75 v/v, after 50 min it is 85 v/v, and at 55 min it is 95 v/v, for the optimum separation. Photo diode array detector was set to measure spectra from 200 to 400 nm.

Quantification of gymnemic acid

The quantification of gymnemic acid, a method described previously by Manohar *et al.* (2009), was followed with minor modifications.[12] Five hundred milligrams of dried leaf sample was put into a 100-mL round bottom flask and 50 mL of extraction solvent was added (volume ratio of methanol to water is 1:1) with 10 mL of 11% potassium hydroxide solution. The mixture was refluxed for an hour. The 9 mL concentrated HCl was added and refluxed again for 1 h and the mixture was cooled to room temperature. The extract was filtered through 0.45 µm nylon filter (Millipore), the volume was made up to 100 mL with extraction solvent, and the clean supernatant was used for HPLC analysis. The isocratic mobile phase composition (acetonitrile-A and water-B, 80:20 [v/v]) with elution rate of 1 mL/min, data acumination and quantification was done at 210 nm. Gymnemagenin standard was purchased from Sigma Aldrich, USA. The conversion of gymnemagenin to gymnemic acid was done using the equation $C = X(809.0 / 506.7)$, where C is the content of gymnemic acid in the sample, X is the content of gymnemagenin present in the sample, 506.7 is the molecular weight of gymnemagenin, and 809.0 is the molecular weight of gymnemic acid. The chromatogram of gymnemic acid standard shown in Figure 1, with a retention time of 1.83 min.

Data analysis

All the genotypes were scored for presence and absence of the ISSR bands. The data were entered into binary matrix as discrete variable (1) for the presence of amplification product or band and (0) for the absence of the band and this matrix were subjected to further analysis. Scores of individual bands were used to create data matrix. The similarity indices (SI) were computed as the ratio of number of similar bands to total number of bands in pair wise comparison of all accessions/genotypes. A dendrogram constructed based on Nei and Li's coefficient [13] with unweighted pair group method and arithmetic average analysis (UPGMA) using Fig Tree Version 1.3.1 software.

Similar methodology was applied for chemical fingerprinting, where presence of peak represented as *variable* (1) and *absence* as (0). To measure the informativeness of markers, the polymorphic information content (PIC) for each ISSR marker was calculated according to formula: $PIC = 1 - €Pi^2$, where PI is the frequency of the *i*th allele. Mantel test[14] was performed using 10 000 permutations carried out in XLSTAT-Pro (Version 7.5, 2004; Addinsoft Inc., Brooklyn, NY, USA) software; the significance level was set at $a = 0.05$, to compute the matrix correlation (r) between the similarity matrices generated from different assays (genetic and chemical fingerprinting) to test the goodness of fit.

RESULTS AND DISCUSSION

ISSRs involve amplification of DNA segments present at an amplification distance in between two identical microsatellite repeat regions oriented in opposite directions. ISSR uses long poly nucleotide chain (15-30 mers) , which permit the subsequent uses of high annealing temperature, leading to high stringency and reproducibility. It has been used in genetic fingerprinting, phylogenetic studies, gene tagging, and genome mapping in many plant and animal species.[15,16] The versatility of this genotyping technique makes ISSR useful for studies in various species.

In this study, 14 genotypes were surveyed with 40 ISSR (microsatellite) markers for their distribution and informativeness and polymorphism for the assessment of genetic diversity among them. Among the 40 primers, 15 were polymorphic and produce scorable, unambiguous bands, of which 12 were anchored-type dinucleotide repeat, one anchored-trinucleotide repeat, and one non-anchored tetra nucleotide repeat. A total of 124 scorable alleles were generated and of which 89 were found to be polymorphic (75.89%). The high level of polymorphism observed with the primers used in this study indicated a high level of genetic variation among the 14 genotypes analyzed in agreement to Rakoczy-Trojanowska and Bolibok (2004), who reported high polymorphic patterns when reaction primers based on microsatellite sequences in plants were used.[17] The number of alleles were produced by different primers ranged from 1 to 14 with an average of 8.26 alleles per primer [Table 2]. The mean number of allele per locus and allele size generated in this study was in close agreement with earlier studies[18,19] on *G. sylvestre*. The results clearly indicate that the *Gymnema* accession could be easily distinguished using these ISSR primers. The maximum number of amplicons (14 alleles) were demonstrated in DNA profiles generated by ISSR-15 number primer whereas minimum number of amplicons (5 alleles) were noted for profile of ISSR-11. The banding pattern using ISSR-15 was shown in Figure 2. Primers ISSR-11 and ISSR-13 revealed 100% polymorphism, showing their ability for discriminating the accessions of *G. sylvestre*. The results with these primers were in consonance with earlier studies that marker techniques which amplifies microsatellite regions viz. SSR and ISSR is a potentially highly polymorphictool, which was applied in several DNA marker studies.[20-25] The highest PIC value recorded for primer ISSR-12 is 0.99 and minimum for primer ISSR-13 is 0.33, with a mean of 0.69. The higher PIC value of any ISSR primer indicates the more informativeness for genotype discrimination and diversity studies[26] for that particular primer. The 15 ISSR primers collectively yielded nine unique accession-specific bands. Out of nine, four unique bands noted for accessions 3, two for accession 8, one for accession 3, one unique band each for accessions 1 and 14 [Table 3]. These accession-specific bands could be used as a descriptor for plant accessions protection and could also be converted

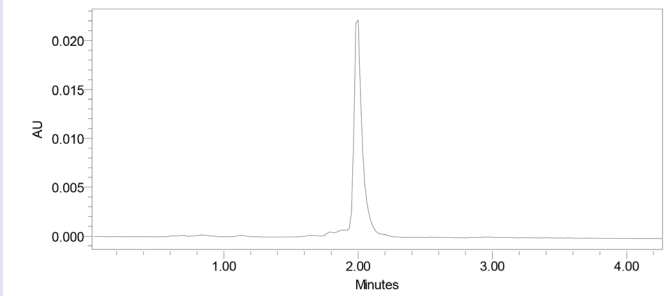

Figure 1: HPLC chromatogram for gymnemic acid in standard gymnemagenin

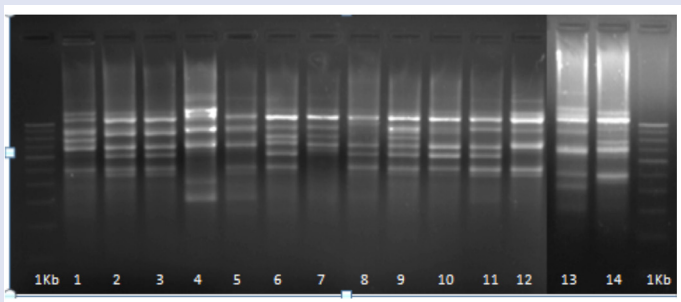

Figure 2 : ISSR marker profiles of 14 accessions generated by primer ISSR-15 in 2% agarose gel

Table 2: ISSR primers used in this study and different parameters of genetic diversity.

S.N.	ISSR primer name	Primer sequence (5' to 3'direction)	Total no of bands with primer	Number of polymorphic bands	Number of monomorphic bands	% of polymorphim	PIC
1.	ISSR-1	$(AC)_8T$	9	5	4	55.55	0.950
2.	ISSR-2	$(CAC)_7T$	7	6	1	85.71	0.540
3.	ISSR-3	$(CA)_6AC$	11	10	1	90.90	0.604
4.	ISSR-4	$(CAG)_5$	7	4	3	57.14	0.702
5.	ISSR-5	$(TC)_8CC$	9	6	3	56.67	0.500
6.	ISSR-6	$(GACA)_4$	10	7	3	70.00	0.500
7.	ISSR-7	$(GA)_9T$	6	4	2	66.67	0.480
8.	ISSR-8	$(GT)_8C$	10	7	3	70.00	0.540
9.	ISSR-9	$(AG)_{10}T$	7	5	2	71.47	0.530
10.	ISSR-10	$(AG)_8C$	8	5	3	62.50	0.573
11.	ISSR-11	$(GA)_8C$	5	5	0	100	0.740
12.	ISSR-12	$(AC)_8T$	6	1	5	16.67	0.990
13.	ISSR-13	$(GT)_8C$	6	6	0	100	0.330
14.	ISSR-14	$(AG)_8CT$	9	8	1	88.88	0.540
15.	ISSR-15	$(TG)_8G$	14	10	4	71.42	0.430
	Total		124	89	35	70.90	0.596

Table 3: Details of observed unique bands

S.N.	Present in accession	Number of unique band	Unique band size	Name of ISSR primer
1	Gym-1	1	< 1.0kb	ISSR-7
2	Gym-4	1	>1.5 kb	ISSR-12
3	Gym-4	1	>1.5 kb	ISSR-14
4.	Gym-4	1	= 2.0 kb	ISSR-15
5.	Gym-5	1	<1.0 kb	ISSR-5
6.	Gym-6	1	>0.5 kb	ISSR-6
7.	Gym-8	1	>0.5 kb	ISSR-9
8.	Gym-8	1	< 1.0 kb	ISSR -3
9.	Gym-14	1	<1.0 kb	ISSR-2

into sequence tagged sites markers of great value to detect between any mixing up of cultivars as an DNA fingerprinting.[27]

The sufficient number of accession-specific bands signifies the power of ISSR markers in fingerprinting and diversity analysis within *G. sylvestre*. The binary data from the polymorphic primers used for computing Nie and Li's similarity indices. The similarity coefficient based on ISSR markers ranged from 0.70 to 0.94 [Table 5]. Two genotypes (Gym 2 and Gym 3) showed highest similarity index, which indicates that both shares common hypothetical ancestor. Gym 14 showed lowest similarity index. The ISSR marker showed very prominent dissimilarity between the accessions. Higher dissimilarity among the genotypes provide better scope for identifying the accessions with desired characteristics. Cluster analysis based on Nie and Li's similarity using UPGMA grouped 14 accessions into three major clusters. Clusters I, II, and III contains 4,5, and 5 accessions, respectively. Interestingly, cluster I was characterized by moderate, cluster II by low, and cluster III by high gymnemic acid containing accessions [Figure 3].

The HPLC profile of methanolic extract showed that all the accessions possess the secondary metabolites of mid-polarity with considerable variability. The content of gymnemic acid varied from 0.02 to 0.64%, as observed for accession Gym 2 and Gym 10, respectively [Table 4]. The content of gymnemagenine varied from 0.014 to 0.407% and its peak was utilized as standard for chemical fingerprinting [Table 4].

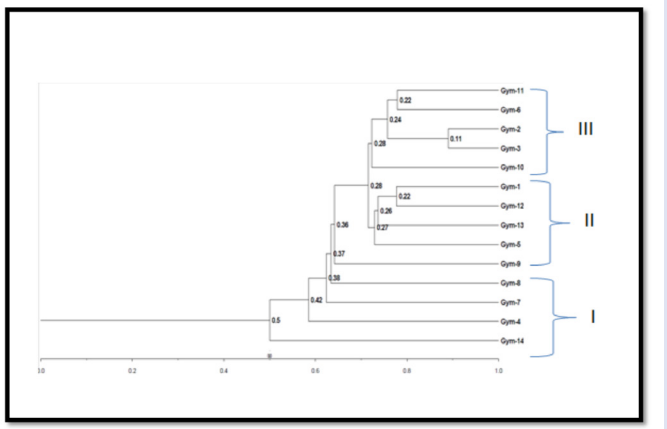

Figure 3 : Dendrogram of 14 accession constructed on the basis of ISSR variation pattern

Table 4: Percentage of gymnemic acid in leaf samples of different accessions of *G. sylvestre*.

S.N.	Accession number	Percentage of gymnemic acid	Percentage of gymnemagenine
1.	Gym-1	0.035	0.022
2.	Gym-2	0.022	0.014
3.	Gym-3	0.361	0.226
4.	Gym-4	0.130	0.081
5.	Gym-5	0.072	0.045
6.	Gym-6	0.154	0.096
7.	Gym-7	0.085	0.053
8.	Gym-8	0.053	0.033
9.	Gym-9	0.047	0.030
10.	Gym-10	0.649	0.407
11.	Gym-11	0.481	0.301
12.	Gym-12	0.053	0.033
13.	Gym-13	0.042	0.032
14.	Gym-14	0.063	0.040

The study of chromatogram indicates that peaks with retention time 2.63, 3.41, 23.83, 24.50, and 44.67 min were of universal type, as observed in all the accessions during analysis [Figure 4 and Figure 5]. These peaks were selected as marker peaks, which could be utilized in identification and standardization of *G. sylvestres* germplasm and based herbal preparations/formulations. Hierarchical cluster analysis based on chromatographic fingerprint (38 scorable peaks) grouped all the accessions in three major groups. In cluster I four, in cluster II four, and in cluster III six accessions were grouped, respectively. This analysis also indicated that high-yielding accessions Gym 11, Gym 7, Gym 10, and Gym 11 are closely related with each other as observed in phylogenetic tree [Figure 6]. The similarity coefficient based on HPLC fingerprints ranged from 0.24 to 1.0 [Table 6]. Gym 12 and Gym 13 accessions were almost similar with respect of chemical profiles, therefore both may shares common hypothetical ancestor. It is interesting that, accessions with low gymnemic acid content belongs to cluster I, moderate content in cluster II, and higher belongs to cluster III. HPLC-based chemical fingerprinting was performed in several medicinal plants viz. *Vitex rotundifolia*,[28] *Berberis vulgaris*,[29] *Andrographis paniculata*,[30] *Pyrola* species,[31] and wild Jujuba leaf tea.[32] Comparative study of results of genetic and chemical fingerprinting indicates that both techniques have potential to discriminate the *G. sylvestre* accessions according to gymnemic acid content, an antidiabetic active principle. However, clustering diagram based on both fingerprinting analysis were not perfectly matched with each other. This may be due to fact that ISSR measures genetic variation, mainly noncoding sequences which probably have a minor impact of the phenotypic and physiochemical behavior of plant on the other hand chemical constituents and their availability are affected by environmental conditions and show considerable variation.[28]

Table 5: Similarity matrix for 14 *G. sylvestre* accessions based on ISSR finger printing.

Accession	Gym-1	Gym-2	Gym-3	Gym-4	Gym-5	Gym-6	Gym-7	Gym-8	Gym-9	Gym-10	Gym-11	Gym-12	Gym-13
Gym-2	0.855												
Gym-3	0.877	0.943											
Gym-4	0.776	0.806	0.783										
Gym-5	0.834	0.876	0.852	0.840									
Gym-6	0.867	0.864	0.898	0.782	0.842								
Gym-7	0.783	0.805	0.790	0.770	0.795	0.815							
Gym-8	0.786	0.807	0.828	0.762	0.821	0.793	0.792						
Gym-9	0.797	0.820	0.818	0.702	0.798	0.818	0.792	0.795					
Gym-10	0.848	0.857	0.855	0.764	0.824	0.845	0.821	0.822	0.837				
Gym-11	0.852	0.860	0.871	0.791	0.839	0.885	0.800	0.778	0.841	0.866			
Gym-12	0.884	0.846	0.844	0.822	0.881	0.857	0.822	0.835	0.813	0.838	0.854		
Gym-13	0.851	0.847	0.846	0.802	0.860	0.847	0.824	0.826	0.778	0.852	0.867	0.875	
Gym-14	0.756	0.743	0.727	0.746	0.722	0.713	0.710	0.741	0.763	0.780	0.724	0.759	0.726

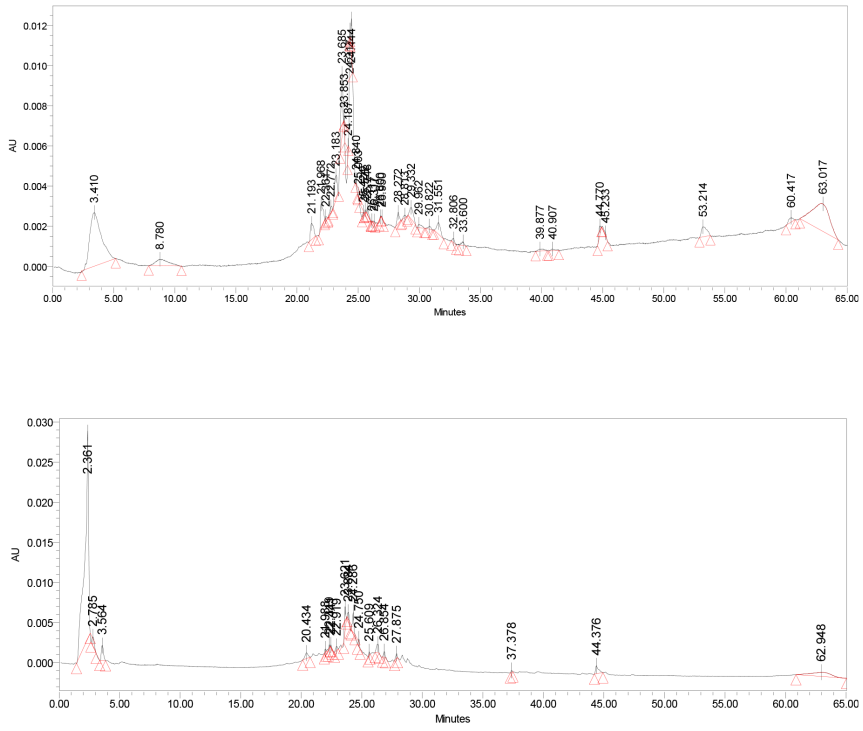

Figure 4 : A representative HPLC chromatograms of *G. sylvestre* leaf samples

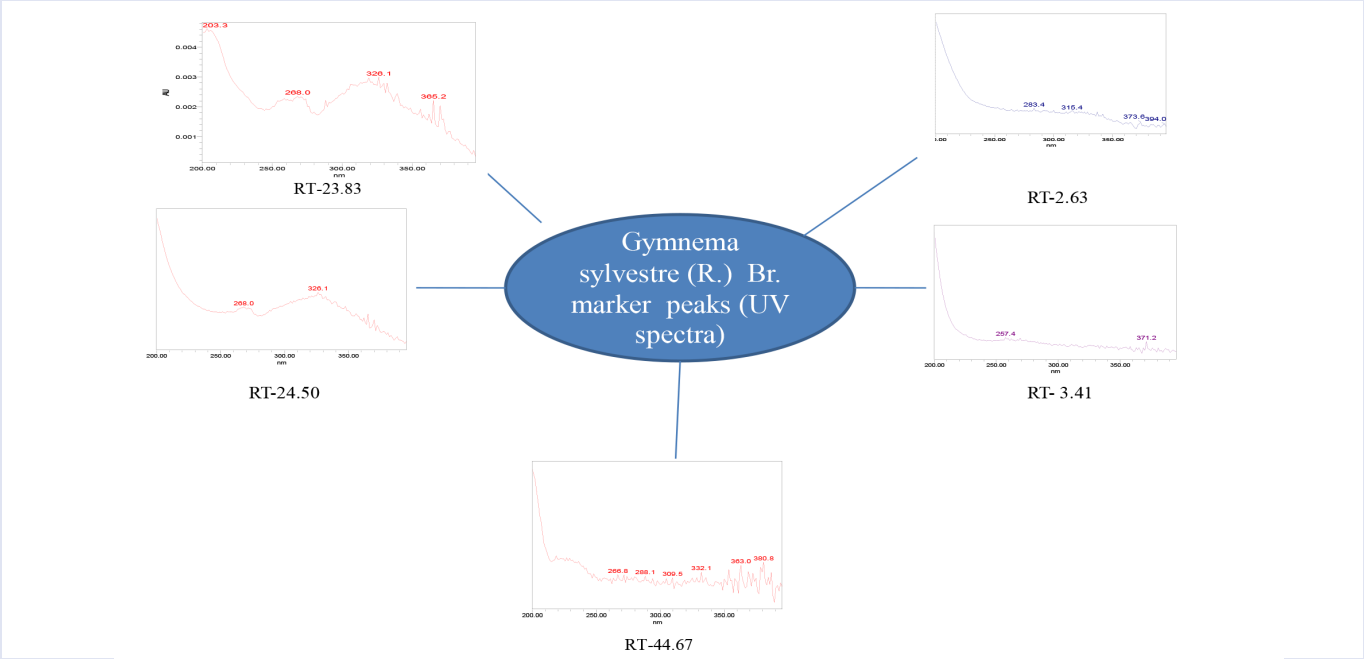

Figure 5 : UV spectra of five unknown marker peaks

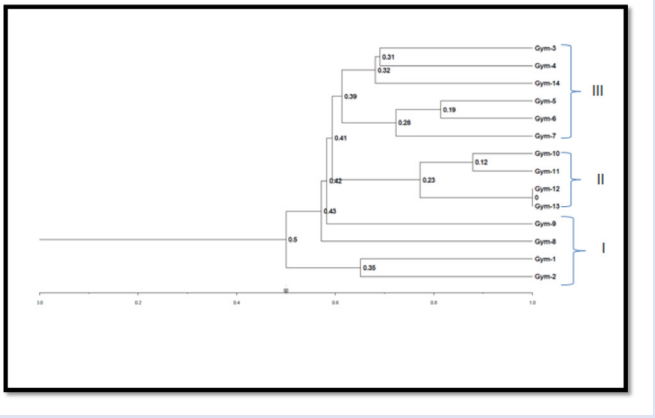

Figure 6 : Dendrogram of 14 accession constructed on the basis of variation obtained by HPLC chromatography

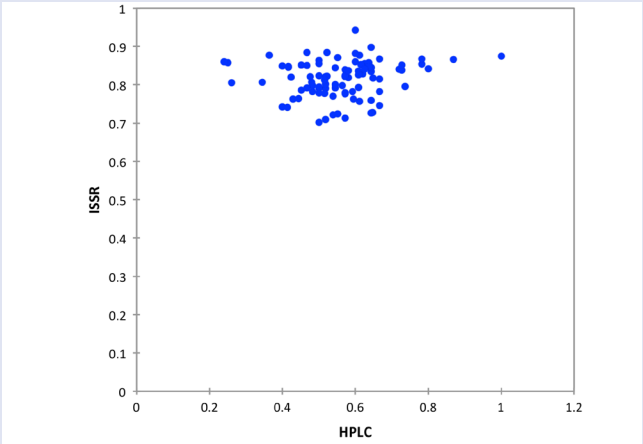

Figure 7 : Scatter plot represents mental correlation between similarity matrix of genetic and chemical fingerprints

Table 6: Similarity matrix for 14 *G. sylvestre* accessions based on HPLC finger printing.

Accession	Gym-1	Gym-2	Gym-3	Gym-4	Gym-5	Gym-6	Gym-7	Gym-8	Gym-9	Gym-10	Gym-11	Gym-12	Gym-13
Gym-2	0.625												
Gym-3	0.611	0.600											
Gym-4	0.571	0.345	0.667										
Gym-5	0.643	0.364	0.615	0.720									
Gym-6	0.667	0.500	0.643	0.593	0.800								
Gym-7	0.483	0.261	0.519	0.538	0.737	0.667							
Gym-8	0.452	0.480	0.621	0.429	0.571	0.609	0.545						
Gym-9	0.564	0.424	0.649	0.500	0.483	0.581	0.467	0.500					
Gym-10	0.400	0.250	0.500	0.444	0.500	0.545	0.476	0.522	0.581				
Gym-11	0.452	0.240	0.552	0.500	0.571	0.522	0.545	0.500	0.625	0.870			
Gym-12	0.467	0.417	0.643	0.519	0.600	0.636	0.571	0.609	0.516	0.727	0.783		
Gym-13	0.467	0.417	0.643	0.519	0.600	0.636	0.571	0.609	0.516	0.727	0.783	1.000	
Gym-14	0.611	0.400	0.647	0.667	0.538	0.571	0.519	0.414	0.595	0.571	0.552	0.643	0.643

Mantel correlation analysis between the similarity matrix based on genetic and chemical fingerprints indicates positive and significant correlation (r = 0.150) between them, which signified the scope of simultaneous study of genetic and chemical finger printing in various medicinal plant species [Figure 7]. Comparative study also indicated that chemical fingerprinting was more suitable than genetic fingerprinting for distinguishing accessions according to their agro-climatic / natural habitat / collection site.

Experimental observations of ISSR and HPLC fingerprinting studies concluded that both approaches were suitable for identification of high-yielding accessions and different chemotypes, but their simultaneous application provides more insight to understand evolutionary trends, chemotypic variation, adaptation, and so on. Used ISSR markers were highly polymorphic and could be utilized as molecular probes for further selecting high-yielding accessions / genotypes. Although target metabolite, that is gymnemic acid in *G. sylvestre*, demonstrates the specific chemotype, but the selection of additional five peaks could also be utilized as standards to define the chemical diversity in the specific accession. Adaptation of both ISSR and HPLC fingerprinting-based methods simultaneously can ensure the consistent quality and therapeutic action of herbal drugs[33].

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Conflicts of interest

There are no conflicts of interest.

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