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Sequestration of the plant secondary metabolite, colchicine, by the noctuid moth *Polytela gloriosae* (Fab.)

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Abstract

Colchicine, a well-known alkaloid, is a potent inhibitor of polymerization of tubulin leading to mitotic arrest. It is highly toxic to eukaryotic cells but also widely used in the field of medicine and plant breeding. *Gloriosa superba* (family: Colchicaceae) is an important natural source of colchicine. The seeds, tubers and leaves of this plant contain about 0.8, 1.2 and 0.014% colchicine by dry weight respectively. A noctuid moth, *Polytela gloriosae* (family: Noctuidae), feeds voraciously on leaves of *G. superba* without any adverse effect. However, the fate of colchicine and the mechanisms by which the insect is able to overcome the toxicity of the metabolite is not known. Here, we trace the fate of colchicine in both, the larva and moth of *P. gloriosae*. Colchicine was quantified in different body parts of the larvae and moth by high performance liquid chromatography, liquid chromatography mass spectrophotometry and nuclear magnetic resonance methods. Of the total colchicine taken in by the larva, a larger portion was excreted, while the rest was sequestered in its cuticle. In the moths however, the wings, legs and antennae were found to accumulate high amount of colchicine. The sequestered colchicine, in both the larva and adult, were chemically identical to that found in the plant. Negligible amounts of demethyl-(−)-colchicine, a less toxic derivative of colchicine was also detected. We discuss the probable adaptive significance of sequestration of colchicine by the insect.

Keywords Colchicine · *Gloriosa superba* · *Polytela gloriosae* · Sequestration

Introduction

Plants produce a number of secondary metabolites as a defense against herbivores. These metabolites range from being simple anti-feedants (such as sugar-mimic alkaloids) to Na^+, K^+-ATPase inhibitors (such as cardiac glycosides) or cell division arresters (such as taxol) (Aharoni et al. 2005; Howe and Jander 2008; Taiz and Zeiger 2010; Rosenthal and Berenbaum 2012; Fürstenberg-Hägg et al. 2013). However, over evolutionary time, in an obvious arms race, several herbivores adapt and often evolve mechanisms to overcome plant defenses. The evolution of resistance of monarch butterflies against cardenolide glycosides, for instance is a classic example of an insect overcoming plant defensive secondary metabolites (Marty and Krieger 1984; Malcolm et al. 1989; Holzinger and Wink 1996; Helmus and Dussourd 2005; Després et al. 2007).

One such plant secondary metabolite known for its anti-mitotic activity in eukaryotic systems is colchicine. The isoquinoline alkaloid has been well exploited therapeutically as a mitotic poison and also in inducing polyploidy

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in crop plants (Nautiyal 2011; Larsson and Ronsted 2014). In actively dividing cells, colchicine binds to tubulin and thereby inhibits polymerization at the growing end of microtubules. This irreversible binding inhibits the metaphasic stage of cell mitosis (Inoue 1981; Caperta et al. 2006). Colchicine was approved by the Food and Drug Administration (FDA, USA) in 2009 for the treatment of gout, familial Mediterranean fever, amyloidosis, sarcoidosis, Behcet's syndrome and scleroderma (Levy et al. 1991; Buskila et al. 1997; Gökel et al. 2000; Ghosh and Jha 2008; Ade and Rai 2010; Slobodnick et al. 2015).

Colchicine was first discovered from the tubers of *Colchicum autumnale* (family: Colchicaceae) (Hartung 1954; Ghosh and Jha 2008; Slobodnick et al. 2015). Later, it was reported from several members of Colchicaceae of the genera *Colchicum, Merendera, Androcymbium, Gloriosa* and *Littonia* (Ghosh and Jha 2008; Vinnersten and Larsson 2010; Larsson and Ronsted 2014). Among the various species known, *Gloriosa superba* L., is an important source of colchicine. The seeds and tubers of the plant contain 0.8 and 1.2% of the alkaloid respectively while the leaves have about 0.014% (Finnie and Van 1989; Rajagopal and Kandhasamy 2009). The plant is known to grow naturally in parts of Asia and Africa and is traditionally regarded as one of the most poisonous plants (Ade and Rai, 2010; Kavina et al. 2011). The lethal dosage (LD 50) of colchicine is reported to be 1492 mg/kg for *Galleria mellonella* larvae and 5.8 mg/kg (oral) for mouse and is considered to be highly toxic to eukaryotic cells (Allegra et al. 2018).

Despite its toxicity, *G. superba* is defoliated by several insects, predominant among them being *Polytela gloriosae* (Fab.) (Hanumanthaswamy and Rajgopal 1995). Commonly known as the lily moth, this lepidopteran species belong to the family Noctuidae. *P. gloriosae* larvae feed on several species of plants belonging to the families Liliaceae and Amaryllidaceae (Sathe 2015). It defoliates the plant by chewing on soft shoot tips and leaves. Besides, the larvae have also been found to feed on tubers of *G. superba* under laboratory conditions (personal observation). However, the mechanism by which these larvae overcome the toxicity of ingested colchicine has yet not been unraveled.

Several studies have suggested that insects feeding on plants producing toxic secondary metabolites might have evolved mechanisms over time, to avoid, excrete, sequester or detoxify them (Nishida 2002; Després et al. 2007, Heckel 2014). For example, the polyphagous larvae, *Trichoplusia ni* and *Danaus plexippus* cut trenches on host plant leaves to drain allelochemicals such as lactucin and cardenolides, before feeding on the distal portion, as an avoidance mechanism (Dussourd 2003; Helmus and Dussourd 2005). Sequestration of toxic plant metabolites by larvae and adults has been well-studied across different insect families. *Kanarella unicolor*, a chrysomelid beetle and the larvae of *Lymantria* sp., a lymantriid moth sequester camptothecin, a potent eukaryotic topoisomerase I inhibitor. The latter also detoxifies camptothecin from its active lactone form to the inactive carboxylate form through a gut pH-mediated process (Ramesha et al. 2011; Sajitha et al. 2018). Insects might also deploy multiple mechanisms to overcome the toxicity of plant secondary metabolites. A well-known example is *Danaus plexippus*, which feeds on milkweeds (*Asclepias* sp.) containing toxic cardenolides. The insects deploy mechanisms of ingestion avoidance, sequestration, detoxification and target-site mutation of Na^+, K^+-ATPase during its life cycle to overcome the toxicity of cardenolides (Marty and Krieger 1984; Rothschild et al. 1984; Malcolm et al. 1989; Holzinger and Wink 1996; Helmus and Dussourd 2005; Després et al. 2007).

In this study, we determined the fate of colchicine in the larvae and the adult of *P. gloriosae* that were captively reared on *G. superba* leaves. We hypothesized that *P. gloriosae* may use one or several of the mechanisms cited above to overcome the toxic effects of colchicine, in its larval and adult stages. Colchicine was detected and determined in various tissues of the insect by high performance liquid chromatography (HPLC), liquid chromatography mass spectrophotometry (LC–MS) and nuclear magnetic resonance (NMR) analysis. The results obtained have been discussed considering the probable adaptive significance of sequestration of colchicine by the insect.

Material and methods

Study site and system

Gloriosa superba is a perennial tuberous plant distributed in most parts of India including the Western and Eastern Ghats (Kavina et al. 2011; Sarvalingam and Rajendran 2016). The study was conducted in several regions of the Western Ghats, South India during the months of October to January for three consecutive years, 2012, 2013 and 2014. The plants were observed for herbivorous insects and their feeding patterns were recorded.

Several insects were found on the leaves of *G. superba* during the field survey. Among them, one of the prominent herbivorous insect was *P. gloriosae*. The larvae were collected and transported in insect polypropylene boxes to the laboratory at School of Ecology and Conservation, University of Agricultural Sciences (UAS), Bengaluru, India. The larvae were reared in insect rearing plastic boxes (22 × 8 cm) and fed ad libitum on fresh *G. superba* leaves obtained from the Medicinal Plants Garden of UAS, GKVK campus, Bengaluru, India. The emerging moths were maintained in insect cages and fed on sugar solution (10%).

Extraction of colchicine

Colchicine was extracted from *G. superba* leaves, parts of larval and adult body and whole larva. A total of 15 larvae of the 2nd instar were randomly selected and separated into three groups of five larvae each. The first and second groups were fed on *G. superba* leaves for 5 days until they reached the 4th instar following which they were used for colchicine extraction. The third group of larvae was fed on *G. superba* leaves until they developed into adults (approximately 15–16 days from 2nd instar).

Sample preparation. From the first group, three larvae of 4th instar were selected randomly for dissection. Hemolymph was drawn out using a hypodermic syringe, the volume was recorded and then diluted with a known amount of 100% methanol in Eppendorf tubes (Merck). The gut was excised and plant material within it was removed carefully using the blunt end of a scalpel. This along with fat body, cuticle and frass collected over 5 days were used for the analysis. The second group of larvae was used for analyzing total colchicine in its whole body along with the gut contents. Three larvae (4th instar) were randomly selected for the extraction without removing the gut contents. The third group was moths that had emerged from the larvae which were fed on *G. superba* leaves. These moths ($n=3$) were separated into forewings, hindwings, legs-antennae (pooled) and head-thorax-abdomen (pooled). All the above samples were used for colchicine extraction.

Sample processing. *G. superba* leaves and tissue samples from each group of larvae and adults were macerated to fine powder in liquid nitrogen using mortar and pestle. The samples were extracted in 5 ml of 100% methanol and incubated at 60 °C for 2 h in a shaking water bath (Remi, India). After cooling to room temperature, the extract was centrifuged at 10,000 rpm for 10 min at 10 °C (5430 R, Eppendorf, Germany). The supernatant was passed through a 0.2 µm filter (Tarsons, India) and analysed for presence of colchicine by reverse phase high performance liquid chromatography (HPLC).

HPLC–DAD analysis

The extracts were analyzed using reverse-phase HPLC (LC-20AD, Shimadzu, Japan). The separation was performed on a RP-18 column (250 mm × 4.6 mm, 5 µm) (Phenomenex), using a SPD-M20A photodiode array detector with a detection wavelength of 238 nm for colchicine. The separation was carried out using 20 µl of injection volume at a flow rate of 0.8 ml/min. The isocratic mobile phase of 30% acetonitrile for pump A and 70% water containing 0.1% trifluro-acetic acid for Pump B was maintained with a total run time of 20 min. A calibration curve was prepared using colchicine standards ranging from 7 to 250 µg/ml. The amount of colchicine in the samples was calculated based on the regression curve obtained (Sajitha et al. 2018).

LC–ESI–MS analysis

LC–ESI–MS analysis was carried out to further confirm the presence of colchicine in the various insect samples (LC–MS-2020, Shimadzu, Japan). The LC system was attached to an ion trap mass analyzer which was in turn equipped with atmospheric pressure ionization source electrospray ionization (ESI). High purity nitrogen was engaged as both the nebulizer and drying gas. The conditions used for mass spectrum analysis during the study was RP-18 column (250 mm × 4.6 mm, 5 µm) (Phenomenex) with a UV–visible detector set to a wavelength of 238 nm. The flow rate was maintained at 0.3 ml/min and an injection volume of 10 µl was used. The isocratic mobile phase was set at 30% acetonitrile for pump A and 70% water with 0.1% formic acid for Pump B. The total LC analysis was set to 20 min. The conditions for mass spectrum analysis were set as: dry gas flow rate 10 l/min, nebulizing gas flow 1.5 l/min, nebulizer pressure 35 psi, DL Temp 250 °C and mass range 100–700 m/z (Ramesha et al. 2013; Sajitha et al. 2018).

NMR spectroscopy

Equal weight of all the tissue samples, along with *G. superba* leaf sample was extracted in methanol (as described above in the extraction of colchicine subsection) for NMR spectroscopic analysis. The supernatant from methanol extracts were first vacuum dried and the residues obtained were then re-dissolved in deuterated methanol to make the total volume to 600 µl per sample. The samples were then transferred to a 5-mm NMR tube for NMR analysis. NMR spectra were recorded on a Bruker Biospin 600-MHz Avance-III spectrometer that operated at 1H NMR frequency of 600.13 MHz at 298 K and equipped with 5 mm QXI probe. Prior to signal acquisition gradient shimming was performed and deuterated methanol was used as an internal lock. Water suppression of residual water signal was attained with a pre-saturation sequence having low-power selective irradiation at 4.9 ppm through the recycle delay. The proton spectra were gathered with a 90-degree pulse width of 9.15 µs, a recycle delay of 2 s, 2 K data points, 16 scans and a spectral width of 12 ppm. Data were zero-filled by factor of 2 and the FIDs were multiplied by an exponential weighting function corresponding to a line broadening of 1 Hz preceding to Fourier

transformation. The spectra were both phase and baseline corrected and referred to the TMSP resonance at 0.00 ppm. For resonance allocation and metabolite identification, two-dimensional NMR spectra were documented, including 1-1H correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), and 1H-13C heteronuclear coherence spectroscopy (HSQC, HMQC). 2D 1H-13C heteronuclear spectra were acquired with a spectral width of 12 ppm and 220 ppm in the proton and carbon dimensions respectively, 16 scans, 1 K data points, 256 t1 increments and a recycle delay of 2 s. The COSY and TOCSY spectra were obtained with a spectral width of 12 ppm in both dimensions, 1 K data points, 16 scans and 256 t1 increments (Sajitha et al. 2018).

Colchicine dose tolerance by *P. gloriosae* larva

To examine the tolerance of larvae to increasing dosage of colchicine, 30 larvae of 3rd instar (newly molted) were selected at random. These were divided into six treatments with five larvae in each. The following treatments were imposed: control (*G. superba* leaves), solvent blank (*G. superba* leaves dabbed completely on both sides using a cotton swab dipped in methanol, 100%) and *G. superba* leaves dabbed completely on both sides using cotton swab dipped in 50, 100, 200, 300 µg/ml of standard colchicine (Sigma Aldrich) solution respectively. These concentrations were derived from a stock solution of standard colchicine (Sigma grade) prepared in 100% methanol. The larvae were fed daily on *G. superba* leaves imposed with different treatments as mentioned above and the weight of each larva was recorded daily over a period of 5 days. The difference in the weight of the larvae fed with different concentrations of colchicine against blank and control was calculated.

Statistical analyses

The data were statistically analysed using MS excel (2007 version). Data analyses included one-way and two-way analysis of variance (ANOVA) and least significant difference (LSD).

Results

Among several herbivorous insects observed on the plant, *P. gloriosae* was predominant in most locations. Both the larval and adult stages were found. The larvae fed voraciously and often defoliated the entire plant. *P. gloriosae* has a life cycle (egg to adult) ranging from 18 to 20 days with five instars of larval stage. The moths have a life span of 6–8 days.

The larval and adult stages of *P. gloriosae* were analysed for the presence of colchicine. In the first group, colchicine was found to be high in frass (0.836 µg/mg) followed by cuticle (0.323 µg/mg) and fat body (0.297 µg/mg). Hemolymph and gut tissue had relatively lower amounts of colchicine with 0.27 and 0.09 µg/mg respectively. The leaf tissue of *G. superba* (fed to the larvae) was estimated to contain 0.59 µg/mg colchicine which was significantly lower than that estimated in the frass (One-way ANOVA, $p < 0.01$; Fig. 1). The second group in which the whole larva was analysed, it was found to accumulate 1.04 µg/mg of colchicine which is much higher than the concentrations in individual tissues. This could be due to fact that the gut content was not removed for the whole larva analysis. In the third group having adult moths, concentration of colchicine was found to be high in the forewings (3.16 µg/mg) as compared to legs-antennae (2.46 µg/mg) or hindwings (1.76 µg/mg). The rest of the adult body, head-thorax-abdomen contained 0.848 µg/mg of colchicine significantly lower than that estimated in its wings (One-way ANOVA, $p < 0.01$; Fig. 2). The absolute amount of colchicine present in different tissues of both larva and moth (mg/insect) has also been provided in Fig. S1.

LC–MS analysis of different parts of larval and adult body further confirmed the presence of colchicine (*m/z* 400). Besides, a molecular ion at *m/z* 386 corresponding to demethyl-(–)-colchicine (DMC), a less toxic derivative of colchicine was also recorded in *G. superba* leaf, larval frass and parts of the insect body, viz., gut tissue, fat body, forewing, head-thorax-abdomen (Figs. S2, S3). There was also no significant difference in the body weight of the larvae fed on leaves smeared with increasing concentrations of colchicine compared to that of the control (Two-way ANOVA, $p > 0.05$; Fig. S4). Though this result suggest that the larvae are insensitive to the increasing concentration of colchicine, it cannot be unequivocally established, because of lack of data on the exact enrichment of colchicine through such application.

The presence of colchicine in different samples of larvae and adults were also confirmed by comparing the NMR spectra recorded for *P. gloriosae* samples with the NMR spectra of pure colchicine, recorded at 600 MHz, using methanol-d4 as a solvent. Several colchicine peaks with no peak overlaps could be easily identified and used as characteristic peaks for the identification and confirmation of the presence of colchicine in the larva and adult samples, such as the *N*-acetyl CH3 proton peak obtained at 2.00 ppm and other multiplet peaks. However, some other peaks such as methoxy methyls, shown as singlets at 3.6 ppm and 4.0 ppm in both Figs. 3 and 4 could not easily be identified in the larva and adult samples due to peak overlaps with other metabolite peaks. This was overcome by increasing the

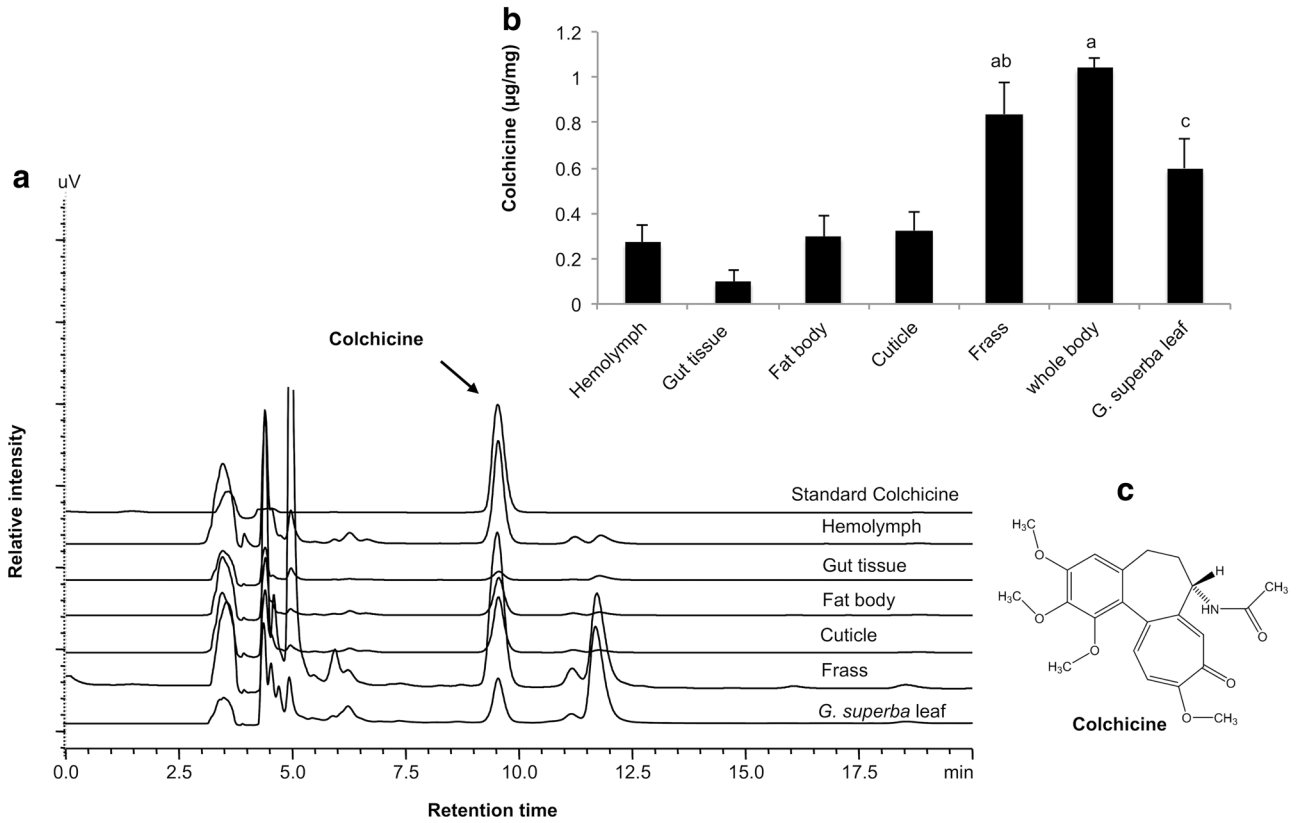

Fig. 1 **a** RP-HPLC profile showing colchicine in different body parts of larvae, frass and *G. superba* leaves, **b** concentration of colchicine in parts of the larval body, frass and *G. superba* leaves. Bars with dissimilar superscripts are significantly different at $p < 0.01$, **c** structure of colchicine

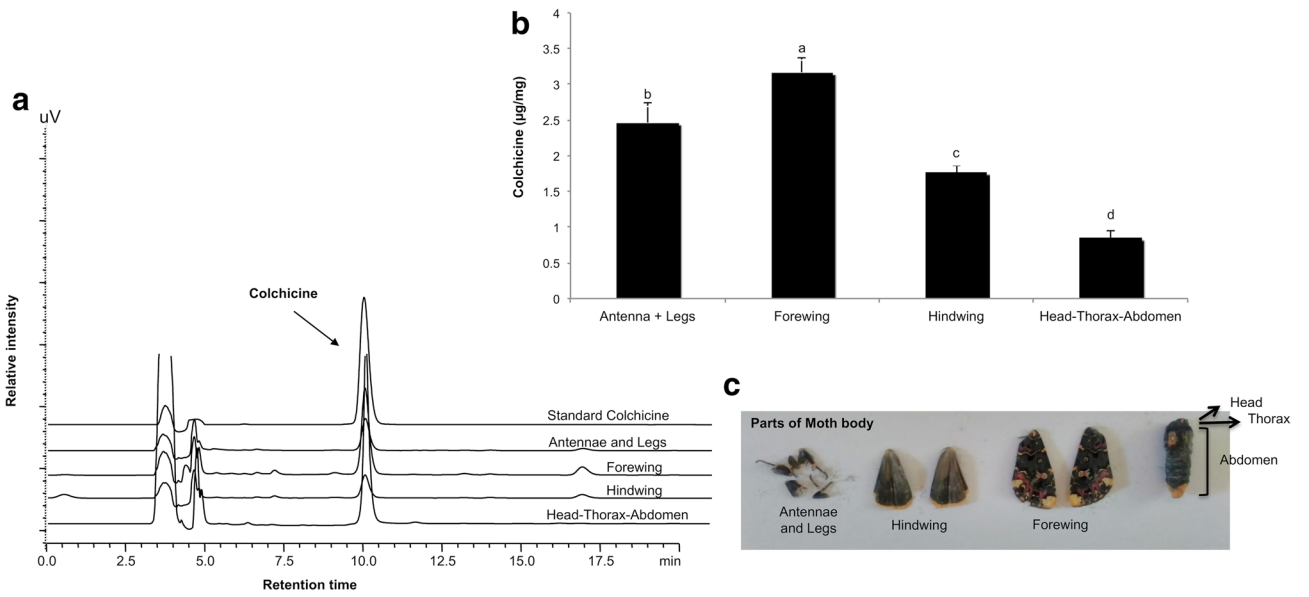

Fig. 2 **a** RP-HPLC profile showing colchicine in different body parts of the moth, **b** concentrations of colchicine in parts of the *P. gloriosae* adult body. Bars with dissimilar superscripts are significantly different at $p < 0.01$, **c** parts of adult body

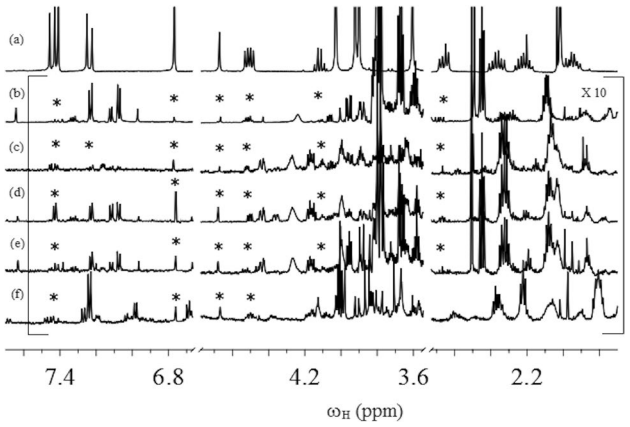

Fig. 3 Regions from 1D 1H NMR spectra of **a** standard colchicine, **b** hemolymph, **c** gut tissue, **d** cuticle, **e** frass and **f** leaf of *G. superba* recorded at 600 MHz

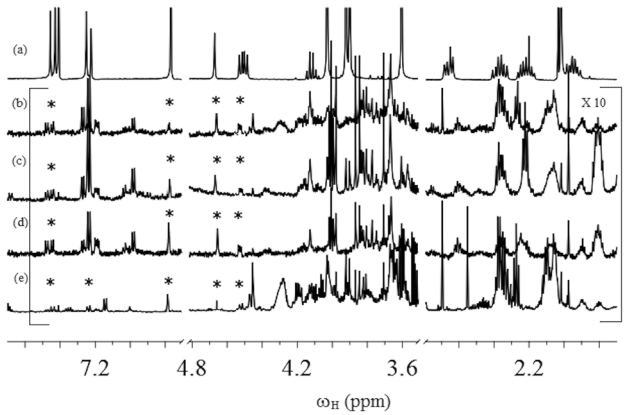

Fig. 4 Regions from 1D 1H NMR spectra of **a** standard colchicine, parts of *P. gloriosae* adult body—**b** legs and antennae, **c** forewing, **d** hindwing, **e** head-thorax-abdomen recorded at 600 MHz

signal intensity to reveal smaller peaks of colchicine and was further confirmed using 2D homo nuclear and heteronuclear experiments.

Discussion

This is perhaps the first time, that sequestration of the potent anti-mitotic alkaloid, colchicine, is being demonstrated in an insect system, *P. gloriosae*. The larvae of *P. gloriosae* feed profusely on leaves of *G. superba* without any apparent adverse effect. Although the content of colchicine estimated in the leaves was 0.59 µg/mg, a much higher amount (0.836 µg/mg) is excreted by the larvae. The significantly high amount of colchicine in the frass compared to that in leaves is likely because, the former is a function of the feeding activity of the larvae over the entire day and reflects the colchicine in the frass, over and above that, which might have been sequestered or degraded. Thus, while the concentration in the leaf offers a static measure (instantaneous), that in the frass reflects a cumulative measure of successive feeding bouts by the larvae. Elimination of secondary metabolite burden through frass is an efficient strategy used by several insect herbivores to overcome host plant chemicals (Heckel 2014; Heidel-Fischer and Vogel 2015). Studies have shown that insects readily transport metabolites in the hemolymph to the malphigian tubules from where they are excreted, thus causing minimum damage to its active tissues. Active excretion has been experimentally validated for alkaloids such as nicotine, morphine and atropine (Maddrell and Gardiner 1976; Nishida 2002). Low levels of colchicine were recovered from the hemolymph and the gut tissue. However, it is interesting to note that about 31% of the sequestered alkaloid was found in the exoskeletal tissues of the larva (Fig. 5). This could serve, as widely suggested in literature, to exclude toxins from metabolically active tissues and also as a possible anti-predatory strategy by the insect (Nishida 2002; Opitz and Müller 2009; Després et al. 2007).

Disregarding the substantial loss of colchicine through frass, *P. gloriosae* larva was found to accumulate 0.564 mg (equivalent to 990 mg/kg) of colchicine in its body tissues. This is much lower than the lethal dose reported for *G. mellonella* (1492 mg/kg) (Allegra et al. 2018). The movement of colchicine across tissues (gut-hemolymph-exoskeleton) may be aided by passive or active transport systems. In fact, studies have suggested the role of multidrug resistant (MDR) gene of the ATP-binding cassette (ABC) transporter family in facilitating such transport. For example, P-glycoprotein transporter, mdr4 is shown to be involved in 80% of the cell lines resistant to colchicine (Schibler et al. 1989; Hari et al. 2003). Studies are underway in our laboratory to examine this possibility of ABC transporters in regulating the movement of colchicine across tissues in *P. gloriosae*.

The larvae of *P. gloriosae* developed into moths over a period of 2 weeks (from 2nd instar) and these were maintained on sugar solution in the laboratory. The moths were found to accumulate proportionately higher concentrations of the metabolite in their wings (60%) and legs-antennae (30%) as compared to only 10% in the head-thorax-abdomen (Fig. 5). The amount of colchicine sequestered in the wings and antennae were much higher than those retained in the cuticle of larva (31%). As a whole, a single *P. gloriosae* moth accumulated 1.4 mg (8240 mg/kg) colchicine, which is much higher than the lethal dose reported in *G. mellonella* (1492 mg/kg) (Allegra et al. 2018).

Many members of Lepidoptera and Coleoptera are known to store large concentrations of toxic plant metabolites in their integuments and wings as a deterrent to predators. Often they advertise their unpalatability by the display of warning coloration (Rothschild et al. 1984; Von

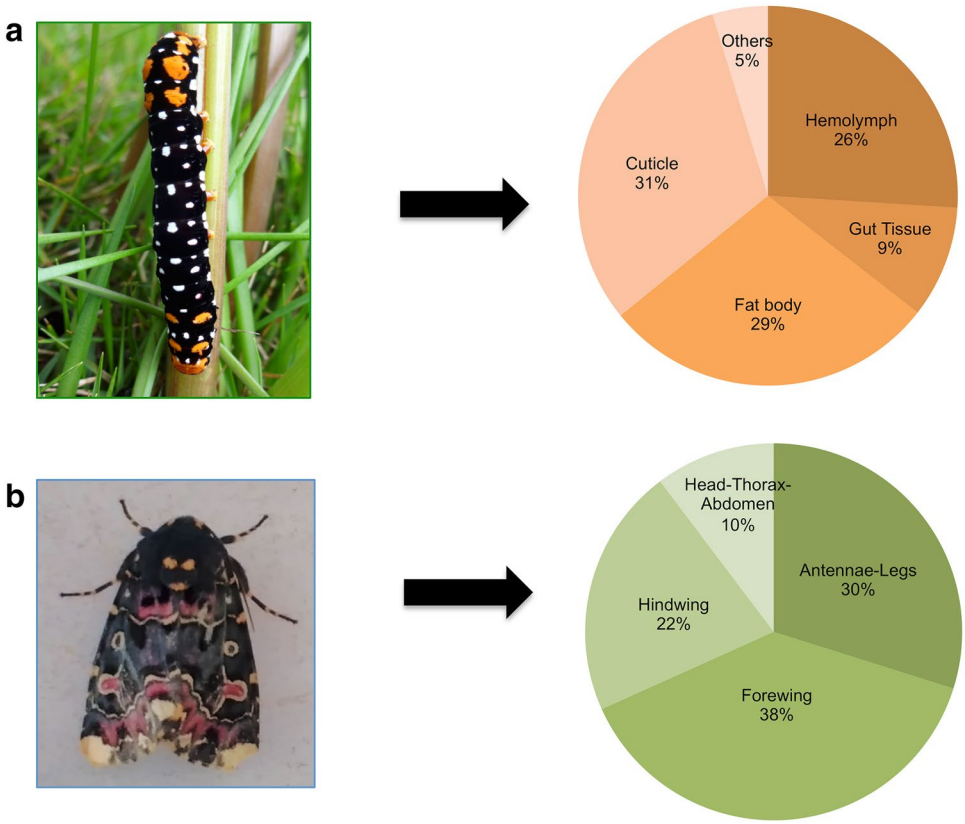

Fig. 5 Distribution of colchicine in **a** larva ($n=3$) and **b** moth ($n=3$) of *P. gloriosae* fed on *G. superba* leaves. Others (5%) in larva may include the head and thorax region which were not analysed. SE (larva) = ±0.0308 (hemolymph), ±0.0098 (gut tissue), ±0.0402 (Fat body), ±0.0180 (cuticle). SE (moth) = ±0.0370 (antennae-Legs), ±0.0694 (forewing), ±0.0392 (hind-wing), ±0.0734 (head-thorax-abdomen). All values are in mg/larva or mg/moth

and Wink 1993; Nishida 1994; Harvey and Paxton 1981; Rothschild 1985; Brown 1988; Bowers 1993). Although there is lack of evidence, it is likely that the sequestration of high amount of colchicine in its exoskeletal tissues coupled with the bright display of colour may have been selected as anti-predatory behaviour by *P. gloriosae* (Sillen-Tullberg and Leimar 1988; Tullberg and Hunter 1996; Nishida 2002).

In summary, our study on the larva and moth of *P. gloriosae* offer the first documented example of sequestration of colchicine in this insect system. It would be interesting to examine, besides sequestration, other mechanisms that might help the insect in circumventing colchicine toxicity. Also of interest would be to identify critical mutations if any, in colchicine binding domain of the tubulin protein, in both the insect and the plant that might render them insensitive to the toxin. Besides, the possible role of ABC transporters in the transport of colchicine across different tissues in the insect could further enrich our understanding of the process of sequestration of colchicine in *P. gloriosae*.

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