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VITAMIN D_3 AND ITS METABOLITES IN THE TOMATO PLANT

T. P. PREMA and N. RAGHURAMULU*

Department of Endocrinology and Metabolism, National Institute of Nutrition, Jamai Osmania PO, Hyderabad-500 007, India

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Key Word Index—*Lycopersicon esculentum*; Solanaceae; leaves; fruit; vitamin D_3; 25-OH-D_3; 1,25-$(OH)_2D_3$; free metabolites; glycosidic metabolites.

Abstract—The tomato plant has been demonstrated to have vitamin D-like activity. The activity was present in the leaves but not in the fruit of the plant. The chloroform extract of the leaves (containing free vitamin D and its metabolites) and the ethanol extract of the residue (containing the glycosidic forms) were partially purified by column chromatography. The fractions corresponding to authentic vitamin D_3, 25-hydroxy vitamin D_3 and 1,25-dihydroxy vitamin D_3 were tested for biological activity and analysed by HPLC. The results indicate that the plant contains vitamin D_3, 25-hydroxy vitamin D_3 and 1,25-dihydroxy vitamin D_3 and their glycosidic forms. Free vitamin D_3 was observed to be the major active principle and the concentration of the free forms of the metabolites was higher than the corresponding glycosides.

INTRODUCTION

Ingestion of certain plant species is known to cause 'Calcinosis' in grazing animals, which was attributed to the vitamin D-like activity of these plants [1–3]. Studies carried out in the calcinogenic plants *Solanum malacoxylon* and *Cestrum diurnum* have revealed the active principle to be a glycoside of 1,25-dihydroxy vitamin D_3 (1,25-$(OH)_2D_3$), the hormonally active form of vitamin D_3 in higher animals [4, 5]. In *S. malacoxylon*, in addition to 1,25-$(OH)_2D_3$ glycoside, glycosides of vitamin D_3, 25-hydroxy vitamin D_3 (25-OH-D_3) and 24,25-dihydroxy vitamin D_3 (24,25-$(OH)_2D_3$) were also present [6–8]. Furthermore, Weissenberg *et al.* [9] recently demonstrated 1,25-$(OH)_2D_3$ activity both in aglycone and glycoside forms not only in leaves but also in berries, stems and roots of *S. malacoxylon*. In *C. diurnum*, although earlier work had shown the presence of only a glycoside of 1,25-$(OH)_2D_3$, we recently demonstrated the presence of free 1,25-$(OH)_2D_3$ in a much higher concentration than the glycosidic form [10]. In addition, we also detected vitamin D_3 and 25-OH-D_3 and their glycosides in this plant.

Most of the calcinogenic plants so far discovered belong to either the Solanaceae or the Gramineae. Since some of the vegetable plants also belong to family Solanaceae, we investigated whether the vegetable plant *Lycopersicon esculentum* (tomato) has any vitamin D-like activity. In the present paper, we report for the first time the occurrence of vitamin D-like activity in the leaves of this plant. However, the fruit of the plant was devoid of vitamin D-like activity. This report also presents evidence for the presence of the free and glycosidic forms of vitamin D_3, 25-OH-D_3 (only free) and 1,25-$(OH)_2D_3$, the major active principle being free vitamin D_3.

*Author to whom correspondence should be addressed.

RESULTS AND DISCUSSION

A vitamin D-deficient rat model was used to test for the presence of vitamin D-like activity in *L. esculentum* leaves and fruit. Rats raised on a vitamin D-deficient diet for four to five weeks developed vitamin D deficiency characterized by reduced calcium transport activity, serum calcium, bone ash and elevated serum phosphorus and alkaline phosphatase levels (Table 1). These effects were reversed by supplementation with vitamin D_3 (Table 1). Incorporation of *L. esculentum* leaf powder in the diet at the 2% level significantly corrected the altered vitamin D-dependent parameters (Table 1), though the effect was not comparable to that observed in vitamin D_3 treated rats. The partial reversal of the alterations observed in vitamin D deficiency on leaf powder feeding could be due to the lower concentration of the active principle in the test material. On feeding a higher level (5%) of *L. esculentum* leaf powder, a further elevation in the serum calcium and correction of other parameters was observed (Table 1) which was comparable to vitamin D_3 treated rats. The calcium transport activity, however, remained almost the same at both the levels tested. The reason for this is not clear. But, the fact that near normalcy was achieved by feeding *L. esculentum* leaf powder at the 5% level clearly indicated the presence of vitamin D-like activity in the leaves. The fruit of the plant had no effect on vitamin D-deficient rats (Table 1) suggesting the lack of vitamin D-like activity.

Table 1. Effect of *L. esculentum* leaf and fruit powders on vitamin D-deficient rats

Group	Body weight gain (g)	Calcium transport (S/M)	Serum Ca (mg dl^{-1})	Serum P (mg dl^{-1})	Serum alkaline phosphatase (U/L)	Bone ash (%)
Control	68.7±1.2*	3.0±0.15*	10.9±0.23*	9.0±0.12*	102.8±7.01*	51.3±0.82*
Vitamin D-deficient	30.9±1.55†	1.7±0.07†	5.2±0.17†	11.7±0.03†	220.1±14.13†	41.0±0.80†
Replet with vit D_3	71.3±2.81*	3.4±0.19*	9.9±0.14*	8.8±0.16*	91.9±4.38*	48.3±0.74*
+ 2% *L. esculentum* leaf	55.1±3.38‡	3.2±0.32*	6.7±0.21‡	11.0±0.40†	129.6±4.59‡	45.5±0.46‡
+ 5% *L. esculentum* leaf	55.3±3.25‡	3.1±0.11*	8.5±0.08§	8.8±0.38‖	99.7±6.7*	51.3±0.31*
+ 2% *L. esculentum* fruit	28.7±2.72†	1.8±0.11†	4.4±0.17‖	12.9±0.62‡	163.2±5.5‖	41.9±0.41†

All values are mean±S.E.M.

S/M – ratio of ^{45}Ca from the serosal side to the mucosal side.

Values bearing different symbols are significantly different as determined by ANOVA ($P < 0.05$).

The identification of the active principle in the leaves of *L. esculentum* was next attempted. Some of the calcinogenic plants were earlier demonstrated to contain vitamin D_3 metabolites as glycosides, which were soluble in polar solvents but not in $CHCl_3$ [11–13]. Evidence which supported the glycosidic nature of the active principle were (1) the higher biological effectiveness of the leaf extract when given orally rather than parenterally [14] and (2) glycosidic cleavage being a prerequisite for the active principle to bind to the 1,25-$(OH)_2D_3$ receptor [15, 16]. However, some studies have reported the biological effectiveness of the plant factor when given intraperitoneally [17, 18]. Furthermore, Procsal *et al.* [19] demonstrated that the unhydrolysed *S. malacoxylon* factor could compete with ^{3}H-1,25-$(OH)_2D_3$ for its receptor. These observations suggest the possible presence of free vitamin D_3 metabolites in calcinogenic plants. In fact, the aglycone activity of vitamin D_3 was demonstrated in *S. malacoxylon* leaves, berries, roots and stem in addition to the glycoside forms [9]. Also, we have recently shown the occurrence of free vitamin D_3 metabolites in $CHCl_3$ extract of *C. diurnum* leaves and the corresponding glycosides in the residue [10].

In the present study, the $CHCl_3$ extract of *L. esculentum* leaves increased the serum calcium levels significantly within 48 hr when administered intraperitoneally to vitamin D-deficient rats (Table 2). In addition, the residue (residue I, after $CHCl_3$ extraction) was also biologically active (Table 2). The vitamin D activity of residue I was soluble in EtOH as shown by the absence of any effect of the residue (residue II) remaining after EtOH extraction on vitamin D-deficient rats (Table 2). Based on these observations, it was presumed that the $CHCl_3$ extract contained free vitamin D metabolites while the EtOH extract contained the corresponding glycosides. The plant sterols in the $CHCl_3$ extract were partially purified by column chromatography and the fractions corresponding to authentic vitamin D_3, 25-OH-D_3 and 1,25-$(OH)_2D_3$ were collected. All three fractions increased the serum calcium levels significantly in vitamin D-deficient rats when administered intraperitoneally (Table 3), lending further support to our contention that the $CHCl_3$ extract contained free vitamin D metabolites. The chemical identity of these fractions was also established by HPLC. These fractions showed similar elution properties as their respective standards in reverse phase and normal phase HPLC systems which separate vitamin D_3 metabolites with high resolution. In addition, they comigrated with respective authentic unlabelled and radiolabelled standards. It has been reported earlier that a clear separation between vitamin D_2 and vitamin D_3 metabolites can be achieved on normal phase HPLC [20]. In the present study, the sample peaks of various fractions tested had the exact R_fs as the authentic standards of vitamin D_3 or its metabolites. This strongly suggests that the plant contains vitamin D_3 but not vitamin D_2 metabolites.

The EtOH extract, after glycosidase treatment and partial purification by column chromatography, was also analysed by HPLC. The fractions corresponding to vitamin D_3 (only reverse phase) and 1,25-$(OH)_2D_3$ (both reverse and normal) had UV absorbing peaks identical to those of their respective standards and co-eluted with corresponding authentic unlabelled and radiolabelled standards. However, the fraction corresponding to 25-OH-D_3 did not give a peak corresponding to its standard in either reverse phase or normal phase systems. The untreated EtOH extract of the leaves contained no detectable levels of vitamin D_3 or its metabolites. This establishes that the residue, in

Table 2. Effect of the $CHCl_3$ extract and the residues of *L. esculentum* leaves on serum calcium levels in vitamin D-deficient rats

Test material	Serum calcium (mg dl^{-1}) Before	After
$CHCl_3$	5.5±0.29*	8.2±0.20†,§
Residue I (after $CHCl_3$ extraction)	5.2±0.39*	7.2±0.40‡,‖
Residue II (obtained by EtOH extraction of Residue I)	7.0±0.20*	6.9±0.07*,‖

Values are mean of three determinations. The serum calcium levels were not altered by vehicle treatment alone. Variation in superscripts between mean values indicates significant differences when compared horizontally (paired 't' test).

†$P < 0.001$, ‡$P < 0.05$ } compared to *.

§Estimated 48 hr after intraperitoneal injection.

‖Estimated after 4 days of feeding.

Table 3. Effect of Sephadex LH-20 fractions corresponding to authentic vitamin D_3, 25-OH-D_3 and 1,25-$(OH)_2D_3$ of *L. esculentum* leaves ($CHCl_3$ extract) on serum calcium levels in vitamin D-deficient rats

	Serum calcium (mg dl^{-1})	
Fraction tested	Before	After 48 hr
Vitamin D_3	5.3±0.15*	7.5±0.45†
25-OH-D_3	4.9±0.10*	6.3±0.17†
1,25-$(OH)_2D_3$	5.1±0.29*	7.1±0.19†

Values are mean of three determinations.

The serum calcium levels were not altered by vehicle treatment alone. Variation in superscripts between mean values indicates significant differences when compared horizontally (paired 't' test).

*$P < 0.01$ compared to †.

fact, contains the glycosides of vitamin D_3 or its metabolites. On quantitation, it was observed that of all the various metabolites detected, the concentration of vitamin D_3 was the highest (Table 4) indicating that it could be the major active principle in *L. esculentum* leaves. Further, the concentration of the free forms of vitamin D_3 and its metabolites were higher than their corresponding glycosides.

In conclusion, the leaves of tomato have been shown to be a good source of vitamin D and its metabolites. The leaves of this plant which are usually discarded thus appear to have a potential use in both human and veterinary medicine.

EXPERIMENTAL

Young apical leaves and fruit of *L. esculentum* (variety pusa ruby) were obtained from plants grown in the Institute garden. The leaves and the fruit were either dried at room temp and used for animal feeding experiments or, in the case of leaves, lyophilized and used for identification of the active principle. The calcium and phosphorus content of the leaves was estimated to be 2% and 0.5% of the dry wt respectively, while the fruit had 0.5% calcium and 0.3% phosphorus (expressed as dry wt).

Vitamin D-like activity in L. esculentum *leaves and fruit.* Vitamin D-deficient rats were used to test for the presence of vitamin D activity in the leaves and fruit of *L. esculentum.* Vitamin D deficiency was developed in male weanling Wistar/NIN rats as described earlier [21]. Feeding a vitamin D-deficient diet to weanling rats resulted in hypocalcaemia (indicator of vitamin D deficiency) by 4 weeks. A group of 10 rats fed a vitamin D_3 replete diet from weanling served as control. The vitamin D-deficient rats were divided into 5 groups of 10 rats each. The first group of rats were kept on the vitamin D-deficient diet while the second group received the same diet with an oral dose of 25 IU vitamin D_3/day in cottonseed oil. The third and fourth groups of rats were given the vitamin D-deficient diet supplemented with either 2% or 5% *L. esculentum* leaf powder. The fifth group of rats received 2% fruit powder mixed in the vitamin D-deficient diet. After two weeks, the animals were sacrificed for the measurement of vitamin D-dependent parameters. Intestinal calcium transport was determined in everted gut sac as described by Martin and Deluca [22]. Serum calcium was estimated by atomic absorption spectroscopy [21], serum phosphorus by the method of Chen *et al.* [23], serum alkaline phosphatase by the method of King and Armstrong [24] and bone ash as described earlier [21].

The effect of the calcium and phosphorus contributed by the leaf powder (2% or 5%) and fruit powder was compensated for by the addition of similar amounts to the diet of the animals on the vitamin D deficient regime.

Biological activity of the $CHCl_3$ extract and the residue of L. esculentum *leaves.* Lyophilised leaves of *L. esculentum* were extracted with 10 vol. of $CHCl_3$ (Extract I). The residue (Residue I) was further extracted with 10 vol. of EtOH (Extract II). The $CHCl_3$ extract, the residue after $CHCl_3$ extraction (Residue I) and the residue after EtOH extraction (Residue II) were tested for biological activity in vitamin D-deficient rats. The $CHCl_3$ extract after evaporation under N_2 was reconstituted in 0.3 ml propylene glycol and administered intraperitoneally to vitamin D-deficient rats. Serum calcium levels were determined before and 48 hr after administration in blood obtained by orbito sinus puncture using atomic absorption spectroscopy [21]. Control animals were administered the vehicle alone. The residues I and II were incorporated into the diet at the 2% level and fed to vitamin D-deficient rats. The serum calcium levels were determined before and after 4 days of feeding the test materials.

Vitamin D metabolite profile in L. esculentum *leaves.* The lyophilized leaf powder was initially extracted with $CHCl_3$ as described earlier (Extract I) [10]. After ensuring that all free vitamin D_3 metabolites were extracted (i.e. the last $CHCl_3$ extract had no tritiated vitamin D_3 metabolites), the residue was allowed to dry. It was then extracted with 10 vol. EtOH (Extract II) after addition of trace amounts (20 000 dpm each) of [1α, 2α, (n)^{3}H] vitamin D_3 (Sp. act. 10 Ci $mmol^{-1}$), 25-OH-[23,24 (n)^{3}H] D_3 (Sp. act. 107 Ci $mmol^{-1}$) and 1,25-$(OH)_2$ [23,24 (n)^3 H] D_3 (Sp. act. 101.5 Ci $mmol^{-1}$) to monitor recoveries. The EtOH extract was evapd *in vacuo* and the residue was reconstituted in citrate-Pi buffer (pH 5.0) and incubated with almond β glucosidase (1 mg) as described earlier [5]. The free sterols released were extracted by the method of Bligh

Table 4. Concentration of free and glycosidic vitamin D_3 and its metabolites in *L. esculentum leaves*

	*Concentration ($\mu g\ kg^{-1}$ dry leaf)	
Metabolite	Free	Glycosides
Vitamin D_3	778	18
25-OH-D_3	22	N.D
1,25-$(OH)_2D_3$	100	18

*Values are average of two determinations

N.D.: not detectable.

and Dyer [25] and the $CHCl_3$ phase was collected.

The plant sterols of the $CHCl_3$ extract and those of the residue were partially purified on Sephadex LH-20 (1 × 15 cm) [26], alumina (modified procedure of Rambeck *et al.* [27]) and Sephadex LH-20 (1.5 × 3.9 cm) [26] columns successively. The columns were calibrated for the elution positions of vitamin D_3, 25-OH-D_3 and 1,25-$(OH)_2D_3$ using radioactive standards prior to sample analysis. The $CHCl_3$ extract was initially chromatographed on a Sephadex LH-20 (1 × 15 cm) column with $CHCl_3$–*n*-hexane (13:7) as the eluting solvent. The pooled fractions corresponding to authentic vitamin D_3 and 25-OH-D_3 from this column was further chromatographed on an alumina column as described earlier [10]. The frs corresponding to vitamin D_3 and 25-OH-D_3 from this column and 1,25-$(OH)_2D_3$ from the Sephadex LH-20 column (1 × 15 cm) were further rechromatographed individually on a longer Sephadex LH-20 column (1.5 × 39 cm) with $CHCl_3$–MeOH–*n*-hexane (75:2:23) as the eluting solvent. The frs corresponding to authentic vitamin D_3, 25-OH-D_3 and 1,25-$(OH)_2D_3$ were collected and the biological activity of these frs was tested on vitamin D-deficient rats as described for the $CHCl_3$ extract. The free sterols released by glycosidase treatment of the EtOH extract were also partially purified employing Sephadex LH-20 (1 × 15 cm and 1.5 × 39 cm) columns. An alumina column was not employed for this extract. The recoveries of various metabolites tested were calcd by determining the radioactivity in a suitable aliquot at all steps of purification and the overall recovery ranged between 85–98%.

The various frs collected from Extracts I and II were analysed both on reverse phase (Zorbax-ODS, 15 × 0.46 cm–modified procedure of Takeuchi *et al.* [28]) and normal phase (Zorbax-SIL, 15 × 0.46 cm) [20] HPLC. The solvent system used for reverse phase was MeOH-MeCN (1:1). In normal phase, iso-PrOH–*n*-hexane (1:9) was used for analysis of 1,25-$(OH)_2D_3$ and iso-PrOH–*n*-hexane (1:39) for 25-OH-D_3 analysis. Vitamin D_3 and its metabolites were identified by UV monitoring at 265 nm and comparison of the R_fs with those of corresponding authentic standards. Further identification was achieved by co-chromatography with respective authentic and radiolabelled standards. The various metabolites tested were quantitated by comparing the sample peak areas from respective standard plots and after appropriate recovery correction.

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