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RESEARCH ARTICLE

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Nectar Sugar Composition, Standing Nectar Crop and Floral Visitor Diversity of Three Endemic Plant Species from Western Ghats Biodiversity Hot-Spot of India

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Plant insect interactions are governed by various factors. Nectar availability and floral nectar composition play a significant role in deciding the pollinator pool that visits a particular plant species. This study investigates nectar sugar composition and volume from three endemic species from Western Ghats of India viz. *Canthium dicoccum* (Gaertn.) Teijsm. & Binn., *Ligustrum perrottetii* A. DC., and *Wendlandia thyrsoidea* (Roth) Steud., in their natural habitats. Our results demonstrate intraspecific variation in nectar sugar composition in these endemic plant species. Fructose, mannose and glucose sugars were found in the nectar of all three species. In addition to these three, arabinose was found in *Ligustrum* and sucrose in *Canthium*. Nectar volume showed variations in bagged and unbagged conditions. The highest average nectar quantity was found in *Canthium* (1.27 μl/flower), followed by *Ligustrum* (0. 31 μl/flower), and *Wendlandia* (0.14 μl/flower). Floral visitor diversity with a specific emphasis on butterflies showed the highest number of visitors on *Ligustrum* i.e., 42 out of 45 total butterfly species across all three plant species. This is the first report of standing nectar crop and nectar-sugar composition data compiled for these plant species.

Keywords: standing nectar crop, nectar sugar composition, endemic plants, floral visitors, biodiversity hot-spot.

Introduction

Nectar is an aqueous solution that varies from species to species and is made up of sugars, proteins, and amino acids. It is usually produced by the floral nectary, although in some species extra-floral nectaries are also present. Floral nectar studies are conducted for understanding the relationships of floral visitors with the plant. Nectar is produced by the plants as a reward for the visitors.[1] The 'visitors' in turn help with pollination by carrying, and thereby transferring pollen. Nectar is consumed primarily by Hymenopterans, Lepidopterans, Dipterans, and birds. Some Lepidopterans use flower nectar as their primary food source. Floral nectar characteristics such as sugar composition, sucrose-hexose proportions, concentration, volume, and time of nectar secretion, are often related to the interaction of flowers and their pollinators.[2,3,4,5,6,7,8,9] In phylogenetically related taxa, nectar sugar composition is similar.[5,10] Nectar produced by different plant species varies in composition of sugar content, sugar type, amino acids, vitamins, and minerals.[11,12,13] The sweetness is due to sugars like sucrose, glucose and fructose (sugar percent of nectar varies from 15% to 75% by weight).[14] Apart from nectar sugar composition, factors such as number of open flowers and plant size, also affect pollinator visit rate due to their influence on nectar production volume.[15,16,17] Many studies have shown that there is an adaptation to specific pollination syndrome.[4,18,19,20] Pollination syndromes are a set of

Supporting information for this article is available on the WWW under https://doi.org/10.1002/cbdv.202200001 floral characters including color, presence of nectar guides, odor, nectar, and flower shape, that play a vital role in attracting a particular type of pollinator towards the plant.[21] Pollination syndromes are often named after the most typical pollinator.[22,23]

Several endemic and threatened plant species from the Western Ghats biodiversity hot-spot of India are currently showing a decline in population. The main reasons for this decline are habitat loss, overexploitation, immature harvest time, and reproductive biology constraints. Lack of knowledge about species-specific floral visitors/pollinators is compounding this. Scanty information is available about the nectar dynamics of three endemic tree species viz. *Canthium dicoccum* (Gaertn.) Teijsm. & Binn, *Ligustrum perrottetii* A. DC. and *Wendlandia thyrsoidea* (Roth) Steud. This is a first of a kind of study where nectar dynamics and associated floral visitors have been documented for these three plant species from the northern Western Ghats, a global biodiversity hot-spot. This study further investigates in detail a) standing nectar crop of bagged and unbagged flowers, b) nectar sugar composition, and c) butterfly visitors of all the three species.

Results and Discussion

Floral Attributes

Various floral attributes exhibited by these three plant species were studied in detail. *Table 1* provides data on flower morphology, flower color, flower type, odor, primary attractants, sexual organs, and types of floral visitors.

Nectar Composition

Nectar sugar composition was studied using thin layer chromatography and high-performance liquid chromatography (*Table 2*). Refractometer analysis revealed that all three species had nectar with greater than 30% sugar. Variation in nectar sugar composition was observed in the species under investigation. Though fructose and glucose were common to all the three species, the composition and concentration of sugars varied from species to species. *Canthium* nectar contains fructose, mannose, glucose and sucrose, with fructose being a major component, whereas arabinose, fructose, mannose and glucose, with mannose as a major component were found in *Ligustrum*. Nectar of *Wendlandia* was composed of fructose, mannose and glucose, with fructose as a major component.

Table 1. Floral attributes of plant species under investigation.

Sr. No.	Plant Species	Family	Flower Morphology	Color	Type	Odor	Primary attractants	Sexual organs	Types of floral visitors
1	*Canthium dicoccum* (Gaertn.) Teijsm. & Binn.	Rubiaceae	Actinomorphic	White	Tube	Strong sweet	Odor and Nectar	Exposed	Lepidoptera and Hymenoptera
2	*Ligustrum perrottetii* A. DC.	Oleaceae	Actinomorphic	White	Tube	Not significant	Nectar	Exposed	Lepidoptera, Hymenoptera, Coleoptera, and Diptera
3	*Wendlandia thyrsoidea* (Roth) Steud.	Rubiaceae	Actinomorphic	White	Tube	Mild sweet	Odor and Nectar	Concealed	Lepidoptera and Hymenoptera

Table 2. Nectar sugar composition of *Canthium*, *Ligustrum* and *Wendlandia*.

Sr. No.	Plant Species	TLC	HPLC	Ara (%)	Fruc (%)	Man (%)	Glu (%)	Suc (%)
1	*Canthium dicoccum* (Gaertn.) Teijsm. & Binn.	Glu, Fruc	Fruc, Man, Glu, Suc	–	52	17	13	9
2	*Ligustrum perrottetii* A. DC.	Glu, Fruc	Ara, Fruc, Man, Glu	1	42	49	8	–
3	*Wendlandia thyrsoidea* (Roth) Steud.	–	Fruc, Man, Glu	–	61	33	6	–

*Ara – Arabinose, Fruc – Fructose, Man – Mannose, Glu – Glucose, Suc – Sucrose.

Standing Nectar Crop

Table 3 gives standing nectar crop estimates of the plant species studied. Experimental bagging resulted in a roughly 2-fold increase in nectar volume as compared to unbagged open-pollinated field flowers (63.5 μl in bagged flowers as against 36.5 μl in the case of unbagged flowers), in the case of *Canthium*. It was 1.5 times more in the case of *Ligustrum* (14 μl in bagged flowers as against 9 μl in the case of unbagged flowers). The nectar volume of *Wendlandia* was 5.8 μl (bagged flowers) as against 1.3 μl (unbagged flowers) showing the 4-fold difference. The number of flowers with nectar decreased in the order of *Canthium* > *Ligustrum* > *Wendlandia* when bagged. In the case of *Canthium*, the total nectar quantity collected was 63.5 μl from 50 flowers (approx.1.27 μl per flower) in the case of bagged flowers as opposed to 36.5 μl from 33 flowers (approx. 1.1 μl per flower), in the case of unbagged flowers. The percentage of nectarless flowers was zero in the case of bagged flowers, whereas unbagged flowers had 34 % flowers that were nectarless. The total nectar quantity collected in the case of bagged flowers was 14 μl from 44 flowers (approx.0.31 μl per flower), whereas unbagged flowers possessed only 9 μl nectar from 33 flowers (approx. 0. 27 μl per flower) in the case of *Ligustrum*. The percentage of nectarless flowers was 12 % in the case of bagged flowers, whereas unbagged flowers had 34 % flowers that were nectarless. For *Wendlandia*, the total nectar quantity collected in the case of unbagged flowers was 1.3 μl from 12 flowers (approx. 0. 1 μl per flower) and 5.8 μl from 41 flowers (approx. 0.14 μl per flower) in the case of bagged flowers. In all the species studied, the high number of nectarless flowers in the unbagged condition is indicative of the fact that the floral visitors have foraged on them for their nectar.

Floral Visitors of Canthium, Ligustrum and Wendlandia

Floral visitors generally include Hymenopterans (honey bees, wasps), Lepidopterans (butterflies, moths), Coleopterans (beetles, bugs), Dipterans (flies), and small birds. Floral visitors of *Ligustrum* belonged to Lepidoptera, Hymenoptera, Diptera and Coleoptera; whereas flowers of *Canthium* and *Wendlandia* were visited by members of Lepidoptera and Hymenoptera (data provided as supplementary information). In the current study, we focused on Lepidopterans, specifically butterflies (*Table 4*). *Ligustrum* attracted the most number (42) of butterflies. The maximum number of

Table 3. Standing nectar crop in bagged *vs.* unbagged flowers of *Canthium*, *Ligustrum* and *Wendlandia*.

Sr. No.	Plant Species	Bagged flowers						Unbagged flowers					
		No. of flowers sampled	Flowers with nectar	Flowers without nectar	% of nectar less flower	Total volume of nectar (μl)	Avg. nectar per flower (μl)	No. of flowers sampled	Flowers with nectar	Flowers without nectar	% of nectar less flower	Total volume of nectar (μl)	Avg. nectar per flower (μl)
1	*Canthium dicoccum* (Gaertn.) Teijsm. & Binn.	50	50	0	0	63.5	1.27±0.25	50	33	17	34	36.5	1.1±0.52
2	*Ligustrum perrottetii* A. DC.	50	44	6	12	14	0.31±0.16	50	33	17	34	9	0.27±0.21
3	*Wendlandia thyrsoidea* (Roth) Steud.	50	41	9	16	5.8	0.14±0.09	50	12	38	76	1.3	0.1±0.05

butterfly species visiting *Ligustrum* was from the family Hesperidae (15). *Canthium* was visited by 4 butterfly species from the Nymphalidae family, whereas *Wendlandia* was visited by 9 species from families Nymphalidae (4), Papilionidae (1), Lycaenidae (3) and Hesperidae (1).

CEPF (2007) report highlighted that the northern Western Ghats have the presence of more fragmented forests patches than the southern Western Ghats and are under the pressures of selective logging, excessive grazing, fire, and road construction. In a damaged ecosystem, knowing about nectar variation and floral visitor diversity would help us understand the likely damage to insect pollinators (due to habitat degradation) and could also provide insights regarding mitigating factors.[24]

Nectar volume and nectar composition are affected by environmental conditions as well as by pollinators.[25] Nectar mediates the interaction between the plant and its visitors. Floral nectar studies have shown that flowers producing high-volume of nectar are usually brightly colored and preferred by larger visitors such as hummingbirds, whereas flowers producing low-volume of nectar are usually pale colored and visited by smaller visitors such as honey bees, butterflies and ants.[26,27] *Canthium*, *Ligustrum* and *Wendlandia* have small pale-colored flowers with low nectar volumes. Detailed studies of other floral visitors of these plant species will help understand nectar dynamics better.

Nectar composition varies as per ecological constraints such as the flowering time of the plant. Plants flowering in summer/springs have high sucrose-containing nectar whereas the ratio is reversed in winter flowering plants.[28] However, in this study, out of the three plant species, two species – *Canthium dicoccum* and *Wendlandia thyrosidea*, which had peak flowering during the summer (February-March) showed high percentage of fructose in the nectar. Both these species belong to the family Rubiaceae. Nectar composition and nectar volume studies have been performed for some other members of family Rubiaceae.[9,29] Raju A et al. (2011) have studied butterfly visitors of *Wendlandia tinctoria* (Roxb.) DC. (Rubiaceae) and have shown that this species with its massive summer flowering in the dry deciduous forest ecosystem of Seshachalam Hills, India, is a keystone tree species for butterflies, as it provides nectar.[30] Our study is the first of its kind for *Canthium dicoccum* and *Wendlandia thyrosidea*. The notable difference in nectar composition among these two species was the presence of one additional sugar i.e., sucrose in

Table 4. Butterflies visiting *Canthium*, *Ligustrum* and *Wendlandia*.

Family	Name of Butterflies	*Canthium dicoccum* (Gaertn.) Teijsm. & Binn.	Ligustrum perrottetiiA. DC.	*Wendlandia thyrsoidea* (Roth) Steud.
Papilionidae	*Graphium agamemnon* (Tailed jay)	0	+	0
	Graphium teredon (Narrow banded blue bottle)	0	+	+
	Papilio dravidarum (Malabar raven)	0	+	0
	Papilio helenus (Red helen)	0	+	0
	Papilio paristamilana (Paris peacock)	0	+	0
	Papilio polymnestor (Blue mormon)	0	+	0
	Papilio polytes (Common mormon)	0	+	0
Pieridae	*Belenois aurota* (Pioneer)	0	+	0
	Catopsilia pomona (Common emigrant)	0	+	0
	Delias eucharis (Common jezebel)	0	+	0
	Ixias marianne (White orange tip)	0	+	0
	Cepora nadina (Lesser gull)	0	+	0
	Hebomoia glaucippe (Great orange tip)	0	+	0
Nymphalidae	*Danaus chrysippus* (Plain tiger)	+	+	+
	Euploea core (Common crow)	0	+	+
	Hypolimnas bolina (Great Egg Fly)	0	+	0
	Junonia iphita (Chocolate pansy)	0	+	0
	Melanitis leda (Common evening brown)	0	+	0
	Parantica aglea (Glassy tiger)	+	+	+
	Tirumala limniace (Blue tiger)	+	0	+
	Ypthima baldus (*Common five ring*)	0	+	0
	Danaus genutia (Striped tiger)	+	0	0
Lycaenidae	*Cigaritis lohita* (Long banded silver line)	0	+	0
	Jamides bochus (Dark cerulean)	0	+	0
	Jamides celeno (Common cerulean)	0	+	0
	Nacaduba sp. (Six-line blue)	0	+	0
	Rapala varuna (Indigo flash)	0	+	+
	Rapala manea (Slate flash)	0	+	+
	Tajuria cippus (Peacock royal)	0	0	+
	Virachola perse (Large guava blue)	0	+	0
Hesperiidae	*Badamia exclamationis* (Brown awl)	0	+	0
	Burara awlet (Orange awlet)	0	+	0
	Caltoris philippina (Philippine swift)	0	+	0
	Celaenorrhinus ruficornis (Tamil spotted flat)	0	+	0
	Parnara swift spp.	0	+	0
	Hasora chromus (Common banded awl)	0	+	0
	Hasora taminatus (White banded awl)	0	+	0
	Lambrix salsala (Chestnut Bob)	0	+	0
	Odontoptilum angulatum (Chestnut angle)	0	+	+
	Pelopidu sagna (Obscure banded swift)	0	+	0
	Tagiades litigiosa (Water snow flat)	0	+	0
	Baoris farri (Paintbrush swift)	0	+	0
	Orions goloides (Indian dartlet)	0	+	0
	Celaenorrhinus amabariesa (Malabar spotted flat)	0	+	0
	Celaenorrhinus leucocera (Common spotted flat)	0	+	0
	Total	4	42	9

Canthium. Bees and wasps prefer high sucrose-containing flowers, whereas butterflies prefer low sucrose-containing nectar.[6] In the present study, we have also observed that *Ligustrum* which showed no sucrose was visited by 42 different butterfly species. Out of 42 butterfly species, 15 belonged to Hesperidae family. Hesperidae family butterflies i.e skippers prefer tube-like flowers. Similar results have been reported previously by Bauder et al. 2015.[31] It would be interesting to study the kind of preference the butterflies of a certain family show for a particular nectar composition. Our study is a preliminary report of three plant species whose nectar volume and nectar composition are reported for the first time. This preliminary information would enable researchers to conduct in-depth studies of floral visitors and nectar composition variations with respect to changing landscapes and habitats in which these plants are found.

Conclusion

Nectar composition and nectar volume play an important role in deciding the pollinators and visitors of the plant. There is a strong relationship between floral characteristics and the type of visitors. In this study, we observed that as the volume of nectar changes, the diversity of visitors also changes. All three plant species under investigation have small tubular pale-colored flowers. Even though the morphology is similar, there is a significant difference in nectar volumes and composition. *Ligustrum* with arabinose, fructose, mannose and glucose sugars in nectar attracts 42 different species of butterflies from 5 families out of which 15 are from the family Hesperidae. *Canthium* which is rich in fructose was visited by 4 butterfly species from the family Nymphalidae. *Wendlandia* with 61 % fructose is visited by 9 species from four families. Our study also supports the observation that if the nectar volume is low, the primary pollinators and visitors were bees (*Wendlandia* has the lowest nectar volume in our case). In our study, *Ligustrum* had the highest number as well as largest variety of visitors for which its floral attributes, nectar volume and nectar composition, can be considered as key contributors. These types of studies will reveal different facets of insect-plant interactions.

Experimental Section

Study Area

Our study was conducted in the evergreen forests of *Amboli*, located in northern Western Ghats (NWG). *Amboli* (15°57′′ N, 74° 00′′ E), situated 700m above the mean sea level and located in Sawantwadi Taluka of Sindhudurg District of Maharashtra (*Figure 1*). Forests in this region are seasonal with an annual rainfall

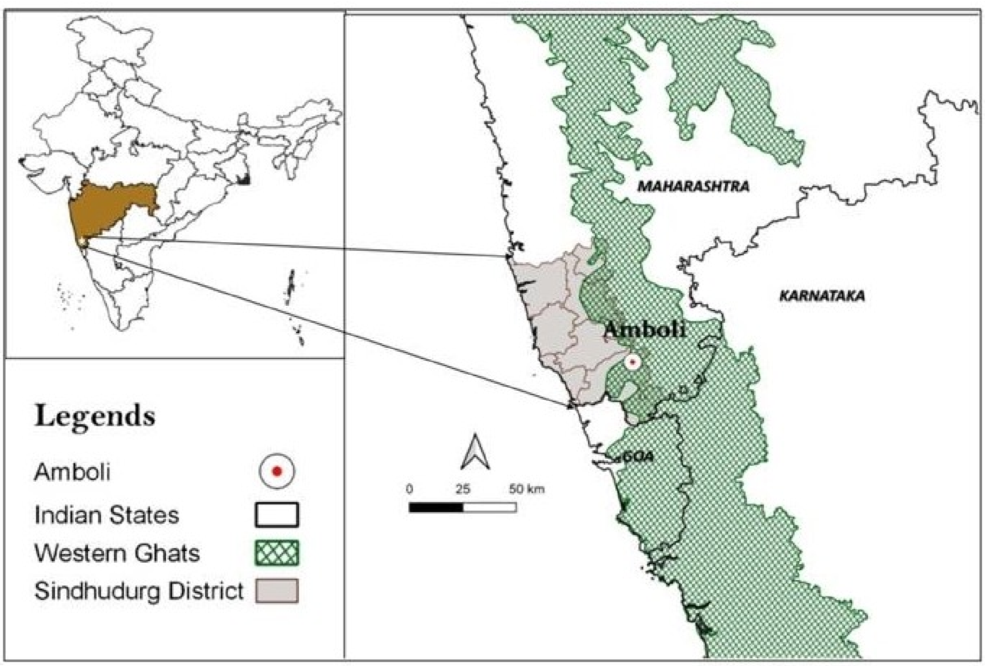

Figure 1. Study area.

range of 6000–7000 mm, dry period length (DPL) of 7–8 months, and temperatures ranging from 8° to 35 °C. The primary vegetation type is evergreen. The forests harbour several endemic and threatened plant species. The area is proposed as ecologically sensitive and also forms a part of the geographically and ecologically important Sahyadri-Konkan Ecological corridor.[24]

Plant Species Under Investigation (Figure 2)

Plant species were collected during the field work and identified with the help of standard taxonomic literature.[32] The specimens were deposited in AHMA – Agharkar Research Institute Herbarium, Pune.

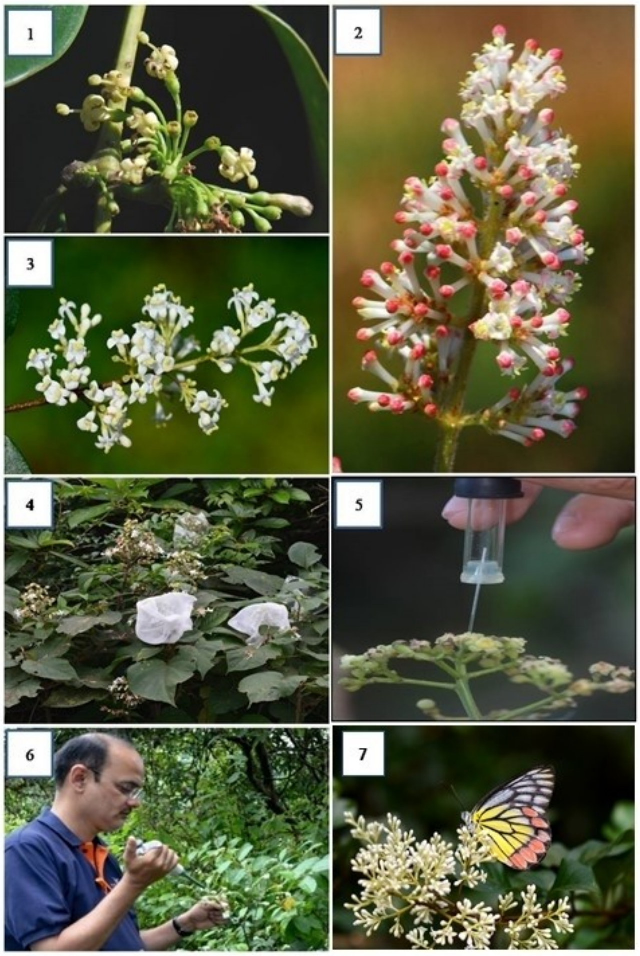

Figure 2. Plant species under investigation and nectar collection – 1. *Canthium dicoccum* 2. *Wendlandia thyrsoidea* 3. *Ligustrum perrottetii* 4. Bagged flowers 5. Nectar collection by Microcapillary 6. Nectar collection by Micropipette 7. Common jezebel butterfly visiting *Ligustrum perrottetii*.

(i) *Canthium dicoccum* (Gaertn.) Teijsm. & Binn. AHMA (34128): Small tree. Leaves olive-green, opposite, 7.0–12.7×3.4–7.0 cm, elliptic or ovate, smooth, shining, coriaceous, apex acute; petiole 0.5 cm long; stipules connate, ovate, with a dorsal subulate hard point. Flowers in axillary clusters, white, fragrant, 5-merous; corolla tube narrow. Drupe's sub-globose, 1.3×0.7 cm, prominently rugose, pericarp thin, black when ripe.

Flowering – December to March

(ii) *Ligustrum perrottetii* A. DC. AHMA (34129): Trees. Leaves 3.0–7.5×1.8–5.0 cm, elliptic, obtuse, acute or acuminate at apex, acute at base. Flowers white, in terminal, thyrsoid panicles. Drupes c 0.9×0.5 cm, obovoid.

Flowering – August – October

(iii) *Wendlandia thyrsoidea* (Roth) Steud. AHMA (34130): Small tree. Bark orange-red; branches, leaves and inflorescence pubescent. Leaves 10.5 –14.5×3.5-4.0 cm, elliptic-lanceolate, apex acuminate, base tapering into a short petiole of 0.5 cm, glabrous above, tomentose below. Flower white, fragment, in dense pyramidal hirsute panicles, 15–30 cm long. Capsules globose, 0.2 across, pubescent, crowned with calyx-teeth.

Flowering – January – March

Nectar Collection and Standing Nectar Crop Estimation

Nectar production was measured in the above-mentioned species from January to December by collecting nectar samples from flowers from the wild. Nectar was sampled between 7 am to 10 am. For each species, flowers were 'bagged' the previous evening, i.e., covered with bags to ensure that the nectar was not robbed by the pollinators/floral visitors before sampling, and to get accurate estimates of nectar volumes per species.[33] Other flowers were left as is ('not bagged'). The volume of nectar was measured the next morning. Nectar was collected from bagged and unbagged flowers by probing each flower using a calibrated micro-capillary tube and a micropipette (details given below) (*Figure 2*). At least 50 individual flowers per species were measured.

(i) Nectar collection using microcapillary tube: This was carried out using fixed bore calibrated 0.5 μL microcapillary tubes (Drummond scientific company). The capillary was inserted in the flower and nectar was drawn into the tubes by capillary action. While using the microcapillary tube, particular care was taken to avoid damage to floral tissue. The volume of nectar drawn was quantified by measuring the column of nectar within the tube and calculating the volume of the entire column filled.

(ii) Nectar collection using micropipette: Micropipettes of 0.1 to 10 μL were used and nectar was directly collected from the floral nectaries.

Total Sugar Estimation

Collected nectar was diluted with 2 mL of distilled water. For estimation of sugar concentration on the field, 0.5 ml of nectar was placed on a handheld Refractometer (Erma Inc. Tokyo Japan).

Storage of Nectar Samples

Nectar samples were stored in a −20 °C refrigerator in a non-climate-controlled room until they were analyzed by Thin layered Chromatography and High-Performance Liquid Chromatography.

Nectar Analysis

(i) **Thin Layered Chromatography**: TLC sheets were cut horizontally into plates of 5 cm length and variable width. Chloroform: Methanol: Water (65 : 25 : 10) solvent system was used for separation of sugars. The developing stain was prepared using 98 % sulfuric acid and distilled water (1 : 1).

(ii) **High-Performance Liquid Chromatography**: Determination and quantification of specific reducing sugars: arabinose, xylose, fructose, mannose, galactose and glucose were performed by HPLC RI (Jasco, Japan) using COL-AMINO 250×4.6 mm column (Kromasil). The analysis was performed with a flow rate of 1.5 ml min^{-1} using isocratic elution with 75 % Acetonitrile (MeCN):25 % water (H_2O) mixture as a mobile phase.

Sugar standards for HPLC RI analysis: Standard stock solutions of individual sugars ((+)-L-Arabinose, (+)-D-Xylose, (+)-D-Fructose, (+)-D-Mannose, (+)-D-Galactose, (+)-D-Sucrose and (+)-D-Glucose was prepared in separate volumetric flasks. Working solutions were prepared by diluting the stock solutions with the same solvent to contain (0.5–50 mg ml^{-1} final concentration). Linearity was established by triplicate injections of different concentrations of the standards obtained by dilution in water. Calibration curves were obtained by plotting peak area versus amount injected. For optimization of sugar separation protocol, effect of different solvents and sample flow rate was analyzed. Each sample was tested by triplicate injections. The blank and control solutions were analyzed with each series of samples to verify the accuracy of the obtained results. Chromatograms of each specific reducing sugar in samples were compared with the retention time and resolution of sugar standards.

Floral Visitor Documentation

For species-specific floral visitor documentation, individual plants were selected based on peak flowering season, flowering percentage, and accessibility to the flowering branch. Floral visitors were observed primarily in the morning (7.00 am to 10.00 am) and evening sessions (4.00 pm to 6.00 pm). All floral visitors were systematically observed and documented with naked eyes and with the help of binoculars (Nikon Action 8X40). Digital SLR camera (Canon 700 D and Canon 1200 D) with 18X55 mm lens and 55×250 mm telephoto lens was used for photo-documentation.

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contribution Statement

TP – Data validation, methodology design for standing nectar crop estimation, data analysis, original draft preparation. MS – Data collection and curation regarding standing nectar crop estimation. AB – Data collection regarding standing nectar crop estimation. KP – Data collection regarding standing nectar crop estimation. SS – Data collection regarding standing nectar crop estimation. PM – Data collection, organization and curation pertaining to floral visitors. SK – Data collection, organization and curation pertaining to floral visitors. HO- Data curation. AP – Conceptualization, methodology, investigation and supervision, funding acquisition, manuscript checking.

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