Reproductive ecology of the semi-evergreen tree *Vitex negundo* (*Lamiaceae*)

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Abstract. *Vitex negundo* is a small aromatic semi-evergreen tree often cultivated as an ornamental plant. It sheds foliage and produces new foliage almost simultaneously in December-April and flowers in June-November. The flowers are homogamous, herkogamous, self-compatible and exhibit a facultative xenogamous breeding system. They are bi-labiate, gullet type and are pollinated nototribically by bees, wasps and butterflies, while collecting nectar, and also by bees, which pollinate it sternotribically, while collecting pollen. *Trigona* bee is the primary nectar robber, because it bores holes at the base of the corolla tube to collect nectar. Other bees and wasps are principally legitimate foragers, but they collect nectar illegitimately, if they happen to reach the hole in the corolla tube made by *Trigona*. Nectar robbing by *Trigona* indirectly increases the pollen dispersal distance by pollinators and promotes outcrossing rate in this tree. Fruits are non-fleshy and disperse by gravity, and subsequently become hydrochorous and anthropochorous. The seeds did not germinate naturally during the study period and, hence, detailed studies on seed dormancy and seed viability are suggested to understand the seed biology.

V. negundo is grown commercially as a crop in certain parts of the world and for ornamental purposes in parks, gardens and the backyards of houses both in urban and rural areas. Therefore, the reproductive biology information reported now is useful to understand the various aspects of its sexual reproduction and, accordingly, to take measures for the protection of its pollinator fauna for the production of genetically superior seeds in natural areas. Finally, it is suggested that *V. negundo* should be used in traditional medicine or for other purposes without affecting the surviving individuals in natural areas.

Key words: anthoropochory, entomophily, facultative xenogamy, hydrochory, *Vitex negundo*

Introduction

Vitex is the largest genus in the subfamily *Vitioicoideae* of the family *Verbenaceae*, which comprises 250 deciduous shrub species distributed all over the world. Recently, it has been placed with *Lamiaceae* on the basis of DNA sequence data (Thomas & al. 2012). According to the APG IV System of Flowering Plants Classification 2016, it has been also classified under *Lamiaceae*. The genus is widely distributed in the tropical and subtropical regions of Australia, Asia, Africa, with a few South American species (Munir 1987). *Vitex* species generally exhibit hermaphroditism (Schmidt 2000). A few species of *Vitex* have been studied for their pollination biology and seed dispersal. Dirr (1998) noted that *V. rotundifolia* exhibits herkogamy and also mentioned that self-pollination is unlikely. Abe (2006a,b) reported that this plant is pollinated by diverse groups of pollinators such as flies, honeybees, beetles, butterflies, and ants. Escobin & Cervancia (1998) wrote that *V. parviflora* is dichogamous, self-incompatible, outcrossing and entomophilous. Viviane & al. (2016) reported that *V. doniana* is hermaphroditic, homogamous, and self-compatible, presenting a predominant selfing through geitonogamy. The flowers show a generalist pollination syndrome and are pollinated by a wide array of insects

and sunbirds. Anderson (2003) noted that *V. lucens* is hermaphroditic and exclusively pollinated by endemic honeyeaters in New Zealand. Ahenda (1999) reported that *V. fischeri* (= *V. keniensis*) is hermaphrotiditic, homogamous, self-compatible and pollinated mainly by bees, especially *Apis mellifera*. Vishwanathan & Basavaraju (2010) stated that *V. negundo* is distributed in Afghanistan, India, Pakistan, Sri Lanka, Thailand, Malaysia, East Africa, and Madagascar. It is grown commercially as a crop in parts of Asia, Europe, North America, and the West Indies. It is a source of timber and also cultivated as a food crop. These authors reviewed its ethnobotanical knowledge and listed it as an important medicinal plant. Byragi Reddy & Subba Reddi (1994) reported that *V. negundo* is self-compatible and exhibits a mixed breeding system; it is pollinated by bees, wasps and butterflies. Bhattacharya & Mandal (1998) wrote that *V. negundo* is pollinated also by ants and flies.

Ingrid (1987) reported that *V. stahelii* produces fleshy drupes for dispersal by animals. Ahenda (1999) pointed out that *V. keniensis* produces pulpy fruits for dispersal by hornbill birds, monkeys and even human beings. Tiffney (2004) reported that *V. lucens* produces large bright-red drupes; flying frugivorous animals probably disperse them. Charles-Dominique (1993) noted that the New Zealand Pigeon, *Hemiphaga novaeseelandiae,* consumes the fruits of *V. lucens* and causes occasional long distance dispersal via endozoochory. Bass & al. (2006) noted that if the fruits of *V. lucens* are not consumed by birds, they fall to the ground from the tree and ground foraging animals may then disperse the seeds. Cousins & al. (2009; 2010) stated that *V. rotundifolia* is most likely hydrochorous. Sato (2012) reported that the Common Brown Lemur is the sole disperser of the fruits of *V. beraviensis* in Madagascar. Chakravarthy & Ratnam (2015) reported that *V. glabrata* fruits are consumed by the Common Palm Civet and frugivorous birds, but the civet is the effective seed disperser. Zhi-Yong & al. (2007) wrote that animals are probably involved in the dispersal of fruits or seeds of *V. negundo*.

The present study is aimed at providing information on phenology, floral biology, pollination, pollinators, sexual system, breeding system, fruiting behaviour and seed dispersal of *Vitex negundo* L. The information will help understand its sexual reproduction and the reasons for its rarity.

Material and methods

Study site: Srungavarapukota is part of the long stretch of Eastern Ghats Forest (18°07' N latitude and 83°10' E longitude), at an altitude of 70 m (232 ft) in Vizianagaram district. The soils are loamy, with medium fertility. There are red loamy soils in the drylands and clay loamy in the wetlands. The climate is characterized by high humidity nearly round the year, with oppressive summer and good seasonal rainfall. The summer season from March to May is followed by the southwest monsoon season, which continues up to September. October and November mark the retreat of the monsoon season. The climate of the hilly regions of the district stands out with heavier rainfalls and is cooler than in the plains. The average maximum temperature is 39.6 °C in May and the minimum temperature is 17.1 °C in December. Twenty-five trees of *Vitex negundo,* interspersed here and there with other plant species in this hilly area, were used for the study.

Phenology: Prior to collecting the data, visits were made to the study areas to get an idea of the timing of leaf fall, leaf flushing and flowering of *V. negundo*. Subsequently, regular field visits were made to record the timing of these three phenological events. The inflorescence type and the number of flowers per inflorescence were noted. Ten inflorescences prior to commencement of their flowering were tagged and followed daily to record the flowering duration.

Flower morphology: Twenty-five fresh flowers were used to record the flower type, sex, shape, colour, odour, symmetry, calyx, corolla, stamens, and style. Floral configuration and rewards presentation aspects were examined in relation to the forage collection activity of insects.

Floral biology: Anthesis was initially recorded by observing the marked inflorescences in the field. Later, the observations were made 3 to 4 times on different days, in order to record an accurate anthesis schedule. Similarly, the mature buds were followed to record the time of anther dehiscence. Pollen presentation pattern was also investigated by recording how anthers dehisced and that was confirmed by observing the anthers under a 10× hand lens. Twenty-five mature buds, five on each of the five plants were bagged and tagged to measure the nectar after anthesis. The presence of nectar was determined by gently pulling a flower from its calyx

and firmly pressing its base against a hard surface. The protocols provided by Dafni & al. (2005) were used for measuring the nectar volume, sugar concentration, sugar types, and amino acid types. A micropipette was inserted into the flower base to extract nectar for measurement. An average of ten flowers was taken as the total volume of nectar/flower and expressed in µl. Similarly, a sample of nectar was used for measuring the nectar sugar concentration at selected intervals of time; the Hand Sugar Refractometer (Erma, Japan) was used for this purpose. Nectar was spotted on Whatman No. 1 filter paper, along with the standard samples of glucose, fructose and sucrose. The paper was run ascendingly in chromatography chamber for 24 hours with a solvent system of n-butanol-acetone-water (4:5:1), sprayed with aniline oxalate spray reagent and dried at 120 °C in an electric oven for 20 minutes for the development of spots from the nectar and the standard sugars. The developed spots were compared with the spots of the standard sugars. Then, the present sugar types were recorded and also the dominance of each sugar type was noted based on the extent of spot development against the standard sugar type. The sugar content/flower is expressed as the product of nectar volume and sugar concentration per unit volume, mg/µl. This was done by first noting down the conversion value for the recorded sugar concentration on the refractometer scale and then by multiplying it with the volume of nectar/flower. Table 5.6 given in Dafni & al. (2005) was followed for recording the conversion value to mg of sugars present in one µl of nectar. Nectar was spotted on Whatman No. 1 filter paper along with the standard samples of twenty one amino acids, namely, arginine, tyrosine, alanine, aspartic acid, butyric acid, cysteine, cystine, glutamic acid, glycine, histidine, hydroxyl-proline, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, and valine. The paper was run ascendingly in chromatography chamber for 24 hours with a solvent system of n-butanol-acetic acid-water (4:1:5). The chromatogram was detected with 0.2 % ninhydrin reagent and dried at 85 °C in an electric oven for 15 minutes for the development of spots from the nectar and the standard amino acids. The developed nectar spots were compared with the spots of the standard amino acids and recorded the amino acid types present in the nectar. Further, the dominance of amino acids was also recorded based on the extent of spot developed against the standard amino acid type. A number of mature but un-dehisced anthers was collected from different individuals and placed in a Petri dish. Later, each time a single anther was taken out and placed on a clean microscope slide (75×25 mm) and dabbed with a needle in a drop of lactophenol-aniline blue. The anther tissue was then observed under the microscope for pollen. The pollen mass was drawn into a band, and the total number of pollen grains was counted under a compound microscope (40× objective, 10× eye piece). This procedure was followed for counting the number of pollen grains in each anther collected. Based on these counts, the mean number of pollen grains produced per anther was determined. The mean pollen output per anther was multiplied by the number of anthers in the flower for obtaining the mean number of pollen grains per flower. Another set of dehisced anthers was collected in a Petri dish and the pollen grains removed from these anthers were examined under microscope for recording the pollen grain features. The pollen-ovule ratio was determined by dividing the average of the number of pollen grains per flower by the number of ovules per flower. The value thus obtained was taken as pollen-ovule ratio (Cruden 1977). The stigma receptivity was observed visually and by H_2O_2 test. In visual method, the stigma physical state (wet or dry) and the unfolding of its lobes were considered to record the commencement of receptivity; withering of the lobes was taken as loss of receptivity. H_2O_2 test, as given in Dafni & al. (2005) was followed for noting down the stigma receptivity period.

Breeding systems: Mature flower buds of some inflorescences on different individuals were tagged and enclosed in paper bags. They were tested in the following way for each mode of pollination.

1. The stigmas of flowers were pollinated with the pollen of the same flower manually by using a brush and bagged to observe manipulated autogamy.
2. The flowers were fine-mesh bagged as such without hand pollination to detect spontaneous autogamy.
3. The emasculated flowers were hand-pollinated with the pollen of a different flower on the same plant and bagged to test geitonogamy.
4. The emasculated flowers were pollinated with the pollen of a different individual and bagged to detect xenogamy.

All these categories of flower pollination were kept under regular observation until fruit set. Then, the percentage of fruit set and seed set was calculated. The flowers/inflorescences were tagged on ten individuals prior to anthesis and followed for fruit and seed set in open-pollinations. The resulting fruit and seed output were pooled up for calculating fruit and seed set rates.

Foraging activity and pollination: After making preliminary observations on the foraging activities of insects, a thorough knowledge of the local insect species was obtained by observing the representative species available with the Department of Environmental Sciences, Andhra University, Visakhapatnam. All butterflies were identified to species level by consulting the books of Kunte (2007) and Gunathilagaraj & al. (1998) while other insects, some to species level while a few others to genus level only. The efforts to get the specimens identified to species level for the species which were identified up to genus level by Zoological Survey of India, Government of India were not successful during the study period. The insect species were observed visually and by using binoculars; the insect species that could not be identified on spot were captured and later identified with the help of the identified specimens available in the Department. The foraging activities of insects were recorded for 10 min at each hour for the entire day on 3 or 4 occasions depending on the possibility and the data was tabulated to use the same for further analysis, especially to understand the foraging activity rate at different times of the day. For each species, 20–30 inflorescences were selected to record the foraging visits of insects. The data thus obtained was used to calculate the percentage of foraging visits made by each category insects per day to evaluate their association and pollination role. The insects feeding on nectar and/or pollen were carefully observed to assess their role in effecting pollination. They were observed on a number of occasions for their foraging behaviour such as mode of approach, landing, probing behaviour, contact with essential organs to result in pollination, inter-plant foraging activity in terms of cross-pollination, etc. Based on this data, the association between floral rewards and insects was assessed. Further, nectar robbing activity of insects bypassing pollination apparatus was also observed. Ten individuals of each insect species were captured while collecting pollen and/or nectar on the flowers; the collection was done during their peak foraging activity period. The captured specimens of insects were brought to the laboratory, washed in ethyl alcohol, stained with aniline-blue on a glass slide and observed under microscope to count the number of pollen grains present and evaluate their relative pollen carryover efficiency and pollination role.

Fruiting behavior: Fruit maturation period, the fruit and seed characteristics were recorded. Field observations were made regularly to record fruit and seed dispersal modes. Casual observations were also made to record whether the seeds germinate immediately after they are dispersed or not.

Results

Phenology: It is a small aromatic semi-evergreen tree with a spreading crown and grows in moisture-rich open habitats and waste lands. Leaf fall and leaf flushing occur almost simultaneously during December-April and flowering occurs June-November. The flowering extends into December in certain individuals which delay leaf fall and leaf flushing events. Inflorescence is a terminal panicle which produces 73 to 201 flowers over a period of two to four weeks, each day producing a mean number of 5.8 flowers (Range 3–15) (Fig. 4a,b).

Flower morphology: The flowers are pedicellate, small, pale purple lacking perceptible smell, bisexual and somewhat zygomorphic. The calyx is green, campanulate, obscurely bi-lipped, upper lip 2-lobed, lower lip 3-lobed and stellate pubescent. The corolla is light purple, tubular at base, obscurely bi-lipped; upper lip is 2-lobed, sub-orbicular, apex emarginate while lower lip is 3-lobed and obovate. The corolla tube is densely hairy from the base to the throat. The stamens are 4, inserted at the middle part of the corolla tube by slender light purple filaments, didynamous, exserted (Fig. 4g); anthers are dithecous, light purple and have versatile fixation. The ovary is superior, globose, puberulous, bicarpellary syncarpous with four locules by a false septum; each locule is 1-ovuled, erect and anatropous on axile placentation (Fig. 4l,m). The style is linear and stigma shortly and unequally bifid (Fig. 4j). The stigma is situated beyond the height of long stamens (Fig. 4e,f). The style tip and stigmatic lobes are curved towards the broad lobe of the lower lip.

Floral biology: The mature buds open during 0900–1100 h by diverging petal lobes (Fig. 4c,d).

Petals slightly stretch out and reflex exposing the stamens, style and stigma slightly beyond the rim of the corolla tube; the sex organs are positioned adjacent to the upper lip. The curved style tip with bifid stigma is situated beyond the height of long stamens. The anthers dehisce during mature bud stage by longitudinal slits exposing the creamy white pollen (Fig. 4h). At the same stage, the stigma also attains receptivity by stretching out its lobes gradually and ceases its receptivity by the noon of 2nd day (Fig. 4k). While the stigma is still in receptive phase, the corolla and stamens fall off together by the evening of the day of anthesis. The style and stigma wither and dry up on 3rd or 4th day. The calyx is persistent and grows further partly enclosing the growing fruit. The flowers are morphologically and functionally hermaphroditic. Homogamy facilitates self-pollination but it prevents autonomous autogamy due to lack of physical contact between the stamens and stigma. However, the flowers are compatible to facilitated autogamy, geitonogamy and xenogamy mediated by insect vectors.

The pollen output per anther is 377±85.81. The total pollen productivity in individual flowers is 1,508±343.24. The pollen-ovule ratio is 377:1. The pollen grains are spheroidal, tricolpate, 24.9 μm long and 21 μm wide; exine supra-rugulate, muri rounded, tightly packed together (Fig. 4i). Nectar is secreted around the ovary inside the corolla tube from mature bud stage and until noon after anthesis. Its secretion is gradual from anthesis onwards, reaches its peak after three hours and decreases gradually thereafter towards evening. A flower produces 2.6±0.52 μl of nectar. Half of the tube part of the corolla is filled with nectar. The nectar sugar concentration varied from 24–29 % and it averaged to 26.6±1.71 %. The sugars present in the nectar include sucrose, glucose and fructose; the first sugar is the most dominant. The total sugar content in the nectar of a flower is 0.77 mg. The nectar also contains the essential amino acids such as arginine, isoleucine, leucine, methonine, phenylalanine, and tryptophan. The non-essential amino acids present were alanine, aspartic acid, aminobutyric acid, cystine, serine and tyrosine.

Breeding systems: The results of breeding systems indicate that the fruit set is absent in spontaneous selfing, 32 % in hand-pollinated selfing, 60 % in geitonogamy, 89 % in xenogamy and 48 % in open pollinations. Seed set rate is constant and it is 25 % in all modes of pollinations due to production of only one seed per fruit (Table 1).

Table 1. Breeding systems in *Vitex negundo*.

Mode of breeding system	Number of flowers sampled	Number of flowers set fruit	Fruit set (%)	Seed set (%)
Spontaneous selfing	25	0	0	0
Hand-pollinated selfing	25	8	32	25
Geitonogamy	50	30	60	25
Xenogamy	62	55	89	25
Open pollination	2258	1079	48	25

Foraging activity and pollination: The flowers expose the stamens and stigma slightly beyond the rim and lobes of the corolla upon anthesis. The corolla lobes stretch out but do not reflex backwards. The flowers were foraged by five bee species, four wasp species and 18 butterfly species during day-time; all bee species foraged for both nectar and pollen while all other insects foraged exclusively for nectar (Table 2). The bees were *Apis dorsata* (Fig. 5a,b), *A. florea* (Fig. 5c,d), *Trigona iridipennis* (Fig. 5e), *Ceratina* sp. (Fig. 5f) and *Pithitis smaragdula* (Fig. 5g). The wasps were *Eumenes conica* (Fig. 5h), *E. petiolata* (Fig. 5i), *Rhynchium* sp. (Fig. 5j) and *Bembix* sp. (Fig. 5k). The butterflies were *Pachliopta aristolochiae* (Fig. 6a), *Graphium agamemnon* (Fig. 6c), *G. nomius* (Fig. 6d), *Papilio polytes* (Fig. 6b) (Papilionidae), *Catopsilia pomona* (Fig. 6e), *C. pyranthe* (Fig. 6f), *Cepora nerissa* (Fig. 6g), *Anaphaeis aurota* (Fig. 6h), *Colotis fausta* (Fig. 6i) (Pieridae), *Junonia lemonias* (Fig. 6j), *Hypolimnas misippus* (Fig. 6k), *H. bolina* (Fig. 6l), *Danaus chrysippus* (Fig. 6m), *Euploea core* (Fig. 6n) (Nymphalidae), *Tarucus nara* (Fig. 7a), *Zizyla hylax* (Fig. 7b), *Lampides boeticus* (Fig. 7c) and *Spindasis vulcanus* (Fig. 7d) (Lycaenidae). All these insects foraged during 09:00–16:00 h; but bees and wasps showed more activity between 11:00–12:00 h, while the butterflies showed more activity between 10:00–11:00 h. However, individual species of all insects showed variation in their activity by visiting the flowers either continuously, or with one or two hours break, or stopped after noon time (Figs 1, 2). All these insects were consistent and regular foragers during the entire flowering period. The bees accounted for 23 %, wasps for 18 % and butterflies for 59 % of all foraging visits (Fig. 3). The body washings of these foragers collected from the flowers at peak foraging activity revealed that they carried pollen on their bodies. The mean number of pollen grains varied from 76.6 to 181.6 in case of bee species, from 37.5 to 55.2 in case of wasps, and from 19.5 to 58.5 in case of butterflies (Tables 3, 4).

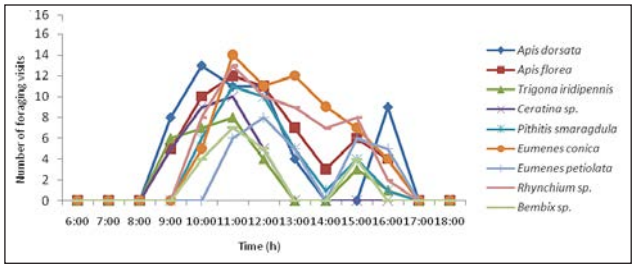

Fig. 1. Hourly foraging activity of bees and wasps on *Vitex negundo*.

Table 2. List of insect foragers on *Vitex negundo*.

Order	Family	Genus	Species	Common name	Forage sought
Hymenoptera	Apidae	*Apis*	*dorsata* F.	Rock Bee	Pollen + nectar
		Apis	*florea* F.	Dwarf Honey Bee	Pollen + nectar
		Trigona	*iridipennis* Smith	Stingless Honey Bee	Pollen + nectar
		Ceratina	sp.	Small Carpenter Bee	Pollen + nectar
		Pithitis	*smaragdula* F.	Green Metallic Bee	Pollen + nectar
	Eumenidae	*Eumenes*	*conica* F.	Potter Wasp	Nectar
		Eumenes	*petiolata* F.	Potter Wasp	Nectar
	Vespidae	*Rhynchium*	sp.	Black Potter Wasp	Nectar
	Crabronidae	*Bembix*	sp.	Sand Wasp	Nectar
Lepidoptera	Papilionidae	*Pachliopta*	*aristolochiae* F.	Common Rose	Nectar
		Graphium	*agamemnon* L.	Tailed Jay	Nectar
		Graphium	*nomius* Esper	Spot Swordtail	Nectar
		Papilio	*polytes* L.	Common Mormon	Nectar
	Pieridae	*Catopsilia*	*pomona* F.	Common Emigrant	Nectar
		Catopsilia	*pyranthe* L.	Mottled Emigrant	Nectar
		Cepora	*nerissa* F.	Common Gull	Nectar
		Anaphaeis	*aurota* F.	Caper White	Nectar
		Colotis	*fausta* Olivier	Dakhan Large Salmon Arab	Nectar
	Nymphalidae	*Junonia*	*lemonias* L.	Lemon Pansy	Nectar
		Hypolimnas	*misippus* L.	Danaid Egg Fly	Nectar
		Hypolimnas	*bolina* L.	Great Egg Fly	Nectar
		Danaus	*chrysippus* L.	Plain Tiger	Nectar
		Euploea	*core* Cramer	Common Indian Crow	Nectar
	Lycaenidae	*Tarucus*	*nara* Kollar	Striped Pierrot	Nectar
		Zizula	*hylax* F.	Tiny Grass Blue	Nectar
		Lampides	*boeticus* L.	Pea Blue	Nectar
		Spindasis	*vulcanus* F.	Common Silverline	Nectar

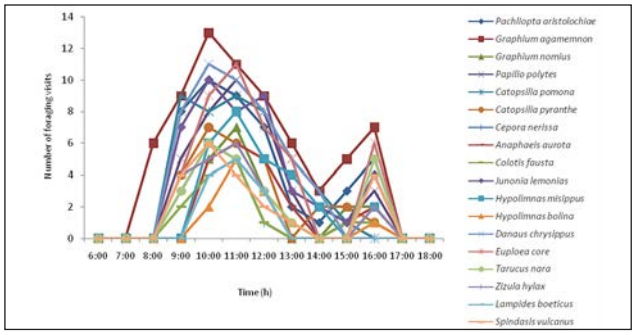

Fig. 2. Hourly foraging activity of butterflies on *Vitex negundo*.

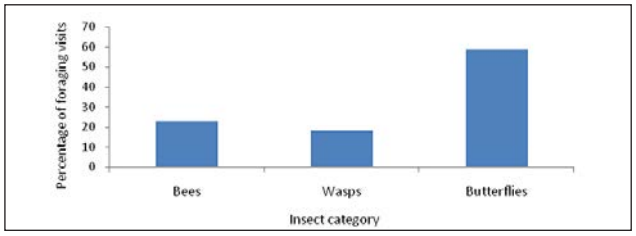

Fig. 3. Persentage of foraging visits of diffirent categories of insects on *Vitex negundo*.

Table 3. Pollen recorded in the body washings of bees and wasps on *Vitex negundo*.

Insect species	Sample size (N)	Number of pollen grains		
		Range	Mean	S.D
Apis dorsata	10	94–231	181.6	36.85
Apis florea	10	66–156	117.7	24.68
Trigona iridipennis	10	52–103	76.6	12.88
Ceratina sp.	10	63–115	97.1	14.12
Pithitis smaragdula	10	87–162	121.5	23.77
Eumenes conica	10	34–60	44.5	8.30
Eumenes petiolata	10	26–52	37.5	7.13
Rhynchium sp.	10	45–71	55.2	7.50
Bembix sp.	10	30–67	48.2	9.49

Table 4. Pollen recorded in the body washings of butterflies on *Vitex negundo*.

Butterfly species	Sample size (N)	Number of pollen grains		
		Range	Mean	S.D
Pachliopta aristolochiae	10	25–61	47.1	9.29
Graphium agamemnon	10	43–74	58.5	8.79
Graphium nomius	10	31–53	42.7	5.47
Papilio polytes	10	35–62	50.4	8.23
Catopsilia pomona	10	29–51	39.8	6.30
Catopsilia pyranthe	10	22–55	42.8	8.59
Cepora nerissa	10	16–40	30.1	6.52
Anaphaeis aurota	10	27–50	38.3	7.81
Colotis fausta	10	20–41	32.6	5.54
Junonia lemonias	10	32 –68	49.6	9.33
Hypolimnas misippus	10	25–63	46.5	10.05
Hypolimnas bolina	10	19–47	33.6	7.36
Danaus chrysippus	10	36–65	50.2	8.28
Euploea core	10	24–54	42.5	9.24
Tarucus nara	10	17–40	28.5	6.93
Zizula hylax	10	8–26	19.5	4.64
Lampides boeticus	10	11–31	21.7	5.27
Spindasis vulcanus	10	15–37	25.9	7.04

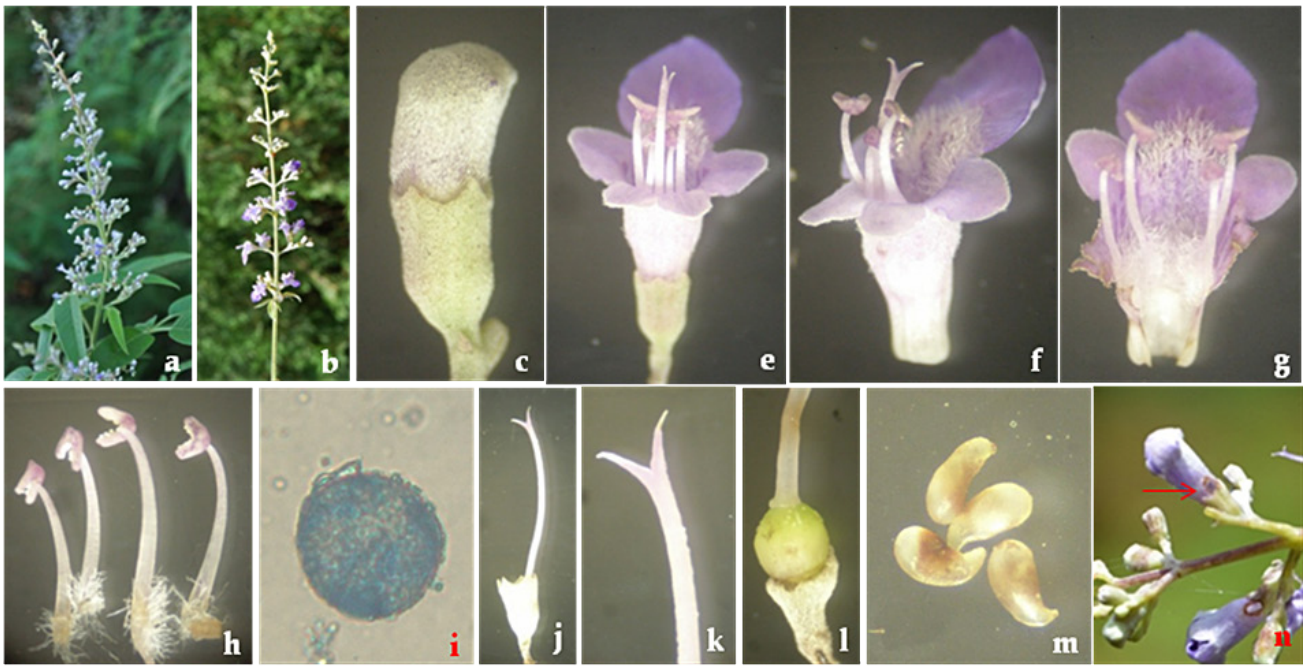

Fig. 4. *Vitex negundo*: **a.** & **b.** Flowering inflorescence, **c.** Mature bud, **d.** Flower, **e.** & **f.** Exstension of bifid stigma beyond the height of stamens, **g.** Epipetalous and didynamous stamens, **h.** Dehisced anthers, **i.** Pollen grain, **j.** Ovary, style and stigma, **k.** stigma, **l.** Ovary, **m.** Ovules, **n.** Holes made by *Trigona iridipennis* at the base of the corolla tube to rob nectar.

Fig. 5. *Vitex negundo*: **a.** & **b.** *Apis dorsata*, **c.** & **d.** *A. florea*, **e.** *Trigona iridipennis*, **f.** *Ceratina* sp., **g.** *Pithitis binghami*, **h.** *Eumenes conica*, **i.** *E. petiolata*, **j.** *Rhynchium* sp., **k.** *Bembix* sp.

Fig. 6. *Vitex negundo*: **a-d:** Papilionids – **a.** *Pachliopta aristolochiae*, **b.** *Papilio polytes*, **c.** *Graphium agamemnon*, **d.** *G. nomius*, **e-i:** Pierids – **e.** *Catopsilia pomona*, **f.** *C. pyranthe*, **g.** *Cepora nerissa*, **h.** *Anaphaeis aurota*, **i.** *Colotis fausta*, **j-n:** Nymphalids – **j.** *Junonia lemonias*, **k.** *Hypolimnas missipus* (male), **l.** *H. bolina* (female), **m.** *Danaus chrysippus*, **n.** *Euploea core*.

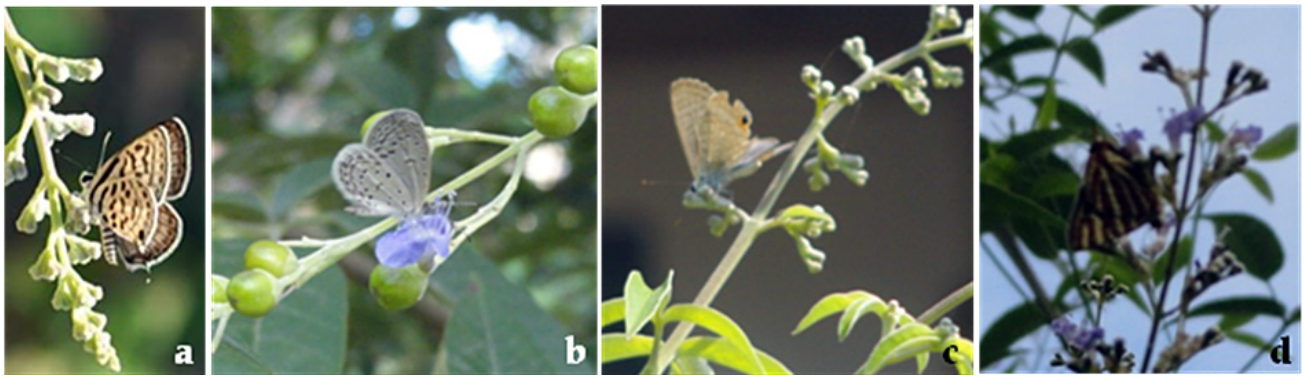

Fig. 7. *Vitex negundo*: Lycaenids – **a.** *Tarucus nara*, **b.** *Zizula hylax*, **c.** *Lampides boeticus*, **d.** *Spindasis vulcanus*.

The bees and wasps probed both mature buds and flowers for forage collection. Of these, *T. iridipennis* never visited the flowers in the legitimate way. It made a single round or ovoid hole on the basal part of the corolla tube of both mature buds and flowers to collect nectar in the illegitimate way, without contributing to pollination (Fig. 4h). This bee approached the flowers in upright position, landed on the corolla tube and bored it to collect nectar. To collect pollen, this bee landed on the stamens and collected pollen from the anthers. It did not discriminate between the anthers and stigma; in effect, the bee moved from anther to anther and to stigma for pollen collection. The moment it perceived that the stigma is not the source for pollen, it immediately shifted to anthers for pollen collection. It gathered pollen voraciously. Its nectar feeding activity by robbery resulted in emptying completely the flower of nectar, or greatly depleting

Fig. 8. *Vitex negundo*: **a.** Fruiting phase with last flowers, **b.** Mature fruits with fruiting calix, **c.** Dray fruits, **d.** Dry fruits.

the nectar volume. More than 50 % of the flowers were bored by this bee. The flowers emptied of nectar compelled the legitimate nectar foragers to pay multiple visits to the same plants and also to make frequent inter-plant foraging visits in order to quench their thirst for nectar. Such a foraging activity was considered to be promoting geitonogamy and xenogamy.

All other bees and wasps used the lower corolla lip as landing platform and probed the mature buds and flowers in the legitimate way for nectar. As the corolla tube is short and its throat is broad, these foragers inserted their proboscis through the hairy part of the corolla and collected nectar with great ease. Then, the back of head and thorax of these foragers contacted the stigma first and then the anthers in a spontaneous way. In this process, the stigma brushed against the back of the forager facilitating pollen transfer from the bee (if it carried it from the previously visited flowers) and the occurrence of self- or cross-pollination. This was immediately followed by the nototribic transfer of pollen from the anthers of the visited flower to the back of the forager. The foragers withdrew their proboscis after nectar collection and moved backwards from the corolla throat to depart from the flower. While visiting mature buds, these foragers directly landed on the top of the bud, inserted their proboscis through the corolla lobes and collected nectar. However, in this foraging, pollen was transferred from the dehisced anthers to the back or ventral side of the foragers, depending on their posture in relation to the position of anthers. The stigma at this stage was receptive and, hence, there was a likelihood of pollination occurrence. The bees moved towards the anthers and held the filaments with their legs to collect pollen; in so doing, the pollen was transferred sternotribically. During pollen collection, the contact between the bee and the stigma facilitating pollination was occasional and related to the posture of the bee. Further, when bees and wasps happened to move towards the base of the corolla tube in approaching the flowers and found the hole made by *Trigona* bee, they inserted their proboscis and collected nectar. Usually, both bees and wasps probed the flowers from the front only. They never visited the flowers that did not have corolla with the stamens and stigma intact. However, they had contact with the flowers that have only the ovary with style and stigma intact while approaching and searching for the fresh and rewarding flowers for nectar and/or pollen; such a contact was considered to be facilitating the occurrence of pollination.

All butterflies recorded were found to collect only nectar. They approached the flowers in upright position, landed and/or hovered at the flowers to collect nectar. They fluttered their wings in vertical position and inserted their proboscis through the hairy throat of the corolla tube to collect nectar. The length of proboscis of all butterflies enabled them to reach the flower base with great ease. During search and probing the flowers, their proboscis and forehead had contact with the stamens and stigma facilitating

the occurrence of pollination. They never visited the flowers lacking corolla, stamens and stigma. They never visited the flowers that did not have corolla with stamens and stigma intact. However, their body parts, especially ventral side and wings had contact with the flowers that have only the ovary with style and stigma intact while approaching and searching for the fresh and rewarding flowers for nectar; such a contact was considered to be facilitating the occurrence of pollination.

All insects visited the flowers in quick succession and swiftly moved from one panicle to another on the same and different plants for nectar and/or pollen and such a foraging activity was considered to be effecting both self as well as cross-pollinations. Several individuals of these insects visited the flowers of the same plant at the same time harmoniously and such an activity was very much pronounced during peak flowering phase. Such an intense foraging activity was found to be important to drive them off to different plants in search of more forage enabling them to promote outcrossing rate. Further, the nectar thievery by *Trigona* bee was also found to be amplifying the intra- and inter-plant foraging activity and the associated self and/or cross-pollination rate.

Fruiting behavior: Pollinated and fertilized flowers initiate fruit development immediately and take approximately four weeks to produce mature fruits (Fig. 8a). Fruit is nearly a globose indehiscent drupe, green initially and light brownish-black when ripe. The drupe produces one fully developed seed enclosed by hard seed coat and basally sheltered by fruiting calyx (Fig. 8b). The seed is oblong to ovoid, smooth and light brown. Fruit dispersal occurs during July–January (Fig. 8d). The ripe fruits along with fruiting calyx fall to the ground (Fig. 8c) and remained in the vicinity of the parental plants. Rain water disperse fruits the fallen fruits during wet season. Local people use the leaf of this plant in traditional medicine dispersing fruits. Hence, hydrochory and anthropochory are functional. The fruit pericarp is very hard and, hence, the seed is not liberated. The seed coat is also very hard and highly impermeable. Natural seed germination has not been found during the study period. Further studies are suggested for detailed information on seed dormancy, seed germination and establishment. Locals informed us that this plant has the ability to regenerate by vegetative propagation through stem cuttings.

Discussion

The flowering season and the habitat of a few *Vitex* species have been reported by different workers. Bello & Go (1978) reported that *V. parviflora* sheds foliage either partially or entirely during late dry season and flowers during rainy season. Viviane & al. (2016) reported that *V. doniana* is a dry season bloomer. Large & Mabberley (1995) reported that *V. negundo* is a variable taxon in both leaf and flower form. Serviss et al. (2007) recognized *V. negundo* has three varieties, *negundo, cannabifolia* and *heterophylla* which occur as spontaneous populations. Thomas & al. (2012) noted that this species grows along marshy slopes, banks of streams, edges of rivulets, lakes, dry sandy places, along road-sides and cultivated mostly as a hedge plant in India. Vishwanathan & Basavaraju (2010) stated that *V. negundo* thrives well in humid places or along water courses in wastelands and mixed open forests. Byragi Reddy & Subba Reddi (1994) reported that *V. negundo* flowers twice in a year, once during July–November and again during March–May. Jain & al. (2013) reported that *V. negundo* blooms during January–March in Gwalior, Madhya Pradesh, India. The present study reports that *V. negundo* grows in moisture-rich open habitats and waste lands as isolated individuals. The leaves and flowers do not show any variation to classify them into different forms. The plants shed foliage and produce new foliage almost simultaneously during December–April and flower during June–November. However, certain individuals extend flowering into December and in effect delay leaf fall and leaf flushing by about a month. The terminal panicles producing numerous flowers for two to four weeks serve as units of attraction because the flowers are small and are not conspicuous from a long distance. The extended period of flowering throughout rainy season is advantageous for the plant to maximize fruit set rate in areas where pollinators are either scarce or the co-flowering plants outcompete for the same pollinator fauna.

Schmidt (2000) reported that *Vitex* species generally exhibit hermaphroditism, where both functional male and female organs are in the same flower. Dirr (1998) reported that *V. rotundifolia* is herkogamous and autonomous self-pollination is unlikely. Escobin & Cervancia (1998) noted that this species is dichogamous, self-incompatible and out-crossing. Viviane & al. (2016) reported that *V. doniana* is hermaphroditic,

homogamous, and self-compatible species, presenting a predominant selfing through geitonogamy. Ahenda (1999) reported that *V. fischeri* and *V. keniensis* are taxonomically similar; their flowers are hermaphrotiditic, homogamous and partially self-compatible. Large & Mabberley (1995) reported that *V. negundo* is widely cultivated in Sri Lanka and in effect, it may possibly exhibit apomixis. Byragi Reddy & Subba Reddi (1994) reported that the flowers of *V. negundo* are self-compatible and fruit through facilitated autogamy, geitonogamy and xenogamy but fruit set is the highest in the last mode of pollination. The present study also agrees with this report that *V. negundo* is self-compatible and exhibits facultative xenogamous breeding system involving the operation of facilitated autogamy, geitonogamy and xenogamy with the last mode setting the highest fruit set. The pollen-ovule ratio also substantiates the operation of this breeding system and the ratio falls in the range of pollen-ovule ratio (244.7–2,588) for facultative xenogamy provided by Cruden (1977). But, Bhattacharya & Mandal (1998) reported that the pollen-ovule ratio in this species is 1,000:1. This breeding system functional in this tree species facilitated natural fruit set rate to 48 % with the pollinator activity level at the study area. Bhattacharya & Mandal (1998) reported that *V. negundo* flowers set fruit to 61 % in open-pollinations. It suggests that *V. negundo* has the ability to set fruit to more than 60 % depending on the pollinator activity levels and also on other abiotic factors such as soil moisture and nutrient environment. The homogamous nature of the hermaphroditic flowers facilitates autonomous autogamy but herkogamy prevents it due to spatial separation of the stamens and stigma. The facultative xenogamy is completely pollinator-dependent and hence the reproductive success of the plant is totally dependent on the availability of pollinator fauna. However, this breeding system enables the plant to set fruit through self-pollination in isolated plants if pollinators are available in the habitat. Therefore, the reproductive traits of *V. negundo* flowers assure sexual reproduction even for isolated and distant trees in small, remnant populations.

Abe (2006a,b) noted that *V. rotundifolia* is pollinated by diverse groups of pollinators such as flies, honey bees, beetles, butterflies and ants. Escobin & Cervancia (1998) noted that this species is entomophilous, especially pollinated by *Xylocopa* bees. Viviane & al. (2016) reported that *V. doniana* is pollinated by a wide array of insects and sunbirds. Anderson (2003) noted that *V. lucens* is exclusively pollinated by endemic honeyeaters in New Zealand. Ahenda (1999) considered *V. fischeri* and *V. keniensis* as a single species and reported that they are pollinated mainly by bees. Wasps and spiders also visit flowers but they are not pollen vectors. Byragi Reddy & Subba Reddi (1994) reported that *V. negundo* is pollinated by bees, wasps, and also visited by butterflies without contributing to pollination. Bhattacharya & Mandal (1998) reported that *V. negundo* is pollinated by bees, wasps, ants, butterflies and flies. Jain & al. (2013) reported that *V. negundo* is principally visited by honey bees and wasps in Gwalior, Madhya Pradesh, India. Gaur & al. (2014) reported that *V. negundo* is a potential bee forage plant in Garhwal Himalaya. The present study reports that *V. negundo* flowers being zygomorphic and bilabiate with the stamens and stigma situated adjacent to upper corolla lip and the lower corolla lip serving as landing platform for the forager conform to the gullet type blossoms described by van der Pijl (1972). The gullet type blossoms with the sex organs situated dorsally are primarily adapted for nototribic pollination which is naturally performed by advanced hymenopterans, especially bees. In this pollination mechanism, pollen is deposited precisely on the back of the insect preventing the wastage or loss of pollen (van der Pijl 1972). In *V. negundo*, the characteristics such as purple flowers, no perceptible smell, tubular corolla, and concealed nectar covered by the hairs at the base of the corolla tube allow only select foragers which can perform a more elaborated intra-floral behavior. The lower corolla lip being elaborate provides a comfortable landing place for the appropriate foragers. The plant is polyphilous because bees, wasps and butterflies utilize the flowers of *V. negundo* as forage source; all these insects, except the bee, *Trigona* pollinated the flowers nototribically while collecting nectar. All the bees also pollinated the flowers sternotribically while collecting pollen. The varying amounts of pollen found in the body washings of these insects conform their prime role in pollination. Such dual pollination systems effected by bees increases pollination rate and assures reproductive success. The foraging activity of these insects is in tune with the state of standing crop of nectar in particular which in turn is linked to the flower-opening schedule. As the corolla together with stamens fall off by the end of the day of anthesis, the floral parts still in place become unattractive to the foraging insects on the following day

and hence they do not visit them. However, these insects pollinate sternotribically while searching for the fresh flowers upon landing on the panicles. The insects benefit from the aggregate arrangement of flowers into panicles because that reduces flight time and search time and hence is energetically profitable. Petanidou (2005) reported that bees and wasps prefer "high sucrose" nectars while butterflies prefer "lower sucrose" nectars. Baker & Baker (1982) reported that floral nectar sugar concentrations vary with climatic conditions. The sugar concentrations of nectar from flowers adapted to different classes of flower visitors range widely (Baker & al. 1983) while the volumes of nectars in flowers with different pollinatory adaptations range over several degrees of magnitude (Baker 1978). Sugar concentrations of tropical forest nectars range from 5 to 80 %. The nectar volume and sugar concentration vary with each pollinator class (Baker & al. 1983). These variations in both volume and sugar concentrations appear to have evolved as adaptations to particular classes of animal pollinators. The present study indicates *V. negundo* flowers with small volume of sucrose-rich nectar with sugar concentrations ranging from between 24–29 %, appear to be an adaptive response for utilizing different groups of insects that need different sugar concentrations. Further, the range of sugar concentrations recorded for nectar in *V. negundo* may optimize the net energy gain by the visiting foragers (Kingsolver & Daniel 1979). In this plant, the nectar also provides certain essential amino acids along with some non-essential amino acids. Therefore, *V. negundo* flowers with flexible nectar characteristics satisfy the foraging insects which reciprocate them with pollination service.

Inouye (1983) reported that nectar robbing is a behavior exhibited by some bee species, when nectar is obtained through holes bored near the bases of the corolla tubes. Nectar robbers are subdivided into primary nectar robbers, which make the holes and then extract the nectar, and secondary nectar robbers, which obtain nectar by using holes made by primary robbers. Barrows (1976; 1980) stated that Carpenter Bees are the most notorious nectar robbers and make perforations with their maxillae, when they are unable to access nectar from tubular flowers. Van der Pijl (1954) opined that this method is probably used by all *Xylocopa* bees. Solomon Raju & Rajendra Kumar (2016) reported that *Xylocopa* and *Anthophora* bees bore holes into the corolla tube of *Clerodendrum inerme* to steal nectar and, hence, they act as primary nectar robbers; secondary robbers of nectar are absent on the flowers. Prasad & Sunojkumar (2013) stated that *Trigona* bees visit a wide range of flowers and are well known for stealing nectar. These authors reported that the Digger Bee, *Amegilla,* bores holes into the corolla tube of *Orthosiphon aristatus* to steal nectar and, hence, acts as a primary nectar robber. Byragi Reddy & Subba Reddi (1994) reported that the wasps *Rhynchium metallicum* and *Ropalidia* sp. puncture the corolla tube just above or at the juncture of the calyx and corolla tube of *Vitex negundo* to steal nectar, without effecting pollination and, hence, they act as primary nectar robbers. Other wasps and bees as secondary nectar robbers also collect nectar through such holes, if they come across them, without providing pollination service, otherwise they collect nectar from the flower entrance and effect pollination. Butterflies always use the flower entrance to collect nectar. The present study observed that *Trigona* bees are the primary nectar robbers as they make holes in the corolla tube of *V. negundo* to steal nectar without effecting pollination due to their inability to collect nectar legitimately. *Rhynchium* wasp, as a regular forager of *V. negundo,* always collected nectar legitimately and thus this study refutes the report by Byragi Reddy & Subba Reddi (1994) that wasps make perforations on the corolla tube of *V. negundo* to steal nectar. However, all other bees and wasps stole nectar only if they came across the hole on the corolla tube made by *Trigona* bees.

Pleasants (1983) stated that the removal of floral nectar by robbers decreases the standing crop and in some cases changes the sugar concentration of nectar available to other pollinators. Fenster (1991) mentioned that longer pollinator flight distances generally translate into increased pollen flow and increased outcrossing rates. If nectar robbers are the cause of longer flight distances by the legitimate pollinators, they could be increasing the fitness of the robbed plants by promoting outcrossing. The robbers could then be considered as mutualists. Guitian & al. (1994) observed that nectar robbing by Carpenter Bees had a positive effect on the seed set in *Pterocoptis grandiflora*. Zimmerman & Cook (1985) and Castro & al. (2008; 2009) stated that, besides influencing the host female fitness, nectar robbing could potentially enhance male fitness and increase the outcrossing rate through forcing legitimate pollinators to fly farther in

search of nectar, thus expanding the pollen dispersal distance and neighbourhood size, and reducing geitonogamy. In the present study, nectar robbing by swarms of *Trigona* bees from *V. negundo* could potentially enhance male fitness by driving the legitimate pollinator bees, wasps and butterflies to fly farther and farther in search of nectar. Such a foraging behavior by the pollinators expands the pollen dispersal distance and promotes the outcrossing rate in *V. negundo* enabling the latter to produce genetically superior seeds.

Ingrid (1987) reported that *Vitex stahelii* produces ovoid fleshy drupes and they are dispersed by animals in Surinam. Ahenda (1999) wrote that *V. keniensis* fruits are pulpy and mostly dispersed by hornbill birds, monkeys and even human beings. Tiffney (2004) reported that *V. lucens* produces large bright-red fleshy drupes and they are probably dispersed by flying fruit-eating animals. Charles-Dominique (1993) reported that the New Zealand Pigeon consumes the fruits of *V. lucens* and causes occasional long distance dispersal after the passage of the seed through the gut. Bass & al. (2006) noted that if the fruits of *V. lucens* are not consumed by birds, they fall to the ground from the tree and ground foraging animals may then disperse the seeds further. Sato (2012) reported that the Common Brown Lemur is the sole disperser of the fruit of *V. beraviensis* in Ankarafantsika, Madagascar; the seeds that pass through the lemur gut show improved germination rate. Chakravarthy & Ratnam (2015) reported that *V. glabrata* fruits are single-seeded drupes dispersed by the nocturnal mammal, Common Palm Civet, and frugivorous birds such as Lineated Barbet, Blue-Throated Barbet, Black-Crested Bulbul, Pin-Tailed Green Pigeon, and Wedge-Tailed Green Pigeon. Sundarapandian & al. (2005) stated that *V. altissima* is anemochorous. Zhi-Yong & al. (2007) reported that seed dispersal of *V. negundo* occurs through animal ingestion and execretion. They also stated that they have not observed bird feeding activity on the fruits and seeds of this plant. The present study indicates that *V. negundo* produces ripe non-fleshy fruits within a month and disperses them throughout wet and winter seasons. Each drupe invariably produces only one well developed seed with hard seed coat, partially covered by fruiting calyx despite the production of four ovules per flower; the other three seeds also initiate growth but subsequently abort, probably in response to post-zygotic competition within the developing fruit. In effect, seed set rate percentage remains constant in all modes of reproduction. Such a situation has also been reported for the drupes of *V. doniana* by Viviane & al. (2016). The present study has also recorded that *V. negundo* displays fruit dispersal by gravity and the fallen fruits remain in the vicinity of the parental plants. The fruits disperse by rain water and also by humans, who use the plant parts in traditional medicine. Therefore, *V. negundo* exhibits hydrochory and anthropochory.

Cousins & al. (2009; 2010) stated that *V. rotundifolia* is most likely hydrochorous, which enables the seeds to disperse over long distances. Fruits are covered with thick coatings of hydrophobic cuticular alkanes, which allow them to resist water penetration for extended periods. Seeds possess physical and physiological dormancy mechanisms. Physical dormancy mechanism is enforced in part by cuticular alkanes that prevent water penetration. Physiological dormancy mechanism is believed to be allowing the establishment of a substantial soil seed bank that is capable of surviving and producing new seedlings beyond four years after all vegetation has been removed. Collectively, these two mechanisms allow the plant to undergo long-distance dispersal. Viviane & al. (2016) reported that *V. doniana* seeds have a hard seed coat which prevents the entry of water and oxygen to break seed dormancy. The seeds have low level of natural seed germination and poor recruitment. But, imbibed seeds extracted from fruits have high germination rates. Sahoo & Chand (1998) reported that propagation through seed is hindered due to poor germination and that the conventional method of propagation is rather slow in *V. negundo*. In the study area, *V. negundo* seeds did not germinate during the entire study period suggesting that their hard seed coat is highly impermeable and prevents the entry of water and oxygen to break seed dormancy for extended periods to enable hydrochory more effectively and establish substantial soil seed bank that is capable of surviving and producing new seedlings after a long time. However, physical and physiological dormancy mechanisms regulating the seed germination in *V. negundo* need to be studied in detail before breaking seed dormancy and producing new plants. The plant has the ability to regenerate by vegetative propagation through stem cuttings, as the local people insist. However, in the long run this method is not a viable one for propagating this tree species as it would lead to inbreeding depression and associated deleterious

effects. Therefore, further research should focus on seed dormancy and seed viability in *V. negundo* for restoring genetically viable populations in natural areas.

Vitex negundo is grown commercially as a crop in certain parts of the world. It is a source of timber and also is cultivated as a food crop. The plant is used to treat asthma, cancer, allergy, wounds, body aches, toothache, joint pains, jaundice, and is also used as antidote for snake bites, respiratory disorders, fever, headache, and eye pain in various parts of India (Vishwanathan & Basavaraju 2010). These various uses for this tree species indicate that it is highly valued in traditional medicine and also has commercial value to some extent**.** Therefore, the reproductive biology information on this tree species is useful to understand the various aspects of its sexual reproduction and, accordingly, take measures for the protection of its pollinator fauna for the production of genetically superior seeds in natural areas. Finally, it is suggested that *V. negundo* should be used in traditional medicine or for other purposes without affecting the surviving plants in natural areas.

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