Formation of calcium oxalate nanoparticles in leaves: significant role of water content and age of leaves

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Calcium oxalate monohydrate (COM) was synthesized in the presence of leaves which act as a source of oxalate ions. Three different leaves, viz. *Ficus religiosa* (peepal in India), *Heterophragma adenophyllum* (Katsagon) and *Spinacia oleracea* Linn. (spinach) were used for the synthesis, which led to nanoparticles of COM of size 100–300 nm, 40–70 nm and 150–200 nm respectively. The particles are highly monodisperse, spherical and uniform in size as shown by TEM studies. The amount of COM formed varied significantly (35–80%) with the type of leaf used. The yield could be inversely correlated with the water content of the leaves; though water is essential for the formation of COM (dry leaves do not yield COM). Our studies show that the secretion of oxalate ions (to form COM) from the cells occurs even in leaves which have been removed from the living plants and the ability to secrete oxalate ions increases with the age of the leaf. The young leaves of *F. religiosa* do not show COM formation. In the presence of chelating agents (like citric acid) which can bind to Ca^{2+} ions, COM is absent, suggesting that the secretion of oxalate ions is prevented.

Keywords. Biomineralization, calcium oxalate monohydrate, *Ficus religiosa*, leaf age, nanoparticles.

CALCIUM oxalate is an important biomaterial. It is the main constituent in kidney stones[1] and plants. In nature, calcium oxalate exists in three different forms: monoclinic calcium oxalate monohydrate or COM (whewellite) tetragonal calcium oxalate dihydrate or COD (weddellite) and triclinic calcium oxalate trihydrate or COT[2–4]. Among these oxalates, the monohydrate is the thermodynamically stable form at room temperature and is found in abundance in plants[3,4]. The crystals of calcium oxalate can be found at all taxonomic levels from small algae to higher gymnosperms. Leaves play an important role in the formation of calcium oxalate in plants. These are found to have the highest oxalate concentrations compared to roots and other parts. Oxalic acid is synthesized via a number of pathways, viz. conversion of glycolate

and glyoxalate to oxalate, ascorbic acid catabolism or cleavage of isocitrate and oxaloacetate[5–8]. The presence of excess calcium ions, though vital for proper functioning of the bioprocesses, is toxic to plant cells at higher concentration. The cells secrete oxalic acid to sequester calcium ions by precipitating calcium oxalate and control the toxicity[9]. It is also a well-known fact that the presence of complexing agents such as citric acid prevents the formation of oxalates[10,11]. In the present study we have investigated the formation of COM by leaves (of three different plants) in the presence (or absence) of calcium and carbonate ions. Note that we do not add any oxalate ions to obtain calcium oxalate (apart from what is provided by the leaves).

Three different leaves were used to synthesize calcium oxalate, viz. *Ficus religiosa*, *Heterophragma adenophyllum* and *Spinacia oleracea* Linn. Four procedures were undertaken: (A) reaction of Ca^{2+} and CO_3^{2-} in the presence of leaves (all the above-mentioned leaves were used); (B) reaction of Ca^{2+} and leaves (*F. religiosa*) in absence of CO_3^{2-} ion; (C) only *S. oleracea* Linn. leaves in the absence of ions and (D) reaction of Ca^{2+} and leaves (*F. religiosa*) in presence of citric acid (Table 1).

In procedure (A), 15 g of *F. religiosa* leaves were mixed with 85 ml of 0.1 M calcium nitrate tetrahydrate (Merck). The whole system was stirred for 24 h on a magnetic stirrer. In another beaker 15 g of *F. religiosa* leaves were mixed with 85 ml of 0.1 M ammonium carbonate solution (Merck) and stirred for 24 h on a magnetic stirrer. The two systems were subsequently mixed and stirred for another 24 h at pH 6.8. The leaves were separated from the solution and charred at 350°C for 6 h (Table 1, A-1). The resulting powder was subsequently heated at 400°C/10 h, 450°C/12 h, 700°C/12 h, 800°C/12 h and 950°C/12 h. The procedure was repeated with *H. adenophyllum* (pH = 6.9) and *S. oleracea* Linn. (pH = 7.2; Table 1, A-2 and A-3). However, with *S. oleracea* Linn., the leaves could not be separated after the two systems were mixed and stirred for 24 h. The resulting system was centrifuged. The residue was dried at 200°C for 18 h.

In procedure (B), the procedure used in (A) was repeated with only *F. religiosa* leaves and a solution of Ca^{2+} ion (CO_3^{2-} was not added; Table 1).

In procedure (C), *S. oleracea* Linn. leaves were taken without Ca^{2+} or CO_3^{2-} ions. The procedure was the same as that followed for (A) (Table 1).

In a room-temperature synthesis of COM from *F. religiosa* leaves, 1 g of the leaves (three different stages of the leaves, viz. young leaves, intermediate leaves and mature leaves; Figure 1) was mixed with 50 ml of 0.1 M calcium nitrate tetrahydrate (Merck). The whole system was stirred for 24 h on a magnetic stirrer. In another beaker 1 g of *F. religiosa* leaves was mixed with 50 ml of 0.1 M ammonium carbonate solution (Merck) and stirred for 24 h on a magnetic stirrer. The two systems were

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Table 1. Details of the formation of calcium oxalate monohydrate (COM) using different types of leaves. A, B, and C refer to experimental procedures whereas, 1, 2 and 3 refer to type of leaves (1, *Ficus religiosa*; 2, *Heterophragma adenophyllum*; 3, *Spinacia oleracea* Linn.)

Sl. no.	Reactions		Heating condition	XRD
A-1	(I)	15 g of leaves (*F. religiosa*) + 85 ml of 0.1 M $Ca(NO_3)_2 \cdot 4H_2O$	$350^\circ C/6$ h	$CaC_2O_4 \cdot H_2O$ (monoclinic)
	(II)	15 g of leaves (*F. religiosa*) + 85 ml of 0.1 M $(NH_4)_2CO_3$		(80%) + calcite (20%)
		Both systems (I and II) are stirred for 24 h separately and mixed along with solutions and stirred for 24 h again. After 24 h, leaves are separated from the solution.		
A-2	(I)	15 g of leaves (*H. adenophyllum*) + 85 ml of 0.1 M $Ca(NO_3)_2 \cdot 4H_2O$	$350^\circ C/6$ h	$CaC_2O_4 \cdot H_2O$ (monoclinic)
	(II)	15 g of leaves (*H. adenophyllum*) + 85 ml of 0.1 M $(NH_4)_2CO_3$		(50%) + calcite (50%)
		Both systems (I and II) are stirred for 24 h separately and mixed along with solutions and stirred for 24 h again. After 24 h, leaves are separated from the solution.		
A-3	(I)	15 g of leaves (*S. oleracea* Linn.) + 85 ml of 0.1 M $Ca(NO_3)_2 \cdot 4H_2O$	$200^\circ C/18$ h	$CaC_2O_4 \cdot H_2O$ (monoclinic)
	(II)	15 g of leaves (*S. oleracea* Linn.) + 85 ml of 0.1 M $(NH_4)_2CO_3$		(35%) + calcite (65%)
		Both systems (I and II) are stirred for 24 h separately and mixed along with solutions and stirred for 24 h again. The resulting system was centrifuged after 24 h.		
B-1		15 g leaves (*F. religiosa*) + 85 ml of 0.1 M $Ca(NO_3)_2 \cdot 4H_2O$	$350^\circ C/6$ h	$CaC_2O_4 \cdot H_2O$ (monoclinic)
C-3		15 g of leaves (*S. oleracea* Linn.) + 85 ml of double distilled water	$350^\circ C/6$ h	$CaC_2O_4 \cdot H_2O$ (monoclinic)
D-1	(I)	15 g of leaves (*F. religiosa*) + 85 ml of 0.1 M $Ca(NO_3)_2 \cdot 4H_2O$ + 85 ml of 0.1 M citric acid.	$350^\circ C/6$ h	Amorphous
	(II)	15 g of leaves (*F. religiosa*) + 85 ml of 0.1 M $(NH_4)_2CO_3$		
		Both systems (I and II) are stirred for 24 h separately and mixed along with solutions and stirred for 24 h again. After 24 h leaves are separated from the solution.		

Figure 1. The leaves of different ages.

subsequently mixed and stirred for another 24 h at pH 7.312. The leaves were separated from the solution and dried at 60°C in an oven. Control experiments without Ca^{2+} or CO_3^{2-} and in the absence of both the ions were carried out.

The water content of each of the above leaves was determined by heating the leaves in an oven. Fifteen grams of each type of leaf (*F. religiosa*, *H. adenophyllum* and *S. oleracea* Linn.) was taken in a beaker and kept in an oven at 60°C for 48 h. The weight losses were found to be 48%, 60% and 90% in the leaves of *F. religiosa*, *H.*

adenophyllum and *S. oleracea* Linn. respectively. Leaves are easily crushed by hands after drying at 60°C for 48 h.

Powder X-ray diffraction studies (PXRD) were carried out on Bruker D8 Advance diffractometer using Ni-filtered CuK_α radiation. Thermogravimetric analysis (TGA)/differential thermal analysis (DTA) experiments were carried out on a Perkin Elmer Pyris Diamond TGA/DTA system on well-ground samples in flowing nitrogen atmosphere with a heating rate of 5°C/min. IR studies were carried out on a Nicolet Protege 460 FTIR Spectrometer. The data were recorded (with KBr disk) in the range 400–4,000 cm^{-1}.

Transmission electron microscopy (TEM) studies were carried out on FEI Technai G^2 20S-TWIN. Atomic absorption spectroscopy (AAS) was used to obtain the metal content (Ca^{2+} and Zn^{2+}) in the charred leaves using a Perkin Elmer, Analyst 100, Atomic Absorption Spectrometer. Flame photometry studies (sodium and potassium content in the charred product) were performed using CL22A Flame Photometer (Elico, India).

The formation of COM was observed with three different stages of the leaves (*F. religiosa*) – young leaves, intermediate leaves and mature leaves, when Ca^{2+} and CO_3^{2-} solutions containing leaves are mixed together. The formation of COM was observed in the presence of mature leaves up to 46%; the remaining being calcium carbonate (calcite) at room temperature (Table 2). It is known that oxalic acid is present in plants. Excess of free calcium ions is toxic to the plants and in order to reduce the toxic

Table 2. Details of product formation at room temperature

System	Product obtained from supernatant liquid	Residue
Young leaves	100% $CaCO_3$	100% $CaCO_3$
Intermediate leaves	93% $CaCO_3$ and 7% $CaC_2O_4 \cdot H_2O$	58% $CaCO_3$ and 42% $CaC_2O_4 \cdot H_2O$
Mature leaves	84% $CaCO_3$ and 16% $CaC_2O_4 \cdot H_2O$	54% $CaCO_3$ and 46% $CaC_2O_4 \cdot H_2O$

effect of the calcium ions, COM is precipitated and stored in vacuoles from the cells[9]. In plants, specialized cells and/or organic molecules in or around specialized cells govern and mediate crystal formation. The same mechanism is possibly responsible for the high fraction of COM being formed in this case. It is to be noted that there is only calcium carbonate (calcite) formation (Table 2) in the reaction in case of young leaves, whereas COM is present in all three stages of the leaves (young, intermediate and mature). In the mature leaves, there is 16% COM formation in the supernatant liquid (Table 2), which implies that either oxalate ions come out of the leaves or there may be COM formation inside the leaves which is then excreted from the leaves. The PXRD patterns of COM formation are given in the **Supplementary Information (Figures S1–S3)**.

To understand the exact mechanism for formation of COM inside and outside of the leaves, two different types of leaves, viz. *F. religiosa* and *H. adenophyllum* (separate reactions) were stirred with Ca^{2+} and CO_3^{2-} ions at room temperature. The leaves were then charred at 350°C (Table 1, A-1 and A-2). A similar reaction was carried out with *S. oleracea* Linn. (Table 1, A-3) at pH 7.20, where the charring was done at 200°C. The charred products in all the three cases led to COM and calcite (Figure 2 *a–c*). The ratio of calcium oxalate and calcite varied with different leaves. It is to be noted that in spite of mixing calcium and carbonate ions, we obtain monoclinic COM as the major phase, while $CaCO_3$ was the minor phase. Note that similar reaction was also carried out using dry leaves of *S. oleracea* Linn. (dried at 60°C/48 h) with Ca^{2+} and CO_3^{2-}, which results in only calcium carbonate (calcite) at 200°C. IR studies clearly show the absence of $C_2O_4^{2-}$ band. This suggests that calcium oxalate present in the leaves has decomposed or the oxalate ions cannot be released from the dry leaves of spinach. Note that the formation of COM is inversely related to the water content.

The charred product obtained from fresh *F. religiosa* leaves (for procedure (A)) was heated at several temperatures 400°C/10 h, 450°C/12 h, 700°C/12 h, 800°C/12 h and 950°C/12 h. Pure calcite phase was obtained at 400°C. The amount of CaO (formed at 700°C) increases with temperature and the calcite phase decreases. An impurity phase is also seen increasing with temperature. The results obtained are shown in the **Supplementary Information (Table S1)**. Figure 3 *a–c* shows the PXRD pattern obtained from the three different types of leaves after heating the charred product at 450°C.
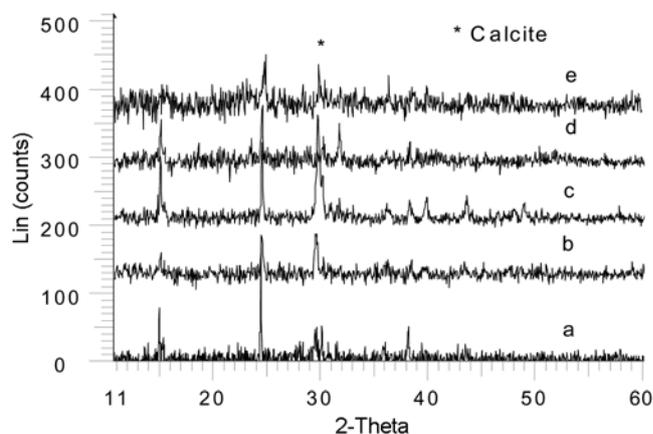

Figure 2. Powder X-ray diffraction pattern of calcium oxalate monohydrate synthesized at 350°C with (a) *Ficus religiosa* (pH = 6.80), (b) *Heterophragma adenophyllum* (pH = 6.90), (c) *Spinacia oleracea* Linn. in the presence of ions (pH = 7.20), (d) *S. oleracea* Linn. in the absence of ions and (e) *F. religiosa* in the absence of CO_3^{2-} ions.
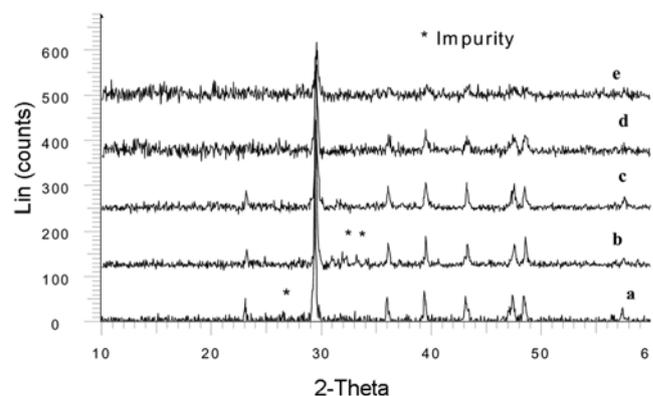

Figure 3. PXRD pattern of sample heated at 450°C with leaves (in the presence of Ca^{2+} and CO_3^{2-} ions) of (a) *F. religiosa*, (b) *H. adenophyllum*, (c) *S. oleracea* Linn., (d) *F. religiosa* in the absence of CO_3^{2-} and (e) *F. religiosa* with ions in the presence of citric acid. All the major lines correspond to calcite (rhombohedral).

We have also carried out the reaction using dry leaves of *F. religiosa* (dried at 60°C/48 h) with Ca^{2+} and CO_3^{2-}. The procedure was the same as that followed for A-1 (Table 1). The PXRD pattern of the product charred at 350°C for 6 h was found to correspond to an amorphous material. However, from IR studies bands corresponding to oxalate group were present, which indicates the presence of calcium oxalate. TGA/DTA studies show only one transition in the range 150–450°C, which corresponds to the loss of one mole of carbon dioxide. The precursor was heated at 450°C for 12 h, which led to the

formation of monophasic calcium carbonate (calcite). This implies that the precursor may be anhydrous calcium oxalate. Hence contrary to the previous case (using dry spinach leaves), even dry leaves of *F. religiosa* are able to release oxalate ions.

The charred product (after heating at 350°C) after the reaction was carried out in the absence of ions (C-3, Table 1) was identified from XRD studies as COM (Figure 2 *d*). This suggests that both the calcium and oxalate ions are provided by the leaves. Further heating to 450°C led to an unidentified compound. Elemental studies (atomic absorption spectroscopy and flame photometry) of the charred product of the pure leaves (after 350°C) show 48.26 mg/g of calcium ions. Our studies also show that zinc is absent, whereas 9.58 mg/g of sodium and 3.67 mg/g of potassium are present, which are higher than the expected amount reported[12] in the *S. oleracea* Linn. leaves. It is possible that the concentration of the above ions varies depending on the soil in which the plants are grown.

In order to study the role of leaves in the formation of calcium oxalate, we carried out a reaction in the absence of carbonate ions with *F. religiosa* leaves (procedure (B)), where we could obtain monoclinic COM (54%) and calcite (46%) (Figure 2 *e*). It is possible that the formation of calcite results from the partial decomposition of COM during charring at 350°C. Further heating at 450°C led to the formation of pure calcite phase (Figure 3 *d*).

There is a competitive formation of $CaCO_3$ and COM in the presence of leaves, which however is crucially dependent on the amount of water present. When only dry leaves (dried at 60°C) were used in the presence of carbonate ions, we could obtain pure calcium carbonate (no COM), which suggests the significant role of water for the formation of COM. XRD data showed that 80% COM and 20% of calcium carbonate is formed in the presence of carbonate ions and normal (not dried) leaves. In the absence of carbonate ions, using the same leaves only COM is formed. We conclude there is no conversion of CO_3^{2-} ions to oxalate ions, using even in the absence of carbonate ions the oxalate (COM) can be obtained.

In the formation of COM, the role of water content appears to be of significance as COM formation is dependent on the age of the leaves (Table 2). In young leaves water content is quite high compared to the intermediate and mature leaves. As the water content is low in the mature leaves, COM formation is much higher. The water content of the different leaves was found by drying the leaves at 60°C/48 h in an air oven. The water content varied from 48%, 60% and 90% in *F. religiosa*, *H. adenophyllum* and *S. oleracea* Linn. leaves respectively. It is interesting to note that the formation of COM decreased, whereas the calcite phase increased as the water content of the leaves increased (Figure 4). Thus it appears that though water is important for oxalate formation, the amount of oxalate is inversely proportional to the amount

of water in the leaves. This is an important result, which may have significant implications. Water is important for stabilizing calcium oxalate in the leaves. However, the amount of calcium oxalate formed may depend on the water content, which varies with the type of leaf.

IR studies for the reaction carried out in the presence of leaves and ions (after heating at 350°C) show the presence of a broad absorption at 3341 cm⁻¹, which could be assigned to O–H stretching frequency (**Figure S4; See Supplementary Information**). Strong bands at 1614 and 1317 cm⁻¹ are also observed. These bands correspond to antisymmetric and symmetric C–O stretching respectively, for the coordinated oxalate group. Bands at 781, 662 and 514 cm⁻¹ were also observed which are characteristic of COM[13].

TGA/DTA studies in all the cases (other than reaction (D)) showed the loss of one water molecule at around 150°C (TG/DTA plot for sample synthesized using *S. oleracea* Linn. leaves is shown in **Figure S5; see Supplementary Information**). Beyond this temperature weight loss is continuous and no sharp transition is observed. From TGA and XRD studies, loss of one molecule of carbon dioxide is confirmed (from anhydrous calcium oxalate), which is observed at around 430°C. The loss of the second molecule of carbon dioxide is observed above 700°C and this leads to the formation of CaO as confirmed by XRD studies.

When the reaction of Ca^{2+} and CO_3^{2-} ions and *F. religiosa* was carried out in the presence of citric acid, we could not obtain calcium oxalate at 350°C (Table 1, D-1). PXRD pattern of the charred product was amorphous. It is possible that initially calcium oxalate did not crystallize since calcium would be bound to citric acid as a complex and hence, the leaves did not sense the increase in calcium ions and did not trigger the release of oxalic acid to form calcium oxalate complex. However, at 450°C the complex decomposed to form calcium carbonate (calcite) (Figure 3 *e*). From TGA data (assuming calcium oxalate as the initial compound) we find that the weight loss is only 5%, which corresponds to 0.37 mol of water molecules per mole of calcium oxalate. Thermal
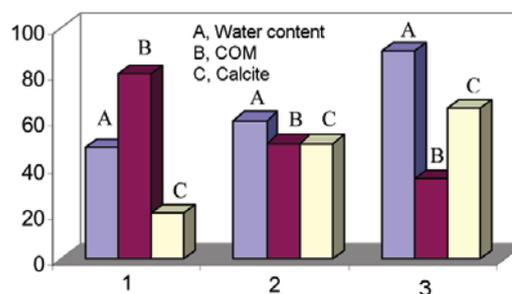

Figure 4. Plot for variation in the percentage of water contents (at 60°C for 48 h) (1, *F. religiosa*; 2, *H. adenophyllum* and 3, *S. oleracea* Linn.) in the leaves and the formation of COM and calcite with different leaves.
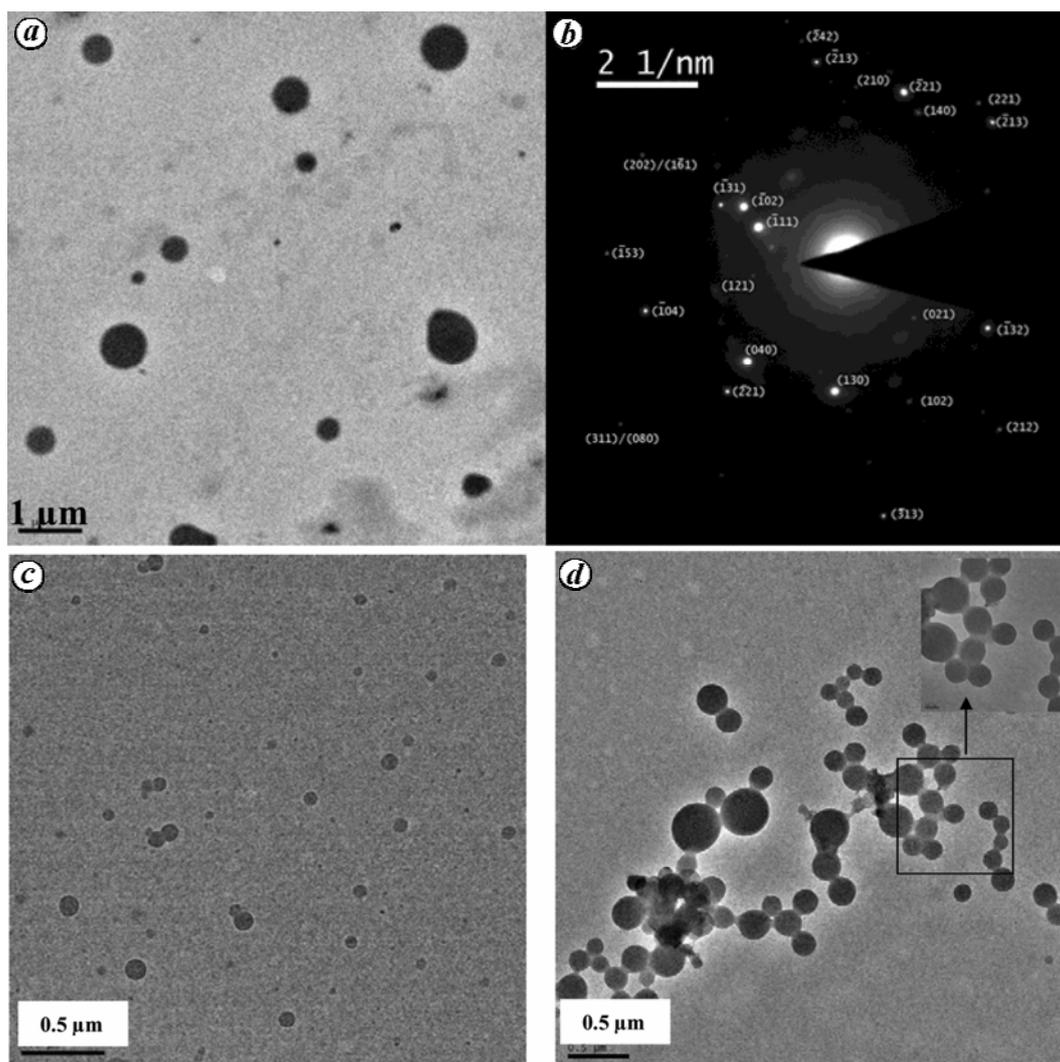
Figure 5. (*a*) TEM micrograph of the product (sample details: Table 1, A1). (*b*) Electron diffraction pattern of COM. (*c*) TEM micrograph of COM (sample details: Table 1, A2). (*d*) TEM micrograph of COM (sample details: Table 1, A3).

analysis thus confirms that the amorphous compound is not COM. A band at 1608 cm^{-1} indicates the presence of C–O stretching. It is possible that the amorphous compound has a carboxylate group, which shows this band.

TEM image (obtained from the reaction A-1 after charring at 350°C) shows that the nanoparticles of COM are monodisperse and spherical in shape (Figure 5). The particles are of 100–300 nm in size. When *H. adenophyllum* leaves were used for the synthesis of COM (A-2), the particles were also monodisperse, spherical and nearly uniform in shape and the average size of the particles was found to be 40–70 nm (Figure 5 *c*). The electron diffraction pattern (Figure 5 *b*) also shows the formation of COM. In the case of *S. oleracea* Linn. leaves (A-3), particles form aggregates (5–6 spherical particles) in a curvilinear fashion and the average size of each particle of this aggregate was found to be around 150–200 nm as observed from TEM (Figure 5 *d*).

From solutions of calcium and carbonate ions in the presence of leaves, COM along with calcite was obtained. COM formation in the supernatant reveals that either oxalate ions are coming out of the leaves (in the presence of excess Ca^{2+} ions), or COM is formed inside the pores of the leaves and then excreted into the supernatant. The ratio of COM to calcite varies with different templating leaves due to variation in the water content in the leaves. COM formation is inversely proportional, whereas the calcite phase is directly related to the water content of the leaves, which is evident from the reactions with leaves of different ages. Dry leaves of spinach (with Ca^{2+} and CO_3^{2-}) did not yield COM, implying water in the leaves is important for oxalate formation, though the amount of COM formed is inversely related to the water content as mentioned above. It was found that in the presence of a chelating agent (citric acid which binds to calcium ions) secretion of oxalate ions from the leaves is prevented.

This is an evidence of the calcium sensing mechanism present in the leaf. The size, morphology and ratio of COM depend on the type of leaf used as template. Spherical COM particles (40–70 nm) were obtained from reactions in the presence of *H. adenophyllum* leaves, whereas spinach leaves led to the formation of aligned nanostructures consisting of spherical particles of diameter of 100–150 nm.

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A comparative study of deglaciation in two neighbouring basins (Warwan and Bhut) of Western Himalaya

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Glaciers of the Himalaya contribute significantly in the processes linking atmosphere, biosphere and hydrosphere, thus need to be monitored in view of the climatic variations. In this direction, many studies have been carried out during the last two decades and satellite-based multispectral data have been used extensively for this purpose throughout the world. The present study is aimed at mapping of glaciers in two adjacent basins (Warwan and Bhut) of the Western Himalaya with almost similar altitude and latitude and comparing the changes in the two time-frames with respect to three parameters, i.e. area, debris cover and area–altitude distribution of glaciers. The two time-frames are topographical maps of 1962 and IRS LISS III images of 2001/02. Deglaciation was observed in both the basins with 19% and 9% loss in the glaciated area in Warwan and Bhut respectively. This difference may be due to: (i) the smaller size of the glaciers of the Warwan Basin (e.g. 164 glaciers having < 1 sq. km area in comparison to 101 glaciers in the Bhut Basin), (ii) lower percentage of moraine cover in Warwan (18) than in the Bhut Basin (30) and (iii) higher percentage of glaciated area lying below 5100 m (80) in Warwan than in the Bhut Basin (70).

Keywords: Bhut, deglaciation, glacier, Warwan.

GLACIERS of the Himalaya contribute significantly to the processes linking atmosphere, biosphere and hydrosphere. The processes are realized through: (i) occurrence of high albedo from the snow and glacier cover; (ii) perennial flow of freshwater to all the rivers originating in the mountainous region; (iii) variation of glacier extent with respect to climate change, thus acting as sensitive indicators; (iv) percolation of melt water to groundwater storage in the mountains; (v) transportation of enormous sediment load; (vi) reshaping the peri-glacial geomorphology and (vii) maintaining and regulating the weather pattern through the frozen state. The water resource produced by the melting of snow and glaciers of the Himalaya has sustained a large population since historical times (currently almost 800 million people) living in the