Spectroscopic Studies on Dual Role of Natural Flavonoids in Detoxification of Lead Poisoning: Bench-to-Bedside Preclinical Trial

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ABSTRACT: Ubiquitousness in the target organs and associated oxidative stress are the most common manifestations of heavy-metal poisoning in living bodies. While chelation of toxic heavy metals is important as therapeutic strategy, scavenging of increased reactive oxygen species, reactive nitrogen species and free radicals are equally important. Here, we have studied the lead (Pb) chelating efficacy of a model flavonoid morin using steady-state and picosecond-resolved optical spectroscopy. The efficacy of morin in presence of other flavonoid (naringin) and polyphenol (ellagic acid) leading to synergistic combination has also been confirmed from the spectroscopic studies. Our studies further reveal that antioxidant activity (2,2-diphenyl-1-picrylhydrazyl assay) of the Pb–morin complex is sustainable compared to that of Pb-free morin. The metal–morin chelate is also found to be significantly soluble compared to that of morin in aqueous media. Heavy-metal chelation and sustainable antioxidant activity of the soluble chelate complex are found to accelerate the Pb-detoxification in the chemical bench (in vitro). Considering the synergistic effect of flavonoids in Pb-detoxification and their omnipresence in medicinal plants, we have prepared a mixture (SKP17LIV01) of flavonoids and polyphenols of plant origin. The mixture has been characterized using high-resolution liquid chromatography assisted mass spectrometry. The mixture (SKP17LIV01) containing 34 flavonoids and 76 other polyphenols have been used to investigate the Pb detoxification in mouse model. The biochemical and histopathological studies on the mouse model confirm the dual action in preclinical studies.

INTRODUCTION

Lead (Pb), one of the most widely used metals in the industry and simultaneously a versatile, subtle, and persistent poison present in Earth’s crust, can induce a wide array of physiological, biochemical, and behavioral dysfunctions affecting almost every organ system (central and peripheral nervous, hematopoietic, respiratory, cardiovascular, renal, hepatic and reproductive system) cumulating in death.1–4 Although Pb toxicity has been documented as early as 2000 BC and found to be prevalent, victims of such toxicity in human civilization are evident in recent times.5 Even in 2015, 494 550 deaths occurred due to Pb exposure, which is 0.6% of the global burden of diseases and 9.3 million disability-adjusted life years.4,6,7 Of particular concern is the role of Pb exposure in the development of intellectual disability in children.7 Though there is wide recognition of this problem and many countries have acted to stop its use, exposure to Pb, particularly in childhood, remains a key concern for health care providers and public health officials worldwide. Though Pb itself is a nonredox metal and does not possess any prooxidant catalytic activity, it rather converts to some indirect mechanisms [e.g., auto-oxidation of hemoglobin (Hb), accumulation, and subsequent auto-oxidation of δ-aminolevulinic acid induced by Pb] for imparting pathogenesis via oxidative disturbances.8–10 Thus, controlling the free-radical-induced intracellular damage is equally important in combating Pb poisoning alongside the removal of the same from organs. On the other hand, the currently approved treatment for Pb toxicity (use of chelating agents such as 2,3-dimercaptosuccinic acid and CaNa2EDTA) can neither be used at a therapeutically adequate dose for a prolonged period of time owing to a number of shortcomings and inherent toxicity (e.g., nephrotoxicity, cardiotoxicity, zinc diuresis, nausea, fever, and breathing trouble) nor reduce the significant oxidative stress.
generated due to cellular Pb exposure. Therefore, considering the indispensable necessity of an alternative therapeutic approach, an exogenously supplied chelator with sustainable antioxidant activity even after complexation with Pb can be an ideal therapeutic detoxification strategy.

Flavonoids, a class of natural polyphenols with a benzo-γ-pyrone structure ubiquitously present in plants, are widely known for their antioxidant activity. Since their discovery in 1936, several studies have determined the flavonoid antioxidative activity, many of which have used pure compounds, calculated individual antioxidation power, and performed structure–activity relationship studies. The other most studied property of flavonoids is the metal ion chelation ability. Several studies have reported interactions between flavonoids and metal ions leading to chelate formation, which are only slightly active in the promotion of free-radical reactions. Although several studies have shown that plants use this chelation property for the detoxification of heavy metals, in-depth study on the interaction of heavy metals with flavonoids is sparse in contemporary literature. Some of the recent studies focus on the antioxidation properties of flavonoid–transition-metal complex (e.g., Fe, Cr, Cu, and Al). However, to the best of our knowledge, no study has addressed the sustainable antioxidation activity imparted due to heavy-metal chelation to flavonoid alone or in mixture with other polyphenols and is one of the motives of the present study.

Here, we have studied a model heavy-metal (Pb) chelating efficacy of a model flavonoid (morin) using steady-state and picosecond-resolved optical spectroscopy. The efficacy of morin in the presence of other flavonoid (naringin) and polyphenol (ellagic acid, EA) leading to synergistic combination has also been confirmed from the spectroscopic studies. This is particularly important because previous observations suggest that polyphenolic mixtures show fewer side effects than the pure compounds due to interactions among themselves. Our studies reveal that the antioxidative activity [2,2-diphenyl-1-picrylhydrazyl (DPPH) assay] of the Pb–morin complex is sustainable compared to that of Pb-free morin. We have also found that the solubility of the Pb–morin complex increases significantly compared to that of morin in an aqueous environment. Heavy-metal chelation and sustainable antioxidant activity of the chelate complex are found to accelerate the Pb detoxification in the chemical bench (in vitro). Considering the synergistic effect of flavonoids in Pb detoxification and their omnipresence in medicinal plants, we have prepared a mixture (SKP17LIV01) of flavonoids containing 34 flavonoids and 76 other polyphenols and investigated the Pb detoxification in mouse model. The biochemical and histopathological studies confirm the dual action in preclinical studies. Our studies on biodistribution of Pb in various target organs of the intoxicated mice using inductively coupled plasma-assisted atomic emission spectroscopy (ICP-AES) confirm that SKP17LIV01 efficiently facilitates excretion of Pb from mouse body after heavy-metal chelation and sustainable reactive oxygen species (ROS) scavenging in vivo.

### MATERIALS AND METHODS

**Materials.** Morin, EA, naringin, quercetin, lead nitrate (Pb(NO₃)₂), sodium chloride (NaCl), sodium hydroxide (NaOH), and DPPH were purchased from Sigma (St. Louis, MO, USA). All solvents were obtained from Merck (NJ, USA) unless otherwise stated. All reagents were of analytical grade and used without further purification. Nanopure water (resistivity ≥ 18 MΩ cm) from Milli-Q system (Millipore GmbH, Germany) was used whenever required. In order to prepare a mixture of flavonoids and polyphenols, we have used a well-characterized (by using ultra-high-performance liquid chromatography mass spectrometry; UHPLC–MS) extract from eight medicinal plants (Table S1). We call the mixture to be SKP17LIV01. The detailed characteristics and systems pharmacology of the extract will be published elsewhere.

**Fingerprint Analysis Using UHPLC–MS.** For complete chemical characterization of SKP17LIV01, we used fingerprint analysis by ultra-high-resolution LC–MS. Mass spectra were recorded by electrospray ionization in both negative and positive modes using an Agilent 1290 Infinity UHPLC System (MS Q-ToF, model G6550A, Agilent Technologies, CA, USA) equipped with a C-18 stainless steel column (30 cm × 0.46 cm). The capillary voltage was kept at 80 V, and the air (nebulizing gas) pressure was 35 psig. Full scan data acquisition was performed by scanning from m/z 50 to 1000 with isolation width ≈ 4.0 amu. The oven temperature was set at 40 °C, and mobile phase A consisted of 100% water, whereas mobile phase B consisted of a mixture of 90% acetonitrile, 10% water, and 0.1% formic acid. Sample (0.02 mL) was injected. A flow rate of 0.2 mL/min was used. An elution gradient ranging from 5% B to 95% B from 0 to 20 min was used. Identification of major compounds was accomplished by analyzing the molecular ion peak and base peak using an Agilent MassHunter Workstation (Agilent Technologies CA, USA).

**UV–Vis and Fluorescence Spectroscopy.** Optical absorbance spectra were recorded on a double-beam spectrophotometer (model UV-2600, Shimadzu, Japan) in the 200–800 nm range. Fluorescence spectra were obtained with a fluorescence spectrophotometer (LifeSpec-ps, Edinburgh Instruments, UK) equipped with a microchannel plate–photomultiplier tube (MCP–PMT, Hamamatsu, Japan). The excitation wavelength was set at 375 nm. Fluorescence spectra were corrected for variations with wavelength in source intensity, photomultiplier response, and monochromator throughput.

**Solutions and Experimental Method.** Stock solutions of morin, naringin, and EA were prepared in 0.01 N NaOH. They were further diluted using water as a solvent. NaCl was used to keep the ionic strength constant (0.1 M). Incremental volumes of Pb(NO₃)₂ stock solution (0.15 M) were added to the polyphenol reaction mixture in order to vary the molar ratio, and the pH was maintained constant (pH = 7.4) by small additions of NaOH. A syringe pump (Pump 11 Pico Plus Elite, Harvard Apparatus, MA, USA) was used to circulate the solution from the titration beaker to the experiment cell. UV–vis absorbance and fluorescence spectra were recorded for each molar ratio and used for further calculations. All measurements were performed in a 1 cm quartz cuvette, hermetically closed, and thermostatted at 298.0 ± 1.5 K.

**Time-Correlated Single-Photon Counting Measurement.** PL transients of the solutions were measured and fitted using a commercially available picosecond diode laser-pumped laser.
time-resolved fluorescence spectrophotometer (LifeSpec-ps, Edinburgh Instruments, UK) [excitation wavelength 375 nm, 80 ps instrument response function (IRF)] with a temperature controller attachment (Julabo, model F32). Time-correlated single-photon counting (TCSPC) technique was used to capture all transients. Photoluminescence was detected from the samples using a MCP–PMT (Hamamatsu, Japan) after dispersion through a grating monochromator (polarizer was set at 55°). The observed fluorescence transients are fitted by using a nonlinear least-square fitting procedure to a function

\[ q(t) = \int_0^t E(t') R(t-t') \, dt' \]

comprising convolution of IRF \((E(t))\) with a sum of exponentials

\[ \rho(t) = X + \sum_{i=1}^n Y_i e^{-t/\tau_i} \]

with pre-exponential factors \((Y_i)\), characteristic lifetimes \((\tau_i)\), and a background \((X)\). Relative percentage in a multi-exponential decay is finally expressed as

\[ c_n = \frac{Y_n}{\sum_{i=1}^n Y_i} \times 100 \]

The quality of the curve fitting is evaluated by reduced chi-squared and residual data.

**DPPH Assay.** The free-radical scavenging activity of the samples of interest was determined using the DPPH method. Sample (0.5 mL, in varied concentration) was added to 3.0 mL of freshly prepared ethanolic DPPH solution (0.1 mM). The change in absorbance was monitored at 535 nm for 60 min. EC\(_{50}\) values were measured from the percentage inhibition at different concentrations.

\[ \text{Percentage inhibition} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100\% \]

EC\(_{50}\) is the concentration value which scavenged 50% of the DPPH radicals. Trolox was used as the reference compounds.

For DPPH assay, synthesis of Pb(II)–morin complex was done by the addition of solid Pb(NO\(_3\))\(_2\) (0.1 M final concentration) in ethanolic solution of morin (0.01 M). The mixture was stirred for 1.5 h. Then, it was filtered and subsequently evaporated on a rotary evaporator. The resulting product was washed with 1:1 t-butanol/chloroform to remove unreacted morin. An olive green product Pb(II)–morin complex was then obtained, which was used for further studies.

**Animal Model and Treatment.** Six-week-old healthy Swiss albino mice weighing 25.1 ± 2.0 g, maintained in polypropylene cages under 12 h light/dark cycle in a temperature (25.0 ± 1.5 °C) and humidity (50.0 ± 5%)-controlled room with food (Hindustan Lever, New Delhi, India) and water ad libitum, were randomly divided into four groups \((N = 10/\text{group})\) after 2 weeks of acclimatization. Group I served as control and received physiological saline (0.9% NaCl) by oral gavage during the whole course of experiment. Groups II–IV intraperitoneally received an aqueous solution of Pb(NO\(_3\))\(_2\) [50 mg/kg body weight (BW)] in every alternative day for 4 weeks for the induction of oxidative stress and associated liver damage. Then, group II was left untreated and served as a negative control. After induction, groups III and IV daily received (orally) SKP17LIV01 (3.5 mL/kg BW) and silymarin (100 mg/kg BW), respectively. All animal experiments were performed according to the guidelines recommended by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India, and approved by the Institutional Animal Ethics Committee (approval no.—JU-Dey’s/IAEC/09/14, dated 31.01.2014).

**Blood Collection and Serum Isolation.** At the end of requisite fasting period, blood was collected from each mouse by retro-orbital venous puncture and kept in microcentrifuge tubes with and without ethylenediaminetetraacetic acid (2.0%). The tubes were placed in slanting position at room temperature (RT) for 2 h. Then, they were centrifuged at 3500 g for 10 min. Serum was separated and used for further analyses.

**Assessment of Hematological and Liver Function Parameters.** Biochemical parameters such as serum enzymes, aspartate aminotransferase (AST), alanine aminotransferase (ALT), serum alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), and total and direct bilirubin along with total protein (TP) were assayed using assay kits (Span Diagnostic, Surat, India) following the protocol prescribed by manufacturers. The European standardized method was used to determine the blood delta-aminolevulinic acid dehydratase (ALAD) activity. For hematological studies, the blood was collected in heparinized tubes. Hematological parameters were obtained by an automated cell counter (Medonic CA 620, Boule Diagnostics, Sweden). Parameters studied were Hb, total red blood cell, reticulocyte, hematocrit, mean corpuscular volume, mean corpuscular Hb, mean corpuscular Hb concentration, platelets, total white blood cell, and differential count.

**In Vivo Distribution of Pb in Blood and Liver.** The Pb(II) contents in the liver and blood were estimated using ICP-AES (ARCOs, SPECTRO Analytical Instruments GmbH, Germany). The samples were prepared using the open acid digestion method. In brief, dried tissues were dissolved in HNO\(_3\) (15 mL), H\(_2\)SO\(_4\) (10 mL), and H\(_2\)O\(_2\) (5 mL), heated at 120 °C until only a residue remained, and then diluted with deionized water to 10 mL.

**Histopathology.** For microscopic evaluation, a conventional technique of paraffin wax sectioning and differential staining was used. Liver tissues were excised, fixed in 10% neutral buffered formalin saline for 72 h, dehydrated in graduated ethanol (50–100%), cleared in xylene, and embedded in paraffin. Sections (4–5 μm thick) were cut using microtome, stained with hematoxylin and eosin (H&E), and examined under an Olympus BX51 fluorescence microscope (Olympus Optical, Tokyo, Japan). In this staining process, hematoxylin gives deep blue-purple color to nucleic acids, whereas eosin provides pink color to the proteins.

**Tissue Homogenate Preparation.** Tissues from the four groups of animals were collected, minced, and lysed in radio immunoprecipitation assay buffer (Sigma, USA) (5 mg tissue in 0.5 mL buffer) for 30 min. After centrifugation at 12000 g for 15 min at 4 °C, supernatants were collected and stored in aliquots at −80 °C for further use. Protein concentration of the aliquots was determined according to Bradford method.

**Antioxidant Enzyme Activity and Lipid Peroxidation.** The activity of superoxide dismutase (SOD) and catalase (CAT) was measured following reported methods. Reduced glutathione (GSH) was determined using Ellman’s method. For activity measurement of glutathione peroxidase...
(GPx), we followed a modified method of Lawrence and Burk.39 To assess the extent of lipid peroxidation, the level of malonyldialdehyde (MDA), a substance that reacts with thiobarbituric acid, was determined in the homogenates of organs and in serum according to the method of Buege.40

Statistical Analysis. All quantitative data are expressed as mean ± standard deviation (SD) unless otherwise stated. One-way analysis of variance followed by Tukey’s multiple comparison tests was executed for comparison of different parameters between the groups using a computer program GraphPad Prism (version 5.00 for Windows), GraphPad Software, California, USA. p < 0.05 was considered significant.

■ RESULTS AND DISCUSSION

Interaction of Morin: A Model Flavonoid with Heavy-Metal Lead (Pb2+). Primarily, we chose morin (3,5,7,2′,4′-pentahydroxyflavone) (Figure 1a) as a representative of flavonoid class for understanding its heavy-metal chelating activity. Morin is very special among flavonols as it is one of those few natural flavonols that bears a OH group in position 2′ that can be involved in a hydrogen bond with 3-OH, leading to far reaching consequences.13 Despite being the most abundant polyphenol in our diet or in nature, this unique structural feature along with previously reported antiradical activity41 made morin our molecule of interest. The absorbance spectra of morin (Figure 1b) show two absorption maxima centered at ∼255 nm (band II) and ∼354 nm (band I) corresponding to the Π → Π* electronic transition originating at benzoyl (flavonoid ring A) and cinnamoyl (flavonoid ring B) systems, respectively. The interaction of morin with Pb2+ produced large bathochromatic shift (∼45 nm) in band I maxima (Figure 1b). In contrast, band II remained unaffected. The exclusive change in band I indicates the involvement of cinnamoyl system in the complexation process. Among the three probable binding sites (3-OH and 4-oxo, 3-OH and 2′-OH, or 5-OH and 4-oxo) present in morin, 3-OH and 4-oxo is the most preferable site of co-ordination because of the presence of highly acidic proton, stronger chelation ability, and greater delocalization of the oxygen at 3-OH.42,43 The stoichiometry of complexation was investigated by using Job’s method (the method of continuous variation).44 Considering a global equilibrium of Pb(II) and ligands (L)

\[
Pb + nL = PbL_n
\]

where n is determined from the plot of the absorbance as a function of the mole fraction, \( f \), of the added ligand. In the absorbance maximum

\[
\frac{n}{1 - f_{\text{max}}} = f_{\text{max}}
\]

Inset of Figure 1b represents a typical Job plot for the Pb(II)–morin system, in which the transition point for absorbance appeared at the molar fraction of 0.33, suggesting that morin (L) bound to Pb2+ with a 2:1 ratio in the complex

Figure 1. Interaction of Pb(II) with morin, a flavonol. (a) Chemical structure, numbering system, and UV–vis absorption bands of morin. (b) Absorption spectra of 4.09 × 10−5 M morin in the presence of varying concentrations (0.15 × 10−3 to 4.14 × 10−4 M) of Pb(NO3)2. Arrows indicate the increase in the concentration of Pb2+. Inset shows the Job plot for binding of Pb2+ to morin. (c) Fluorescence spectra of morin (2.66 × 10−4 M) in the presence of different concentrations (0.75 × 10−4 to 37.74 × 10−4 M) of Pb(NO3)2 (λem = 375 nm). Inset shows Stern–Volmer and modified Stern–Volmer plots for the interaction (λem = 550 nm). (d) Time-resolved fluorescence transients of morin (2.66 × 10−4 M) in the absence and presence of Pb2+ (37.7 × 10−4 M).
the concentration of aqueous Pb\(^{2+}\) ion added. The binding quenching constant, \(K\) and presence of a quencher, \([Q]\), constant (discernible shift in emission maxima (\(\lambda\)) or shape of emission at \(375\) nm (Figure 1c) or shape of emission at \(375\) nm (Figure 1c).

Interaction of Morin with Pb\(^{2+}\) in the Presence of Other Flavonoid Naringin and Polyphenol EA. Generally, the observed new band at ~413 nm (Figure 1b) due to complexation corresponds to the formation of Pb–3-OH, forming a big extended \(\Pi\)-bonding system with the inclusion of C ring leading to further stabilization.\(^{46}\) The electronic transition within morin shifts from n–\(\Pi^*\) to lower energy \(\Pi–\Pi^*\) favoring the development of a new band at higher wavelength.\(^{46}\) The binding constant of the Morin–Pb\(^{2+}\) complex was found to be \(1.803 \times 10^4\) M\(^{-1}\) (\(R^2 = 0.987\)) using the Benesi–Hildebrand equation:\(^{48}\)

\[
\log \left( \frac{A - A_0}{A_t - A_0} \right) = \log([\text{Pb}^{2+}]) + \log K_b
\]

where, \(A_0\), \(A\), and \(A_t\) are the absorption values, in the absence of, at the intermediate, and at the saturation of the interaction of Pb\(^{2+}\) ion, respectively, and \([\text{Pb}^{2+}]\) represents the concentration of aqueous Pb\(^{2+}\) ion added. The binding constant (\(K_b\)) was determined by linear fitting of absorption titration curve (Figure S1).

In the photoluminescence study, morin shows a strong emission at ~550 nm when excited at ~375 nm (Figure 1c). The observed fluorescence is probably originating from the anion form of the 7-OH group of the pyrone ring, as it gets deprotonated first owing to its low \(pK_a\) of deprotonation (\(pK_a\) 3.80), and as a result, morin stays as monodeprotonated species in a solution of physiological pH.\(^{49,50}\) In the presence of Pb\(^{2+}\), the fluorescence quenched (Figure 1c). The phenomenon of fluorescence quenching can be attributed to several molecular mechanisms including excited-state reactions, molecular rearrangements, energy transfer, ground-state complex formation, and collisional quenching. In the case of morin, no discernible shift in emission maxima (~550 nm) or shape of the fluorescence spectrum accompanied quenching and the quenching behavior generally adhered to the Stern–Volmer equation (eq 1).

\[
\frac{F_0}{F} - 1 = K_{sv}[Q] = k_q[Q] = \frac{r_0}{\tau} - 1
\]

where \(F_0\) and \(F\) are the fluorescence intensities in the absence and presence of a quencher, \([Q]\), \(K_{sv}\) is the Stern–Volmer quenching constant, \(k_q\) is the bimolecular quenching rate constant, and \(r_0\) and \(\tau\) are the fluorescence lifetimes of the fluorophore in the absence and presence of the quencher, respectively.\(^{51}\) The linearity in the Stern–Volmer plot (Figure 1c, inset) indicates the existence of a single type of quenching, either static or dynamic.\(^{51}\) To get further insight into the phenomena, we measured the excited-state fluorescence lifetime of the fluorophore (morin) with increasing concentrations of the quencher, Pb\(^{2+}\) (Figure 1d). It is well-known that in the case of dynamic quenching, the fluorescence lifetime decreases with increasing concentrations.\(^{51}\) However, in this case, no change in fluorescence lifetime (Table 1) was observed, which clearly specifies the quenching mechanism to be static. This was further supported by the calculation of the bimolecular quenching constant, \(k_q\) (\(K_{sv}/\tau_0\)), which was found to be ~7.69 \times 10^{12}\) M\(^{-1}\) s\(^{-1}\) higher than that of diffusion-controlled limit (~10^{10} M^{-1} s^{-1}).\(^{51}\) This along with no change in fluorescence lifetime indicates ground-state complex formation between Pb\(^{2+}\) and morin (association constant, \(K_a\) = \(K_w = 1.819 \times 10^6\) M\(^{-2}\), similar to that found in the absorbance experiment). Probably, binding of Pb\(^{2+}\) to the carbonyl and 3-OH groups of the pyrone ring inhibits the intramolecular proton transfer, resulting in the formation of a nonemissive or low-emission phototautomer at RT.\(^{50}\) Thus, it can be inferred from optical spectroscopic studies that morin acted as a bidentate ligand and formed a mononuclear complex with Pb\(^{2+}\), where one ion is bound to two ligands (Figure 2).

**Figure 2.** Three possible structures of morin–Pb\(^{2+}\) complex. Complex 1 is the most probable one.

| Table 1. Fluorescence Lifetime Components of Polyphenols and Pb(II)–Polyphenol Complexes\(^{\text{a}}\) |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| morin          | 97.3 189.90     | 2.7 1858.50     | 234.95          |
| morin–Pb(II)   | 97.3 189.90     | 2.7 1858.00     | 234.95          |
| naringin       | 90.1 127.09     | 9.8 378.04      | 151.47          |
| naringin–Pb(II)| 27.56           | 5.5 127.00      | 0.5 378.00      | 34.94           |
| EA             | 100 3072.45     |                 | 3072.45         |
| EA–Pb(II)      | 75.6 88.59      | 24.4 3072.00    | 816.54          |
| MNEA           | 96.2 141.38     | 3.8 2088.00     | 213.29          |
| MNEA–Pb(II)    | 82.6 30.83      | 16.1 141.00     | 1.2 2088.84     | 73.22           |
| SKP17LIV01     | 65.7 38.03      | 15.7 475.00     | 18.5 3165.55    | 683.52          |
| SKP17LIV01–Pb(II)| 77.4 32.77   | 9.7 475.46      | 12.7 3165.00    | 472.32          |

\(^{\text{a}}\)\(r\) represents the time constant and \(\alpha\) represents the relative contribution of the component. \(\lambda_{em} = 375\) nm for all systems; \(\lambda_{em}(\text{morin}) = 550\) nm; \(\lambda_{em}(\text{naringin}) = 500\) nm; \(\lambda_{em}(\text{EA}) = 440\) nm; \(\lambda_{em}(\text{MNEA}) = 550\) nm; \(\lambda_{em}(\text{SKP17LIV01}) = 550\) nm.
in natural products, flavonoids never stay as a single entity, rather as a mixture of flavonoids and other polyphenols. Therefore, we prepared an equimolar mixture of morin, naringin (a flavonoid glycoside; Figure S1a), and EA (an ellagitannin; Figure S2a) to see whether the blending exerts any effect on heavy-metal binding property. Naringin and EA were selected as representatives of two different classes of polyphenols commonly found in natural products. Figure 3a shows the absorbance spectra of the mixture (we call it MNEA). Addition of Pb\(^{2+}\) to MNEA caused minimal or no change in absorbance. However, in fluorescence studies (Figure 3b), quenching phenomena was clearly evident at both \(\lambda_{en} = 450\) (probably originating from EA) and \(\lambda_{em} = 550\) nm (a tail probably originating from morin). Positive deviation of \(F_0/F\) from linearity indicates the possible involvement of combined static and dynamic quenching. The Stern–Volmer constant for both types of quenching can be determined from equation (eq 2)

$$\frac{F_0}{F} = (1 + K_{SV}[Q])(1 + K_S[Q])$$

(2)

where \(K_{SV}\) is the dynamic quenching constant and \(K_S\) is the static quenching constant. The double logarithmic plot (Figure 3b, inset) reveals the association constant \((K_S = 3.16 \times 10^4 \text{ M}^{-1})\) and number of binding sites \((n = 1.25)\) using the following equation (eq 3).

$$\log \left(\frac{F_0 - F}{F}\right) = \log K_s + n \log([Q])$$

(3)

where \(K_S\) is the association constant and \(n\) is the number of possible binding sites. The decrease in lifetime, as observed in TCSPC (Table 1), also supports our conjecture. The ground-state complex formation between morin and Pb\(^{2+}\), observed in the earlier section of this study, is primarily responsible for the static part of the observed quenching phenomena. To find out the origin of the dynamic quenching component, we further investigated the interaction of Pb\(^{2+}\) with the other two compounds in the mixture.

In the case of naringin (Figure S2a), as there was observable changes in the absorbance at \(\lambda_{en} (375 \text{ nm})\) or \(\lambda_{em} (500 \text{ nm})\) (Figure S2b), we employed inner filter effect correction using the following equation (eq 4)

$$F_{obs} = F_{corr} \times 10^{-A_a \times d_a / 2 - A_e \times d_e / 2}$$

(4)

where \(F_{obs}\) is the observed fluorescence intensity, \(F_{corr}\) is the corrected fluorescence intensity after correction of inner filter effect, \(d_a\) and \(d_e\) are the path lengths in the excitation and emission detection (in cm), respectively, and \(A_a\) and \(A_e\) are the detected changes in the absorbance value at the excitation and emission wavelengths, respectively, caused by ligand addition.\(^{51,52}\) The Stern–Volmer plot (Figure S2d) constructed using the corrected fluorescence intensities showed linearity. The significant change in the lifetime (Table 1) describes the quenching process to be dynamic in nature. For EA (Figure S3a) also, the inner filter correction was employed because of the presence of significant change in absorbance (Figure S3b). Addition of Pb\(^{2+}\) in the solution caused quenching of the fluorescence (Figure S3c) and the Stern–Volmer plot showed an upward curvature without any dependence onto [Pb\(^{2+}\)] (Figure S3d). Thus, neither simple dynamic (linear \(F_0/F\) vs [Pb\(^{2+}\)]) nor combined static and dynamic (second-degree polynomial) quenching model was accurately able to describe the data. Rather, a combined “sphere of action” dynamic quenching model was helpful. This includes, by means of a Poisson distribution, the probability that a Pb\(^{2+}\) ion is close enough to instantaneously quench the

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**Figure 3.** Interaction of Pb(II) with MNEA, an equimolar mixture of three polyphenols morin (a flavanol), naringin (a flavonoid glycoside), and EA (an ellagitannin). (a) Absorption spectra of MNEA (0.62 \(\times\) \(10^{-4}\) M) in the presence of varying concentrations (0.91 \(\times\) \(10^{-4}\) to 1.46 \(\times\) \(10^{-4}\) M) of Pb(NO\(_3\))\(_2\). Arrows indicate the increase in the concentration of Pb\(^{2+}\). (b) Fluorescence spectra of MNEA (0.62 \(\times\) \(10^{-4}\) M) in the presence of different concentrations (0.75 \(\times\) \(10^{-4}\) to 30.19 \(\times\) \(10^{-4}\) M) of Pb(NO\(_3\))\(_2\), \((\lambda_{en} = 375 \text{ nm})\). Inset shows Stern–Volmer and modified Stern–Volmer plots for the interaction \((\lambda_{em} = 550 \text{ nm})\). (c) Time-resolved fluorescence transients of MNEA (0.62 \(\times\) \(10^{-4}\) M) in the absence and presence of Pb\(^{2+}\) (30.19 \(\times\) \(10^{-4}\) M).
fluorescent species.\(^5\) This deviation from linear Stern–Volmer behavior is plausible for species such as Pb(II), where strong Coulombic interactions are likely to occur between the excited state and the quenching ion.\(^3\) This could further be supported from the time-resolved studies (Figure S3e), which shows a decrease in lifetime of the fluorophore (Table 1). The functional form of the quenching model is

\[
\frac{K_v}{F} = (1 + K_{Q}(Q)) e^{k_{Q}(Q)}
\]

(5)

The constants \(K_v\) and \(K_{Q}\) were found by nonlinear least-squares regression of the data. The dashed line in Figure S2d represents this model equation with the above empirical constants. Therefore, it can be concluded that the excited-state interaction between naringin–Pb(II) and EA–Pb(II) is responsible for the observed dynamic quenching component of MNEA–Pb(II) interaction. In order to investigate the mutual interaction among flavonoids and polyphenols, we have performed spectroscopic studies on the mixture. Although morin–naringin or naringin–EA shows insignificant changes in the steady-state emission and absorption spectroscopic data, mixing of morin with EA resulted in spectroscopic changes as shown in Figure S4a. The steady-state fluorescence spectra reveal the quenching at 550 nm with a subsequent increase at 450 nm with an iso-emissive point at 500 nm (Figure S4b). The Stern–Volmer plot (Figure S4b, inset) indicated the presence of a single type of quenching, which is found to be static in nature as fluorescence lifetime remained unchanged (Figure S4c). Thus, the flavonoids even in the presence of other polyphenols can effectively bind heavy metals and increase the detoxification possibility inside the body. The other polyphenols do not hamper the detoxification procedure rather shows a synergistic effect by chelating the heavy metal. In this process, as many of the polyphenols are potential Pb chelators, competition for metal complexation might occur between these natural chelators, resulting in synergistic effect.

Interaction of Flavonoids with Heavy Metal (Pb\(^{2+}\)) in Real-Life Sample, SKP17LIV01. Next, we proceed to a more complex mixture of flavonoids and polyphenols, SKP17LIV01 (total phenolic content = 4.71 × 10\(^{-3}\) M; total flavonoid content = 3.97 × 10\(^{-3}\) M; morin = 2.9%, naringin = 1.2%, and EA = 1.8%). The mixture was completely characterized by UHPLC–MS (Figure 4). A summary of the results are described in the Supporting Information Datasheet SD1 and SD2. A detailed characterization and computational pharmacological analysis of the mixture are beyond the scope of this study and will be published elsewhere. Although it is hard to draw any conclusion from a mixture of ~100 polyphenols either by absorption or by emission, still we tried to find observable change (if any). In brief, the absorption spectra of SKP17LIV01 show no or minimal changes upon gradual addition of Pb\(^{2+}\) (Figure 5a). However, in steady-state fluorescence spectra, quenching was observed (Figure 5b). The Stern–Volmer plot (Figure 5b, inset) suggests the presence of a single type of quenching, which is static in nature, as is also found from time-resolved studies (Figure 5c and Table 1). This is further supported by the calculation of the bimolecular quenching constant, \(k_{Q}(K_m/\tau_0)\), which is found to be ~4.81 × 10\(^{12}\) M\(^{-1}\) s\(^{-1}\) and higher than that of diffusion-controlled limit (~10\(^{10}\) M\(^{-1}\) s\(^{-1}\)).\(^5\) The association constant (\(K_v\)) of SKP17LIV01 with Pb is found to be, \(K_v = 4.26 × 10^{4}\) M\(^{-1}\). It has to be noted that the mixture (SKP17LIV01) of flavonoids and lots of other polyphenols is able to retain the heavy-metal binding activity of constituent molecules. For the ease of extraction from medicinal plants, low cost, and efficient metal binding, the mixture is more attractive as a therapeutic agent than the individual compounds like morin or naringin.

**Effect on Antioxidant Property of the Flavonoids after Heavy-Metal (Pb\(^{2+}\)) Chelation.** Several studies suggested that polyphenols have a number of potential health benefits due, in part, to their antioxidant activity.\(^8,16,43,54,55\) Even in the case of heavy-metal toxicity, this property is vital along with chelation, as this would help to make the healing faster. Whether the flavonoid will retain the antioxidant property even after heavy-metal chelation was the major question. Therefore, we evaluated the antioxidant activity of model flavonoid, morin, before and after Pb(II) chelation using the DPPH assay. Figure 6a shows that morin was able to retain its free-radical scavenging activity even after Pb(II) chelation. The reaction between morin and DPPH occurs in two steps: (i) DPPH absorbance (\(\lambda_{max} = 535\) nm in methanol) decays quickly (typical time, 60–120 s) and (ii) DPPH absorbance decays slowly in ~1 h to reach a constant value. The fast step essentially refers to the abstractions of the most labile H-atom from the antioxidant (3-OH in the case of morin),\(^43,56\) whereas the slow step reflects the remaining activity in the oxidation–degradation products. Binding of Pb\(^{2+}\) to morin slows down the process. The possible cause for this compromised free-radical scavenging activity may be the binding of Pb\(^{2+}\) to the 3-OH of morin that results in abrogation in coplanarity and conjugation of the molecule. Previous studies have shown that planarity permits conjugation, electron dislocation, and a corresponding increase in the flavonoid phenoxyl radical stability.\(^57,58\) We further studied the recyclability of the compounds in DPPH radical scavenging (Figure 6b–d). Interestingly, although morin scavenged the free radical very fast in the first cycle, it failed in successive cycles. In contrast, Pb\(^{2+}\)–morin complex was able to sustainably scavenge free radicals.
radicals up to three cycles. The EC_{50} value of Pb^{2+}−morin complex remained almost the same as morin (Figure 6e), which further signifies the retention of antiradical activity even after heavy-metal chelation. This comes with an increase in the solubility of the chelate in aqueous media than that of free morin (Figure S5). It may be one of the causes of sustainable antioxidant activity of the chelate. The increased solubility is also important in quick removal of the heavy metal from the body. Similar results of sustainable antioxidant activity after Pb chelation were also obtained in the case of MNEA and...
Thus, the metal chelation imparted sustainability to the flavonoid as well as flavonoid–polyphenol mixtures in their antioxidant activity, leading to higher therapeutic efficacy.

**Effect of SKP17LIV01, Real-Life Complex Flavonoid and Polyphenol Mixture, on in Vivo Mouse Model of Pb Toxicity.** Diverse chemical assays have been developed with an aim to mimic the actual situation in human body. However, chemical models can be realistic assumptions only if a positive correlation between chemical and biological models is established. Therefore, we used an animal (Pb-intoxicated Swiss albino mouse) model that perfectly mimics the pathogenicity of heavy-metal-associated disorders, involving a complexity of physiological, immunological, environmental, and genetic phenomena. Pb intoxication causes severe damage to multiple organs leading to morbidity and mortality. According to many preceding studies, liver is one of the major target organs of Pb(II) toxicity.54,59,60

### Figure 7. Effect of SKP17LIV01 on Pb(II)-intoxicated liver. (a) Morphology of the isolated livers. (b) Relative liver weight (liver weight to BW ratio) in the four groups of mice. (c) Change in the ALAD activity, a marker of Pb toxicity. (d) Results of liver function test. Serum AST, ALT, ALP, GGT, total bilirubin, direct bilirubin, and TP. (e) Distribution of Pb in blood and liver as investigated by the ICP-AES method after acid digestion. (f) Histopathological changes of liver from representative mice from each treatment group as observed after H&E staining (original magnification 200×). The liver of mice treated with SKP17LIV01 did not show the inflammatory or necrotic features distinct in the Pb(II)-treated group. (g) Necroinflammatory score and HAI. All data are presented as mean ± SD (*P < 0.05 compared to control, **P < 0.05 compared to Pb(II)-intoxicated group).

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SKP17LIV01 is an example of a real-life complex flavonoid and polyphenol mixture. It is designed to mimic the actual situation in the human body. Chemical models are often used to study the effects of compounds, but they must be realistic assumptions if they have a positive correlation with biological models. In this study, a mouse model of Pb toxicity was used, as it perfectly mimics the pathogenicity of heavy-metal-associated disorders, involving a complex of physiological, immunological, environmental, and genetic phenomena. Pb intoxication causes severe damage to multiple organs leading to morbidity and mortality.

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and ALT levels by 75 and 70%, respectively, compared to the Pb-intoxicated group ($p < 0.001$). Previous studies suggest that returning of aminotransferase levels to normalcy is associated with the healing of liver parenchyma and regeneration of hepatocytes.60 There was also surge in other liver function parameters such as ALP ($\sim 66\%$), GGT ($\sim 120\%$), total bilirubin ($\sim 320\%$), direct bilirubin ($\sim 135\%$), and marked decrease in TP concentration ($\sim 50\%$) in Pb-treated group ($p < 0.001$ compared to the normal ones). Treatment with SKP17LIV01 recovers all the parameters to normal levels (Figure 7b).

As described in Figure 6e, Pb(NO$_3$)$_2$ exposure increased Pb levels both in the blood and liver of mice ($17.34 \pm 0.91 \mu g$ mL$^{-1}$ and $42.63 \pm 2.1 \mu g$ g$^{-1}$, respectively) compared to control ($0.13 \pm 0.09 \mu g$ mL$^{-1}$ and $0.58 \pm 0.11 \mu g$ g$^{-1}$, respectively; $p < 0.001$). We hereby found that SKP17LIV01 significantly decreased Pb in the blood and liver ($1.69 \pm 0.05 \mu g$ mL$^{-1}$ and $2.58 \pm 0.2 \mu g$ g$^{-1}$, respectively; $p < 0.001$). This is consistent with our in vitro spectroscopic studies, where the ability of SKP17LIV01 to chelate Pb$^{2+}$ is found. The microscopic images of H & E-stained liver sections are depicted in Figure 7f. The control group (group I) showed normal hepatocytes and nuclei where the hepatic lobules are hexagonal, clearly distinguishable, separated by interlobular septa, and traversed by portal veins. In Pb-treated animals, the remarkable degenerative histological changes of liver, such as structural damage, disorganization of hepatic chords, cytoplasmic vacuolation, hepatocellular necrosis, leukocyte infiltration, and massive hemorrhage had been observed.64 The SKP17LIV01-treated Pb intoxicated mice (group III) showed almost normal hepatocytes with a mild degree of mononuclear infiltration and necrosis, almost comparable to the control. The significant tissue damage induced by Pb(NO$_3$)$_2$ intoxication might be attributed to its ability to generate ROS that induce oxidative damage in several tissues by enhancing lipid peroxidation.69 Also, the degradative effect of Pb on cellular enzymes, particularly those associated with energy production, leads to hydropic degeneration of mitochondria and causes cytoplasmic vacuolization.65 We applied modified Ishak and METAVIR histological activity index (HAI) (Figure 7g) to assess the necroinflammatory damage caused by Pb(II). The Pb-intoxicated animals scored 15 and 3 (the maximum possible score is 16 and 3), where SKP17LIV01 treatment decreased it to 3 and 0. Thus, as evident from the microscopic images, SKP17LIV01 is able to protect and preserve the normal liver structure even better than conventional drug silymarin.

The circulatory system is one of the target organs of Pb toxicity.66 The results concerning hematological parameters (Table S4) showed a significant ($p < 0.001$) decline in total erythrocyte count, total leucocyte count, Hb concentration, lymphocyte, and monocyte content in the Pb(NO$_3$)$_2$-treated animals (group II), in comparison to control animals ($p < 0.05$), whereas neutrophil content insignificantly increased in Pb(NO$_3$)$_2$-treated group, when compared with control animals ($p < 0.05$). Recovery of the above-mentioned parameters is observed in the SKP17LIV01-treated group.

**Mechanism Behind the in Vivo Protective Effect of SKP17LIV01 Against Pb Toxicity.** A number of in vitro and in vivo studies indicated that Pb causes oxidative stress by inducing the generation of ROS, including hydroperoxides, singlet oxygen, hydrogen peroxide, and superoxide. It has also been observed that Pb can decrease antioxidant enzymatic activity in liver.64 In the present study, the increase in hepatic ROS production manifested in the depletion of SOD, CAT, and GPx levels, the major enzyme triad responsible for scavenging of free radicals inside the body. To be particular, SOD converts superoxide anions to H$_2$O$_2$, which is further converted to H$_2$O with the help of GPx and CAT.67 In this study, 4 weeks of chronic Pb exposure decreased these enzyme levels by 63, 57, and 45%, respectively, compared to control group ($p < 0.05$) and caused severe oxidative damage to the liver cells. However, treatment with SKP17LIV01 helped the Pb-intoxicated mice to replenish their antioxidant enzyme system to normal (increase in SOD $\sim 132\%$, CAT $\sim 115\%$, and GPx $\sim 50\%$; $p < 0.05$ in all cases) (Figure 8a,b).
Thiol-based antioxidant system plays second line of cellular protection against reactive free-radical-mediated oxidative damage in pathophysiological situation.54 Cellular metabolite such as GSH has been presented in Figure 8a. The level of GSH has been considerably decreased because of intoxication along with the increased level of oxidized glutathione. Post-treatment with SKP17LIV01 after the Pb(II) exposure restored the levels of cellular metabolites close to normal, demonstrating its protective nature. Figure 8b provides a schematic overview of the proposed mechanism of action of SKP17LIV01 in the protection of oxidative stress induced by Pb. The model clearly indicates that SKP17LIV01, despite direct sustainable free-radical scavenging activity, boosts the in vivo enzymatic antioxidant defense system resulting in protection from increased levels of ROS. The model is further supported by the observations of lipid peroxidation and protein carbonylation, the two important markers of oxidative stress. The increase in the MDA level (Figure 8c) in the serum and liver (∼3.8 fold compared to control in both cases, p < 0.01) upon Pb(NO₃)₂ treatment suggests enhanced peroxidation, leading to tissue damage and failure of antioxidant mechanism to prevent the formation of excessive free radicals. Post-treatment with SKP17LIV01 (Pb(NO₃)₂ + SKP17LIV01) significantly attenuated these changes (decrease in MDA level by ∼1.8 folds (liver) and ∼1.5-folds (serum) compared to PbNO₃-treated group, p < 0.05 in all cases). The flavonoids and polyphenols exert their antioxidant effects in the body by preventing the generation of ROS, direct scavenging of it, or through enhancement of cellular antioxidant system. Thus, the observed recovery of antioxidant enzyme pool in the animal model of Pb intoxication after the administration of SKP17LIV01 is the consequence of synergistic activity between its various phytoconstituents as evident from our spectroscopic studies. The flavonoids may have played a crucial role in the reduction of the treatment period by controlling the accumulation and bioavailability of Pb(II) in the tissue through complexation, subsequent sustainable antioxidant activity, and removal.

CONCLUSIONS

The disaster due to Pb poisoning is evident from the ancient human civilization till date, although it is concluded to be preventable. Understanding the mechanism of Pb detoxification by low-cost medicinally important molecules and cost-effective herbal mixture is very important for the promotion of lots of folk medicines in developing countries. Here, we have explored the detailed mechanism of Pb detoxification by morin, a model flavonoid, which is omnipresent in medicinal herbs using steady-state and picosecond-resolved optical spectroscopy. We have shown that Pb-morin chelate becomes a sustainable antioxidant revealing dual role of the flavonoid in the heavy-metal detoxification process. Our spectroscopic studies also reveal that the dual nature in the detoxification remains intact in the controlled mixture of few flavonoid and polyphenol molecules (MNEA), and even in a mixture, many such molecules were extracted from medicinal plants (SKP17LIV01). We have studied the efficacy of the complex mixture in the mouse model for the detoxification of Pb poisoning in detail. Subsequent, biochemical, histopathological, and biodistribution studies confirm that the molecular mixture (SKP17LIV01) facilitates excretion of Pb from the mammal (mice) body after heavy-metal chelation and ROS scavenging. The study is expected to have an impact in the discovery of new drugs of ethnobotanical origin.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b02046.

Composition of ingredient(s) present in SKP17LIV01, AST and ALT activity, summary of the hematological parameters, determination of binding constant of the Pb(II)—Morin complex, interaction of Pb(II) with naringin, interaction of Pb(II) with EA, interaction of EA with morin, effect of Pb2+ chelation on aqueous solubility of morin, compounds and metabolites identified in positive mode, compounds and metabolites identified in negative mode of UHPLC-MS (PDF)

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Notes
The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors thank DAE (India) for financial grant, 2013/37P/73/BRNS and DST, India, for financial grants, SB/S1/PC-011/2013. The authors also thank DBT (WB)-BOOST scheme for financial grant, 339/WBBDC/1P-2/2013. The authors thank SAIF, IIT Bombay, for performing HR-LCMS and estimating the Pb content of liver and blood by ICP-AES. The authors would like to thank Anindita Bhattacharya for careful reading of the manuscript and subsequent modifications.

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