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

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Nepenthes pitchers are leaf-evolved biological traps holding high levels of CO_2 within them. Extrafloral nectar (EFN) secreted by *Nepenthes* pitchers has long been regarded as the major reward to the visiting arthropods, but its chemical constituents and their role in prey capture are least explored. Here we show that *Nepenthes* EFN is a toxic sugar mix devoid of key nitrogenous components (amino acids, proteins) and vitamin C. Fatty acids (*cis*-11-octadecenoic acid (C18:1), palmitic acid (C16:0), stearic acid (C18:0)) and their derivatives were detected in both *N. khasiana* peristome and lid EFNs. Both the nectars showed strong acetylcholinesterase (AChE) inhibition *in vitro* and *in vivo*. Bioactivity guided isolation revealed (+)-isoshinanolone, a naphthoquinone derivative, as the AChE inhibitor in *Nepenthes* EFNs. GC-headspace analysis showed the naphthoquinone, plumbagin, as the major volatile constituent in prey capturing regions of pitchers, along with the VOCs, (Z)-3-hexen-1-ol, 1-hexanol and acetoin. *N. khasiana* pitchers trap a wide spectrum of preys dominated by ants, whereas only limited herbivory was observed on its leaves and pitchers. 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. These leaf-transformed traps with high growth rate and C:N ratio, reduced Rubisco activity, modified stomata, acidic pitcher fluid and absence of key nitrogenous metabolites are model systems displaying the effects of elevated CO_2 within them. We testify that *Nepenthes* EFN is 'a toxic sugar bait' which hinders the neuronal activity and flight of visiting arthropods. These unique traps adopt various deceptive strategies for prey capture, and our discovery abolishes the notion of *Nepenthes* EFN as a 'reward' to the visiting ants and other arthropods.

Key words: *Nepenthes*, pitcher, extrafloral nectar, (+)-isoshinanolone, volatiles, AChE inhibition, toxicity, prey capture.

List of abbreviations

ACh, acetylcholine; AChE, acetylcholinesterase; AOX, alternative oxidase; CO_2, carbon dioxide; EFN, extrafloral nectar; FAME, fatty acid methyl ester; FN, floral nectar; Fru, fructose; GC, gas chromatography; Glu, glucose; PNUE, photosynthetic nitrogen use efficiency; SEM, scanning electron microscopy; Suc, sucrose; VOC, volatile organic compound.

Nepenthes pitchers effect prey capture and nutrient acquisition through various physico-chemical and biological strategies (Bohn and Federle, 2004; Baby *et al.*, 2017; Mithöfer, 2022) (Fig. 1). Their pitcher rim (peristome) is a wettable, low-friction surface with distinct microstructures (Bohn and Federle, 2004; Hsu *et al*., 2015; 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 and 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-encriched cavities, and open pitchers continually emit CO_2 attracting preys (Baby *et al.*, 2017). Over the years researchers reported the role of pitcher colour (Bennet and 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 (Moran and Clarke, 2010; Bauer *et al*., 2011) is considered as the primary reward (and lure) to the visiting ants, insects and small mammals (Bennett and Ellison, 2009; Bauer *et al*., 2008; Bauer *et al*., 2011; Gaume *et al*., 2016). EFN also contributes to the peristome wettability. Bornean montane *Nepenthes* species (*N. lowii*, *N. rajah*, *N. macrophylla*) through mutualistic interactions provide nectar and harvest nitrogenous excreta from tree shrews and other small mammals through their shape-adapted pitchers (Moran and Clarke, 2010; 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 (Chatt *et al*., 2021; Nicolson, 2022). The nectar, being a sweet aqueous secretion, is easily consumed, digested and absorbed. It has a complex chemistry containing sugars, amino acids, proteins, lipids, fatty acids, phenolics, alkaloids, and organic acids (De la Barrera and 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 extrafloral nectar (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 *Nepenthes* pitchers (Baby *et al.*, 2017).

Nectar chemistry

Extrafloral nectars (EFNs) from *N. khasiana* pitchers were water extracted from their peristomes and lids. EFN yields from *N. khasiana* peristomes and lids were 10.00 ± 8.48 mg (n = 6) and 6.23 ± 4.94 mg (n = 6), respectively. EFNs isolated from *N. khasiana* showed Suc-Glu-Fru patterns (peristome, Suc-Glu-Fru: 5.53 ± 3.38%, 6.03 ± 3.38%, 10.27 ± 6.85%, n = 6; lid, Suc-Glu-Fru: 3.67 ± 3.47%, 7.76 ± 5.40%, 15.80 ± 7.00, n = 6), along with a few other minor sugars (Table S1). EFNs of *N. mirabilis* and hybrids *N. mirabilis* × *N. khasiana*, *N. mirabilis* × *N. rafflesiana* also showed sugar patterns (Table S1). *N. mirabilis*, with smaller pitchers, showed relatively low nectar yields (peristome: 1.85 mg (n = 2), lid: 1.95 mg (n = 2)) and sugar patterns as (peristome, Suc: 1.15 ± 1.62% (n = 2); lid, Suc: 3.70 ± 5.23% (n = 2)). Glu and Fru were absent in *N. mirabilis* EFNs. Similarly, in a few EFNs either one or all three sugars were absent (Table S1). Only very low levels of proteins were found in *N. khasiana* EFNs (peristome: 1.85 ± 2.25 g/100 g; lid: 1.20 ± 2.86 g/100 g (n = 6)), *N. mirabilis* × *N. khasiana* (peristome: 0.61 g/100 g; lid: 0.22 g/100 g (n = 1)). No free proteins were detected in *N. mirabilis* and *N. mirabilis* × *N. rafflesiana* peristome, lid EFNs. Further *N. khasiana* peristome and lid EFNs were analyzed for 20 free amino acids (with standards) and none were detected. *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 of amino acids. 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). Toxic metals like cadmium, cobalt, chromium, led and nickel were at below detection level in both nectars. Vitamin C was absent in EFNs of *N. khasiana*, *N. mirabilis* and the hybrids. *N. khasiana* EFNs after FAME-derivatization showed 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%) on gas chromatographic analysis. EFNs of *N. khasiana* peristome and lid 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 GC-headspace 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%). Pitcher fluids of unopen and open *N. khasiana* pitchers 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 also detected in *N. khasiana* tissues. SEM micrographs of *N. khasiana* tendril, pitcher peristome, inner side of the lid, nectary and digestive zone were previously reported (Baby *et al.*, 2017; Sujatha *et al.,* 2023).

Herbivores, preys

In our conservatories, nymphs and adults of *Helopeltis theivora* Waterhouse (tea mosquito bug), a known pest in tea plantations, were found attacking *N. khasiana* at the end of the monsoon season by sucking the sap of its apical buds and young leaves. Severe *H. theivora* infestations caused total damage of the plant. Other herbivore attacks were rarely observed in *N. khasiana*.

N. khasiana pitchers trap a wide spectrum of preys which include arthropods, molluscs and reptiles (Fig. 2, 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 catch

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. 2, Table S2).

AChE inhibition, toxic principle

Two nectar-fed ant species (their homogenates, 5 units/L each, *in vivo*), *Monomorium minutum* and *Anoplolepis gracilipes*, collected from *N. khasiana* pitcher fluids, displayed near 100% AChE inhibition (99.9%) similar to donepezil (10 µg/mL, 94.5%), whereas homogenates of control ants captured from *N. khasiana* stem, leaf surfaces showed very low inhibition (14.5-17.0%) (Table S3, Fig. 3). Again, *N. khasiana* peristome and lid EFNs (1000 µg/mL) displayed 74.5% and 91.0% AChE inhibition (*in vitro*), respectively; and their chromatographic fractions showed varying (a 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 (hydroxyl at C4 instead of carbonyl group), (+)-isoshinanolone (Supplementary information). 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 similar to donepezil (10 µg/mL) (Table S4, Fig. 4). *N. mirabilis*, *N. mirabilis* × *N. khasiana* and *N. mirabilis* × *N. rafflesiana* peristome and lid EFNs at 10-1000 µg/mL showed 50.3-77.9%, 32.9-45.7% and 39.3-53.0% inhibitions, respectively (Table S5, Fig. S1, S2). (+)-Isoshinanolone was also detected in gas chromatographic profiles of peristome and lid EFNs of *N. khasiana*, *N. mirabilis* and the study hybrids (Fig. S3). Further, (+)-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 (Table S6).

Discussion

In angiosperms, extrafloral nectar (EFN) 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. In *Nepenthes*, extrafloral nectaries are present on their tendrils, pitcher peristomes and lids. EFN produced by *Nepenthes* pitchers are long regarded as the major 'reward' for insects and other arthropods that are lured to these traps (Bauer *et al*., 2008; Bennett and Ellison, 2009; Gaume *et al*., 2016). Sugars in floral nectar (FN) meet the energy requirements of pollinators. Unlike Suc being the major carbohydrate in most FNs, *N. khasiana* peristome and lid EFNs are hexose-rich with Suc/(Glu+Fru) ratios of 0.34 and 0.16, respectively, whereas *N. mirabilis* × *N. khasiana* peristome EFN is sucrose-rich (Suc/(Glu+Fru) 1.65). Fru is found to be the dominant sugar in EFNs of *N. khasiana*. Other *Nepenthes* species showed inconsistent Suc-Glu-Fru patterns (even one or more of these sugars were absent) in their EFNs. Variations in content (yield) and Suc-Glu-Fru ratios observed between EFNs secreted by *Nepenthes* species and hybrid pitchers are accounted by varying enzyme activities (Lin *et al.*, 2014; Heil, 2015; Minami *et al.*, 2021; Nicolson, 2022). Fru is the sweetest of naturally occurring carbohydrates, and has the highest solubility of most sugars. All three sugars (Suc, Glu, Fru) are hygroscopic, Fru being the most. They tend to adsorb moisture from the environment (Hanover and White, 1993), which is one of the factors causing peristome surface wetness leading to high prey capture rates (Bauer *et al*., 2008).

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 of amino acids in *Nepenthes* EFNs undermines their nutritional benefits. Fatty acids (C16:0, C18:0, C18:1) were detected in *Nepenthes* EFNs, and along with amino acids, they are used in resting metabolism (Levin *et al*., 2017). 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 and co-workers (2013) demonstrated the substantial decrease of vitamin C in tomato (*Lycopersicon esculentum*, C3 species) under elevated concentration of CO_2 (1000 μmol mol^{-1}). Mineral analysis revealed that *N. khasiana* peristome and lid EFNs are rich in potassium, sodium, and

calcium. The role of minerals in *Nepenthes* EFNs and FNs of 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.

ACh is one of the chief 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 (Xie *et al.*, 2000). Our results showed that *N. khasiana* peristome and lid EFNs, their fractions and the active principle, (+)-isoshinanolone (naphthoquinone derivative), 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, 2007; Colović *et al*., 2013). AChE inhibitors are known insecticides (White, 2002; Colović *et al.*, 2013), and (+)-isoshinanolone is a promising insecticidal entity. AChE inhibition by *Nepenthes* EFNs and its constituents causes the accumulation of ACh, thus inhibiting the locomotion of ants and other visiting insects.

Bioactivity guided isolation of *N. khasiana* EFNs led to the AChE inhibitor, (+)-isoshinanolone, which is known for its fish-stunning (Hanson *et al*., 1981) and mosquito larvicidal activities (Sreelatha *et al*., 2010). We have also isolated (+)-isoshinanolone from *N. khasiana* pitcher tissues. Isoshinanolone has been reported from other *Nepenthes* species/hybrids, *N. gracilis* (Aung *et al.*, 2002), *N. thorelii* (Likhitwitayawuid *et al.*, 1998), *N. mirabilis* (Thanh *et al.*, 2015), *N. thorellii* x (*ventricosa* x *maxima*) (Ou-Yang *et al.*, 2019) and in other genera *viz.*, *Drosera indica*, *D. serpens*, *D. finlaysoniana*, *Dionaea*, and *Drosophyllum* (Schlauer *et al.*, 2021). AChE inhibition assay (*in vivo* and *in vitro*) showed (+)-isoshinanolone as a neuroactive compound which manipulates insect behavior and flight. Our results clearly demonstrate *Nepenthes* EFNs as toxic sugar-rich secretions, which aid the prey capture designs of *Nepenthes* pitcher traps.

In our field study, ants are the most common prey type in the pitchers of *N. khasiana*. 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; Blüthgen and Fiedler, 2004; Di Giusto *et al*., 2008; Bauer *et al.*, 2008; Gaume *et al*., 2016; Renyard *et al*., 2022). EFN is easily accessible and relatively consistent food source for flightless ants. They are attracted to the EFN and due to their presence plants generally benefit a reduction in herbivory; therefore extrafloral nectaries and their associated nectar is an indirect defense mechanism (Pacelhe *et al.*, 2019). We found plumbagin as a volatile entity in the tendril and pitcher tissues in *N. khasiana*. Recent reports demonstrated the protection of *Nepenthes* against insect herbivores by naphthoquinones acting as phytoanticipins (Da´vila-Lara *et al*., 2021b; Rahman-Soad *et al*., 2021). Notably, VOCs, (Z)-3-hexen-1-ol, 1-hexanol and acetoin, were also detected in tissues of tendril, peristome and nectaries of *N. khasiana*. These molecules are known insect attractants; they might also be functioning as indirect defense against herbivore attacks (Katsoyannos and Guerin, 1984; James, 2005; Xiao and Lu, 2014). *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). Herbivory is rarely observed in *Nepenthes* (Merbach *et al.*, 2002; Merbach *et al.*, 2007; Mithöfer, 2022). Ants visiting the extrafloral nectaries, nitrogen deficiency and high naphthoquinone content explain the low-level herbivory observed in *Nepenthes* leaves and traps.

Recently we discovered 2500-5000 ppm respiratory CO_2 within unopened pitchers of *N. khasiana* and hybrids. Open *N. khasiana* 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 (Guerenstein *et al*., 2004; Thom *et al*., 2004; Goyret *et al*., 2008; von Arx *et al*., 2012). 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 substantially 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, like other life forms on Earth, *Nepenthes* pitchers are confronting an elevated atmospheric CO_2 scenario externally (417.06 ppm in 2022; Lindsey, 2023), in addition to the ten-times high CO_2 inside 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 (Conroy and Hocking, 1993; Sardans *et al.*, 2012; Zavala *et al.*, 2013). *Nepenthes* follow the C3 photosynthetic pathway; and similar to CO_2-enrichment experiments, *N. khasiana* leaf and pitcher tissues showed high carbon and low nitrogen contents (Baby *et al*., 2017). Further, Osunkoya and 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 ratios promote the synthesis of C-based metabolites and limit 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, stomatal density and replacement of chlorophyll-containing cells with digestive glands display relatively low photosynthetic rates, and thereby low Photosynthetic Nitrogen Use Efficiency (PNUE). Again these prey traps growing in elevated CO_2 display reduced Rubisco activity (Baby *et al*., 2017). Pavlovič and 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. CO_2 within the pitchers also helps in maintaining the acidic pH of the digestive fluid.

Several alkaloids function as toxins in FNs (Adler, 2000; Grasso *et al*., 2015). A few reports also mentioned anesthetizing alkaloids in *Nepenthes* nectar secretions (Ratsirarson and Silander Jr., 1996; Bohn and Federle, 2004; Raj *et al*., 2011; Baby et al., 2017; Wong *et al*., 2020); but so far phytochemical studies have not reported the isolation of any nitrogenous secondary metabolites (alkaloids) in the genus *Nepenthes* (Schlauer *et al*., 2005; 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 (Schlauer *et al*., 2005; 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 in *Nepenthes* pitchers (Fig. 1). This study clearly demonstrates that *Nepenthes* EFN is not a nutrient, instead a 'toxic bait' helping in trapping the preys and gathering of nutrients for its survival. Unlike in the pollination scenario, wherein FN and pollen are true rewards, and both plant and pollinator acquire the benefits, in prey capture the present discovery of a toxic naphthoquinone derivative 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-Glu-Fru) mix, with the absence of key nectar components, *viz.*, amino acids, proteins (low) and vitamin C. EFN secreted by *Nepenthes* trapping organs is toxic to ants and other insects, and the toxic component (natural AChE inhibitor) has been identified as the naphthoquinone derivative, (+)-isoshinanolone. EFN secreted by *Nepenthes* is merely a 'toxic sugar bait'; and, unlike FNs, no nutritional benefits are provided to the visitors. CO_2 influenced metabolism, naphthoquinones (mainly plumbagin) 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 essentially deceptive, and 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.

Supplementary information

Detailed Methods, Additional Data, Figures and Tables are available in the Supplementary Information file.

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Author contributions

S.B., C.C.L., G.B.S., G.T., A.J.J.: conceived the project and designed most experiments; C.C.L., G.B.S., G.T., A.J.J., 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., 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.

Competing interests

The authors declare no competing interests.

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Figure legends

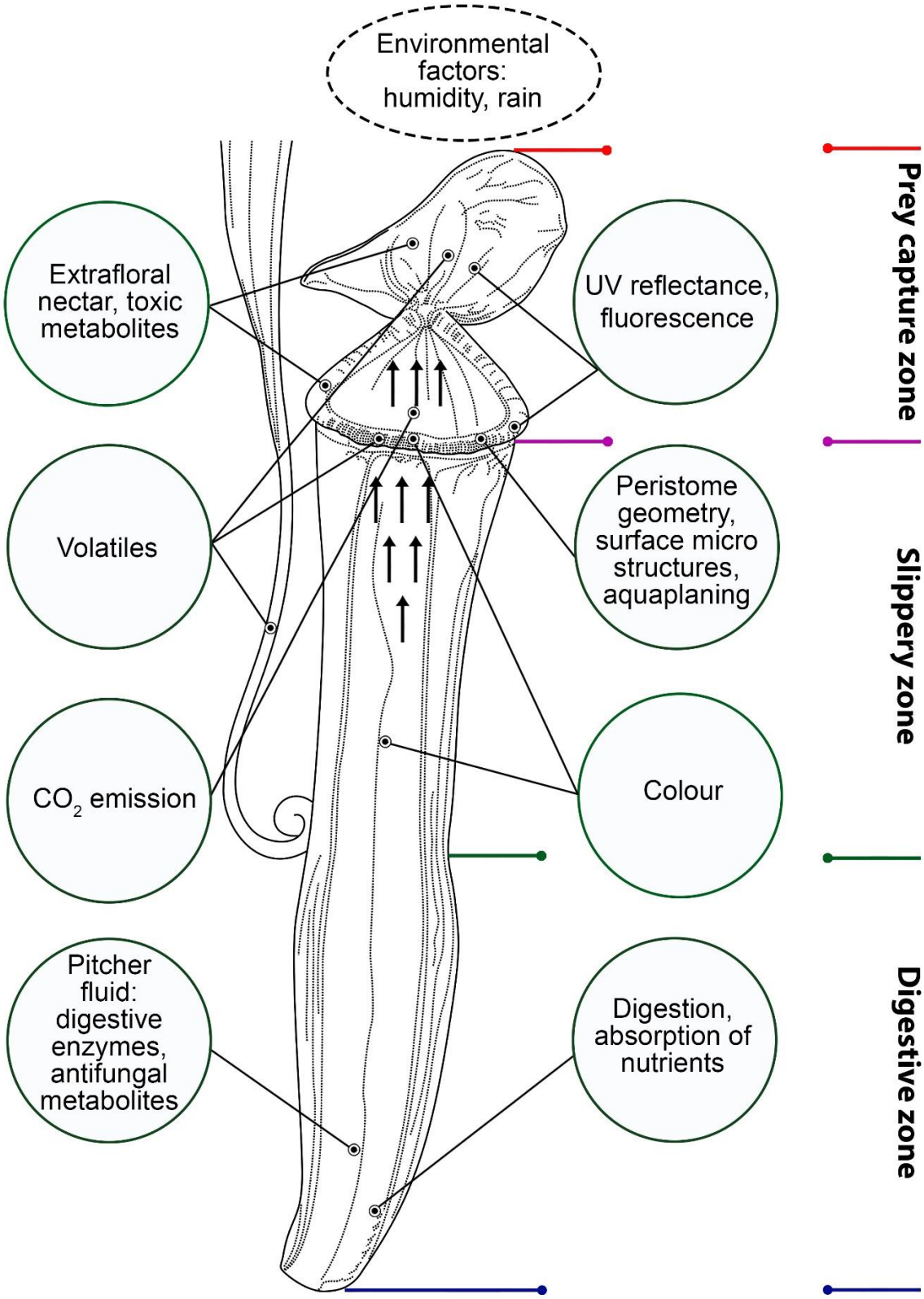

Fig. 1. Prey capture strategies adopted by *Nepenthes* pitchers.

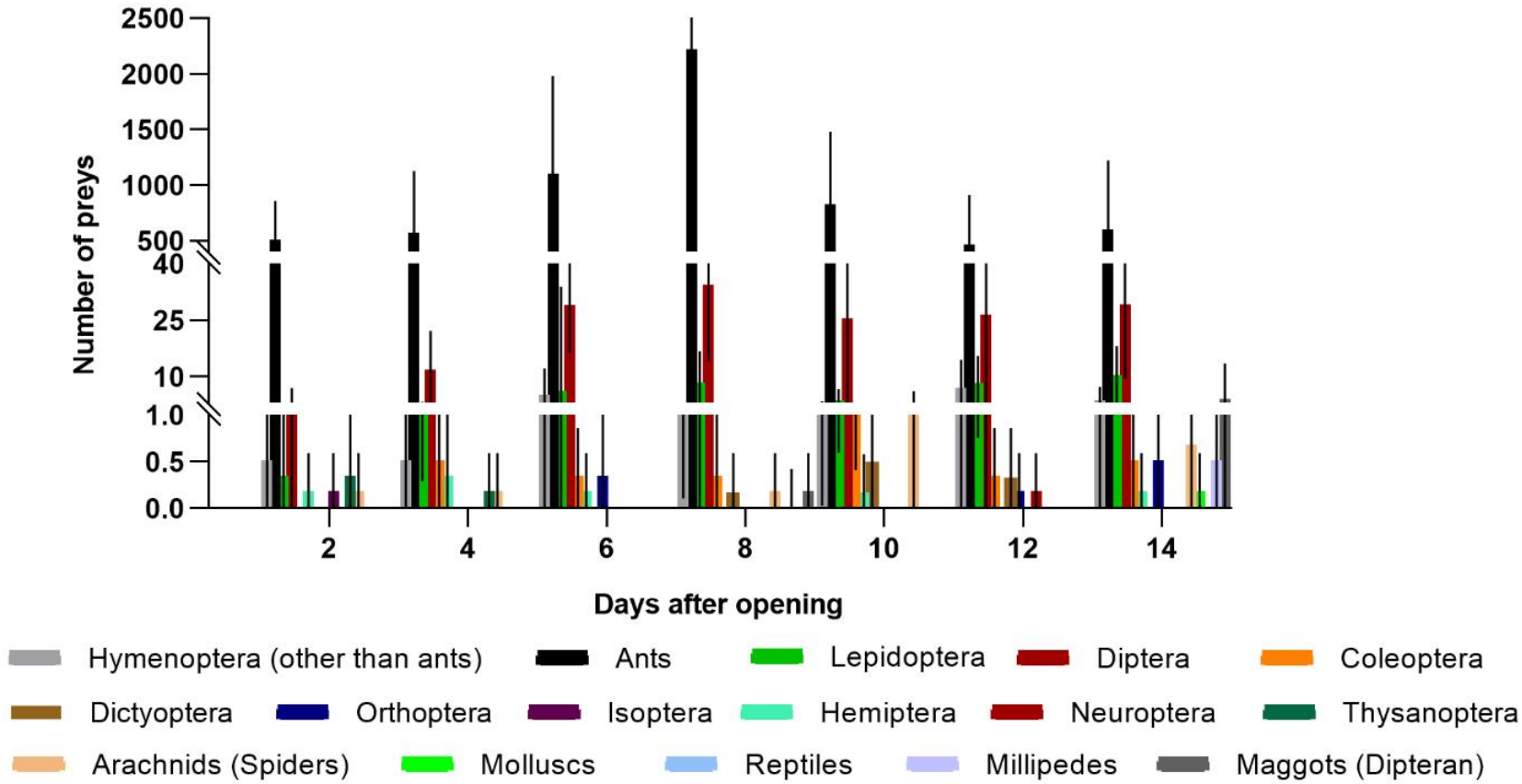

Fig. 2. Insects and other organisms trapped in *N. khasiana* pitchers from 2 days to 14 days after opening (data presented as average prey capture per pitcher, n = 6; details in Table S2).

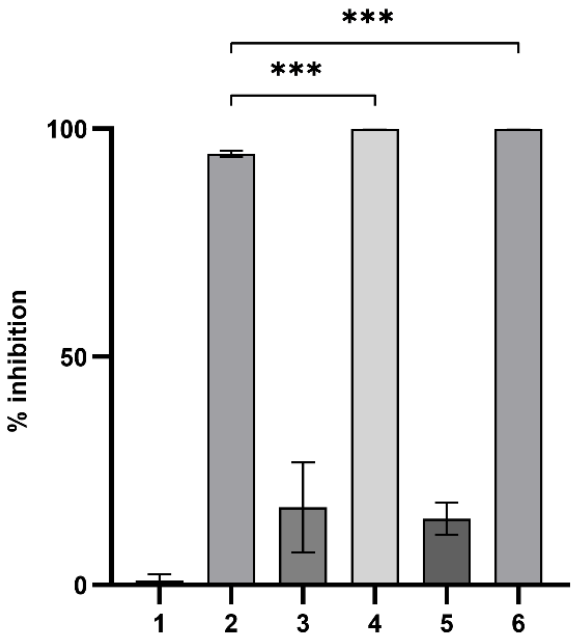

Fig. 3. AChE inhibition by homogenates of ants, *Monomorium minutum* and *Anoplolepis gracilipes*, collected from *N. khasiana* pitcher fluid (i*n vivo*). (**1**: substrate (AChl, 136 µg/mL) + AChE (5 units/L); **2**: substrate (AChl, 136 µg/mL) + drug (donepezil, 10 µg/mL) + AChE (5 units/L); **3**: substrate (AChl, 136 µg/mL) + control-enzyme source (non-EFN fed ant *M. minutum* homogenate) (5 units/L); **4**: substrate (AChl, 136 µg/mL) + test-enzyme source (EFN-fed ant *M. minutum* homogenate, 5 units/L); **5**: substrate (AChl, 136 µg/mL) + control-enzyme source (non-EFN fed ant *A. gracilipes* homogenate, 5 units/L); **6**: substrate (AChl, 136 µg/mL) + test-enzyme source (EFN-fed ant *A. gracilipes* homogenate, 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; *** $P < 0.0001$ (compared to 2).

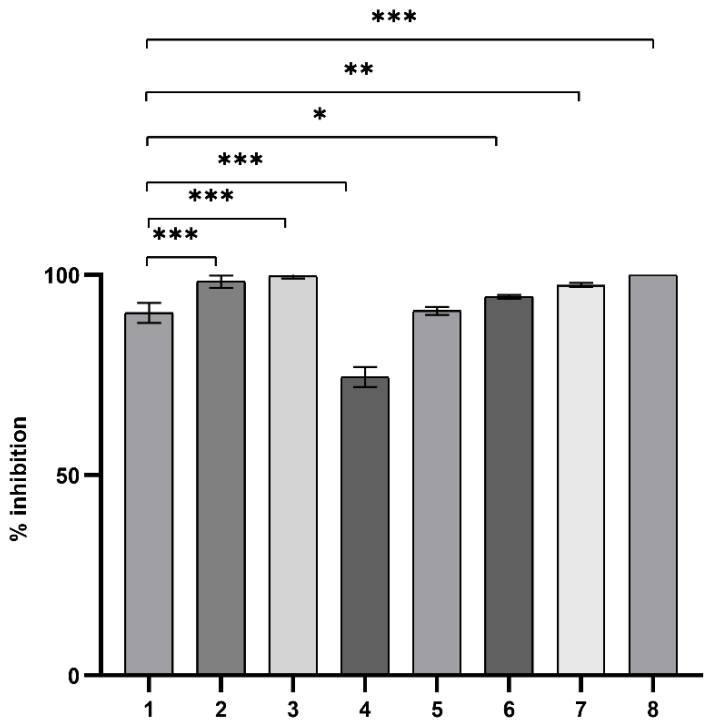

Fig. 4. AChE inhibition of *N. khasiana* peristome and lid EFNs and the active principle, (+)-isoshinanolone (*in vitro*). (**1**: donepezil control (10 µg/mL) + substrate (AChl, 136 µg/mL) + AChE (5 units/L); **2**: lid EFN (4.3 µg/mL) + substrate (AChl, 136 µg/mL) + AChE (5 units/L); **3**: lid EFN (4.3 µg/mL) + donepezil (10 µg/mL) + substrate (AChl, 136 µg/mL) + AChE (5 units/L); **4**: peristome EFN (1000 µg/mL) + substrate (AChl, 136 µg/mL) + AChE (5 units/L); **5**: lid EFN (1000 µg/mL) + substrate (AChl, 136 µg/mL) + AChE (5 units/L); **6**: (+)-isoshinanolone (10 µg/mL) + substrate (AChl, 136 µg/mL) + AChE (5 units/L); **7**: (+)-isoshinanolone (100 µg/mL) + substrate (AChl, 136 µg/mL) + AChE (5 units/L); **8**: (+)-isoshinanolone (1000 µg/mL) + substrate (AChl, 136 µg/mL) + 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; * $P = 0.0257$; ** $P = 0.0001$; *** $P < 0.0001$ (compared to 1).