while the initial lag at the onset was inversely related to it. Similar dependency was observed with substrate concentration. The reaction was inhibited with venom preincubated with antivenom or withdrawal of Ca^{+2} by EGTA. Gas–liquid chromatography of the product showed liberation of myristic acid. The amount of fatty acid released by 1 mg of venom was found to be 3470 and 3680 nmol/min, using scattering mode and pH-stat titrimetric assay respectively, that indicated a good correlation between them. The sensitivity of detection by the scattering mode was double that of the titrimetric assay.

Keywords: Dimyristoyl phosphatidylcholine, light scattering, phospholipase A_2, Russell's viper venom, spectrofluorimeter.

PHOSPHOLIPASE A_2 (PLA_2) is a class of enzymes that hydrolyses the fatty acid ester bonds at position 2 of 1,2 diacyl-*Sn* phosphoglycerides to lysophospholipids and fatty acids[1,2]. Since the solubility of phospholipids in aqueous phase is exceedingly low, the substrate forms micelles and the enzyme interacts at the water–lipid interface. PLA_2 has much higher affinity towards aggregated substrates compared to disperse monomeric ones[3]. X-ray crystallographic structure of this class of enzymes is known at high resolution[4]. PLA_2 requires Ca^{2+} as an essential catalytic cofactor to promote binding to the substrate and for the chemical step of lipolysis[1,5]. Though PLA_2 is required for such essential processes like phospholipid metabolism, signal transudation and other cellular functions, a large number of patho-physiological events are associated with PLA_2, particularly from venom[6].

PLA_2 activities are measured by a large number of procedures[7–11]. In most cases, PLA_2 is assayed by pH-stat, where the released fatty acid is quantified by standard alkali at constant pH. Alternately, changes in the chemical or spectroscopic properties of the substrate or the product are followed by titrimetric or radiometric assay coupled with HPLC or TLC, NMR, polarography, spectrophotometry, fluorimetry, ESR, etc. Sometimes the physical state of the substrate or the product is also monitored, e.g., turbidometric assay. Unfortunately, there is no single procedure that could be applied universally to PLA_2 assay with confidence.

Snake-biting being largely a tropical incidence, several laboratories involved in snake venom studies are situated in those regions. It is a hard reality that many of these laboratories are devoid of adequate facilities, including radiometer pH-stat. In comparison, spectrofluorimeters are common. This led us to explore the possibility of PLA_2 assay using the scattering mode of a spectrofluorimeter, avoiding time-consuming procurement of expensive fluorescence labelled reagents. When the excitation and emission wavelengths of a spectrofluorimeter are the same and test samples are free from 'inner filter effect', the emission 90° (ref. 12) measures Raleigh's scattering at 90°. There are stray reports of application of 90° scattering at near UV as supportive evidences for PLA_2 kinetic studies[11]. Here we report hydrolysis of dimyristoyl phosphatidylcholine (DM-PC) by Russell's viper venom (RVV), which is a rich source of PLA_2, as monitored by this scattering mode.

RVV was provided by Dipak Mitra, a licensed trophy of Calcutta Snake Park, as desiccated yellowish shining crystals containing approximately 75–80% of protein (w/w). RVV (2 mg/ml) was dissolved in 20 mM K-phosphate, pH 7.4, left overnight at 4°C and centrifuged for 10 min at 6000 rpm in a micro centrifuge to discard cell debris. The whitish clear supernatant was used as the stock. Polyvalent aqueous suspension of antivenom was a product of M/s Haffkin Bio-pharmaceutical Corporation Ltd, Mumbai, India. Its neutralization capacity was 0.6 mg of Indian cobra venom and RVV, and 0.45 mg of common krait venom and saw-scaled viper venom/ml. Porcine pancreatic PLA_2, *Echis carinatus* venom, *Crotalus atrox* venom, phosphatidylcholine, and all its derivatives were from Sigma, USA. *Naja kauthia* was taken from Calcutta Snake Park. Absolute ethanol was from Bengal Chemicals and Pharmaceuticals Ltd, Kolkata. About 10 mg of DM-PC, stored desiccated at –20°C, was weighed gravimetrically under anhydrous condition and dissolved in 50 µl absolute ethanol. It was diluted to 1 ml by water to yield a uniform suspension used as the stock[13].

Semi quantitative PLA_2 assay was done with egg yolk as substrate[7,14]. A suspension of 20 ml was made with 9 ml chicken egg yolk, 2.51 ml 34.18 mM NaCl, 1.49 ml 1.34 mM EDTA, 4.44 ml 6.8 mM $CaCl_2$, 2.0 ml 50 mM Tris-HCl, pH 7.5 and 0.56 ml 14.52 mM saline. Venom samples were added to 2 ml egg yolk suspension, mixed well and incubated at 37°C for 1 h. Incubates were placed on a boiling water bath and the time required to coagulate was noted. Pancreatic PLA_2 and 14.52 mM saline served as positive and negative controls respectively.

Scattering measurements were done using a Hitachi F 4500 spectrofluorimeter attached with a constant temperature circulating water bath (Polyscience, USA) and a 3 ml quartz

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cuvette. Unless mentioned, excitation and emission wavelengths and slit widths were set at 650 nm and 2.5 nm respectively. General precautions for laboratory practice and washing of glassware for scattering measurements were followed[15]. For scattering measurement, the lowest recording during 5 min was considered. Since the lipid suspension in the cuvette survived gravitational settling, no magnetic flea was applied. While calculating kinetic parameters, 100% completion of the reaction was assumed from the drop of scattering that remained constant for at least 10 min.

RVV–PLA_2 was also assayed by measuring decrease in turbidity of DM-PC micelle in 20 mM K-phosphate, pH 7.4 containing 0.2 mM $CaCl_2$ at 650 nm in a spectrophotometer[10], where none of the reaction components have absorption. Rate of decrease of absorption per minute for 5 min was noted. The released fatty acids formed after hydrolysis of lipids were esterified and identified by gas–liquid chromatography (GLC) using a 10% DEGS glass 6 mt Chromatopak column at a nitrogen flow of 42 ml/min[16]. Myristic acid, palmitic acid, stearic acid, arachidonic acid and behenic acid served as reference fatty acids.

Release of fatty acids from phospholipids after PLA_2 reaction was estimated by a pH-stat model 751 GPD Trtrino (Metrohm) at 25°C. Typically, in a 1 ml reaction mixture containing 0.29 µmol DM-PC in 5 mM Tris-HCl, pH 7.5 in the presence of 0.2 mM $CaCl_2$, 5–30 µg RVV was added and the constancy of pH was maintained after titration with 0.012 (N) NaOH.

The physical state of the lipid substrates was determined by transmission electron microscopy (TEM) using standard protocols. In short, a sample volume of 10 µl of 5 mg/ml was placed on a plastic-coated, 400-mesh grid for 1 min and the unattached substrate was removed using blotting paper. To stain the adhered particles, they were treated with 1% phosphotungstic acid for 20 s and excess reagent was removed using blotting paper. The grid was dried for 15 min and the sample was served under TEM (JEOL 100 CX, 20,000X) at an opening voltage of 60 kV.

Absorption spectra of RVV and the substrates were scanned between 200 and 800 nm with Analytical Jena Specord 200 (Germany) spectrophotometer. The absorption spectra of 10 mg/ml of RVV in 20 mM K-phosphate, pH 7.4, between 240–800 nm showed a peak at 280.2 nm characteristics of proteins, but no absorption was detected above 320 nm (<0.001). DM-PC (10 mg/ml) in spectral-grade ethanol showed absorption of <0.001 between 400 and 800 nm. This confirmed the absence of 'inner filter effect' in these experiments[12]. Protein estimation was done with Bio-Rad Protein Assay Reagent (Catalog no. 10044), having BSA as reference.

A uniform distribution of the size of DM-PC in the aqueous phase was ensured after repeated dispersion of the stock in water with 100-fold dilution in 20 mM K-phosphate, pH 7.4, followed by measuring turbidity (spectrophotometrically) or scattering (spectrofluorimetrically) at 650 nm. It revealed variation by ± 5% (n = 5). Once dispersed in the buffer, scattering remained constant for at least 30 min justifying avoidance of magnetic flea in the cuvette. A linear dependence of scattering intensity within 400–9000 (arbitrary units) by 0–40 mM of DM-PC was observed (R^2 = 0.9841, where R is the regression coefficient; result not shown). Also, scattering intensity of 0–40 mM of DM-PC was found to be inversely related with the fourth power of wavelength between 400 and 700 nm as per classical Raleigh's equation[17]. This excluded association–dissociation-like phenomenon of lipid particles. Further, when RVV (0.1 mg/ml) was incubated with buffer at 25°C, no change in scattering intensity was observed for 1 h, indicating absence of proteolysis to alter scattering intensity.

The reactivity of PLA_2 is highly dependent on the physical state of its substrate[3]. Therefore, the state of DM-PC under conditions of its hydrolysis was determined by TEM. It showed that the particles formed aggregated micelles having size in the range of 265–440 nm. Electron micrograph of two such micelles has been shown in Figure 1, which corresponded to 400 and 440 nm.

PLA_2s are sub-classified depending on molecular weight, Ca^{+2} ion dependency and mechanism of action[4]. Also the size and polarity of the head group and length of fatty acyl chain at S_N-1 or S_N-2 position are important factors in determining the overall catalytic turnover at the interface[13,18]. Since RVV is known to contain multiple species of PLA_2, suitability of several phospholipids as substrate was tested on the basis of change in scattering intensity. The rate of hydrolysis expressed as change of scattering intensity/s using 0.29 µmol phospholipids and 5–50 µg RVV was as follows: DM-PC, 0.80; phosphatidylcholine, 0.02; phosphatidyl serine, <0.1; phosphatidyl ethanolamine, <0.10 and phosphatidylcholine dipalmitoyl, <0.10. Thus among the five phospholipids tested, DM-PC was the best choice for further studies.

When DM-PC was incubated with RVV, its scattering intensity at 650 nm was reduced by approximately 2.5-fold (Figure 2 *a*). The reduced scattering remained stable for 10–15 min. The rate of hydrolysis was measured from the time zone where the change of scattering intensity with time

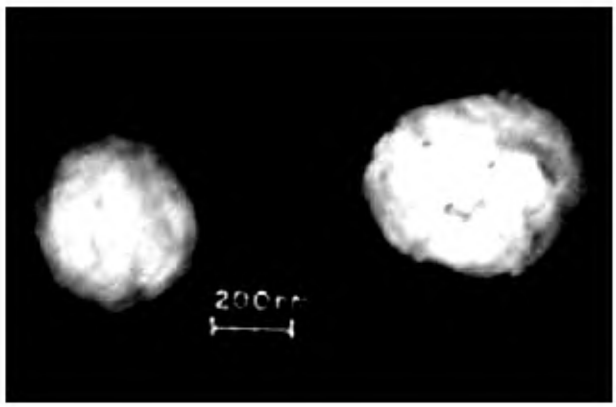

Figure 1. Electron micrograph of DM-PC prepared under conditions as they were applied in scattering assay (x = 40,000).

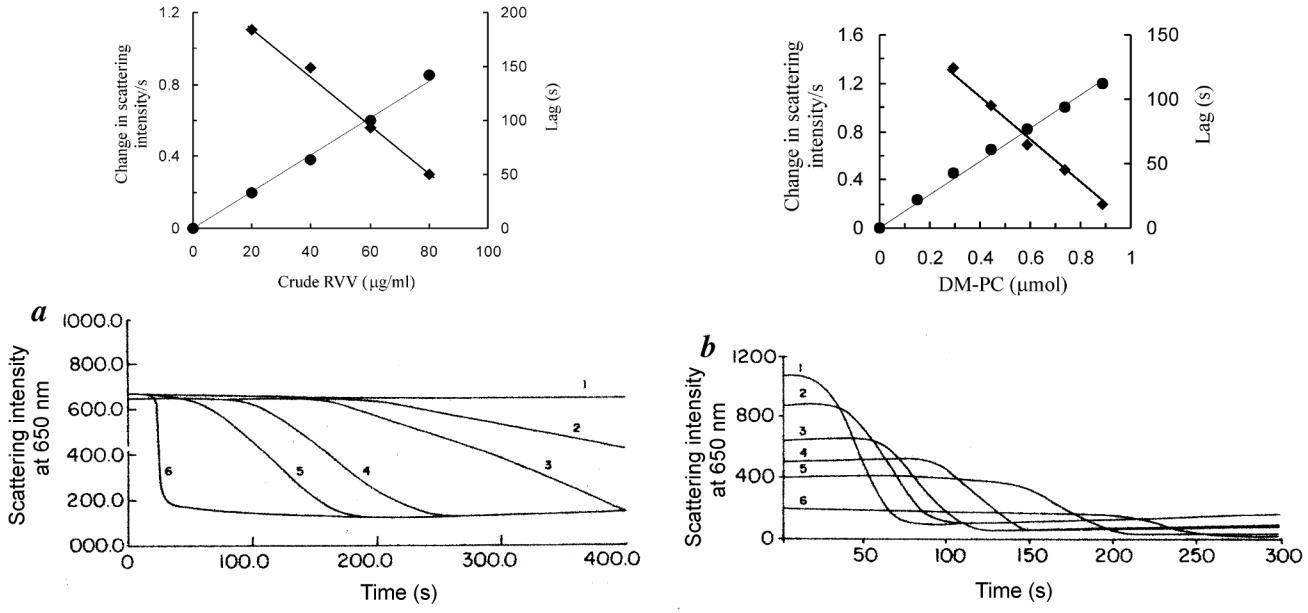

Figure 2. PLA_2 activity of RVV as measured by change in scattering intensity of the substrate DM-PC. ***a***, Time course of change of scattering intensity of 0.59 μmol DM-PC at 650 nm with (1) 0; (2) 10; (3) 20; (4) 30; (5) 40 and (6) 50 μg/ml RVV in 20 mM K-phosphate, pH 7.4 at 25°C. (Inset) Dependence of change in scattering intensity/s (●—●) and duration of initial lag (◆—◆) of hydrolysis reactions with venom concentration. The former was measured from linear portion of the reaction profiles. The latter was calculated by extrapolating linear portion of the reaction profiles to the initial horizontal portion of the curves. ***b***, Time course of change in scattering intensity at 650 nm of (1) 0.885, (2) 0.738, (3) 0.590, (4) 0.443, (5) 0.295 and (6) 0.148 μmol of DM-PC with 40 μg/ml RVV. (Inset) Dependence of change in scattering intensity/s (●—●) and duration of initial lag (◆—◆) of hydrolysis reactions with venom concentration. Conditions of reaction and methods for calculation of parameters are the same as (***a***).

was linear. The rates were found to be linearly dependent on RVV concentration between 5 and 80 μg/ml holding DM-PC concentration at 0.59 μmol ($R^2 = 0.9961$). Decrease in scattering was initiated after experiencing a lag. The duration of lag (50–200 s) was also found to follow an inverse linear relation with RVV concentration ($R^2 = 0.9930$; Figure 2 *a*, inset). In all cases, decrease in scattering was associated with drop in turbidity from 0.351 ± 0.025 to 0.042 ± 0.004 as measured spectophotometrically at 650 nm; thus indicating reduction in size of the particles.

Similar experiments were performed with DM-PC between 0.147 and 0.885 μmol, holding RVV concentration at 40 μg/ml (Figure 2 *b*). In each set, a linear change in scattering intensity was observed at the middle of the reaction time zone. Further, the rate of change in scattering intensity was found to be linearly dependent on substrate concentration ($R^2 = 0.9949$), while the kinetic lag observed at the onset of the reaction was found to follow inverse linear relation on it ($R^2 = 0.9952$; Figure 2 *b*, inset).

Crude RVV, dialysed overnight against buffer failed to abolish the kinetic lag. The lag appeared to be associated with the reaction mechanism and was not an artifact. Supplementing the assay buffer with 0.1 and 0.2 M salts inhibited the rate by 32 and 75% respectively. Similarly, supplementing by 5, 7.5 and 10% methanol inhibited the reaction rate by 46, 59 and 66% respectively. Thus alteration of solvent polarity negatively affected the reaction, similar to earlier reports[18].

Hydrolysis of DM-PC by RVV leading to destabilization of micelles was completely prevented when the venom was heat-treated at 100°C for 10 min or after treatment with antivenom (two-fold excess w/w, 30 min at 25°C). In the presence of EDTA or EGTA (10 mM), hydrolysis of DM-PC by RVV was similarly arrested (result not shown). This observation indicated the necessity of Ca^{2+} ions for PLA_2 activity.

Semi-quantitative assay of PLA_2 using 5–50 μg/ml venom has been done with egg yolk as substrate, to correlate coagulation time with scattering assay. The observed coagulation time varied from 30 to 490 s and was found to be linearly dependent on venom concentration ($R^2 = 0.9906$). With higher concentration of venom at 100 μg/ml, coagulation time was more than 30 min. Coagulation time was similar to the control without venom, i.e. 30 ± 2 s after heating RVV at 100°C for 5 min or preincubation with two-fold molar excess of antivenom at 25°C for 10 min (Table 1).

The GLC profiles of DM-PC, RVV and RVV treated DM-PC are shown in Figure 3. While DM-PC yields only one major peak of retention time (R_t) = 2.54 min, RVV yields a number of volatile products, including one of the said retention time. RVV-treated DM-PC yielded an additional peak of R_t = 1.82 min, which corresponded closely to the myristic acid derivative of R_t = 1.78 min. RVV-treated DM-PC, being a multi-components system, possibly suffered minor alteration of retention time of myristic acid derivative by 0.04 min compared to the reference.

Titrimetric assay for PLA_2 is one of the standard protocols, where the released fatty acids are estimated against standard alkali. PLA_2 assay by scattering mode as described was correlated with pH-stat (titrimetric) method. In pH-stat method, PLA_2 activity of RVV was found to be linearly dependent with the enzyme concentration between 5 and 30 μg (R^2 = 0.9748). The amount of fatty acid released by 1 mg venom was found to be 3470 and 3680 nmol/min using scattering mode and pH-stat titrimetric assay respectively. The comparable figures thus validate the scattering mode with respect to titrimetric assay.

So far results have been presented with crude RVV as a source of PLA_2. To check whether the same scattering mode of assay is sensitive to pure enzymes, the same experiment (Figure 2) was done using porcine pancreatic PLA_2 in the presence of 37.19 μM Na-taurocholate. Pancreatic PLA_2 activity was also found to be linearly dependent on enzyme concentration and inversely related to kinetic lag at the initiation of reaction, as observed in case of crude RVV–PLA_2 (Figure 4).

Table 1. PLA_2 activity of RVV under modified conditions as measured by scattering mode and egg yolk coagulation time

	Coagulation time (s)	Rate of hydrolysis*
Control	30	0.0
RVV	1800	0.2
EGTA/EDTA-incubated RVV	30	0.0
Heat-denatured RVV	30	0.0
AVS-treated RVV	30	0.0

*Rate of hydrolysis was expressed as change in scattering intensity/s. Control refers to a reaction without RVV. In each experiment, 20 μg/ml RVV and 200 μg/ml DM-PC were used.

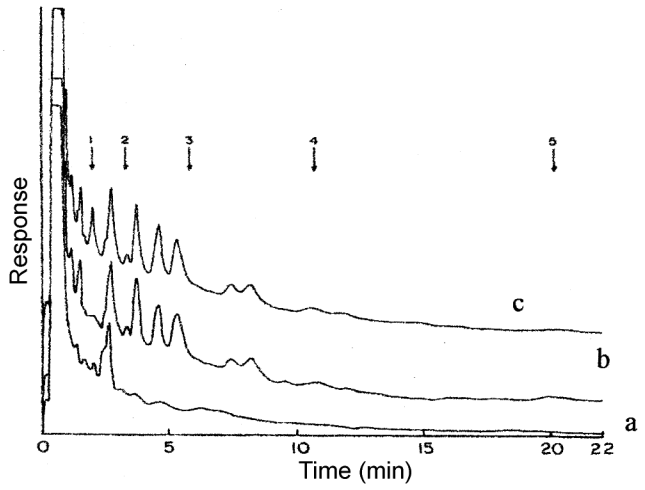

Figure 3. GLC analysis of fatty acids in presence and absence of RVV. Elusion profile of DM-PC (*a*); RVV (*b*) and RVV treated DM-PC (*c*). Elusion positions of standard fatty acid derivatives are (1) myristic acid (C_{14}), R_t (retention time) = 1.637 min; (2) palmitic acid (C_{16}), R_t = 2.925 min; (3) stearic acid (C_{18}), R_t = 5.381 min; (4) arachidonic acid (C_{20}), R_t = 10.224 min and (5) behenic acid (C_{22}), R_t = 20.124 min.

Thus we report the assay of PLA_2 using Raleigh's scattering from a spectrofluorimeter at 650 nm using crude RVV and DM-PC. Since snake venom is a composite mixture of PLA_2s with varying substrate specificity, and venom composition depends on the zoogeographical location[19,20], the animal species was confined to eastern India origin. The rate of hydrolysis was found to be dependent on both enzyme and substrate concentration (Figure 2 *a* and *b*). The observed kinetic lag was a common feature of PLA_2 hydrolysis[21]. Further, actual release of myristic acid from DM-PC after hydrolysis was confirmed by GLC analysis (Figure 3).

An advantage of spectrofluorimetric measurements is increasing sensitivity to several orders by altering slit width. With wider slit width or measuring emission below 650 nm, the concentration of DM-PC could be sufficiently reduced within the limit of detection. However, the reduced reaction rate was unfit for reliable measurements. Therefore, it is the rate constant and not the scattering intensity that limits the assay. This mode has been validated by pH-stat titrimetric assay. The amount of fatty acid released by 1 mg of venom as measured by the two methods was 3680 and 3470 nmol/min. Further, in the pH-stat, 24–144 μg of crude venom could be assayed yielding 80–50 nmol fatty acid/min. In case of scattering mode, these figures stand at 12–60 μg and 40–220 nmol/min respectively, indicating the scattering mode to be over two-fold more sensitive. Applicability of this assay with venom from Russell's viper (western India, Haffkin Institute, Mumbai), *N. kauthia* (eastern India, Calcutta Snake Park), *Crotalus atrox* (Western diamond rattlesnake, Sigma), *Echis carinatus* (Saw-scaled viper, Sigma) and porcine pancreatic PLA_2 (Sigma, in the presence of 0.02% sodium taurocholate) was tested. The dependencies were similar to that seen in Figure 1.

The turbidometric assay of RVV–PLA_2 with DM-PC at 650 nm was found to be inconsistent, though the reaction was completely arrested in the absence of Ca^{2+} ions, after

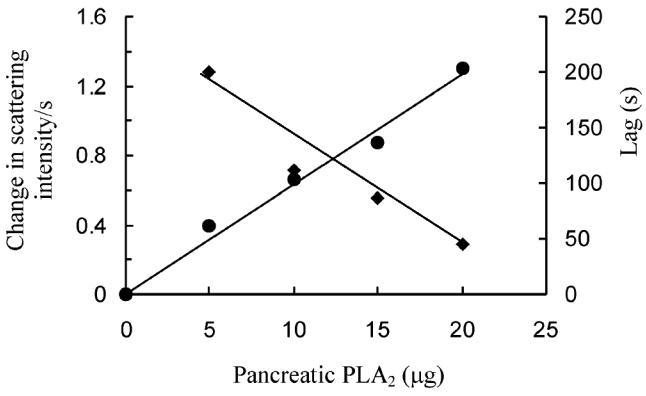

Figure 4. Assay of pancreatic PLA_2 with DM-PC using scattering mode under conditions as stated earlier. Rate of hydrolysis and kinetic lag have been represented by (●—●) and (◆—◆), where R^2 was 0.9859 and 0.9356 respectively.

heat or AVS treatment. This asserts the notion that there is hardly any PLA_2 assay which could be applied universally[11]. The presented procedure has been applied successfully to some plant aqueous extracts popularly known as 'antivenom' and was found to be completely inhibiting hydrolysis. Thus it may serve as an empirical guide for screening similar plants. This is in relation to our long-term interest to address better snake-bite management in the Indian subcontinent[14,22–24].

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Germination improvement in *Swertia angustifolia*: a high value medicinal plant of Himalaya

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The present communication deals with improvement in seed germination of *Swertia angustifolia* via various hormonal treatments (GA_3, IAA and KNO_3). Germination of the species under controlled conditions is found to be low (<32.0%). GA_3 is found to be the best with respect to germination (96.0%) and reducing mean germination time (7.6 days) followed by KNO_3 (81.3%; 8.4 days) and IAA (66.0%; 16.6 days). A high degree of variation with regard to the germination percentage and mean germination time in different populations and treatments is recorded. The possible reasons for such variations are discussed.

Keywords: Conservation, endangered, gibberellic acid, Himalaya, *Swertia angustifolia*.

SWERTIA angustifolia Ham. ex D. Don (family Gentianaceae), an endangered medicinal plant of west Himalaya[1], is listed among medicinal plants prioritized for conserva-

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