Detection of ptaquiloside and quercetin in certain Indian ferns

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Enzootic bovine haematuria (EBH), which is known to be caused by the interaction of bracken fern toxin (ptaquiloside) and bovine papillomavirus-2 (BPV-2) is a chronic incurable neoplastic ailment of urinary bladder in cattle in certain regions of the world. The present study was aimed to quantify the carcinogen, ptaquiloside (Pta) and flavonoid, quercetin in various ferns growing in certain EBH enzootic areas of two mountainous states of northern India, Himachal Pradesh (HP) and Uttarakhand, where this ailment is a major problem in hill cattle. Of the 25 studied samples of 16 species of ferns, 9 samples collected from both the states were found to contain Pta on HPLC and LCMS analyses. The concentration of Pta present in the fern samples ranged from 0.00 to 1182.84 μg/g on a dry-matter basis. Further, seven samples revealed the potential to contain Pta. *Dryopteris cochleata, Hypodematium crenatum, Pseudocyclosorus canus* and *Pteris cretica* were identified to contain Pta for the first time. Quercetin was also analysed by TLC in 32 fern samples and was detected in 17 samples with its concentration ranging from 0.0% to 0.030%. Samples from HP were found to contain a variable range of quercetin in *Christella arida, Deparia japonica, Dryopteris cochleata, Dryopteris juxtaposita, H. crenatum, Polystichum squrrosum* and *Pteridium revolutum.* Similarly, *Polystichum squarrosum* and *Pteris cretica* from Uttarakhand contained higher concentration of quercetin. The study also revealed that ferns like *D. cochleata, H. crenatum, Onychium tenuifrons, Pseudocyclosorus canus, Pteridium revolutum* and *Pteris cretica* contained both Pta and quercetin. The environmental toxin and flavonoid present in these non-bracken fern species are suspected in the causation of EBH along with BPV-2.

Keywords: Fern, haematuria, hill cattle, ptaquiloside, quercetin.

BRACKEN fern is widely distributed in uplands and marginal areas throughout North and South America, Europe, Australia and Asia. In India, it is present in and around grazing lands and forest areas throughout the Himalayas between 1800 masl to 2400 masl. Bracken fern (*Pteridium* spp.), one of the most abundant plants on the planet, is well known to cause cancer naturally in cattle. At certain places, it contains extremely high concentrations of ptaquiloside (Pta), which almost certainly is its major environmental carcinogen. There is epidemiological evidence that the bracken carcinogen, in special situations, may cause cancer in man. Pta in animal models of carcinogenesis also offers a good tool for the study of cancer[1]. Bracken contains many obnoxious metabolites which contribute to its status as one of the five worst weeds in the world. It has been well proved that regular consumption of bracken fern causes haematuria and cancer in cattle in endemic areas[2–4]. Pta, a water-soluble, norsesquiterpenoid glycoside is reported to be clastogenic, mutagenic and teratogenic. It is activated in the alkaline urinary pH of the bovine urinary bladder, thus causing tumours of the urinary bladder in cattle. It can alkylate DNA *in vitro* causing modifications both of bases and phosphate leading to cleavage of DNA notably at N3 alkyl adenine in a sequence-specific manner[5].

Pta concentrations were determined in 26 bracken fern samples of Uttarakhand and Tamil Nadu[6]. These ferns were found to contain a range of 0–1026.40 mg/kg of Pta on a dry-matter basis. Of these, two samples contained 0, 6 < 10, 7 < 50, 2 < 100, 5 < 250, 2 < 500, 1 < 1000 and 1 > 1000 mg/kg Pta. Compared to reports from Australia and New Zealand[7], Indian bracken was shown to contain low levels Pta.

Another potential mutagen present in bracken is quercetin (3,3,4,5,7-pentahydroxyflavone), a well-known flavonoid which has been found to be genotoxic and mutagenic, but its role in carcinogenesis has not been studied extensively[8]. Quercetin is a plant-derived flavonoid found in fruits, vegetables, leaves and grains. It is classified as IARC group-3 substance (no evidence of carcinogenicity in humans). In cattle, there is a synergistic interaction between bovine papillomavirus-2 (BPV-2) infection and exposure to quercetin, promoting bladder neoplasia, clinically presenting as enzootic haematuria.

The mechanism of action of quercetin includes antioxidative[9,10], direct radical scavenging[11], inducible nitric oxide synthesis inhibitory action[12], xanthine oxidase inhibitory action[13], modulation of gene expression[14] and interaction with other enzyme systems[15]. Quercetin raises a paradox in living cells in that the antioxidant directs oxidative damage selectively to thiol arylation. The quercetin paradox is that in the process of offering protection, it is converted into a potential toxic product[16].

Besides bracken, some other ferns were also reported to contain quercetin. Marsileaceous fern, *Pilularia globulifera*, is reported to accumulate the quercetin glycosides: 3-glucoside, 3-rhamnoside, 3-xyloside, 3-arabinopyranoside, 3-arabinofuranoside and 3,7-di-rhamnoside.

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Kaempferol 3-glucoside is also present and kaempferol 3,7-dirhamnoside is its major component. In some members of Marsileaceae, 7-*O*-glycosylation is present[17]. Two new flavonol glycoside compounds of quercetin 3-*O*-glucoside-7-*O*-neohesperidoside (I) and quercetin 3-*O*-galactoside-7-*O*-neohesperidoside (II) have been found in the fern *Cheilanthes fragrans*[18]. Kaempferol 3-*O*-(acetylrutinoside), a new flavonoid and two new fern constituents, quercetin 3-*O*-(acetylglucoside) and 3-*O*-(acetylrutinoside) have been reported from *Dryopteris villarii*[19]. In addition, quercetin 3-*O*-(glucosylrhamnoside) was also reported from *D. villarii*[20]. In *Adiantum lunulatum* 0.051% quercetin was detected[21].

Nineteen of 31 ferns tested by chemotaxonomic methods in Japan have been found to contain Pta and/or related carcinogens, as have *Cheilanthes sieberi* and *Pteridium esculentum*[22]. Besides bracken, some other ferns like *Onychium contiguum* and *C. sieberi* have been suspected to be associated with enzootic bovine haematuria (EBH), causing carcinogenesis in laboratory[23] and farm animals[24]. Pta was estimated in non-bracken fern samples from the northern Indian mountainous state of Uttarakhand[25]. Of these, only *O. contiguum* was found to contain high levels, i.e. 449–595 mg/kg of Pta on a dry-matter basis. The samples of *O. contiguum* were collected from high altitudes of the Himalayas (districts Chamoli and Uttarkashi in Uttarakhand), which are enzootic for EBH. *O. contiguum* has been experimentally proved as carcinogenic in guinea pigs[23]. Samples of *Diplazium esculentum*, *Polystichum squarrosum*, *Dryopteris juxtaposita*, *Cheilanthes farinosa* and *Christella dentata* were found to contain very low levels of Pta (0.40–30.50 mg/kg)[25].

The present study was aimed at determining the levels of Pta and quercetin in bracken and some other ferns from certain areas of Himachal Pradesh (HP) and Uttarakhand in northern India, where EBH is prevalent.

Ferns from certain EBH endemic regions of HP and Uttarakhand were surveyed. Thirty-two samples were collected close to Mukteswar, Nainital District and Ranikhet, Almora District, Uttarakhand and from Chamba and Kullu Districts, HP. Identification of fern samples was done in the Pteridology Laboratory, Department of Botany, Government Post Graduate College, Pithoragarh. These samples were shade-dried and then ground into fine powder. Only 25 samples were sent to Plant and Food Research, Hamilton, New Zealand for Pta estimation. A part of all 32 samples was sent to the National Botanical Research Institute, Lucknow for estimation of quercetin.

The concentration of Pta and its major breakdown product, pterosin B (PtB), in the fern samples was determined using reversed phase HPLC method[26]. Briefly, Pta and PtB were extracted from the milled samples by shaking with Milli Q-water (1 g sample in 25 ml water) at room temperature for 1 h. The Pta concentrations in the aqueous extracts were determined following removal of many co-extractives, including PtB by the clean-up of an aliquot of extract through polyamide 6S resin (Riedel-de Haen, Seelze, Germany) and subsequent conversion by base-heat-acid treatment of the remaining Pta into the more stable PtB for analysis. To determine the total Pta + PtB present in the samples, a second aliquot, without polyamide 6S clean-up, was converted and analysed for PtB. After HPLC analysis, all samples found positive for PtB were reanalysed by LCMS to unequivocally confirm the identity of the PtB peak. LCMS was performed on a LCQ Deca Ion Trap mass spectrophotometer fitted with an ESI interface and coupled to a Surveyor HPLC. MS data were acquired in the positive mode and peak identity was confirmed with MS/MS scans.

For the analysis of quercetin, 1 g of the powdered sample was exhaustively extracted with methanol (4 × 5 ml, each time for 15 min) under reflux in a water bath at 100°C. The combined extracts were filtered through Whatman No. 1 filter paper (separately for each sample), concentrated under reduced pressure and lyophilized. The methanolic extract was defatted with hexane. The weight of the defatted extract was recorded. Accurately weighed 10 mg of the defatted extract was dissolved in 1 ml of methanol and filtered through a 0.45 μm filter membrane; the filtrate was used as the sample solution. A solution (10 mg ml^{-1}) of these extracts was prepared in methanol for quantification of quercetin. A standard solution of quercetin (0.1 mg/ml) was prepared by dissolving accurately weighed 1 mg of quercetin in 10 ml of methanol.

TLC was performed on 10 cm × 20 cm Higlachrosep plates coated with 0.2 mm layers of silica containing UV 254 fluorescent indicator (S.D. Fine Chemicals, India). Samples (15 μl) were applied as bands 5 mm wide, 10 mm apart, 10 mm from the bottom edge, starting 10 mm from the edge of the HPTLC plate with Hamilton syringes (100 μl) using Linomat 5 applicator (CAMAG). Aliquots of standard markers of quercetin (0.5, 1.0 and 1.5 μg/band) were also applied on silica plates by means of a Camag (Switzerland) Linomat V sample applicator. The plates were developed to a distance of 8.0 cm with 25 ml toluene–ethyl acetate–formic acid (7 : 3 : 1 v/v/v/v) as the mobile phase, in a Camag twin-trough chamber previously saturated with mobile phase vapour for 30 min at 24°C. After removal from the chamber, the plates were completely dried in air at room temperature (24°C). Densitometric scanning was performed on CAMAG TLC scanner III with Wincats 3.2.1 software. The plates with the phenolic markers were scanned and quantified at 400 nm. The scanning profiles of the samples showing presence of quercetin were noted. The photographs were taken by the CAMAG Reprostar 3 video documentation unit by illumination at UV 254 nm and 366 nm. The plates were derevatized by dipping them in chromatogram immersion device III (CAMAG) containing anisaldehyde sulphuric acid reagent, dried and heated at

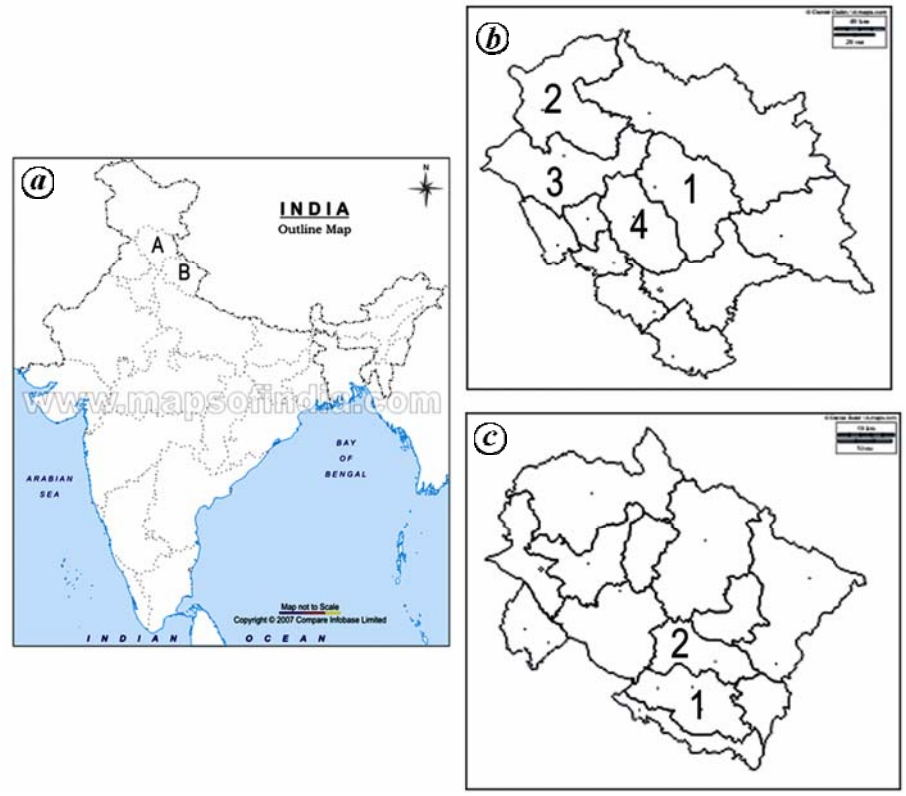

Figure 1. ***a***, Map of India showing sites of collection of fern samples from (A) Himachal Pradesh and (B) Uttarakhand. ***b***, Map of Himachal Pradesh: 1, Kullu; 2, Chamba; 3, Kangra; 4, Mandi. ***c***, Map of Uttarakhand: 1, Nainital; 2, Almora.

110°C for 5 min, and then the photographs were taken under visible light. Photographs of TLC profiles of all samples were recorded.

Various places in the districts of Kullu, Chamba, Almora and Nainital were visited to collect the fern samples (Figure 1). Sarol and Salooni in Chamba District are at relatively higher elevation and rich in ferns. *Christella arida*, *Deparia japonica*, *D. juxtaposita*, *Lygodium japonicum*, *P. squarossum* and *Pseudocyclosorus tylodes* grow in varied habitats and were found at different stages of growth, viz. in a few the leaves were in the crozier stage; in others, they were expanded and in still others the straw-coloured dry leaves were attached with the rhizome. Similarly, *D. japonica*, *D. juxtaposita*, *Hypodematium crenatum*, *Onychium cryptogrammoides* and *P. squarrosum* were found growing in various habitats at Nohani, (Chamba District). Nohani and the adjoining regions are reported to be enzootic for bovine haematuria. In Kullu District, Bajora, Lag valley and Manali were visited to collect *C. arida*, *D. esculentum*, *Dryopteris cochleata*, *Emodiopteris appenticulata*, *H. crenatum*, *Pseudocyclosorus canus* and *Pteridium revolutum*. Most of these were in different stages of growth.

In Uttarakhand, Mukteswar and Ranikhet were surveyed and a variety of fern samples were collected. *P. revolutum* was the most predominant fern in and around Mukteswar, along with *P. squarrosum*. Mature and dry leaves of *Onychium tenuifrons* were also abundant in the grazing areas. Certain ferns such as *Athyrium setiferum*, *D. juxtaposita*, *P. canus*, *Pt. revolutum* and *Pteris cretica* were also collected from areas adjoining Mukteswar (Gardenkral, Latoli and Padampuri; Figure 2). These areas are also known to be enzootic for bovine haematuria. The only fern studied for toxin from Ranikhet was *D. juxtaposita*.

The amount of Pta and/or PtB present in various fern samples collected from Uttarakhand and HP is shown in Tables 1 and 2. Out of 25 samples of the 16 species (9 from Uttarakhand and 16 from HP) studied, 5 samples from Uttarakhand and 4 samples from HP were found to contain Pta. The overall concentration of Pta present in the 25 fern samples ranged from 0.00 to 1182.84 μg/g on a dry-matter basis. In the samples from Uttarakhand, the highest Pta level was found in *P. cretica* (1182.8 μg/g), followed by *P. revolutum* (74.82 μg/g), *O. tenuifrons* (27.8 μg/g; 20.7 μg/g) and *P. canus* (25.6 μg/g). Of the samples from HP, the highest Pta level was found in *D. cochleata* (591.4 μg/g), followed by *O. cryptogrammoides* (38.5 μg/g) and *H. crenatum* (21.6–46.6 μg/g). *P. cretica*, *H. crenatum*, *D. cochleata* and *P. canus* were identified to contain Pta for the first time.

Figure 2. Some of the fern species analysed for toxins. ***a***, Herbarium specimen of mature leaf of *Hypodemantium crenatum* from Chamba District. ***b***, Mature leaf of *Onychium tenuifrons* from Nainital District. ***c***, Herbarium specimen of mature leaf of *Dryopteris cochleata* from Kullu District. ***d***, Mature leaf of *Pteridium revolutum* from Nainital District. ***e***, Herbarium specimen of mature leaf of *Onychium cryptogrammoides* from Chamba District. ***f***, Mature leaf of *Pteris cretica* from Nainital District.

The measurements performed to determine the sum of Pta + PtB in the crude extract were able to show the potential of a species to contain Pta even where most, or all, Pta had been degraded to PtB, whether by standing in the field or during the post-harvest drying. In this respect, *P. cretica* and *P. revolutum* samples from Uttarakhand contained mostly unchanged Pta, whereas the samples of *D. cochleata*, *H. crenatum* and *O. cryptogrammoides* from HP contained relatively high amounts of PtB, which is suggestive of their being a high source of Pta compared to the Pta measurements alone.

In the present study, a number of ferns such as *C. arida*, *D. japonica*, *D. esculentum*, *D. juxtaposita*, *E. appendiculata*, *L. japonicum*, *P. squarrosum*, *P. canus*, *P. tylodes* and *P. revolutum* from HP and *A. setiferum*, *D. juxtaposita* and *P. squarrosum* from Uttarakhand failed to show the presence of Pta, but some of these clearly have the potential to contain Pta since PtB was found in their crude extracts.

The results of quantitative quercetin estimation in the ferns are also presented in Tables 1 and 2 and Figures 3–5. From the 32 samples of 16 species, 11 samples from HP and 6 samples from Uttarakhand were found to contain quercetin. The concentration of quercetin measured ranged from 0.0% to 0.03%, with amounts varying both within species from different localities and in different species. The highest concentration was found in one sample of *P. squarrosum* from Nohani (Chamba District).

The simultaneous presence of both Pta and quercetin was also detected in six fern species (Table 3).

Table 1. Estimation of ptaquiloside and quercetin levels in some ferns from Uttarakhand

Fern	Place	District	Ptaquiloside*		Quercetin**	
			Pta + PtB (μg/g on dry-matter basis)	Pta (μg/g on dry-matter basis)	Methanol extract (wt. mg)	Quercetin (%)
Dryopteris juxtaposita	Padampuri	Nainital	0.0	0.0	89.0	0.0
	Ranikhet	Almora	0.0	0.0	100.0	0.0
Pteris cretica	Padampuri	Nainital	1245.7	1182.8	209.0	0.017
Polystichum squarrosum	Padampuri	Nainital	8.1	0.0	159.0	0.0
	Surkhidhar	Nainital	ND	ND	115.0	0.015
Pseudocyclosorus canus	Gardenkral	Nainital	30.5	25.6	28.0	0.006
Athyrium setiferum	Gardenkral	Nainital	0.0	0.0	117.0	0.0
Onychium tenuifrons	Latoli	Nainital	162.4	27.3	49.0	0.006
	Gardenkral	Nainital	205.0	20.3	32.0	0.004
Pteridium revolutum	Latoli	Nainital	65.3	74.8	48.0	0.007

Pta, Ptaquiloside; Pta B, Pterosin B; *Detection limits about 5 μg/g; **Detection limit about 0.004%; ND, not done.

Table 2. Estimation of ptaquiloside and quercetin levels in some ferns from Himachal Pradesh

Fern	Place	District	Ptaquiloside*		Quercetin**	
			Pta + PtB (μg/g on dry-matter basis)	Pta (μg/g on dry-matter basis)	Methanol extract (wt. mg)	Quercetin (%)
Lygodium japonicum	Sarol	Chamba	0.0	0.0	80.47	0.0
Pseudocyclosorus tylodes	Salooni	Chamba	0.0	0.0	29.53	0.008
Christella arida	Salooni	Chamba	18.7	0.0	50.73	0.015
	Bajora	Kullu	10.9	0.0	44.82	0.016
	Lag valley	Kullu	ND	ND	15.70	0.0
Deparia japonica	Salooni	Chamba	16.7	0.0	20.25	0.010
	Nohani	Chamba	ND	ND	30.18	0.010
Dryopteris juxtaposita	Salooni	Chamba	14.7	0.0	24.97	0.0
	Nohani	Chamba	ND	ND	41.29	0.012
Polystichum squarrosum	Nohani	Chamba	0.0	0.0	45.46	0.030
	Salooni	Chamba	ND	ND	40.88	0.0
Hypodematium crenatum	Nohani	Chamba	266.5	21.6	45.32	0.013
	Shivabadar, Manali	Kullu	1518.3	46.6	29.53	0.0
	Bajora	Kullu	33.4	0.0	42.66	0.0
	Manali	Kullu	ND	ND	42.77	0.0
Onychium cryptogrammoides	Nohani	Chamba	2419.3	38.5	27.0	0.0
Pteridium revolutum	Lag valley	Kullu	7.5	0.0	37.61	0.010
Pseudocyclosorus canus	Lag valley	Kullu	0.0	0.0	64.98	0.0
Diplazium esculentum	Lag valley	Kullu	0.0	0.0	41.69	0.0
Dryopteris cochleata	Lag valley	Kullu	1429.7	591.4	59.99	0.014
	Bajora	Kullu	ND	ND	15.08	0.0
Emodiopteris appendiculata	Shivabadar, Manali	Kullu	0.0	0.0	48.29	0.009

*Detection limits about 5 μg/g; **Detection limit about 0.004%; ND, not done.

D. cochleata, *H. crenatum*, *O. tenuifrons*, *P. canus*, *P. revolutum* and *P. cretica* showed both Pta and quercetin. Higher amounts of both Pta and quercetin were present in *D. cochleata* and *P. cretica*. Two samples of *O. tenuifrons*, one from Latoli (Nainital District) and the other from Gardenkral (Nainital District) were positive for Pta and quercetin.

EBH, known to be caused by long-term consumption of bracken fern (toxins) and infection with BPV-2, is a chronic, incurable, neoplastic ailment of the urinary bladder in cattle in certain geographical regions of world[27]. In cattle, there is a synergistic interaction between BPV-2 infection and exposure to quercetin, promoting bladder neoplasia, clinically presenting as enzootic haematuria. BPV-4 co-carcinogen quercetin was reported[28] to induce cell-cycle arrest and upregulate transcription from the LCR of BPV-4. A similar effect was seen on exposure to the bracken fern (*Pteridium aqualinum*) and the chemical Pta found within it[29].

In the present study, of the 25 studied samples of 16 species of ferns, 9 samples from both the states were found to contain Pta. The concentration of Pta present in the fern samples ranged from 0.00 to 1182.84 μg/g on a dry-matter basis. Further, seven samples demonstrated

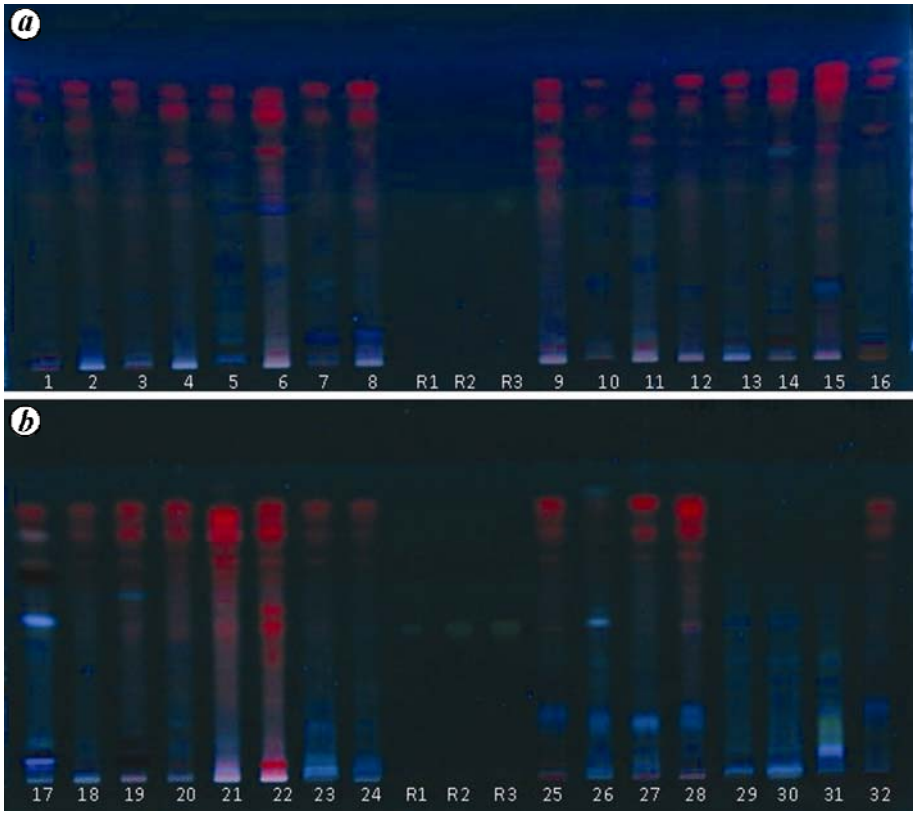

Figure 3. TLC showing detection of quercetin in fern samples (samples no. 1–32; R1–R3, Quercetin) under 366 nm.

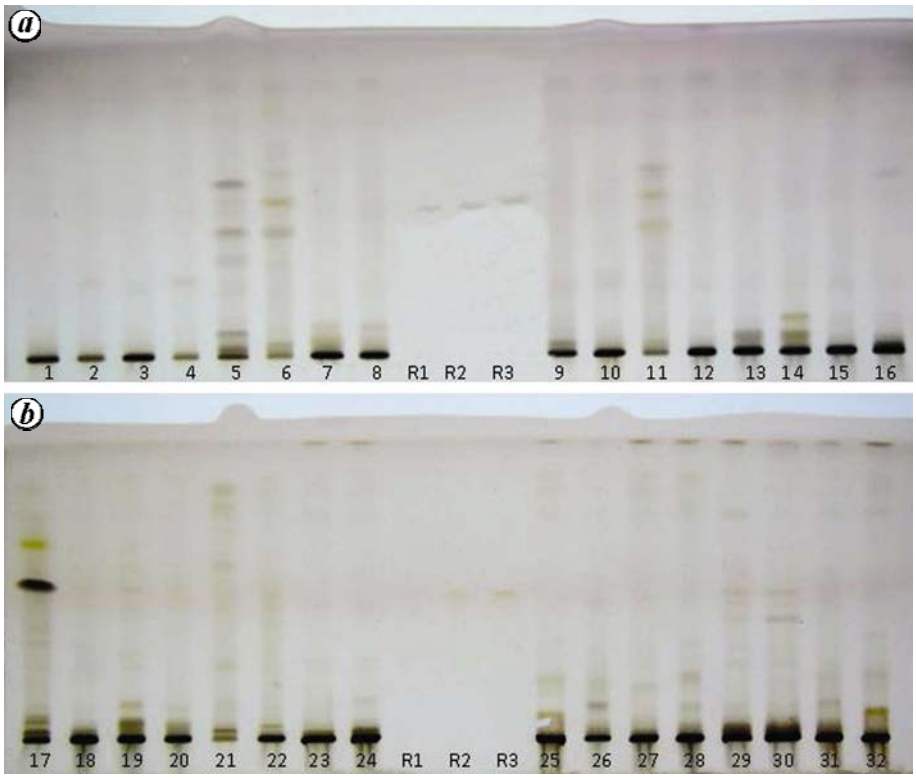

Figure 4. TLC showing detection of quercetin in fern samples (samples no. 1–32; R1–R3, Quercetin) under visible light after derivatization.

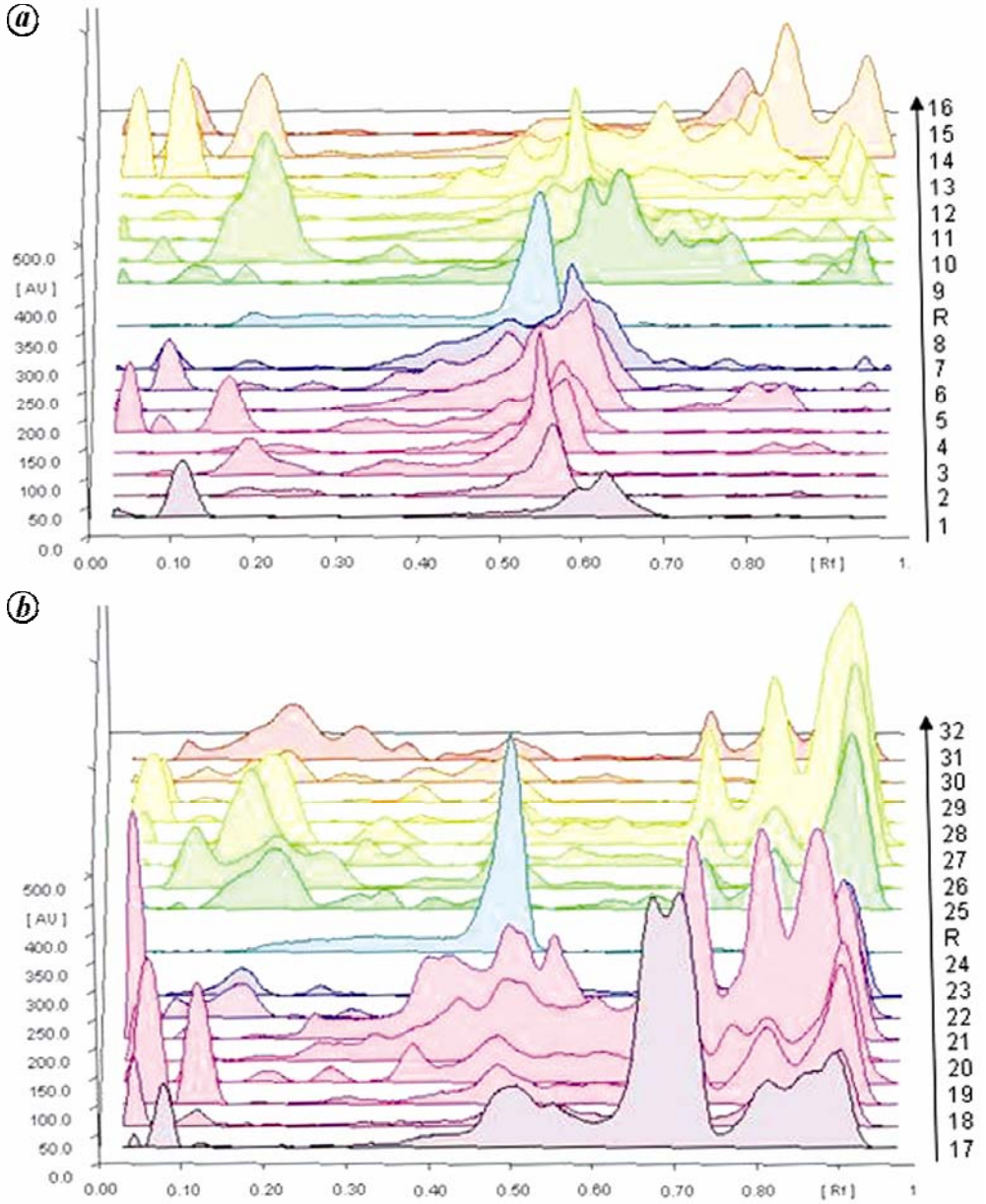

Figure 5. TLC densitometeric scanning profile at 400 nm (samples no. 1–32; R, quercetin).

the potential to contain Pta. *D. cochleata*, *H. crenatum*, *P. canus* and *P. cretica* were identified to contain Pta for the first time. On comparing the Pta values (which showed Pta still present at harvest) with the Pta + PtB values (which give an indication of the potential of a species to contain high levels of Pta at some time prior to harvest and testing) in certain fern samples, it was observed that the amount of latter was higher. This is an interesting fact since the sample of *O. cryptogrammoides*, for example, showed a minor amount of Pta at harvest, but the Pta + PtB results of 2419 mg/kg would have been very high if it was found as non-degraded Pta. If this species was tested in an unfurling or ripen state, it could have shown as a high level of Pta as that found in bracken.

Fern samples from other parts of Uttarakhand were analysed for Pta by earlier workers too[30]. They studied 21 samples of 13 species, out of which 8 samples of 5 species were found to contain Pta. The highest concentrations of Pta were found in *Emodiopteris appendiculata*, *Onychium contiguum*, *Pteris aspercaulis*, *P. biaurita* and *P. wallichiana* with all species showing a high potential to contain Pta, with the concentrations of Pta + PtB found to be much higher than that of Pta alone. *P. aspercaulis*, *P. biaurita*, *P. wallichiana* and *E. appendiculata* were identified for the first time in that study to contain Pta[30]. In the present study it was observed that there was variation in the Pta-potential of two *Onychium* species as demonstrated by the Pta + PtB concentrations measured.

Table 3. Simultaneous detection of ptaquiloside and quercetin in ferns of Uttarakhand and HP

Fern	Place	District	Pta (μg/g on dry-matter basis)	Quercetin (%)
Dryopteris cochleata	Lag Valley	Kullu	591.4	0.014
Hypodermatium crenatum	Nohani	Chamba	21.6	0.013
Pteris cretica	Padampuri	Nainital	1182.8	0.017
Pseudocyclosorus canus	Gardenkral	Nainital	25.6	0.006
Onychium tenuifrons	Latoli	Nainital	27.3	0.006
Onychium tenuifrons	Gardenkral	Nainital	20.3	0.004
Pteridium revolutum	Latoli	Nainital	74.8	0.007

Earlier, Pta alone was estimated in non-bracken fern samples collected mostly from Uttarakhand[25]. In that study[25], only two samples of *O. contiguum* contained high levels of free Pta, i.e. 449–595 mg/kg on a dry-matter basis. The samples of *O. contiguum* were collected from high-altitude areas of the Himalayas (Districts Chamoli and Uttarkashi), which is enzootic for EBH[25]. *O. contiguum* has been experimentally proved as carcinogenic in guinea pigs[23]. Samples of *D. esculentum*, *P. squarrosum*, *D. juxtaposita*, *C. farinosa* and *C. dentata* were also found to contain low levels of free Pta (0.40–30.50 mg/kg), but determination of the Pta potential (Pta + PtB) was not done at that time[25].

In the present study a number of ferns failed to show the presence of Pta, although some, such as *C. arida*, *D. japonica*, *D. juxtaposita* and *P. revolutum* from HP and *P. squarrosum* from Uttarakhand showed the potential to contain Pta along with the presence of PtB. These findings are in accordance with earlier observations[30] where a sample of *O. cryptogramoides* failed to show the presence of Pta, but it did demonstrate a Pta potential with the presence of PtB. It is therefore interesting that in the present study a single sample of this species showed both free Pta and a much higher amount of PtB. This suggests that for future studies of the toxic potential of different fern species, the method used in the present study to measure PtB as well as Pta should be the standard practice.

In some ferns from Darjeeling (eastern Himalayas, India), the toxins either altogether absent or present at very low concentrations[31]. Also, samples of *Adiantum incisum* and *Pteris stenophylla* from other regions failed to show Pta[25]. These observations indicate that the level of Pta varies in particular species occurring at different places and that the amount of toxin also varies from species to species. Australian workers also concluded that Pta is found in all varieties of bracken, but in rather variable amounts[7]. It is also known that relatively lower concentrations of toxin in mature leaves might be due to loss during drying and senescence. Seasonal sampling and toxin estimation from the different areas and species tested will be necessary to establish the carcinogen risk arising from the different growth stages of these ferns.

In the present study, quercetin was analysed in 32 fern samples and was detected in 17 samples with its concentration ranging from 0.0% to 0.030%. Samples from HP were found to contain variable range of quercetin in *C. arida*, *D. japonica*, *D. cochleata*, *D. juxtaposita*, *H. crenatum*, *P. squarrosum* and *P. revolutum*. Similarly, *P. squarrosum* and *P. cretica* from Uttarakhand contained higher levels of quercetin. Perusal of the literature revealed that a number of ferns contain quercetin or its glycosides. Besides bracken some other ferns were also reported to contain quercetin, including *P. globulifera*[17], *C. fragrans*[18] and *D. villarii*[19,20]. The status of quercetin in ferns has not been studied in India, with exception of one report[21], which estimated qualitatively and quantitatively the presence of quercetin in methanolic extract of *A. lunulatum* by HPTLC and detected 0.051% quercetin. *C. sieberi* or mulga fern is attributed to EBH in Australia and New Zealand[24].

In view of the widespread detection of quercetin in different types of plants, it was noticed that little work has been done on the toxicity of this compound. A low toxicity of quercetin for rats and rabbits in the short and long-term studies was observed[32]. Quercetin was reported to be mutagenic in *Salmonella typhimurium* TA 98 and TA 100 strains[33]. Quercetin also showed mutagenic activity in the absence of liver-mediated metabolism. Mutagenic activity was approximately tripled in the presence of liver microsomes. The carcinogenic potential of a mutagen, quercetin, which is present in bracken was studied in rats[34]. These authors demonstrated that quercetin is carcinogenic for the intestinal and bladder epithelium of rats. The neoplasms produced in quercetin-fed rats were grossly and histologically identical to those produced in bracken-fed rats. The ingested quantity of quercetin, found in bracken as the glycosides isoquercitrin and rutin, was about five times that ingested by bracken-fern-fed rats. Recently, it has been reported that quercetin may cause harm through the 'quercetin paradox', i.e. 'in the process of offering protection, it is converted into a potential toxic product'[16].

In the present study, four non-bracken ferns were found to contain Pta. Three of these, *P. cretica*, *H. crenatum* and *D. cochleata*, showed either a high level of Pta

or PtB. The present study thus shows that several other ferns may also contain moderate levels of quercetin.

This study also revealed that non-bracken fern species from both HP and Uttarakhand contain Pta and/or quercetin. The simultaneous presence of Pta and quercetin was also detected in some ferns which are well-known carcinogenic plants. The presence of both Pta and quercetin in certain ferns may act synergistically to cause immunosuppression and thus, predispose the animals to urinary bladder cancers/EBH along with BPV-2 and other co-factors. Further studies are required on this aspect.

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