Lipoxygenase inhibitory activity of some *Sida* species due to di(2-ethylhexyl) phthalate

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Lipoxygenase (LOX) is the key enzyme in the biosynthesis of leukotrienes, postulated to play an important role in the pathophysiology of several inflammatory and allergic diseases. The study of LOX inhibitory activity of some *Sida* species, its isolation, structure elucidation and characterization of the active compound are reported. The LOX inhibitor from Sida species was di(2-ethylhexyl) phthalate. The interaction of di(2-ethylhexyl) phthalate with LOX was demonstrated by its kinetics, isothermal titration calorimetry and molecular docking studies. *In vitro* studies and molecular docking showed that di(2-ethylhexyl) phthalate was binding perfectly into the active site of LOX to inhibit it.

Keywords: Inhibitory activity, lipoxygenase, molecular docking, *Sida* species.

MEDICINAL plants are the main source of chemicals with potential therapeutic applications. The use of medicinal plants for the treatment of many diseases is associated with folk medicine from different parts of the world. Naturally occurring compounds in plants, fungi and microbes are still used in pharmaceutical preparations in pure or crude-extract forms[1]. Lipoxygenase (LOX; EC 1.13.11.12) is the key enzyme in the biosynthesis of leukotriens that has been postulated to play an important role in the pathophysiology of several inflammatory and allergic diseases. The products of LOX-catalysed oxygenation (hydroperoxyeicosatetraenoic acid, hydroxyeicosatetraenoic acid, leukotriens and lipoxins) seem to be involved in rheumatoid arthritis, psoriasis, asthmatic responses, glomerular nephritis and myocardial ischaemia[2,3].

Sida is an important medicinal herb in ayurveda, the Indian system of medicine[4,5]. It is invariably present in most of the herbal preparations prescribed in the ayurvedic system for derangements, implicating inflammation. Hence, *Sida* species was presumed to have compounds or their precursors with anti-inflammatory property. The above presumption encouraged us to study the presence of enzyme inhibitors relevant in inflammatory disorders. Thus, six members of *Sida* species, viz. *S. acuta*, *S. cordata*, *S. mysorensis*, *S. alnifolia*, *S. cordifolia* and

S. rhomboidea were studied for lipoxygenase inhibitory activity. In search of compounds of plant origin with lipoxygenase inhibitory activity, we found that lipoxygenase (soybean lipoxygenase, type I-B) was inhibited by the crude methanolic extracts of some of the *Sida* species mentioned above. Activity-guided fractionation of the whole-plant extracts yielded the active compound which exhibited LOX inhibition. In this communication we describe the identification, isolation, purification, structure elucidation, kinetics of lipoxygenase inhibition and its isothermal titration calorimetric analysis, and molecular docking studies of the purified lipoxygenase inhibitory compound.

Whole plants of *Sida acuta* Burm.f., *Sida cordata* (Burm.f.) Borssum, *Sida mysorensis* Wight & Arn., *Sida alnifolia* L., *Sida cordifolia* L. and *Sida rhomboidea* Roxb. (Malvaceae) were collected from different parts of Kannur and Kollam districts, Kerala, India during the period March 2011–March 2012. Samples were authenticated and voucher specimens were deposited in the Herbarium, Inter University Centre for Bioscience, Kannur University. All the plant materials were collected and categorized based on geographic conditions and collection time. All samples were dried in shade, at room temperature.

Lipoxygenase is known to catalyse the oxidation of unsaturated fatty acids containing 1-4 diene structures. The conversion of linoleic acid to 13-hydroperoxy linoleic acid was followed spectrophotometrically by the appearance of a conjugate diene at 234 nm on a UV/visible spectrophotometer (Hitachi U-2900) as described earlier with some modifications[6]. The enzyme inhibition assays were performed with different concentrations of the isolated compound. The activity of LOX was monitored as an increase in the absorbance at 234 nm, which reflects the formation of hydroperoxy-octadecadienoic acid. The reaction medium (2.0 ml final volume) contained 50 mM Tris HCl buffer (pH 8.5) or 50 mM borate buffer (pH 9.0), 1 mg of enzyme protein and solution of linoleic acid prepared in tween 20 and solubilized in 50 mM Tris HCl buffer (pH 8.5)[7]. Crude extracts (40 mg/ml) were prepared in DMSO and aliquots were taken for assay. The assay mixture consisted of 50 µl of lipoxygenase enzyme solution (200 U/ml) and 50 µl of test solution, and the mixture was incubated for 1 min. After incubation appropriate volumes of buffer were added (1540 µl). The reaction was initiated by adding 360 µl substrate solution (60 µM) and absorbance was recorded at 234 nm for 5 min using UV visible spectrophotometer (Hitachi U2900, Tokyo, Japan). Nordihydroguaiaretic acid (NDGA), a known inhibitor of soybean lipoxygenase, was used as positive control. A graph showing the time-dependent activity of the enzyme was plotted using Origin Pro software.

The plants which showed lipoxygenase inhibitory activity were selected and fractionated using column chroma-

tography. Fractions were collected and further screened for lipoxygenase inhibitory activity to find out the fraction responsible for activity. For the isolation of the fraction responsible for activity, air-dried samples were powdered (50 g) and extracted three times with methanol in a water bath at 55°C. The methanolic extract was evaporated *in vacuo* to leave a greenish residue, which was eluted on silica gel using *n*-hexane and ethyl acetate as eluant with increasing polarity to obtain ten fractions. Fractions 1–3 were further passed through a silica gel column (60–120 mesh) and eluted with *n*-hexane/ethyl acetate (1–5%), which resulted as a colourless, oil-like liquid. Thin-layer chromatography (TLC) was performed using silica gel 60 F_{254} plates (200 μm, Merck, Germany). Column chromatography was carried out on a column packed with silica gel (60–120 μm, Merck). Purity was checked by TLC (mobile phase: *n*-hexane : ethyl acetate, 9.4 : 0.6, v/v). HPLC analysis was carried out on a Shimadzu instrument using a C18 column (Phenomenex C18 250 × 4.6 mm, 5 μm), mobile phase, methanol : water (9 : 1) and detection at UV 210 nm (refs 8 and 9). Quantification of the compound in different samples was done using high performance thin-layer chromatography (Camag, Muttenz, Switzerland) and HPLC.

^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker NMR spectrometer operating at 500 MHz for ^{1}H NMR and 400 MHz for ^{13}C spectra. The chemical shifts were given in ppm (δ) and were referenced relative to CDCl$_3$ (δ 7.26 and 77.24 ppm for ^{1}H and ^{13}C NMR respectively) spectrometer and the chemical shifts were expressed in δ (ppm) values with trimethylsilane as an internal reference (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet and br = broad peak). Mass spectra were recorded on a Hitachi M-80. The IR spectra were recorded on a Bruker FT-IR spectrometer. High-resolution TOF mass spectra (positive ESI mode) were measured on a Waters LCT Premier mass spectrometer coupled with a Waters Alliance HPLC system.

The binding of the active principle to LOX was evaluated by isothermal titration calorimetry (ITC). The calorimetric titrations were performed using VP-ITC isothermal titration calorimeter from Microcal (Northampton, MA, USA) as described in the manufacturer's instruction manual. First, 0.01 mM of protein and 0.2 mM of ligand were prepared in 5% DMSO in 0.2 M borate buffer. Both protein and ligand samples were degassed before loading to the ITC machine. Then 10 μl of ligand solution was added from the rotating syringe to the cell which contains protein solution. Ten seconds time was set for each injection. A time interval of 180 sec was also set between each injection to allow the peak resulting from the reaction to return to the baseline. A total of 29 injections were made. The reference power was set as 10 μcal and the stirring speed was adjusted to 307 rpm. The volume of the first injection was set as 3 μl to avoid inaccuracy. The heat changes between the LOX and 5% DMSO in 0.2 M

borate buffer solution were subtracted from the original value and the final data at the end of the injections were fitted by a nonlinear least square method using ORIGIN software from Microcal. The binding constant (K), enthalpy change (ΔH), entropy change (ΔS) and binding free energy (ΔG) were calculated using the source software.

Molecular docking simulations were performed using Discovery studio 2.5 (Accelrys Software Inc., San Diego, USA). Docking studies were carried out to find the possible orientation and interactions (hydrogen bond and hydrophobic interaction) of the ligand in active site of the enzyme. Human 5-lipoxygenase was used as a target for docking experiments, as the active site and the mode of catalysis are similar for soybean and human LOX[10]. The three-dimensional crystallographic structure of LOX was downloaded from the protein Data Bank (PDB ID 3V99). CHARMm force fields were applied with Momany-Rone partial charge. The ligand from the 3D structure was removed and redocked to standardize the docking protocol. A receptor grid was prepared by selecting residues within 4 Å around the arachidonic acid (or ligand in 3V99) as the centre. All ligands and water molecules were removed and hydrogen atoms were added to the protein. The ligand molecule was prepared using Ligprep module in DS 2.5 and the generated pose was used for further docking studies.

From ^{1}H, ^{13}C NMR spectra and mass determination, the compound was identified as di(2-ethylhexyl) phthalate (Figure 1).
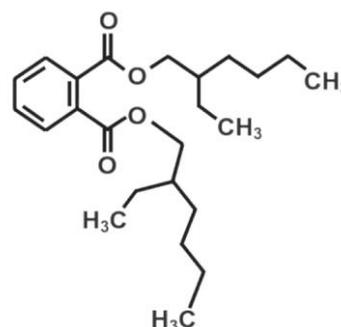

Figure 1. Structure of the isolated compound, di(2-ethylhexyl) phthalate (DEHP).

Table 1. The di(2-ethylhexyl) phthalate (DEHP) content in methanolic extract of different *Sida* species

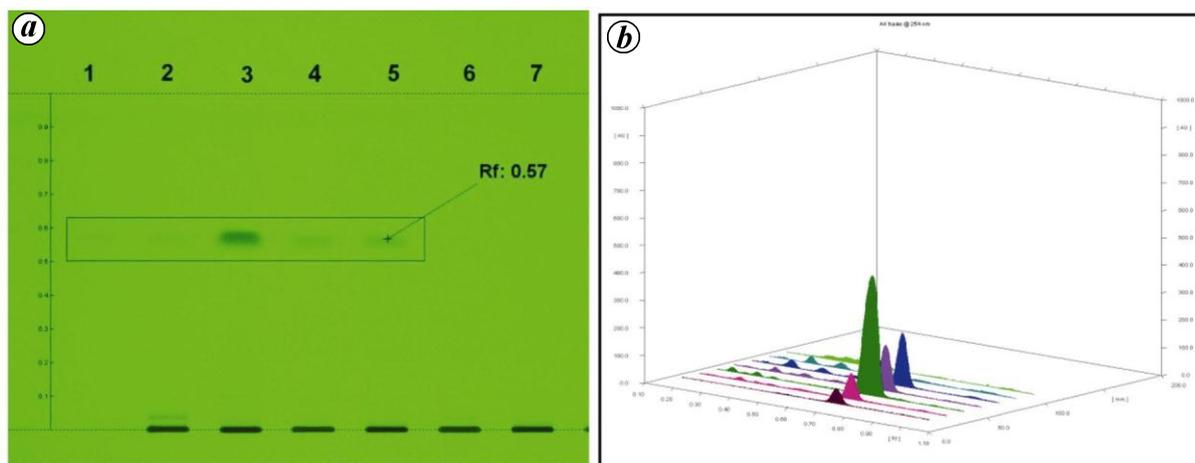| Sample	Average content of DEHP in the sample (%, w/w)
Sida cordifolia	0.36 ± 0.007
Sida alnifolia	0.32 ± 0.008
Sida acuta	0.25 ± 0.011
Sida mysorensis	0.19 ± 0.009
Sida cordata	not detected
Sida rhomboidea	not detected

Figure 2. *a*, TLC visualized after illumination at UV 254 nm. *b*, 3D display of all the samples (methanol extracts). Track 1, Purified compound; track 2, *Sida mysorensis*; track 3, *Sida cordifolia*; track 4, *Sida acuta*; track 5, *Sida alnifolia*; track 6, *Sida rhombodea*; track 7, *Sida cordata.*
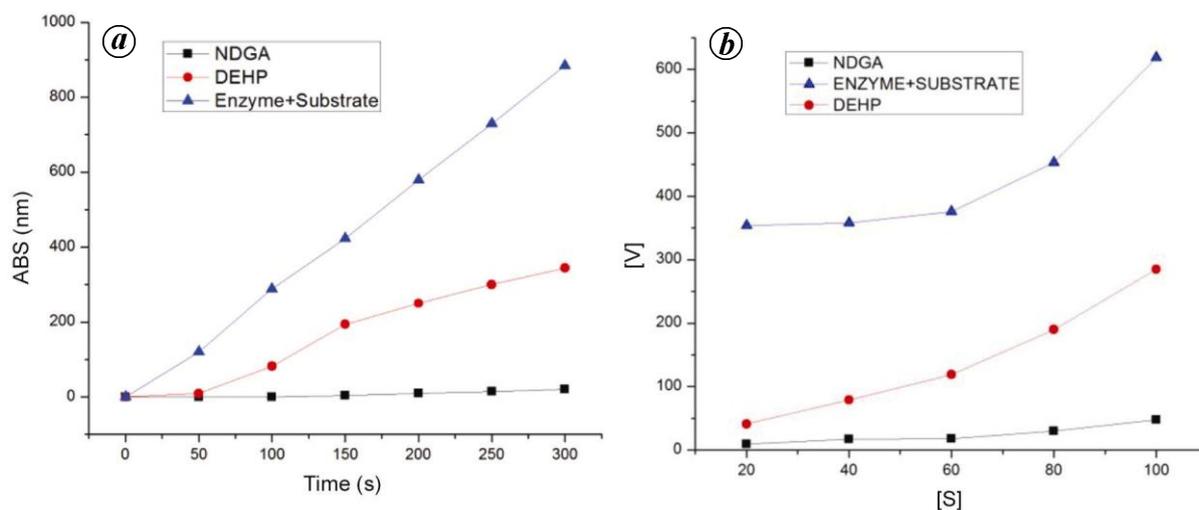

Figure 3. *a*, Progression curve showing the LOX inhibitory activity of the isolated compound di(2-ethylhexyl) phthalate (DEHP) compared with NDGA. *b*, LOX inhibitory activity of DEHP and NDGA at different substrate concentrations.
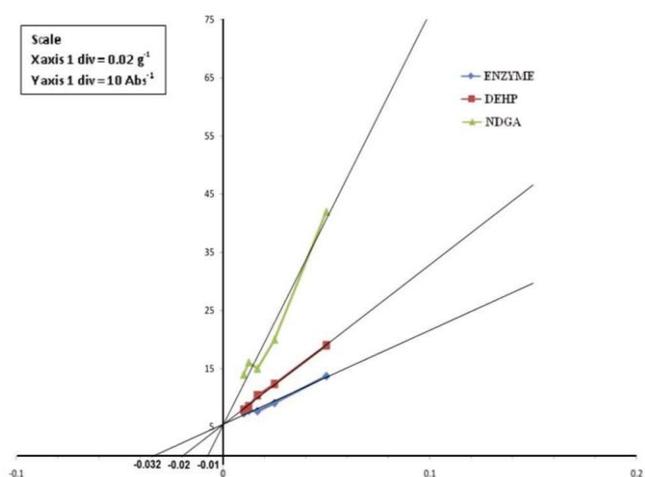

Figure 4. Lineweaver–Burk plot obtained for LOX with DEHP and NDGA.

The highest LOX inhibitory effect was exhibited by *S. cordifolia* methanolic extract followed by *S. alnifolia*, *S. acuta* and *S. mysorensis* (Table 1). TLC fingerprint of crude extracts and the isolated compound are shown as Figure 2 *a*. All the LOX inhibitory fractions of different *Sida* species yielded the same compound. 3D display of all the samples showed (Figure 2 *b*) difference in the active content. Results of the progression curve showing the LOX inhibitory activity of NDGA and DEHP are shown in Figure 3 *a* and LOX activity at different concentrations of DEHP and NDGA is shown in Figure 3 *b*. *S. cordata* and *S. rhomboidea* did not show any LOX inhibitory activity.

Lineweaver–Burk graph was plotted using the values obtained by enzyme kinetics at different substrate concentrations and equimolar enzyme to inhibitor ratio (Figure 4).

The inhibitor constant K_i was calculated using the equation[11]

$$K'_m = K_m (1 + I_0/K_i).$$

From the K_i value obtained, the IC$_{50}$ was calculated using the Cheng–Prusoff equation[12]

$$K_i = IC_{50}/1 + [S]/K_m.$$

K_m of the uninhibited enzyme was 0.115 M, which increased to 0.1783 M under the inhibition exerted by DEHP at equimolar ratio. In the case of NDGA, it was 0.3566 M. The V_{max} did not show any alteration and therefore, the type of inhibition was deduced to be competitive[13]. The IC$_{50}$ values obtained are given in Table 2.

ITC analysis revealed that the isolated compound showed binding with LOX. The binding constant (K), change in enthalpy (ΔH) and change in entropy (ΔS) are obtained from the graph as shown in Table 3. The raw data generated by ITC experiment and the data fitted by nonlinear least squares analysis of DEHP are shown in Figure 5. The binding free energy observed for DEHP is –6.86 kcal/mol (Table 3).

From the molecular docking studies it was identified that DEHP perfectly docked in the active site of LOX with CDOCKER energy of –60.0 kcal/mol and that of NDGA was –49.0 kcal/mol. The C-terminal catalytic domain contains a non-heme iron in the active site, coordinated by His 367, His 372, His 550, Asn 554 and the C-terminal Ile. This iron acts as an electron acceptor or donor during catalysis[14]. The hydrogen bonding of DEHP at the active site with the iron coordinated residues, His 367 and His 550, is different from the hydrogen bonding of NDGA. The possible presence of isozymes in the wet samples may be the reason for the differences/inconsistencies in the binding energies of DEHP and NDGA obtained from wet laboratory experiments and simulated binding studies. The van der Waals contacts found to stabilize the protein–ligand interaction were 63 and 65 for DEHP and NDGA respectively. His 367 and Gln 413, presented on two sides of the active site channel, are the two active site residues seen making hydrogen bonds with NDGA (Figure 6 a). These hydrogen bonds keep the NDGA in extended conformation to form a bridge, across the active site channel, rendering it difficult to dislodge. The closely occupied residues His 367

and His 550 hook the DEHP at its middle part to hang it from the active site cavity (Figure 6 b). This suspension may render it less stable compared to the binding of NDGA, considering the difference in anchoring of the inhibitors at the active site, though the van der Waals contacts are comparable. This explains the inhibition of LOX by DEHP to be low compared to NDGA.

Synthetic phthalate esters are widely used in paints and polymer products as plasticizers[15,16]. Occurrence of phthalate and phthalate esters has been reported from different biological sources such as green plants, fungi, yeasts, red-algae and bacteria, as produced by those organisms[17–19]. Occurrence of DEHP in *Alchornea cordifolia* and its inhibition on 15-LOX have already been reported[20]. The antimicrobial, cytotoxic and anti-inflammatory activity of DEHP had been reported earlier[21,22], with induced inflammation in animal models. It may be stated that DEHP can be considered toxic only based on the evidence in animals of DEHP-induced toxicity, to the liver, kidney, testes, uterus, ovary, foetus and thyroid[23]. No report exists regarding acute DEHP toxicity to humans or animals in conditions other than induced toxi-

Table 2. Comparative inhibition of lipoxygenase (LOX) by DEHP and Nordihydroguaiaretic acid (NDGA) at equimolar concentration of substrate

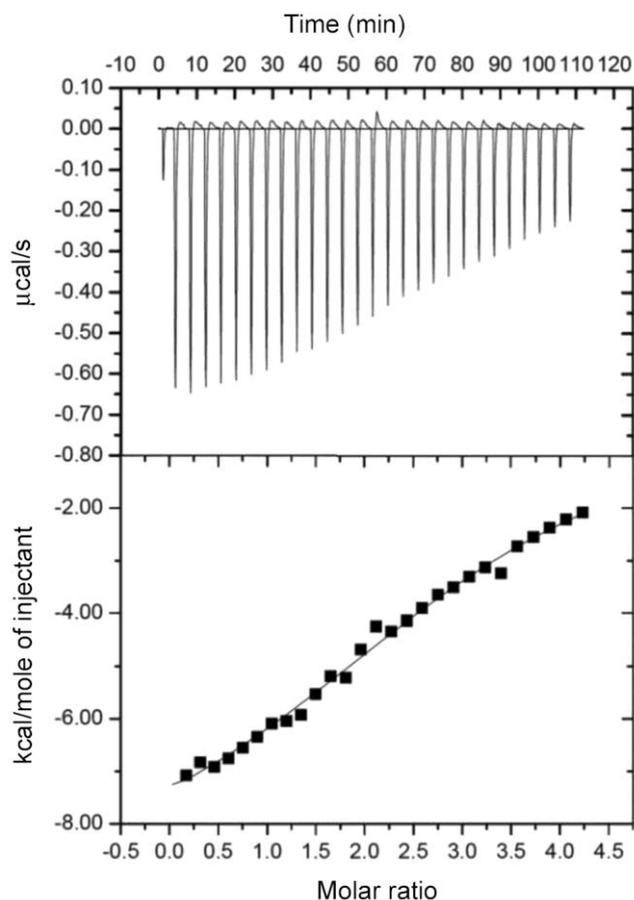| Sample	IC$_{50}$ (μM)
DEHP	0.217 ± 002
NDGA	0.068 ± 005

Figure 5. Isothermal titration calorimetic analysis of LOX with DEHP. Both are with the raw thermal power signals (top), and plot of integrated heat versus ligand/protein molar ratio (bottom).

Table 3. Thermodynamic data (ITC analysis) of DEHP with LOX

Sample	N	K (mol^{-1})	ΔH (cal/mol)	ΔS (cal/mol deg^{-1})	T (K)	ΔG (kcal/mol)
DEHP	3.12	1.08×10^5	−9431	−8.61	298.15	−6.86
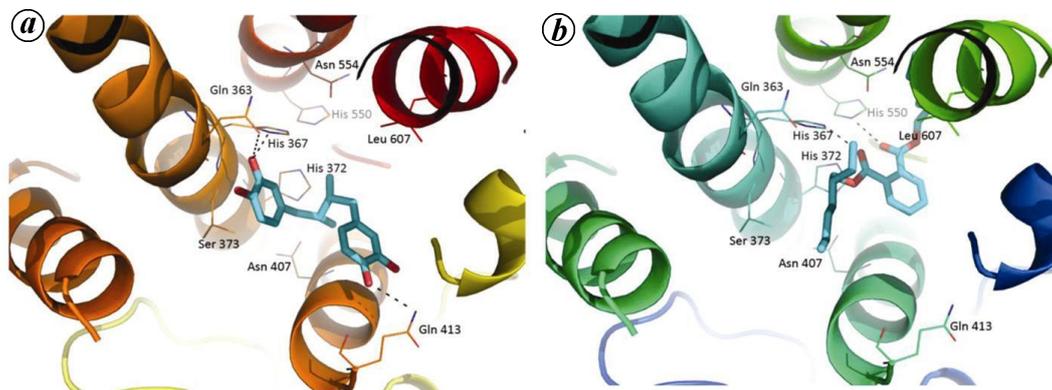
Figure 6. *a*, Illustration of the docked pose of NDGA. *b*, DEHP in the active site of 5-lipoxygenase. Only important amino acids are shown for clarity.

city[23], though biomarker-based exposure estimates for phthalates had been reported[24]. However, DEHP is viewed as a toxic substance in any preparation for human consumption[23]. On the other hand, DEHP is present in *Sida* species as shown in this study and *Sida* is an important herb extensively used in the ayurvedic system of medicine for millennia. It has stood the test of time, without any traditional knowledge on its obvious toxicity. In order to be sure, we followed protocols with utmost rigour to rule out the possibility of finding DEHP in *Sida* as an observation by chance or artifact in analysis. The six *Sida* species were analysed during summer, monsoon and winter of a year and DEHP was observed only in four species growing in dry soil, without its presence in soil or other bio-systems surrounding them. However, the data presented (Figure 1 *a*) show that the purified LOX inhibitor from *Sida* species was DEHP. The above finding suggests the possibility of the presence/absence of this compound in the plants of *Sida* species studied may be due to the genetic variation in them and environmental stress.

The results of comparative analyses of both DEHP and NDGA as inhibitors of LOX, studied by ITC and modelling, show that they are comparable by the mode of enzyme inhibition, DEHP being less inhibitory than NDGA to LOX. The four plants, *S. acuta*, *S. cordifolia*, *S. alnifolia* and *S. mysorensis* showed the presence of DEHP at considerably high concentration (Table 1). Some of them are used as medicinal herbs, under the name 'Bala' in many ayurvedic preparations and are often prescribed for diseases with visible inflamed conditions. The present study, demonstrating the LOX inhibition of the DEHP, suggests a possible molecular mechanism of the anti-inflammatory property of the com-

pound to infer that the DEHP found in *Sida* species may have some role in defining the anti-inflammatory property of 'Bala' in different forms of ayurvedic medical preparations.

The present study has shown that the whole plant of some *Sida* species contain DEHP and the compound is a potent inhibitor of LOX. The enzyme inhibition may partially impart the anti-inflammatory property of the plant. DEHP is generally considered as an unacceptable compound to be present in products for human consumption. *Sida* species containing ayurvedic preparations are widely used as products for consumption as drugs, especially for arthritic symptoms. It implicates the role of DEHP in treating inflammation. This may cast apprehensions for use of such products in societies culturally alien to ours, unless otherwise assured of safety. The toxicity of DEHP is found irrelevant in the context of consumption of Ksheerabala or any other ayurvedic drug containing *Sida* species, since the ingestion of DEHP into the human body is insignificantly low to cause any toxicity and it will not accumulate in the human body to elicit toxicity over a period of time[25,26]. Hence, *Sida* species may be considered as a safe herb. The DEHP present in the *Sida* species might be either deficient to trigger any toxic effect even on prolonged use, or modified into a benign compound by the preparative procedure of *Sida* species containing products. Further studies are needed in this regard.

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Population structure analysis of *Labeo gonius* from three reservoirs of Uttarakhand using RAPD marker

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Population genetic structure of the fish, *Labeo gonius* from three reservoirs, i.e. Dhaura, Baigul and Nanak sagar, Uttarakhand, India was analysed by applying random amplified polymorphic DNA (RAPD) markers. RAPD–PCR products obtained using 15 decamer operon series (OPA, OPB, OPC and OPY) primers, were utilized for analysis of the population of *L. gonius* in each reservoir. Genetic observations based on percentage of polymorphic loci, the number of effective and observed alleles, Nei's genetic diversity and Shannon's information index indicated high level of genetic diversity in stocks of all three reservoirs of *L. gonius*, being more heterozygous in Nanak sagar followed by Baigul and Dhaura reservoirs. The percentage of genetic variation within (86.83) and among (13.17) all the stocks based on coefficient of gene differentiation ($Gst = 0.131$) indicated moderate genetic differentiation likely to be associated with small gene exchange responsible for weak sub-structuring of stocks in all three reservoirs.

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