Bait, not reward: CO_2-enriched *Nepenthes* pitchers secrete toxic nectar

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Summary

- Extrafloral nectar (EFN) secreted by *Nepenthes* pitchers has long been regarded as the major reward to visiting arthropods. Here we report the role of EFN produced by *Nepenthes* pitchers in prey capture by elucidating its chemistry, prey spectrum and insect toxicity. The results are interpreted in the context of the high CO_2 levels within these prey traps.
- *Nepenthes* EFNs were isolated, their sugars, amino acids, proteins, vitamin C and fatty acids were analyzed using various biochemical assays. Minerals and C:N ratio of EFNs were determined by ICP-OES and CHNS analyses, respectively; metabolic profiling was carried out using LC-MS. Volatiles in EFNs and pitcher tissues were analyzed by headspace-GC-MS. Peristome/lid EFNs, their fractions and active constituent were subjected to acetylcholinesterase (AChE) inhibition assays *in vitro* and *in vivo*; AChE inhibitor was characterized by bioactivity guided isolation and spectroscopy. Prey spectrum in *N. khasiana* pitchers was examined in field studies.
- *Nepenthes* EFN is a sugar (Glc-Fru-Suc) mix devoid of key nitrogenous components, amino acids (< detection limit), proteins (low), and vitamin C. Peristome and lid nectars showed strong AChE inhibition; (+)-isoshinanolone has been identified as the AChE inhibitor. Direct (+)-isoshinanolone-feeding bioassay demonstrated dizziness and disruption of motor functions in ants. *N. khasiana* EFNs showed high C:N ratio (peristome: C 24.01%, N 1.77%, lid: C 24.09%, N 2.02%).
- Chemical (EFN, volatiles), gaseous (CO_2), visual (colour, UV reflectance, fluorescence), physical (peristome geometry, surface microstructure, wettable surface-aquaplaning) and environmental (humidity, rain) factors effect prey capture in *Nepenthes* pitchers. We testify that *Nepenthes* EFN is a toxic bait which hinders neuronal activity of the visiting arthropods. Our discovery abolishes the notion of *Nepenthes* EFN as a 'reward' to the visitors. Moreover, our findings infer elevated CO_2 within as the key factor influencing the growth, metabolism, carnivory and herbivory of *Nepenthes* pitchers.

Key words: *Nepenthes*, extrafloral nectar, nectar toxicity, prey capture, elevated CO_2.

Introduction

Nepenthes pitchers effect prey capture and nutrient acquisition through various physico-chemical and biological strategies (Bohn & Federle, 2004; Baby *et al.*, 2017; Mithöfer, 2022). Their pitcher rim (peristome) is a wettable, low-friction surface with distinct microstructures (Bohn & Federle, 2004; Chen *et al.*, 2016), and these are crucial factors deciding the efficiency of prey capture. Environmental factors (humidity, rain) also influence the wettability of prey trapping surface and capture rates (Bohn & Federle, 2004; Chen *et al.*, 2016; Sujatha *et al.*, 2023). In addition, visual signals were reported from prey capturing regions of *Nepenthes* pitchers (Joel *et al.*, 1985; Moran *et al.*, 1999; Kurup *et al.*, 2013). We have described *Nepenthes* pitchers as CO_2-enriched cavities, and open pitchers continually emit CO_2 attracting preys (Baby *et al.*, 2017). Over the years researchers reported the role of pitcher colour (Bennett & Ellison, 2009; Da´vila-Lara *et al.*, 2021a), scent (Di Giusto *et al.*, 2008; Jurgens *et al.*, 2009) and peristome geometry (Moulton *et al.*, 2023) in prey capture in *Nepenthes*.

Extrafloral nectar (EFN) produced by nectaries on the tendrils, peristomes and lids of *Nepenthes* pitchers is considered as the primary nutritional reward to the visiting ants, other insects and small mammals (Bauer *et al.*, 2008; Bennett & Ellison, 2009; Gaume *et al.*, 2016). EFN also contributes to peristome wettability (Bauer *et al.*, 2008; Sujatha *et al.*, 2023). Bornean montane *Nepenthes* species through mutualistic interactions provide nectar and harvest nitrogenous excreta from tree shrews and other small mammals with their shape-adapted pitchers (Greenwood *et al.*, 2011). Non-mutualistic interactions of diverse vertebrates with *Nepenthes* pitchers as their nectar (food) source were also reported from Brunei (Bauer *et al.*, 2016). In angiosperms, floral nectar (FN) is one of the major rewards in pollination (Nicolson, 2022). Nectar, being a sweet aqueous secretion, is easily consumed, digested and absorbed. It has a complex chemistry containing sugars, amino acids, proteins, phenolics, alkaloids, lipids, organic acids and inorganic ions (De la Barrera & Nobel, 2004; Nepi *et al.*, 2012; Nicolson, 2022). However, the composition of nectar produced by floral and extrafloral nectaries in *Nepenthes* is barely investigated (Heil, 2015; Hatcher *et al.*, 2020). In this study, we describe the role of EFN produced by *Nepenthes* pitchers in prey capture by deciphering its chemistry, prey

spectrum and insect toxicity. The results are interpreted in the backdrop of the discovery of high levels of CO_2 within *Nepenthes* pitchers (Baby *et al.*, 2017).

Materials and Methods

Nepenthes species, hybrids

Nepenthes khasiana Hook.f., *N. mirabilis* (Lour.) Druce and hybrids *N. mirabilis* (Lour.) Druce × *N. khasiana* Hook.f., *N. mirabilis* (Lour.) Druce × *N. rafflesiana* Jack pitchers were collected from the conservatories of Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI) (08° 45′00.04″N, 77° 01′41.09″E) from August to October 2023. The plant specimens were authenticated by Dr. K.J. Lathankumar and Dr. Saleem Mohamed, JNTBGRI, and voucher specimens (*N. khasiana* TBGT 39201, *N. mirabilis* TBGT 44774; hybrids *N. mirabilis* × *N. khasiana* TBGT 44776, *N. mirabilis* × *N. rafflesiana* TBGT 44777) were deposited at JNTBGRI Herbarium.

Extrafloral nectar isolation

Nepenthes pitchers of uniform age group (5-8 days after opening) were collected from the field in the morning hours and their physical parameters were noted (Table S1). Pitcher lids were carefully plucked from the peristome-lid intersection; peristomes were circularly (externally) cut from the pitcher using a sharp blade. Whole peristomes and lids (without slicing) were rinsed (individually, separately) with water (5 mL, 15 min each, 5-8 times) and the washes were pooled and concentrated to dryness using a R-210 rotary evaporator (Buchi, Switzerland) at 37°C under reduced pressure. The completion of nectar extraction by water rinsing was ensured by checking the absence of sugars by TLC. Peristome and lid EFN yields were noted.

EFNs in the chosen *Nepenthes* species/hybrids are mostly adhered to the pitcher peristome and lid surfaces, and other methods of nectar sampling (Bauer *et al.*, 2009; Bauer *et al.*, 2016) are not feasible. Therefore, in this study, *Nepenthes* peristomes/lids were water-rinsed repeatedly, water in the washings was removed under vacuum and the ‘dry nectar’ was denoted as ‘extrafloral nectar’ (‘EFN’).

Sugars

Quantitative estimation of Suc, Glc, Fru contents in EFNs of *Nepenthes* pitcher peristomes and lids was carried out by HPTLC-densitometry (CAMAG, Switzerland), comprising of an automatic Linomat V sample applicator, twin trough plate development chamber, TLC Scanner 3, TLC Visualizer 2 and VisionCATS software 3.2, using pre-treated silica gel plates (60 F_{254}, 20 x 10 cm, 0.2 mm thickness; E. Merck, Germany). These TLC plates were modified by dipping in 0.2 M potassium dihydrogen orthophosphate solution (aqueous), wet plates were dried at 90°C for 10 min and then allowed to cool to room temperature in a desiccator (Lee *et al.*, 1979).

Stock solutions of standard Glc, Fru and Suc (Sigma Aldrich, USA) of 10 mg/2.4 mL each in water-methanol mixture (1:2, v/v) were prepared and different concentrations of these solutions were applied onto the pre-treated silica gel TLC plates with the Linomat V sample applicator, fitted with a microsyringe under N_2 flow (application rate 150 nL/s, space between two bands 12.1 mm). Plates were developed three times, each up to 80 mm in a paper-lined twin trough plate development chamber, equilibrated with the mobile phase, acetonitrile:water (8.5:1.5, v/v; 20 mL). For each run the mobile phase was replaced with fresh solvent, and the plates were thoroughly dried in between the runs. After the third run the plates were dried, derivatized using anisaldehyde-sulphuric acid reagent, heated at 110°C for 5 min and scanned densitometrically at 570 nm using TLC Scanner 3 equipped with VisionCATS 3.2 software (slit dimension 6.00 × 0.45 mm, scanning speed 20 mm/s). Standard sugar concentrations (Glc, Fru & Suc,: 0.28, 0.69, 1.39, 2.09, 2.78, 3.48, 4.17 µg) were plotted against peak area(s) to obtain the calibration curves (Glc: $y = 0.0037x + 0.001$, $r^2 = 0.99$; Fru: $y = 0.0027x + 0.0008$, $r^2 = 0.99$; Suc: $y = 0.0039x + 0.0019$, $r^2 = 0.99$).

Nepenthes pitcher peristome and lid EFNs (1 mg each) were dissolved in methanol-water mixture (0.8:0.2, v/v, 1 mL), 10 µL of these solutions were applied to modified silica gel TLC plates as 8 mm wide bands and the plates were developed, derivatized and scanned as in case of the sugar standards; Glc, Fru and Suc contents in EFNs were quantified using the calibration curves. *N. khasiana* (n = 6), *N. mirabilis* (n = 2), *N. mirabilis* × *N.*

khasiana (n = 5), *N. mirabilis* × *N. rafflesiana* (n = 3) EFNs were analyzed and their Glc-Fru-Suc contents (%) are listed in Table S1.

Amino acids

Amino acids in *Nepenthes* pitcher peristome and lid EFNs were tested by HPTLC-densitometry (CAMAG, Switzerland). Stock solutions (1 mg/mL) of twenty standard amino acids (Ala, Arg, Asn, Asp, Cys, Glu, Gln, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val) (Fluka Analytical, USA) were prepared in methanol-water mixture (0.9:0.1, v/v, 1 mL) and applied (2 μL, each) onto a silica gel TLC plate (60 F_{254}, 20 x 10 cm, 0.2 mm thickness; E. Merck, Germany) with the Linomat V sample applicator, fitted with a micro syringe under N_2 flow (application rate 150 nL/s, space between two bands 12.1 mm). Plate was developed up to 80 mm in the twin trough plate development chamber saturated with butanol:acetic acid:water (12:3:5, v/v, 20 mL). The developed plate was dried and derivatized using ninhydrin reagent, heated at 110°C for 5 min. Plates were scanned densitometrically at 570 nm using TLC Scanner 3 equipped with VisionCATS 3.2 software (slit dimension 6.00 × 0.45 mm, scanning speed 20 mm/s).

Nepenthes pitcher peristome and lid EFNs (1 mg each; *N. khasiana* n = 6, *N. mirabilis* n = 3, *N. mirabilis* × *N. khasiana* n = 2, *N. mirabilis* × *N. rafflesiana* n = 3) were dissolved in methanol-water mixture (0.8:0.2, v/v, 1 mL), 5 and 10 μL of these solutions were applied to a silica gel TLC plate as 8 mm wide bands and the plate was developed, derivatized and scanned as in case of the amino acid standards. Peristome and lid EFNs were also subjected to ninhydrin test for amino acids.

Proteins

Total protein contents in *Nepenthes* peristome and lid EFNs (*N. khasiana* n = 6, *N. mirabilis* n = 3, *N. mirabilis* × *N. khasiana* n = 2, *N. mirabilis* × *N. rafflesiana* n = 3) were determined colorimetrically using the Bradford protein assay kit (Thermo Scientific, USA) with bovine serum albumin (BSA) as the standard (Bradford, 1976).

Vitamin C

Vitamin C contents in *Nepenthes* species/hybrid peristome and lid (*N. khasiana* n = 6, *N. mirabilis* n = 3, *N. mirabilis* × *N. khasiana* n = 2, *N. mirabilis* × *N. rafflesiana* n = 3) EFNs were estimated titrimetrically by indophenol method with ascorbic acid as the standard (Nielson, 2019).

Fatty acids

Peristome (145.0 mg) and lid (78.4 mg) EFNs isolated from *N. khasiana* pitchers were dissolved in 10 mL water (each), partitioned with hexane (10 mL × 5) separately and concentrated in a rotary evaporator (Buchi, Switzerland) at 30ºC under reduced pressure to obtain their hexane (peristome 2.4 mg, lid 0.6 mg) fractions. These hexane fractions were dissolved (separately) in 1 mL hexane, added with methanolic NaOH (2 N, 200 µL) and heated on a water bath at 50°C for 20 sec. followed by shaking (10 sec). Methanolic HCl (2 N, 200 µL) was added to these mixtures, their upper layers were collected and dried over anhydrous sodium sulphate (de Melo Soares *et al.*, 2022).

These esterified fractions (1 µL each) diluted in diethyl ether (1:10 dilution, separately) were injected onto a QP2020C NX Gas Chromatograph Mass Spectrometer (Shimadzu, Japan), fitted with an SH-Stabilwax (Crossbond Carbowax polyethylene glycol) capillary column (30 m x 0.32 mm, film thickness 0.25 µm), coupled with a single quadrupole 8030 series mass selective detector. GC-MS parameters: injector temperature 240°C, oven temperature 60-250°C (3°C/min), ion source temperature of mass detector 240°C, and interphase temperature 260°C. Individual compounds were identified using NIIST 17 and Wiley 275 library search. Palmitic acid, stearic acid and *cis*-11-octadecenoic acid standards and their methyl esters were analyzed in GC-MS under similar conditions. Repeated analysis (n = 3) of peristome and lid EFNs of *N. khasiana* pitchers showed identical fatty acid patterns.

Minerals

Mineral contents in *N. khasiana* peristome and lid EFNs (n = 1 each) were analyzed by Avio 200 Inductively Coupled Plasma Optical Emission Spectrometer (PerkinElmer, USA). Spectral range of the equipment was 165-900 nm with a resolution of < 0.009 nm

at 200 nm. Fourth-generation 40 MHz, free-running solid-state RF generator, which is adjustable from 1000 to 1500 watts in 1-watt increments was used. ICP multi-element standard IV was used as the standard, and a five-level calibration was carried out. *N. khasiana* peristome and lid EFNs (0.1 g each, separately) were dissolved in 15 mL ultrapure water and filtered; two drops of acid were added to both samples for analysis.

Volatiles

Headspace GC-MS analysis of fresh *N. khasiana* specimens *viz.*, flower, tendril, peristome, lid, slices of the mid interior portion of peristome (nectary), pitcher fluid, plumbagin standard, was carried out on a QP2020C NX Gas Chromatograph Mass Spectrometer (Shimadzu, Japan) attached to HS 20 head space sampler (Shimadzu, Japan), fitted with an SH-Rxi-5Sil MS (Crossbond similar to 5% diphenyl/95% dimethyl polysiloxane) capillary column (30 m, 0.25 mm i.d., film thickness 0.25 µm). A droplet of EFN collected from the inner side *N. khasiana* lid using a syringe needle in early morning hours was also analyzed. Vial containing each sample was heated at 65°C for 10 min at shaking level 3 and pressurized up to 60 kPa. GC oven temperature: 50-200°C (10°C/min); ionization mode: electron impact ionization (EI) 70 eV, data processing: GC-MS solution Ver. 4. Individual compounds were identified using NIIST 17 and Wiley 275 library search. Repeated headspace GC-MS analysis (n = 2) gave identical results for the listed *N. khasiana* specimens.

CHN analysis

Carbon, hydrogen and nitrogen contents of *N. khasiana* peristome and lid EFNs (n = 1 each) were analyzed on a 2400 Series II CHNS Organic Elemental Analyzer (PerkinElmer, USA). Freeze-dried and crushed EFNs (5-10 mg) were used for the analysis; chromatographic responses were calibrated against pre-analyzed cystine standards, and CHN contents are reported in weight percent.

Prey spectrum

Prey composition of *N. khasiana* pitchers were studied by collecting the pitcher contents from 2 days after opening (DAO) to 14 DAO. Destructive sampling was done at regular

intervals of 2, 4, 6, 8, 10, 12 and 14 DAO of the pitchers. Six pitchers each (total of 42 pitchers) were sampled at the prescribed time intervals from *N. khasiana* plants at the Institute Conservatory. The contents of the pitchers were washed in double distilled water, sorted out using a stereo binocular microscope (Leica MZ6, Germany) and preserved in 75% alcohol for further identification. The insects were identified up to order level and data are listed in Table S2 (n = 6).

Ants

Two ant species visiting *N. khasiana* pitchers were collected and identified as *Monomorium minutum* Mayr. (little black ant) and *Anoplolepis gracilipes* Smith (yellow crazy ant) by Manoj Vembayam, Travancore Natural History Society, Thiruvananthapuram, Kerala, India and Dr. Thania Sara Varghese, one of the authors. Test ants (*M. minutum*, *A. gracilipes*) were collected from opened *N. khasiana* pitchers. For this, *N. khasiana* pitcher fluids with prey capture were transferred to petri dishes, fluid volume(s) were reduced using micropipettes, and these two species of ants were picked with the help of a handheld lens and a light source. The same two species of control ants were captured from the stem and leaf surfaces of *N. khasiana* pitchers. Test (with no pitcher fluid) and control ants were carefully transferred to suitable containers and kept at 4°C for the experiments.

Acetylcholinesterase inhibition by EFN-fed ants

Acetylcholine iodide (AChI), bovine erythrocyte cholinesterase (AChE), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and quinidine sulphate were procured from Sigma-Aldrich (MO, USA). A minimum of 20 ants (*M. minutum*, *A. gracilipes* each) collected from the pitcher fluids of *N. khasiana* were transferred to petri dishes (separately). Crude homogenates of ants were prepared in Eppendorf tubes using a manual homogenizer at 4°C in phosphate buffer (1 mL, 50 mM, pH 7). Homogenates were centrifuged at 12,000 × g for 15 min at 4°C and supernatants were separated; these supernatants of both test ants were used to prepare the AChE enzyme sources at a concentration of 400 units/L as described by Chinyere & Christos (2019). Similarly supernatants of control ants were also prepared.

A reaction mix was prepared by mixing the substrate (AChI, 100 µL, 136 µg/mL), enzyme source (supernatant, 50 µL, 5 units/L) and DTNB (60 µL, 0.4 mg/mL), made up to 500 µL using phosphate buffer (pH 8) and incubated at 30°C for 15 min; quinidine sulphate (0.1%) was added to stop the reaction. Absorbance was measured at 412 nm at 0 min and 10 min intervals for 4 h using a microplate reader (iMark^{TM} Microplate Absorbance Reader, Bio-Rad, Japan), and AChE activity was calculated as % inhibition. Both test and control ants (*M. minutum*, *A. gracilipes*) were subjected to similar AChE inhibition assays (Ellman *et al.*, 1961; Guengerich *et al.*, 2009; Gerczei & Pattison, 2015). Donepezil hydrochloride (10 µg/mL) served as the drug control (Colović *et al.*, 2013; Eddleston & Chowdhury, 2016). All samples were assayed in triplicate (Table S3).

Activity guided isolation

The procedures of active guided isolation of the active principle from *N. khasiana* EFN and peristome tissues, and its quantification are described in the Supplementary Information.

Spectroscopy of active principle

1H-, 13C-NMR (400 MHz, Bruker, Germany), LC-HRMS (Thermo Fisher Scientific, USA), FTIR (Thermo Scientific, USA) spectra and optical rotation (P2000 Polarimeter, Jasco, Japan) of the active molecule were recorded and its structure was elucidated (Hanson *et al.*, 1981) (Table S6).

Acetylcholinesterase inhibition by EFN, EFN- and (+)-isoshinanolone-fed ants

EFNs from *N. khasiana*, *N. mirabilis* and hybrids (*N. mirabilis* × *N. khasiana*, *N. mirabilis* × *N. rafflesiana*) peristomes and lids, active fractions of *N. khasiana* EFNs and active compound were tested for AChE inhibition activity. Briefly, a mix for each reaction was prepared by combining the substrate (AChI, 100 µL, 136 µg/mL), enzyme (AChE, 50 µL, 5 units/L) and DTNB (60 µL, 0.4 mg/mL) in a 12 well plate. Thereafter, EFN or active fraction or compound at various concentrations were added to the respective wells (Tables S4, S5), and the final volumes were made up to 500 µL using phosphate buffer

(pH 8). The samples were incubated at 30ºC for 15 min, and quinidine sulphate (0.1%) was added to stop the reactions. Absorbances were measured at 412 nm at 0 min and 10 min intervals for 4 h using a microplate reader (iMark^{TM} Microplate Absorbance Reader, Bio-Rad, Japan) (Tables S4, S5). Appropriate controls like (substrate (AChI) + enzyme source), (substrate (AChI) + AChE), (drug (donepezil) + substrate (AChI) + enzyme source) and (drug (donepezil) + substrate (AChI)+ AChE) were also evaluated so as to determine specificity of the enzyme (Tables S4, S5). All samples were assayed in triplicate.

A standard curve was generated by preparing reaction mixes consisting of varying concentrations of the substrate (AChI, 136 µg/mL), enzyme (AChE, 50 µL, 5 units/L) and DTNB (60 µL, 0.4 mg/mL), made up to a final volume of 500 µL with phosphate buffer (pH 8) and subjected to similar measurements. Protein contents of enzyme sources were determined using the Lowry assay (Lowry *et al.*, 1951). Hill equation was used throughout for variable cooperativity and non-cooperative model, and % inhibitions were determined by comparing the test and drug controls using GraphPad Prism software (Ellman *et al.*, 1961).

A. gracilipes ants were collected from its natural habitat at the campus sites of JNTBGRI and a minimum of 20 ants each were transferred to glass jars with ventilation holes in their lids. These ants were allowed to feed 1 mL each of sugar mix (Suc 0.55 mg + Glc 0.60 mg + Fru 1.03 mg, in distilled water), (sugar mix + (+)-isoshinanolone), (sugar mix + donepezil), *N. khasiana* peristome and lid EFNs at various concentrations as listed in Table S7. These experimental jars were kept in a temperature (22-24ºC)-controlled room overnight. Suc:Glc:Fru ratio in the sugar mix is same as in *N. khasiana* peristome EFN (Table S1). After 12 h feeding, crude homogenates of these experimental and control ants (unfed ants) were prepared separately in Eppendorf tubes using a manual homogenizer at 4°C in phosphate buffer (1 mL, 50 mM, pH 7), centrifuged at 12,000 × g for 15 min at 4°C and supernatants were separated. These supernatants were used to prepare AChE source at a concentration of 400 units/L from all experimental groups and AChE inhibition assays were carried out as described earlier.

LC-MS-metabolic profiling of EFNs

Stock solutions (1 µg/µL) of *N. khasiana* peristome and lid EFNs were prepared in water and filtered through 0.45 µm syringe filters. Metabolic profiling of the EFNs was carried out using a LC-2050C 3D i-Series liquid chromatography-mass spectrometry system (Shimadzu, Japan) equipped with a C-18 HPLC Shim-pack (5 µm, 250 mm x 4.6 mm) column. Peristome and lid EFNs (25 µL each) were separately injected onto the LC-MS system and a gradient mobile phase with water (solvent A, HPLC grade) and acetonitrile (solvent B, HPLC grade) both containing 0.1% formic acid (flow rate 0.3 mL/min, column temperature 40°C) was applied (initial elution 0% B, increased to 5.05% B in 8.80 min, 17.68% B in 30.70 min, 30.30% B in 52.60 min, 37.88% B in 65.80 min, 38.13% B in 66.20 min and finally to 50.51% B in 87.7 min). Data were acquired on an LCMS-2050 mass spectrometer (Shimadzu, Japan); metabolites were detected in positive ionization mode (scan range 100-400 m/z, MS parameters: gas temperature 450°C, drying gas 5 L/min, nebulizer gas flow 2 L/min).

Statistical analysis

In AChE inhibition assays, quantitative data are presented as the mean ± S.D. of three replicates. Statistical analysis was carried out by one-way ANOVA and Sidak's/Dunnett's multiple comparison tests using GraphPad Prism 8.0.2 software.

Results

Extrafloral nectar chemistry

N. khasiana pitcher peristomes and lids yielded 10.00 ± 8.48 mg (n = 6) and 6.23 ± 4.94 mg (n = 6) EFNs, respectively. EFNs showed Suc-Glc-Fru patterns (peristome, Suc: 5.53 ± 3.38%, Glc: 6.03 ± 3.38%, Fru: 10.27 ± 6.85%, n = 6; lid, Suc: 3.67 ± 3.47%, Glc: 7.76 ± 5.40%, Fru: 15.80 ± 7.00, n = 6), along with a few other minor sugars (Table S1). EFNs of *N. mirabilis* and the two hybrids, *N. mirabilis* × *N. khasiana*, *N. mirabilis* × *N. rafflesiana*, also displayed sugar patterns (Table S1). *N. mirabilis*, with smaller pitchers, showed relatively low nectar yields (peristome: 1.85 ± 0.35 mg (n = 2), lid: 1.95 ± 0.21 mg (n = 2)) and sugar patterns as (peristome, Suc: 1.15 ± 1.62% (n = 2); lid, Suc: 3.70 ± 5.23% (n =

2)). Glc and Fru were absent in *N. mirabilis* EFNs. Similarly, either one or all three sugars were absent in a few EFNs (Table S1).

Only very low levels of proteins were found in *N. khasiana* (peristome: 1.85 ± 2.25 g/100 g; lid: 1.20 ± 2.86 g/100 g (n = 6)) and *N. mirabilis* × *N. khasiana* (peristome: 0.61 g/100 g; lid: 0.22 g/100 g (n = 1) EFNs. No free proteins were detected in *N. mirabilis* and *N. mirabilis* × *N. rafflesiana* peristome, lid EFNs. *N. khasiana* peristome and lid EFNs were analyzed for free amino acids and none were detected (Fig. S1). *N. khasiana* peristome and lid EFNs are devoid of natural and non-protein amino acids. On similar assays, *N. mirabilis* and the two test hybrid EFNs also showed absence (or < detection limits) of amino acids. Vitamin C was absent in EFNs of *N. khasiana*, *N. mirabilis* and the two hybrids. *N. khasiana* EFNs on FAME-derivatization and gas chromatographic analysis showed C16:0, C18:1, C18:0 fatty acids (peristome: palmitic acid methyl ester (C16:0 derivative) 43.34%, *cis*-11-octadecenoic acid (C18:1) 23.33%, palmitic acid (C16:0) 11.96%; lid: *cis*-11-octadecenoic acid (C18:1) 55.79%, palmitic acid (C16:0) 19.73%, stearic acid (C18:0) 13.25%). Mineral analysis revealed *N. khasiana* peristome and lid EFNs as rich in sodium (peristome: 24.21 ppm, lid: 68.67 ppm), potassium (48.14 ppm, 159.80 ppm) and calcium (7.49 ppm, 23.42 ppm). *N. khasiana* EFNs displayed mild green fluorescence at UV 366 nm. Strikingly, *N. khasiana* floral nectar (FN) also showed similar chemistry of a sugary mix devoid of the key components, amino acids and proteins (low) (unpublished data).

Volatiles

Volatiles play crucial role in prey attraction and herbivory. Our headspace-GC-MS analyses showed plumbagin as the major volatile constituent in *N. khasiana* flower (59.21%), tendril (72.55%), peristome (85.47%), lid (74.58%) and slices of the mid interior portion of peristome (nectary, 60.62%). A droplet of EFN collected from *N. khasiana* lid also showed its traces (2.56%). Unopen and open *N. khasiana* pitcher fluids did not show plumbagin. Volatile organic compounds (VOCs), *viz.*, (Z)-3-hexen-1-ol (tendril: 14.72%, peristome: 5.02%, nectary: 16.18%, lid: 7.79%), 1-hexanol (tendril: 12.73%) and acetoin (nectary: 6.60%), were detected in *N. khasiana* pitcher tissues.

Preys, herbivores

N. khasiana pitchers trap a wide spectrum of preys which include arthropods, molluscs and reptiles (Fig. 1, Table S2). Arthropods found in the pitchers include Insecta, Arachnida and Diplopoda. Insects belonging to ten Orders, *viz.*, Hymenoptera, Diptera, Lepidoptera, Coleoptera, Hemiptera, Dictyoptera, Orthoptera, Isoptera, Neuroptera and Thysanoptera were collected and identified. Ants (Formicidae, Hymenoptera) were the predominant preys (96.4% of total capture) in *N. khasiana* pitchers, followed by flies (Diptera, 2.4%). Insects belonging to Hymenoptera (bees, wasps) (0.3%), Lepidoptera (0.6%), Coleoptera (0.05%) and arachnids (spiders, 0.06%) were minimal in pitcher catches from 2 to 14 days after opening. Hemiptera, Orthoptera, Dictyoptera, Isoptera, Neuroptera, and Thysanoptera were noticed occasionally. It was observed that the prey abundance increased from two days after opening and reached maximum on the eighth day (Fig. 1, Table S2). Herbivore attacks were rarely observed in *N. khasiana*.

Acetylcholinesterase inhibition, toxic principle

Two nectar-fed ant species (their homogenates, 5 units/L each, *in vivo*), *M. minutum* and *A. gracilipes* collected from *N. khasiana* pitcher fluids displayed near 100% AChE inhibition (99.9%), better than donepezil (1000 µg/mL, 91.0%), whereas homogenates of control ants captured from *N. khasiana* stem, leaf surfaces showed very low inhibition (14.0-15.3%) (Fig. 2, Table S3). Again, *N. khasiana* peristome and lid EFNs (1000 µg/mL) displayed 87.0% and 96.0% AChE inhibition (*in vitro*), respectively; and their chromatographic fractions showed varying levels of (few fractions > 80%) inhibition (Table S4). The active constituent from most active peristome and lid EFN fractions was isolated by chromatography and characterized by spectroscopy as the naphthoquinone derivative, (+)-isoshinanolone (Table S6). Phytochemical analysis of *N. khasiana* pitcher tissues also yielded (+)-isoshinanolone. This active principle displayed 94.5%, 97.5% and 100.0% AChE inhibition at 10, 100, 1000 µg/mL, comparable or above donepezil (Fig. 3, Table S4). *N. mirabilis*, *N. mirabilis* × *N. khasiana* and *N. mirabilis* × *N. rafflesiana* peristome and lid EFNs at 10-1000 µg/mL showed 55.3-77.9%, 32.9-52.3% and 34.0-53.0% inhibitions, respectively (Table S5, Fig. S2, S3).

(+)-Isoshinanolone was also detected in gas chromatographic profiles of peristome and lid EFNs of *N. khasiana*, *N. mirabilis* and the study hybrids (Fig. S4). (+)-Isoshinanolone in *N. khasiana* peristome and lid EFNs was quantified as 0.25 ± 0.04 and 0.16 ± 0.11 (% dry wt., n = 4 each), respectively, by gas chromatography (Table S8). In addition, LC-MS-metabolic profiling detected (+)-isoshinanolone in *N. khasiana* EFNs (Fig. S5).

In biotests, *N. khasiana* peristome EFN (10-1000 µg/mL)-, *N. khasiana* lid EFN (10-1000 µg/mL)-, sugar mix + (+)-isoshinanolone (10.0 µg/mL)- and sugar mix + donepezil (10 µg/mL)-fed *A. gracilipes* ants showed 52.5-87.0%, 62.5-96.0%, 98.0% and 87.0% AChE inhibition respectively, whereas sugar-fed and untreated control ants did not show enzyme inhibition (Fig. 4, Table S7); IC_{50}s of (+)-isoshinanolone and donepezil were 4.45 µg/mL and 5.25 µg/mL, respectively. Dizziness, low mobility and even deaths were observed in (+)-isoshinanolone-fed ants.

Discussion

Extrafloral nectar chemistry

In angiosperms, extrafloral nectar primarily functions as defense against herbivores; its chemical constituents are mainly carbohydrates along with amino acids, proteins, secondary metabolites, fatty acids and vitamins as minor entities (Nicolson, 2007; Heil, 2015; Nicolson, 2022). It is a persistent food source for tiny arthropods. EFN produced by *Nepenthes* pitchers is regarded as the major ‘reward’ for insects and other arthropods that are lured to these traps (Bauer *et al.*, 2008; Bennett & Ellison, 2009; Greenwood *et al.*, 2011). Sugars in floral nectar (FN) meet the energy requirements of pollinators; similarly, sugars in *Nepenthes* EFNs are an energy source to the ants and other visiting arthropods (Nicolson, 2022). Unlike Suc being the major carbohydrate in most FNs (Nepi *et al.*, 2018; Nicolson, 2022), *N. khasiana* peristome and lid EFNs are hexose-rich with Suc/(Glc+Fru) ratios of 0.34 and 0.16, respectively, whereas *N. mirabilis* × *N. khasiana* EFNs are sucrose-rich (Suc/(Glc+Fru) ratio: peristome 1.65, lid: 1.60). EFNs of other *Nepenthes* species/hybrids showed inconsistent Suc-Glc-Fru patterns (even one or more of these sugars were absent). Variations in yields and Suc-Glc-Fru ratios observed

between EFNs secreted by *Nepenthes* species and hybrid pitchers are due to varying enzyme activities (Lin *et al.*, 2014; Heil, 2015; Minami *et al.*, 2021; Nicolson, 2022). Fru, major sugar in *N. khasiana* EFNs, is the sweetest of naturally occurring carbohydrates, and it has the highest solubility of most sugars. All three sugars (Suc, Glc, Fru) are hygroscopic, Fru being the most. They tend to adsorb and retain moisture from the environment (Hanover & White, 1993), which is one of the factors causing peristome surface wetness leading to high prey capture rates (Bauer *et al.*, 2008; Sujatha *et al.*, 2023).

Strikingly, free amino acids were absent and only very little protein was found in *Nepenthes* EFNs. Amino acids provide taste and nutritional benefits, while proteins function as preservatives in FNs (Grasso *et al.*, 2015; Nicolson, 2022). Absence (or < detection limits) of amino acids in *Nepenthes* EFNs undermines their nutritional benefits. Fatty acids (C16:0, C18:0, C18:1) and their derivatives were detected in *Nepenthes* EFNs; and they are used in resting metabolism in insects (Nicolson, 2022). A recent study showed enhanced carbon assimilation and fatty acid biosynthesis in the C3 species, *Arabidopsis thaliana* (mutant), in high CO_2 (Xi *et al.*, 2023). Vitamin C, a vital nutrient which reduces oxidative damage (Nicolson, 2022), is absent in *Nepenthes* EFNs. Khan & co-workers (2013) demonstrated the substantial decrease of vitamin C in tomato (*Lycopersicon esculentum*, C3 species) under elevated concentration of CO_2. Mineral analysis revealed that *N. khasiana* peristome and lid EFNs are rich in potassium, sodium, and calcium. The role of minerals in FNs and EFNs of *Nepenthes* and other plant species is so far uninvestigated (Nicolson, 2022). Briefly, EFN secreted by *Nepenthes* is a sweet sugary mix, and due to the absence of amino acids, proteins and vitamin C, it is not providing the nutritional requirements for visiting ants and other insects.

Acetylcholinesterase inhibition, toxic principle

ACh is one of the key excitatory central nervous system neurotransmitters in vertebrates and invertebrates; and nerve impulse signal transmission at the synapses is accomplished through its degradation into choline and acetic acid by AChE (Colović *et al.*, 2013). Our results showed that *N. khasiana* peristome and lid EFNs, their fractions

and the active principle, (+)-isoshinanolone, inhibited AChE *in vitro*. Similarly, nectar-fed ants (*in vivo*) proved significant inhibition to the kinetics of AChE. ACh accumulation at the neuromuscular junctions causes symptoms of neuro-muscular degeneration such as cramps, muscular weakness, muscular fasciculation, paralysis and blurry vision (Kalamida *et al.*, 2007; Colović *et al.*, 2013). AChE inhibition by *Nepenthes* EFNs and its constituents results in the accumulation of ACh, thus inhibiting the locomotion of ants and other visiting insects. This was further demonstrated by direct-feeding of the AChE inhibitor, (+)-isoshinanolone, to ants (IC_{50} 4.45 μg/mL) which resulted in dizziness and considerable disruption of their motor function. Honey bees (*Apis mellifera* Linn.) showed similar effects on treatment with AChE inhibitors (Williamson *et al.*, 2013). Ratsirarson & Silander Jr. (1996) reported dizziness in insects on *N. madagascariensis* nectar consumption, enhancing the chances of them falling into the pitchers. Sluggish behavior and hampered flight of toxic nectar-fed pollinators were reported by various groups (Barberis *et al.*, 2023 and references therein). In *Nepenthes*, considering other nutrient acquisition designs listed in Fig. 5, the extent of influence of EFN and its constituents in prey capture is to be further ascertained. AChE inhibitors are known insecticides (Colović *et al.*, 2013), and (+)-isoshinanolone is a promising insecticidal entity. Moreover, this natural naphthoquinone-derivative displayed AChE inhibition comparable to the Alzheimer's disease (AD) drug, donepezil, and therefore, it is a likely candidate for AD therapy (dos Santos *et al.*, 2018).

Bioactivity guided isolation of *N. khasiana* EFNs led to the AChE inhibitor, (+)-isoshinanolone, which is known for its fish-stunning and mosquito larvicidal activities (Hanson *et al.*, 1981). We have also isolated this active principle from *N. khasiana* pitcher tissues. Isoshinanolone, an intermediate in plumbagin biosynthesis (Vasav *et al.*, 2022), has been reported from several other *Nepenthes* species/hybrids (examples: *N. gracilis*, *N. thorelii*, *N. mirabilis*, *N. thorelii* x (*ventricosa* x *maxima*)) and in other genera (*Drosera indica*, *D. serpens*, *D. finlaysoniana*, *Dionaea*, *Drosophyllum*) (Hatcher *et al.*, 2020; Wójciak *et al.*, 2023). Similar to the EFNs, (+)-isoshinanolone showed green fluorescence at UV 366 nm (Hanson *et al.*, 1981). Our data proved (+)-isoshinanolone as a neuroactive compound which manipulates the physical behavior and flight of insects. These results

clearly demonstrate *Nepenthes* EFN as a toxic sugar-rich secretion, which aids prey capture.

Volatiles, preys, herbivores

In our field study, ants were the most common prey type lured to *N. khasiana* pitchers. This observation is in agreement with the previous studies in other native habitats, and the number of ants captured was positively correlated to EFN production (Moran, 1996; Merbach *et al.*, 2001; Bauer *et al.*, 2008; Di Giusto *et al.*, 2008; Gaume *et al.*, 2016). EFN is easily accessible and relatively consistent food source for flightless ants. They are attracted to EFN-bearing tissues, and due to their presence plants generally benefit a reduction in herbivory; therefore, extrafloral nectaries and their associated nectar function as an indirect defense mechanism (Nicolson, 2007; Heil, 2015; Nicolson, 2022;). We found plumbagin as a volatile entity in the tendril and pitcher tissues in *N. khasiana*. Recent studies demonstrated the protection of *Nepenthes* against insect herbivores by naphthoquinones acting as phytoanticipins (Da´vila-Lara *et al.*, 2021b; Rahman-Soad *et al.*, 2021). *N. khasiana* lid EFN on direct headspace-GC-MS analysis showed traces of plumbagin, which is also known for its antifeedant and antimicrobial activities (Raj *et al.*, 2011). Notably, VOCs, (Z)-3-hexen-1-ol, 1-hexanol and acetoin, were detected in tissues of tendril, peristome and nectaries of *N. khasiana*. These volatile molecules might be contributing in plant-herbivore and plant-insect interactions (Dötterl & Gershenzon, 2023). *N. khasiana* EFNs showed high C:N ratio (peristome: C 24.01%, N 1.77%, lid: C 24.09%, N 2.02%); its leaves (C 45.72%, N 2.14%), and pitchers (C 39.07%, N 1.50%) growing under the influence of high CO_2 also showed high C:N ratio (Baby *et al.*, 2017), which causes dilution of nitrogen resulting in reduced palatability and nutritional quality (Nicolson, 2022). Therefore, the reduction in herbivory observed in *Nepenthes* leaves and traps (Merbach *et al.*, 2002; Merbach *et al.*, 2007; Mithöfer, 2022) is explained by ant visitation to these tissues, their nitrogen deficiency and high naphthoquinone contents.

CO_2, metabolism, prey capture

We have discovered 2500-5000 ppm respiratory CO_2 within unopened pitchers of *N. khasiana* and *Nepenthes* hybrids. Open *Nepenthes* pitchers emit CO_2 as plumes above

atmospheric levels (Baby *et al.*, 2017). Studies demonstrated that CO_2 plumes emitted by flowers as indicators to various pollinators (Goyret *et al.*, 2008). These studies infer that plants use CO_2 emission to signal adequate nectar supply to visiting pollinators. In our study, CO_2 streaming through *N. khasiana* open pitchers (620 ppm) caught higher number of aerial preys compared to normal (CO_2 477 ppm) and air (CO_2 413 ppm)-streamed pitchers (Baby *et al.*, 2017). Coincidentally, *Nepenthes* pitchers confront an elevated atmospheric CO_2 scenario externally (419.3 ppm in 2023) (Lindsey, 2024), in addition to the ten-times high CO_2 within their unopen pitchers (Baby *et al.*, 2017).

Elevated CO_2 induces changes in plant metabolism and enhances carbon assimilation demonstrated mainly by increased leaf C:N ratio and non-structural carbohydrates (Sardans *et al.*, 2012; Zavala *et al.*, 2013). *Nepenthes* follow the C3 photosynthetic pathway; and similar to the CO_2-enrichment experiments, *N. khasiana* leaf and pitcher tissues showed high carbon and low nitrogen contents (Baby *et al.*, 2017). Further, Osunkoya & co-workers (2007) recorded low nitrogen contents and high C:N ratios in leaves and pitchers of several *Nepenthes* species/hybrids from Brunei. High C:N ratio promotes the synthesis of C-based metabolites and limits the N-based ones. Again, high carbohydrate contents in the phloem tissues are transported and enzymatically transformed into nectar sugars in *Nepenthes* pitcher nectaries (Lin *et al.*, 2014; Heil, 2015; Minami *et al.*, 2021; Nicolson, 2022). *Nepenthes* pitchers due to low nitrogen, chlorophyll content and stomatal density and replacement of chlorophyll-containing cells with digestive glands display relatively low photosynthetic rates, and thereby low photosynthetic nitrogen-use efficiency. Moreover, these prey traps growing in elevated CO_2 display reduced Rubisco activity (Pavlovicˇ & Saganova, 2015). Pavlovič & Kocáb (2022) reported high level of alternative oxidase (AOX) in *Nepenthes* traps compared to their leaves, and inferred that part of the CO_2 released from the pitchers is from the AOX activity. Another recent transcriptome sequencing study on the hybrid *Nepenthes x ventrata* ascertained the role of CO_2 in pitcher development, function and evolution of its other key features (Shchennikova *et al.*, 2021). CO_2 within the pitchers also helps in maintaining the acidic pH of the digestive fluid (Baby *et al.*, 2017).

Several alkaloids function as toxins in FNs (Adler, 2000; Grasso *et al.*, 2015; Stevenson *et al.*, 2017; Barberis *et al.*, 2023). A few studies also mentioned anesthetizing alkaloids in *Nepenthes* nectar secretions (Bohn & Federle, 2004; Raj *et al.*, 2011). But so far, in genus *Nepenthes*, there are no reports on the isolation of any nitrogenous secondary metabolites (or alkaloids), which are biosynthesized from amino acid building blocks (De Luca & St Pierre, 2000; Nicolson, 2022) (Hatcher *et al.*, 2020; Wójciak *et al.*, 2023). Moreover, our phytochemical analysis on *N. khasiana* whole plant and its pitchers led to the isolation of over 30 secondary compounds (naphthoquinones, phenolics, terpenoids, steroids, fatty acid derivatives); none of them were nitrogen bearing entities (unpublished data). Most secondary metabolites reported from *Nepenthes* are naphthoquinones (Hatcher *et al.*, 2020; Wójciak *et al.*, 2023). They are considered as chemotaxonomic markers of the genus. Phenolics are the second major group of metabolites in *Nepenthes*. Pitcher growth, high respiration, high carbohydrate, low nitrogenous primary and secondary metabolites, modified stomata and acidic pitcher fluid are critical features impelled by the high CO_2 atmosphere within *Nepenthes* pitchers (Baby *et al.*, 2017). In other words, *Nepenthes* pitchers are classic examples of leaf-evolved structures displaying manifestations of an elevated CO_2 atmosphere within them.

Chemical (EFN, volatiles), gaseous (CO_2), visual (colour, UV reflectance, fluorescence), physical (peristome geometry, surface microstructure, wettable surface-aquaplaning) and environmental (humidity, rain) factors are the prey capture strategies adopted by *Nepenthes* pitchers (Fig. 5). This study clearly demonstrates that *Nepenthes* EFN is not a nutrient, instead is a ‘toxic bait’ aiding prey capture. Unlike in the pollination scenario wherein FN and pollen are true rewards and both plant and pollinator mutually benefit, in prey capture, the present discovery of a neurotoxin embedded in *Nepenthes* EFNs nullifies the notion of reward to the visiting ants and other insects.

In conclusion, EFN was believed to be the major reward in *Nepenthes* prey traps. This study demonstrated EFN secreted by nectaries on *Nepenthes* pitchers as a sugar (Suc-Glc-Fru) mix, lacking key nutritional components, *viz.*, amino acids, proteins (low) and vitamin C. Crucially, EFN is toxic to ants and other insects, and its toxic component

(natural AChE inhibitor) has been identified as the naphthoquinone derivative, (+)-isoshinanolone. EFN secreted by *Nepenthes* unveils dual function of a lure and a toxin; and, unlike FNs, it is providing only minimal nutritional benefits to the visitors. Our results impel the chemical characterization and toxicity evaluation of EFNs in other *Nepenthes* species. CO_2 influenced metabolism, naphthoquinones (plumbagin, (+)-isoshinanolone) and ant visitation are the reasons for reduced herbivory in *Nepenthes*, particularly in their pitcher traps. High respiratory CO_2 within *Nepenthes* pitchers is resulting in their high growth rate, C:N ratio, low Rubisco activity, modified stomata, acidic pitcher fluid and absence of key nitrogenous components in its metabolism. The multitudes of physico-chemical and environmental strategies of prey capture in *Nepenthes* are deceptive, and essentially no reward is being offered to the visiting ants and insects. Our findings infer elevated CO_2 within as the single most critical factor influencing the growth, metabolism, prey capture, digestion and herbivory of *Nepenthes* prey traps.

Supporting information

Procedures of activity guided isolation of the active principle from *N. khasiana* EFNs and its quantification.

Table S1. Sugar composition in peristome and lid extrafloral nectars of *Nepenthes* species and hybrids.

Table S2. Insects and other organisms trapped in *N. khasiana* pitchers from 2 days to 14 days after opening.

Table S3. AChE inhibition by homogenates of ants, *Monomorium minutum* and *Anoplolepis gracilipes*, collected from *N. khasiana* pitcher fluid (*in vivo*).

Table S4. AChE inhibition of *N. khasiana* peristome and lid EFNs and the active principle, (+)-isoshinanolone (*in vitro*).

Table S5. Spectral data of (+)-isoshinanolone.

Table S6. AChE inhibition of *Nepenthes* species, hybrids peristome and lid EFNs (*in vitro*).

Table S7. AChE inhibition by *N. khasiana* EFN- and (+)-isoshinanolone-fed ants (*Anoplolepis gracilipes*, *in vitro*).

Table S8. Quantification of (+)-isoshinanolone in *N. khasiana* peristome and lid EFNs.

Fig. S1. Amino acid profiling in peristome and lid EFNs of *N. khasiana*.

Fig. S2. AChE inhibition of *Nepenthes* species peristome and lid EFNs (*in vitro*).

Fig. S3. AChE inhibition of *Nepenthes* hybrid peristome and lid EFNs (*in vitro*).

Fig. S4. Detection of (+)-isoshinanolone in *Nepenthes* species/hybrid peristome and lid EFNs.

Fig. S5. Metabolic profiling of *N. khasiana* pitcher lid EFN by LC-MS.

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Competing interest

None declared.

Author contributions

S.B., C.C.L., G.B.S., G.T. and A.J.J.: conceived the project, designed and performed the experiments; C.C.L., G.B.S., G.T., A.J.J. and S.B.: data acquisition, analysis and organization; GV: biochemical assays, data interpretation; TSV: data acquisition on insect visitors, interpretation; C.C.L., G.B.S., G.T., A.J.J., S.M. and L.A.S.: *Nepenthes* specimen collection, field support; S.B.: fund acquisition; S.B. drafted the original manuscript and all authors contributed in finalizing the manuscript. All authors have read and approved the final manuscript.

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Data availability

The data that support the findings of this study are available within the manuscript and its Supporting Information.

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List of figures

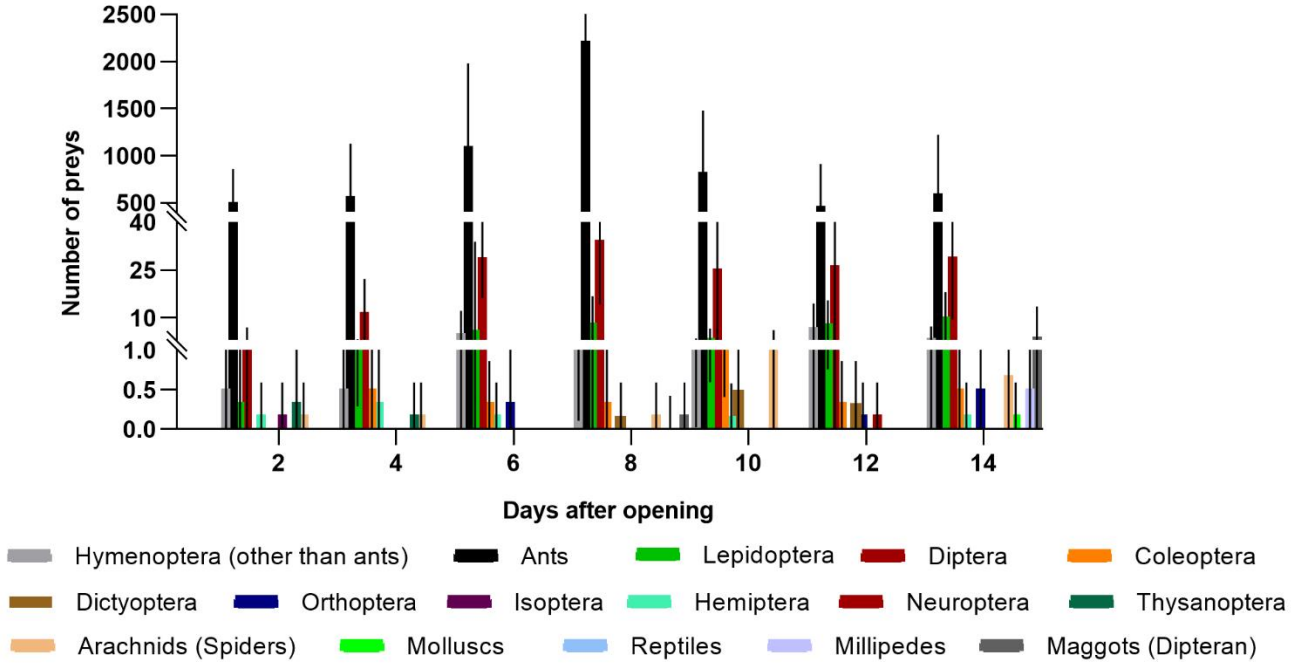

Fig. 1. Prey capture by *Nepenthes khasiana* pitchers. Insects and other organisms trapped in *N. khasiana* pitchers from 2 days to 14 days after opening (data presented as average prey capture per pitcher, n = 6; details in Table S2).

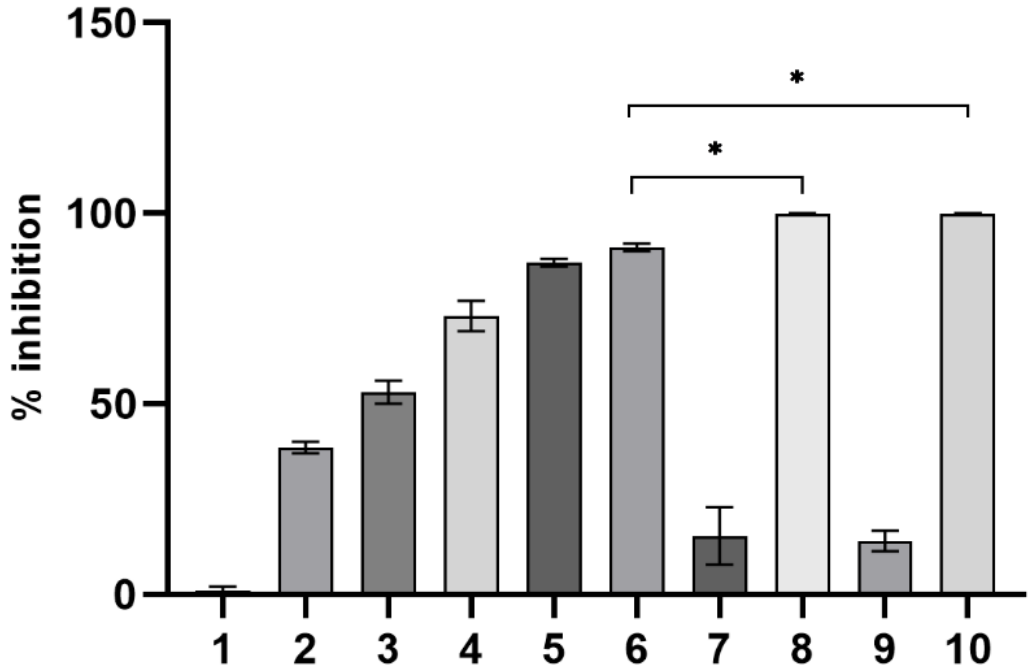

Fig. 2. AChE inhibition by homogenates of ants, *Monomorium minutum* and *Anoplolepis gracilipes*, collected from *N. khasiana* pitcher fluid (*in vivo*). 1: substrate (AChl) + AChE; **2**: AChl + donepezil (2.5 µg/mL) + AChE; **3**: AChl + donepezil (5.0 µg/mL) + AChE; **4**: AChl + donepezil (10 µg/mL) + AChE; **5**: AChl + donepezil (100 µg/mL) + AChE; **6**: AChl + donepezil (1000 µg/mL) + AChE; **7**: AChl + control-enzyme source (non-EFN fed ant *M. minutum* homogenate); **8**: AChl + test-enzyme source (EFN-fed ant *M. minutum* homogenate); **9**: AChl + control-enzyme source (non-EFN fed ant *A. gracilipes* homogenate) (5 units/L); **10**: AChl + test-enzyme source (EFN-fed ant *A. gracilipes* homogenate); AChl (136 µg/mL) and donepezil are substrate and control, respectively; AChE, control and test ant homogenates, 5 units/L. *p* values were calculated using one-way ANOVA and Dunnett's multiple comparisons test using GraphPad Prism 8.0.2; values are mean ± S.D; n = 3; **p* = 0.0130 (compared to 6); data listed in Table S3.

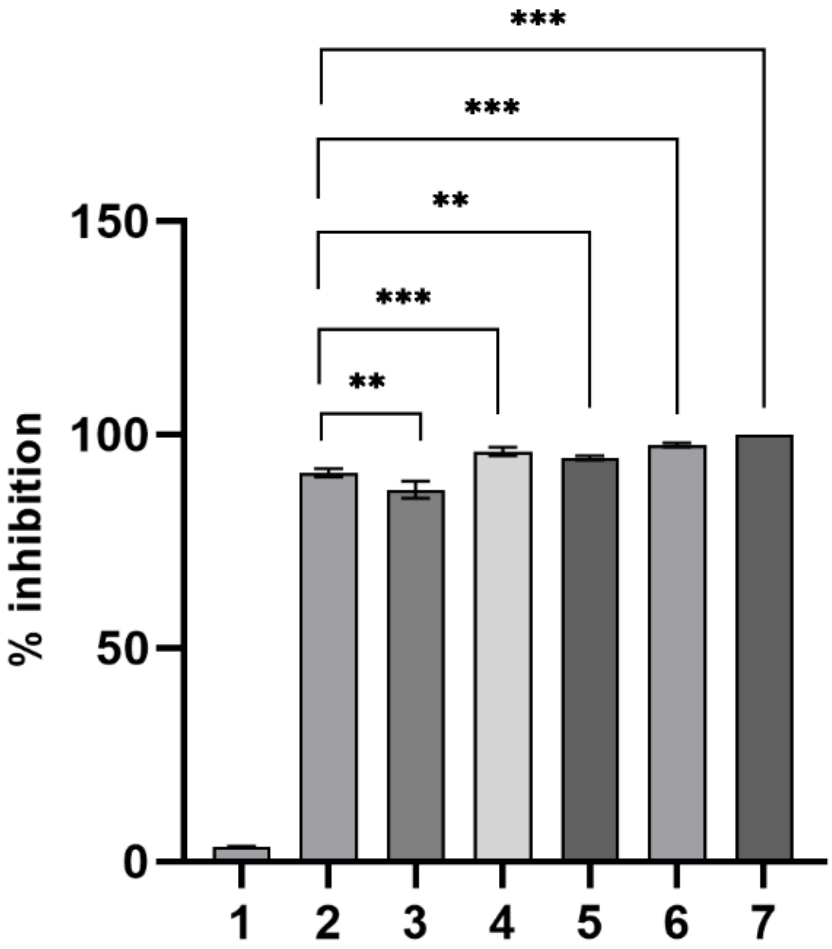

Fig. 3. AChE inhibition of *N. khasiana* EFNs and the active principle, (+)-isoshinanolone (*in vitro*). **1**: AChl + AChE; **2**: AChl + donepezil (1000 µg/mL) + AChE; **3**: AChl + peristome EFN (1000 µg/mL) + AChE; **4**: AChl + lid EFN (1000 µg/mL) + AChE; **5**: AChl + (+)-isoshinanolone (10 µg/mL) + AChE; **6**: AChl + (+)-isoshinanolone (100 µg/mL) + AChE; **7**: AChl + (+)-isoshinanolone (1000 µg/mL) + AChE; AChl (136 µg/mL) and donepezil are substrate and control, respectively; AChE, 5 units/L. *p* values were calculated using one-way ANOVA and Sidak's multiple comparisons test using GraphPad Prism 8.0.2; values are mean ± S.D; n = 3; ***p* = 0.0010, 0.0034; ****p* < 0.0001 (compared to 2); data listed in Table S4.

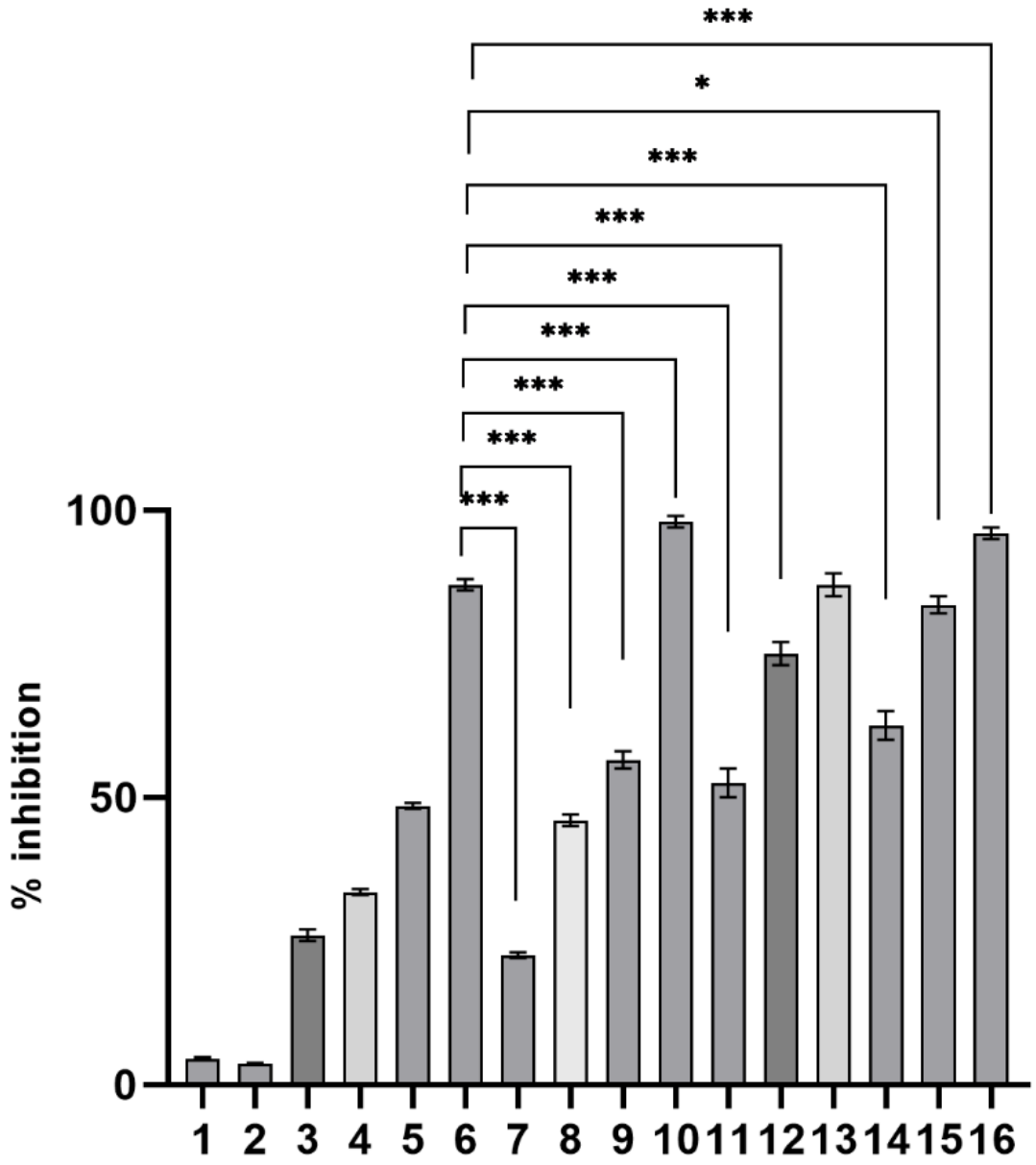

Fig. 4. AChE inhibition by *N. khasiana* EFN- and (+)-isoshinanolone-fed ants (*Anoplolepis gracilipes*). 1: AChl + unfed ant homogenate; **2**: AChl + sugar mix (Suc 0.55 mg + Glc 0.60 mg + Fru 1.03 mg)-fed control ant homogenate; **3**: AChl + (sugar mix + donepezil 1.25 µg/mL)-fed ant homogenate; **4**: AChl + (sugar mix + donepezil 2.5 µg/mL)-fed ant homogenate; **5**: AChl + (sugar mix + donepezil 5.0 µg/mL)-fed ant homogenate; **6**: AChl + (sugar mix + donepezil 10 µg/mL)-fed ant homogenate; **7**: AChl + (sugar mix + (+)-isoshinanolone 1.25 µg/mL)-fed ant homogenate; **8**: AChl + (sugar mix

+ (+)-isoshinanolone 2.5 µg/mL)-fed ant homogenate; **9**: AChl + (sugar mix + (+)-isoshinanolone 5.0 µg/mL)-fed ant homogenate; **10**: AChl + (sugar mix + (+)-isoshinanolone 10.0 µg/mL)-fed ant homogenate; **11**: AChl + peristome EFN (10 µg/mL)-fed ant homogenate; **12**: AChl + peristome EFN (100 µg/mL)-fed ant homogenate; **13**: AChl + peristome EFN (1000 µg/mL)-fed ant homogenate; **14**: AChl + lid EFN (10 µg/mL)-fed ant homogenate; **15**: AChl + lid EFN (100 µg/mL)-fed ant homogenate; **16**: AChl + lid EFN (1000 µg/mL)-fed ant homogenate. AChl (136 µg/mL) and donepezil are substrate and control, respectively; control and test ant homogenates, 5 units/L each. *p* values were calculated using one-way ANOVA and Dunnett's multiple comparisons test using GraphPad Prism 8.0.2; values are mean ± S.D; n = 3; *p = 0.0414; ***p < 0.0001 (compared to 6); data listed in Table S7.

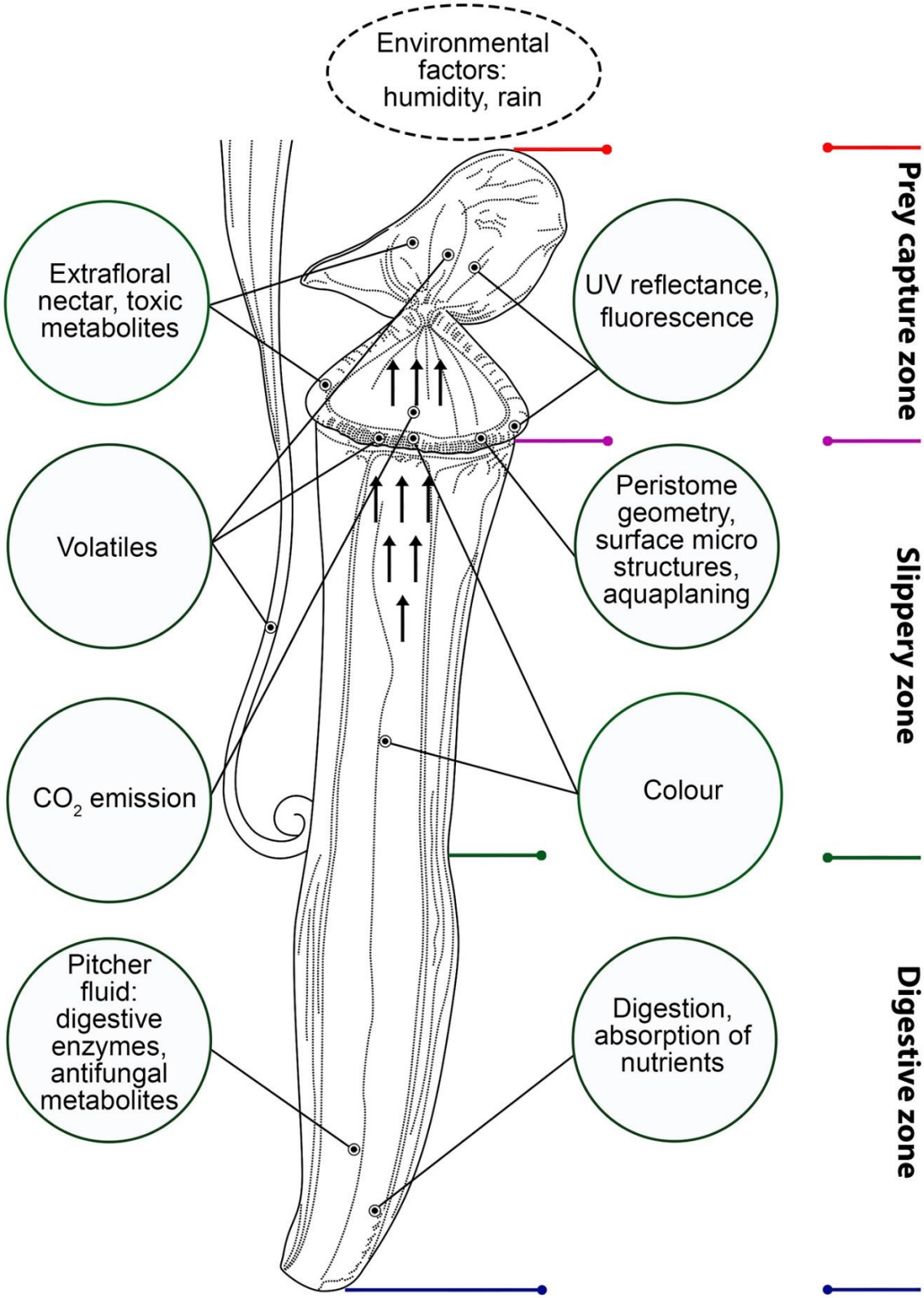

Fig. 5. Prey capture strategies adopted by *Nepenthes* pitchers.