THE POTENTIAL VALUE OF PHYTOCHEMICAL AND MICROMORPHOLOGICAL CHARACTERS IN TAXONOMIC TREATMENT OF GENUS VITEX L. (LAMIACEAE)

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Vitex L. (*Lamiaceae*) in Iran was reviewed using morphological and phytochemical characters. *Verbena officinalis* (*Verbenaceae*) was also studied for comparison. The pollen grains, were examined using SEM and LM Furthermore, the petiolar anatomy, trichome structure and morphology of different plant parts were studied. The essential oils of aerial plant parts were extracted and identified. Pollen grains among the studied species of *Vitex* are tricolpate, isopolar, circular in the polar view, while in *Verbena officinalis* they are tricolporate and triangular. The exine ornamentation provides characteristic features useful for delimitation of the species in our group. The SEM micrographs of fruits, leaves and stems show uni- and tetra-cellular glandular and simple multi-cellular trichomes. The petiolar anatomy of the first to the third lower nodes showed different patterns in the shapes and arrangement of the vascular bundles.

Form phytochemical point of view α-pinene, sabinene and limonene are the characteristic compounds in *Vitex* spp. and are present in examined species in high concentrations. In most species monoterpenoids are the major constituents of the oil. There are differences in mono- and sesquiterpenoid compounds of the oils among species. Examining the morphological and micro-morphological characters of leaf, stem, corolla and calyx in combination with the phytochemical characters revealed the occurrence of four species in the flora of Iran including *V. agnus-castus*, *V. negundo*, *V. trifolia* and *V. pseudo-negundo*. Among these species high morphological, anatomical and phytochemical variations were observed in *V. pseudo-negundo*. Moreover, the affinity of *Vitex* within *Lamiaceae* genera is supported.

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Key words. *Vitex*, anatomy, *Lamiaceae*, Pollen, Phytochemistry, morphlogy.

ارزش بالقوه صفات فیتوشیمیائی و میکرومورفولوژیکی در بررسی تاکزونومیکی جنس Vitex

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در یک بررسی تاکزونومیکی جنس *Vitex* از خانواده *Lamiaceae* با استفاده از صفات مورفولوژیکی و فیتوشیمیائی وگونه *Verbena officinalis* (*Verbenaceae*) نیز به منظور مقایسه، مورد بررسی قرار گرفت. دانه های گرده، میوه، ساقه ها و برگهای چهار گونه از *Vitex* و یک گونه از *Verbena* با استفاده از میکروسکوپ های نوری و SEM مورد بررسی قرار گرفتند. به علاوه آناتومی دمبرگ، ساختمان پوشش های کرکی و ریخت شناسی اندامهای مختلف گیاه مطالعه گردید. دانه‌های‌گرده در گونه‌های *Vitex* سه‌شیاری، جورقطب و در شمای قطبی دایره‌ای شکل هستند. در حالیکه در *Verbena officinalis* سه شیاری، منفذدار و در شمای قطبی مثلثی هستند و به علاوه تزئینات سطح خارجی دانه‌گرده در

گونه‌های مختلف متفاوت ومشخص کنندهٔ محدودهٔ گونه‌ها می باشند. در تصاویر میکروسکوپ الکترونی میوه، برگ، ساقه و کرکهای یک تا چهار سلولی ساده و غده دار مشاهده گردید. در این جنس کرکها و غده ها صفات تاکزونومیکی مفیدی را فراهم می‌سازند. آناتومی دمبرگ مربوط به برگهای گره های اول تا سوم تفاوتهائی را در شکل و آرایش دستجات آوندی نشان می‌دهد. اسانس قسمت های هوائی گیاه استخراج و ترکیبات شیمیائی آن شناسائی‌گردید. در ترکیب اسانس گونه های *Vitex* آلفا-پینن، سابینن و لیمونن ترکیبات شاخص می باشند و در همه گونه‌ها با درصد بالا حضور دارند. در اکثر گونه‌ها منوترپنوئیدها ترکیبات غالب اسانس را تشکیل می‌دهند. از نظر نوع ترکیبات منو- وسزکوئی- ترپنوئیدی در گونه‌های مختلف تفاوتهائی وجود دارد و گونه *Verbena officinalis* دارای ترکیبات شاخص متفاوتی است. بررسی های انجام شده حضور چهارگونه *V. agnus-castus*، *V. negundo* ، *V. trifolia* و *V. pseudo-negundo* در ایران را نشان می‌دهد. در میان این گونه‌ها تنوع بالائی در ساختار آناتومیکی و مورفولوژیکی گونه *V. pseudo-negundo* مشاهده‌گردید. بر این اساس ارتباطات خویشاوندی بین گونه‌ها قابل تفسیر می‌باشد. همچنین تعلق جنس *Vitex* به خانواده *Lamiaceae* موردتأیید قرار می‌گیرد.

Introduction

Vitex L. (*Lamiaceae*) is a large tropical genus with a few temperate species. A total number of 250 species have been considered for it (Boissier, 1888; Bentham & Hooker, 1965; Rechinger, 1967). In Iran it occurs in S, SW, W, NE, E and Central part of the country. The *Vitex* species usually grow in dry river beds, seasonal rivers and stream sides. A combination of *Nerium indicum*, *Tamarix* spp. and *Vitex* spp. are usually observed in the natural habitats of the species in Iran. In previous taxonomic treatments of the genus *Vitex* has been considered in *Verbenaceae* by different authors but in recent works it has been transferred into *Lamiaceae* based on different evidences (Wagstaff et al., 1998; Palmer & Pitman, 1972; Cantino, 1982; Cantino & Sanders, 1986; Cantino et al., 1992 (a); Catino, 1992 (b), 1992).

There is a rather high morphological variation within species which makes the identification difficult. The species boundaries are also unclear. Rechinger (1967) considered three species of *Vitex* in Iran, namely *V. negundo* L., *V. pseudo-negundo* (Hausskn.) Hand.-Mazt., *V. trifolia* L.**.** Boissier (1888) has reported *V. agnus-castus* L. and *V. negundo* from Iran, while Parsa (1950) reported both above mentioned species along with *V. pseudo-negundo* as a variety of *V. agnus-castus* Jafri and Abdul Ghafoor (1974) did the same treatment as for this taxon. Townsend (1980) reported a hybrid between *V. pseudo-negundo* and *V. agnus-castus* in Iraq.

Pollen exine morphology of different genera of *Lamiaceae* and *Verbenaceae* has proved to provide useful characters in taxonomy of these families (Henderson et al., 1968; Raj, 1983; Afzal-Rafii, 1983; Abu-Asab, 1990, 1993; Harley et al.,1992; Jamzad et al., 2000).

The essential oil constituents of *Vitex* spp. have been subject of some previous works (Mallavarapu et al., 1994; Zeng et al., 1996; Moudachirou et al., 1998; Ajiwe & Okeke, 1998; Singh et al., 1999; Hossian et al., 2001). The data provided by these works shows the potential value of terpenoids in taxonomic treatment of *Vitex*.

The anatomical studies on petiole, trichomes, stomata, etc. provide also diagnostically significant characters. In *Verbenaceae* and *Lamiaceae* different genera and species were examined previously and some diagnostic characters were determined (Metcalfe & Chalk, 1950; Metcalfe, 1963; Metcalfe & Chalk, 1983; Phongoudome, 2000).

In this study we aim to identify the *Vitex* spp. in Iran and to define the relationships among them and provide additional information on the species with incorporation of new characters. Taxonomic evidences are obtained from morphology, anatomy, palynology and phytochemistry.

Materials and methods

Herbarium specimens of *Vitex* from the following herbaria were studied: TARI, TUH and IRAN. New collections from western, eastern and northern part of the country were done to provide materials for phytochemical and morphological studies (Tabel 1).

Pollen grains were removed from the herbarium specimens, acetolysed and prepared for light (LM) and scanning electron microscopy (SEM) using methods fully described in Harley et al. (1992). For LM measurements means and ranges of 10-15 pollen grains were taken. Nutlets and leaves were removed from herbarium specimens fixed in SEM stubs and prepared for photography.

Table 1. Materials used for the study. Characters: 1: Pollen; 2: Essential oil; 3: Anatomy

Species	Collectiong data & herbaria	Characters		
		1	2	3
Vitex agnus-castus	Khorassan, Sabzevar, Darvarzan, 900 m, Paryab, 10602.	*	*	*
	Khorassan, Sabzevar, Darvarzan, beside river, 1000 m, Ferduosi University staff, 209.	*		*
	Syria, Jebel ed Druz, E. Suwaydah Richtung Sale, 1450 m, Frey & Kürschner, 83-539, (Her. Hort. Bot. Reg. Edin.).	*		
Vitex negundo	Khorassan, 9 km form Kashmar to Naishabour (FE_2), 1300 m, Assadi & Mozaffarian, 35568 (TARI).	*		*
	Khorassan, 8-9 km for Kashmar to Neishabour, 1280-1300 m, Paryab, 10600.	*	*	*
Vitex trifolia	Sistan va Baluchestan, between Iranshahr and Fannuj, 750 m, Sandoughdaran, 200.			*
	Sistan va Baluchestan, before 1 km to Nik-shahr from Chah-bahar, 600 m, Hashemi, 87602.	*	*	*
Vitex pseudo-negundo	Hamadan, Malayer, above Navleh Village, beside river, 1850 m, Safikhani *et al.* 5574.	*		*
	Kermanshah, 22 km, on the road from Kerend to Sare-pole-zahab to Sorkhe-dizeh, 1200 m, Safavi, 84393 (TARI).		b*	
	Kermanshah, 5 km on the road from Sare-pole-zahab to Ghaser-Shirine, Fatah beg diviation, 700 m, Safavi, 84401 (TARI).		c*	
	Kermanshah, 10 km on the road from Sare-pole-zahab to Ghaser-Shirine, Fatah beg diviation, 700 m, Abbas Azimi & Mohabi, 87611 (TARI).			*
	Kurdestan, Marivan to Dizli after rocky mnt, 1250 m, Safavi & Massoumi, 86034 (TARI).			*
	Lorestan, Nowjian to Keshvar near water fall, 1300-1850 m, Mohabi & Abbas Azimi, 87608 (TARI).		*	
	Lorestan, 15 km on road from Khorramabad to Nowjian and Keshvar, 1500m, Runemark & Lazari, 26154.			a*
Verbena officinalis	Arak, 6 km, S. Arak, Senedjan, near arable-lands & river, Zehzad & Akhani, 412 (TARI).			*
	Khorassan, 14 km form Kashmar to Neishabour (FE2), 1400-1500 m, Assadi & Mozaffarian, 35611 (TARI).	*		

Pollen grain, nutlet and trichomes were examined using an Olympus, BH2-RFCA light Microscope (LM) and a LEO4401 Scanning Electron Microscope (SEM).

For anatomical observations the leaves of the herbarium specimens were prepared using FAA or 70% ethanol and stained with carmine for 10-15 minutes and then in fast green for a few seconds and washed with distilled water. The slides were prepared and studied by Olympus, BH2-RFCA LM. The anatomical characters are determined based on 13 samples.

The aerial parts of the plants of *V. pseudo-negundo* from three different localities and one specimen from each of *V. negundo*, *V. trifolia* and *V. agnus-castus* (Tabel 1) were air-dried and the oils extracted by hydro-distillation using a Clevenger–type apparatus for 2.5-3 hours. The oils were dried over anhydrous sodium sulfate and stored in sealed vials in a fridge before analysis. The oil extracts were analyzed by GC (9A-Shimadzu) and GC/MS (Varian 3400), column (DB1, 60 m x 0.25 mm fused silica capillary column, film thickness 0.25 µm) by temperature program 50º-250º C at a rate of 4º C/ min, injection temperature 260 º C, carrier gas helium, scan range m/z 40-300, ionization energy 70 eV, scan 1 sec^{-1}

Results

Anatomical observations

Trichome

In *Vitex*, the following types of trichomes have been observed by LM on the petiole transverse sections and by SEM of leaf, stem and fruit surfaces.

Type1. non-glandular trichomes including uni- to multi-cellular (2-14 cellular) hairs (Figs. 1& 6).

Type 2. nonglandular multi-cellular curved and straight hairs with a long, thin terminal cell (Fig. 2).

Type 3. peltate trichome composed of one basal epidermal cell and one to four secretory cells with a subcuticular space (Figs. 3 & 7).

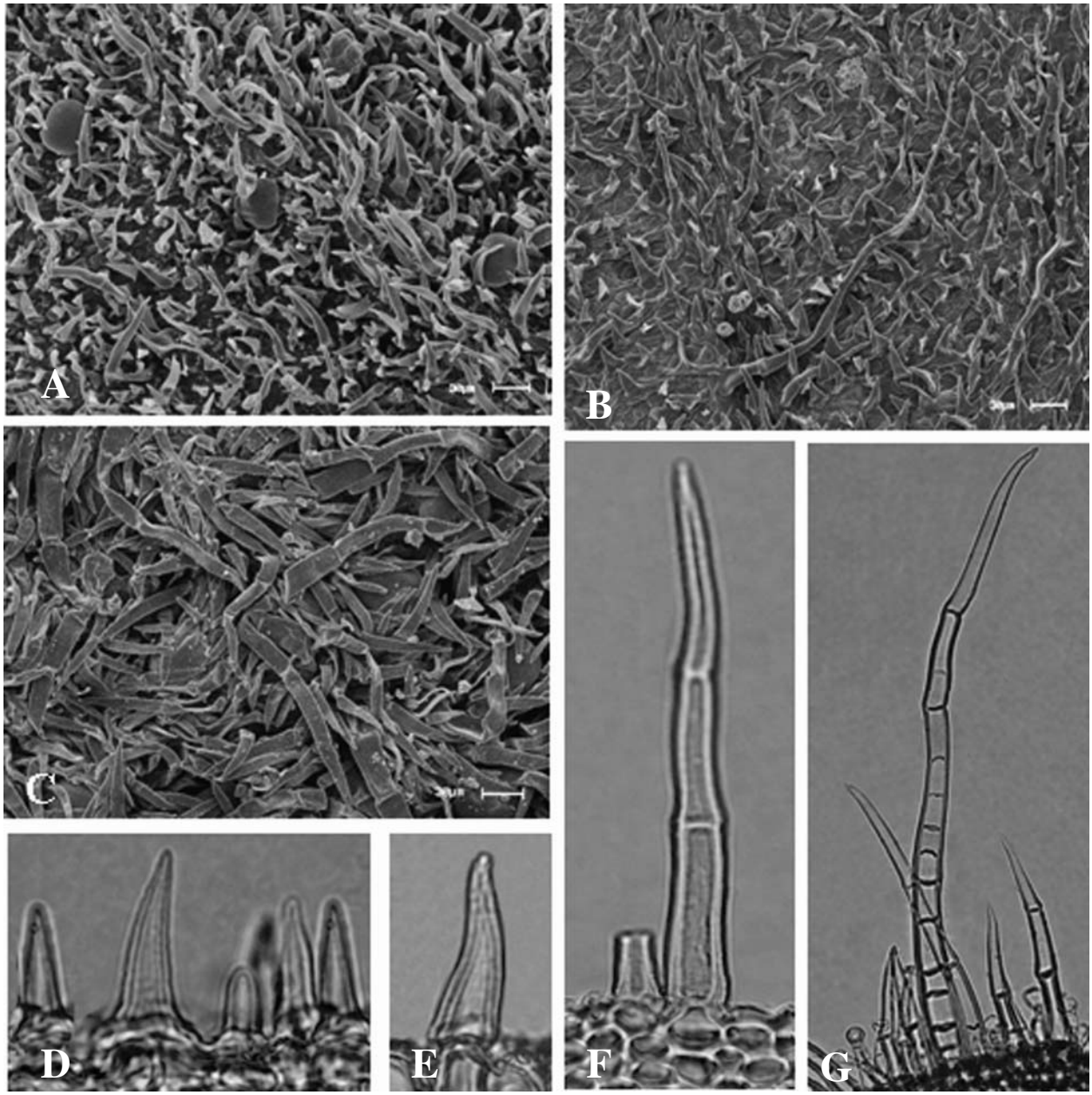

Fig. 1. Trichome type 1 in *Vitex* spp. A & E, *V. agnus-castus*; B, *V. negundo*; C, D, F, G, *V. pseudo-negundo*. Scale bar: A-C=30 μm; D & E=0.03 mm; F=0.05 mm; G=0.15mm.

Type 4. long capitate trichome with a multicellular stalk and one or two more rounded secretory head cells (Fig. 4).

In *Verbena officinalis* simple short and long conical hairs with swollen base, long multi-cellular glandular trichomes and 4-cellular glands with mono-cellular stalk are observed (Figs. 5, 6 &7).

Anatomical structures of petiole

Petiole TS:

The anatomical structure of petiole and petiolule in *Vitex* spp. include epidermal cells, different trichome types, a few layers of angular chollenchyma continued to the sides of petiole and followed by chlorenchyma.

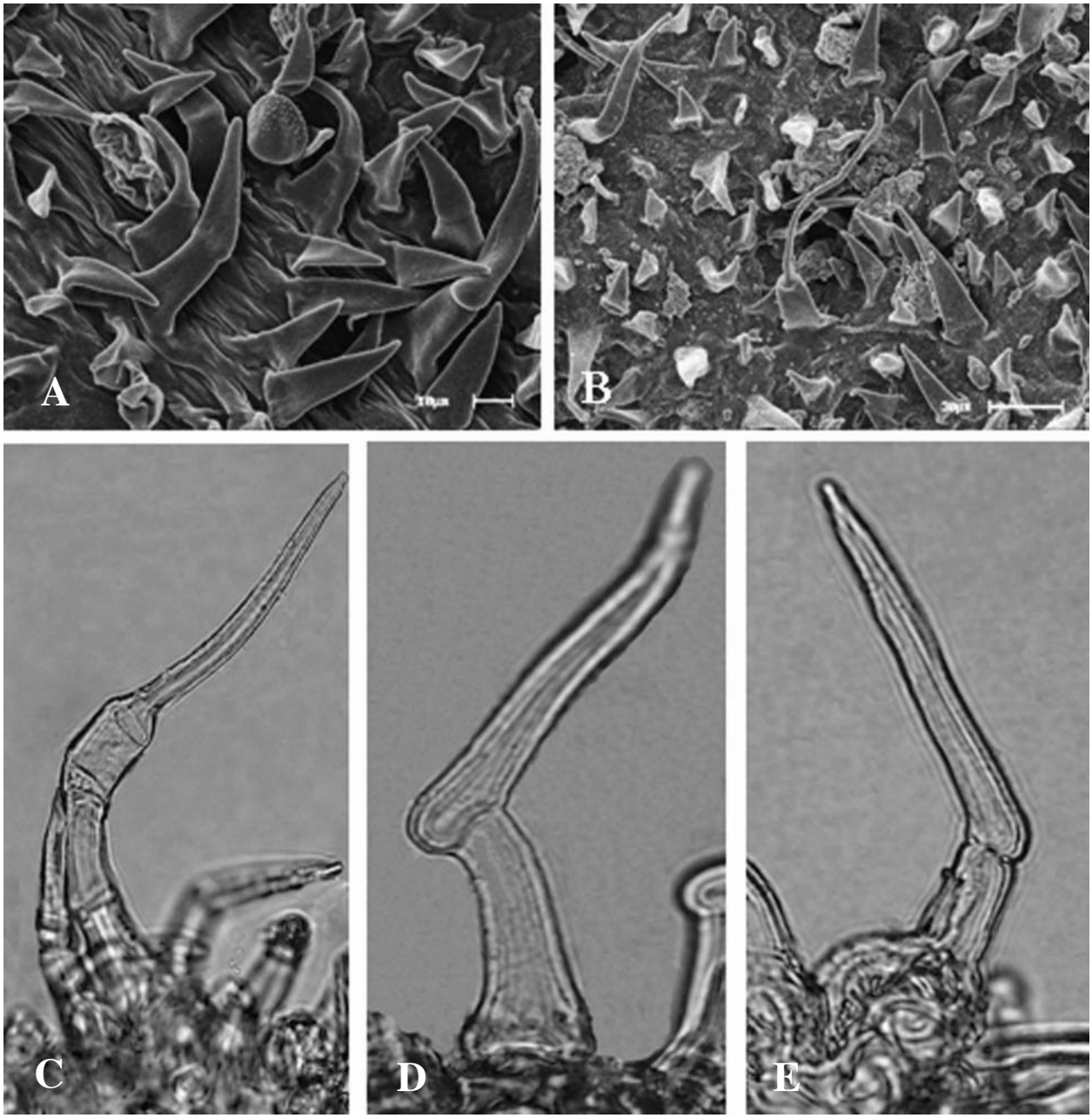

Fig. 2. Trichome type 2 in *Vitex* spp. A & D, *V. negundo*; C & E, *V. agnus-castus*; B, *V. pseudo-negundo*. Scale bar: A=10 μm; B=30 μm; C-E=0.03 mm.

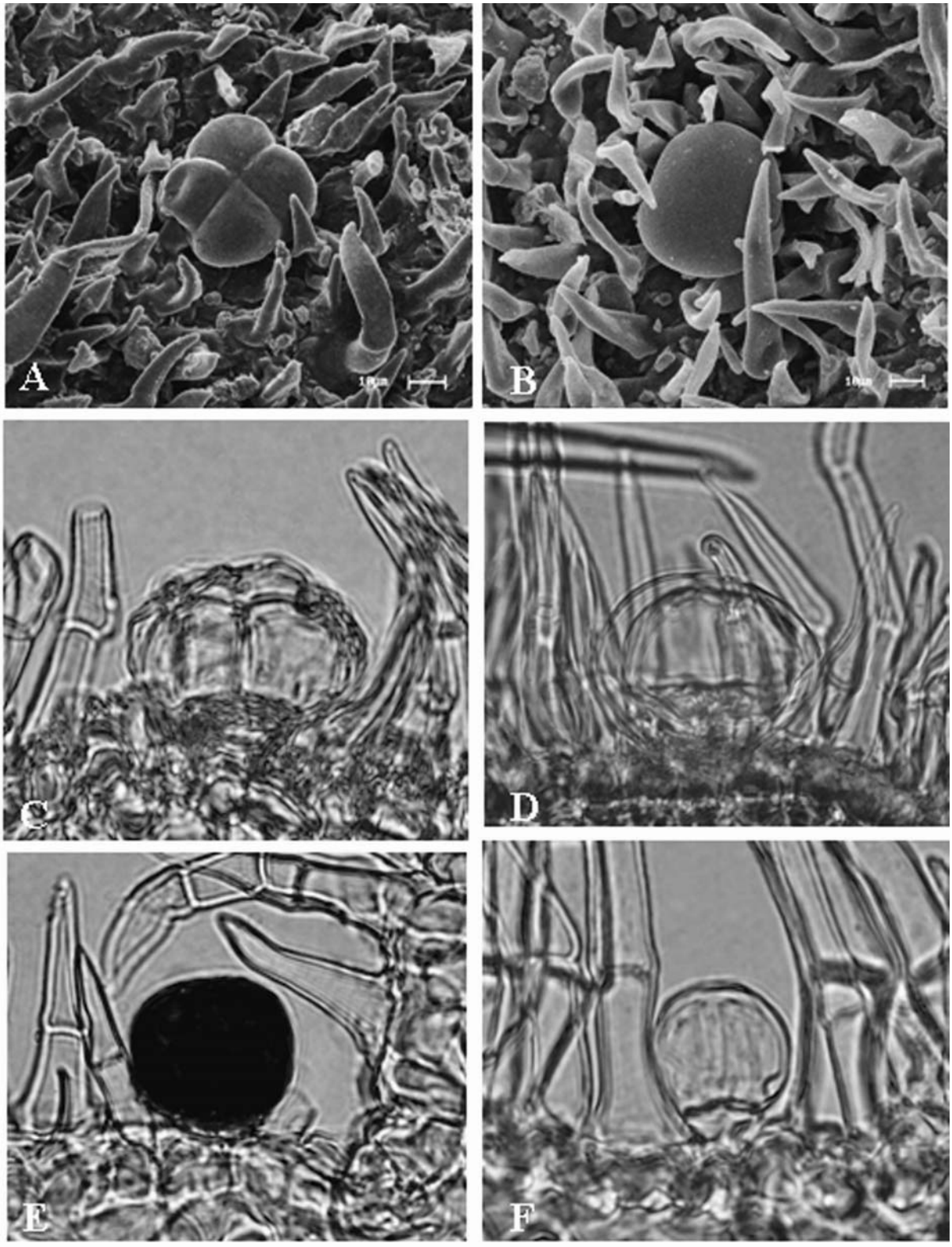

Fig. 3. Trichome type 3 in *Vitex* spp. A, E, F, *V. trifolia*; B & C, *V. agnus-castus*; D, *V. negundo*. Scale bar: A & B= 10 μm; C-F=0.03 mm.

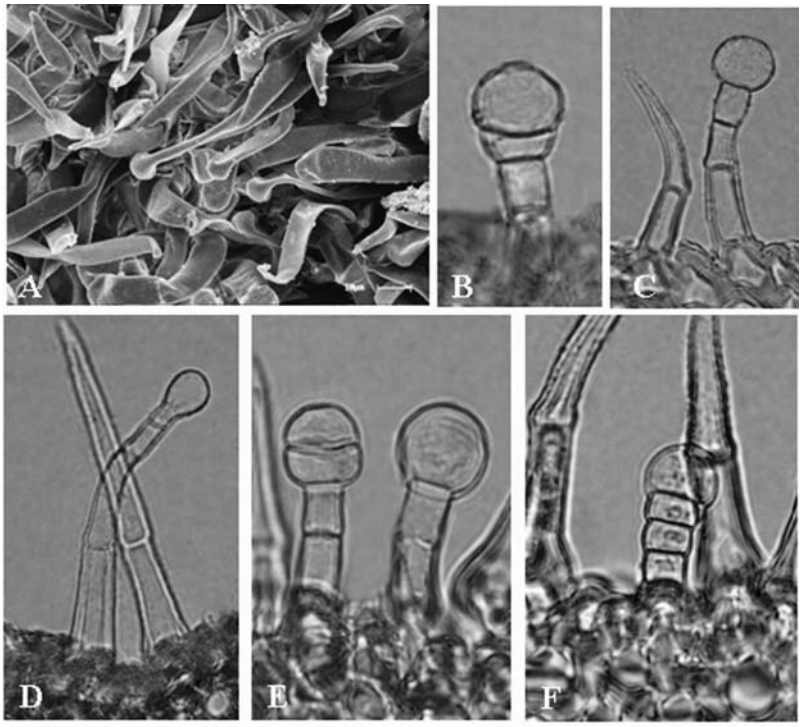

Fig. 4. Trichomes type 4 in *Vitex* spp. A, *V. agnus-castus*; B, C, E, *V. negundo*; D, *V. pseudo-negndo*; F, *V. trifolia*. Scale bar: A =10 μm; B, C, E, F=0.03 mm; D=0.05 mm.

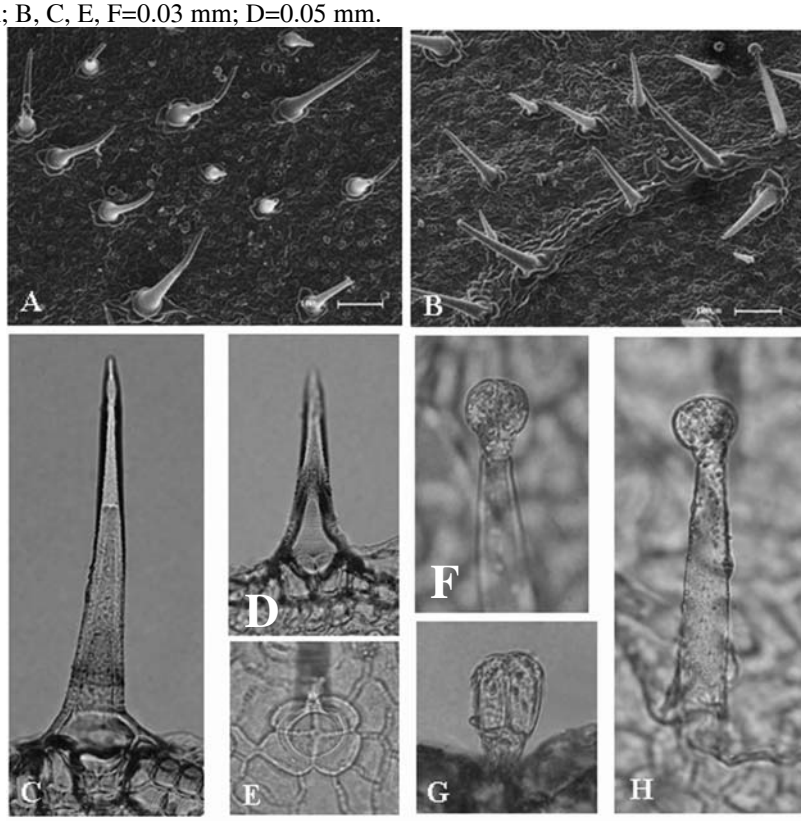

Fig. 5. Leaf trichome of the *Verbena officinalis* A-E, simple hairs with swollen bases; E, basal cells of the trichomes; F-H, glandular trichomes. Scale bar: A & B=100 μm; C, D, E, F=0.03 mm; H=0.05 mm.

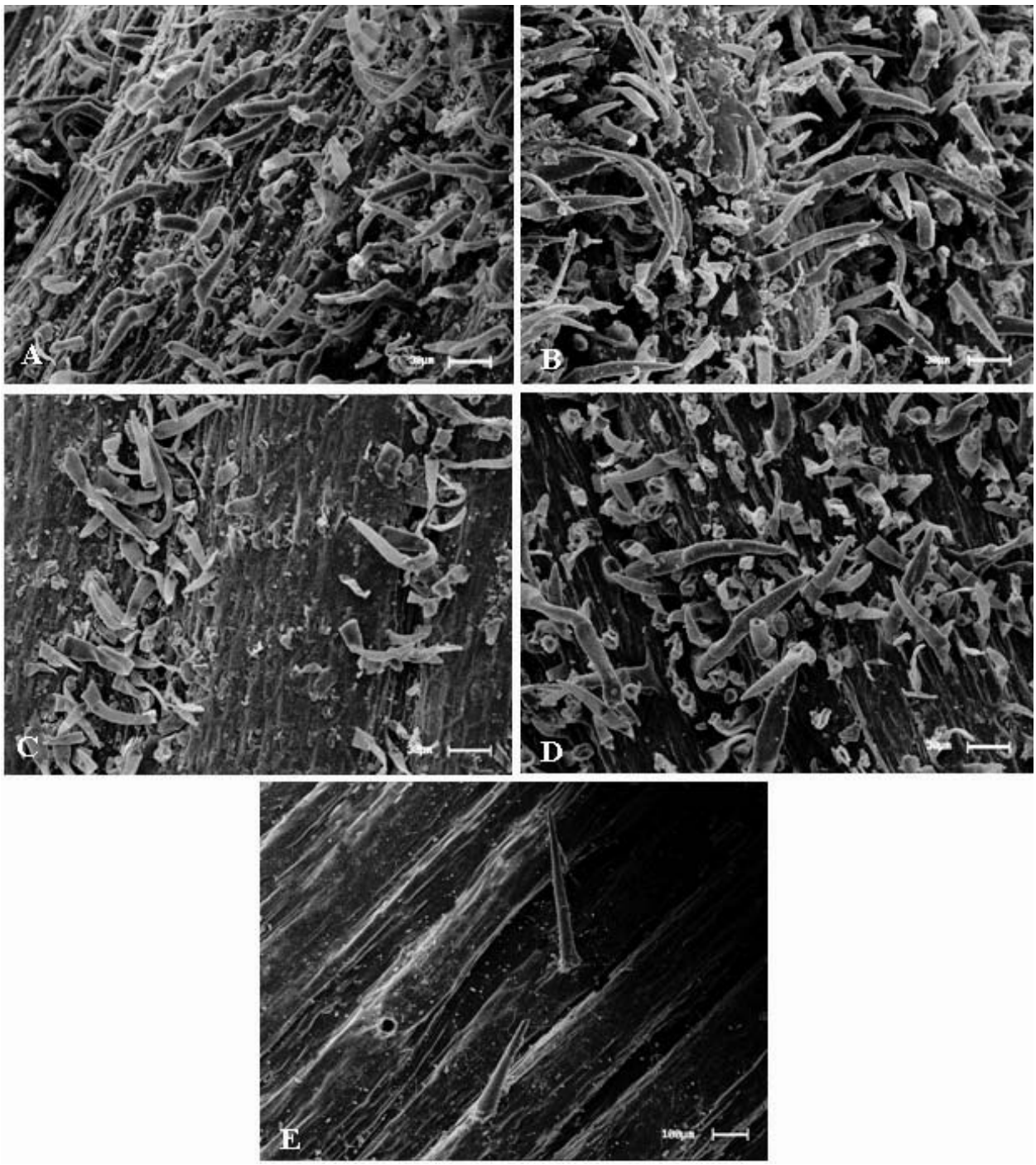

Fig. 6. Stem trichomes: A, *V. agnus-castus*; B, *V. pseudo-negundo*; C, *V. negundo*; D, *V. trifolia*; E, *Verbena officinalis*. Scale bar: A-D=30 µm; E=100 µm.

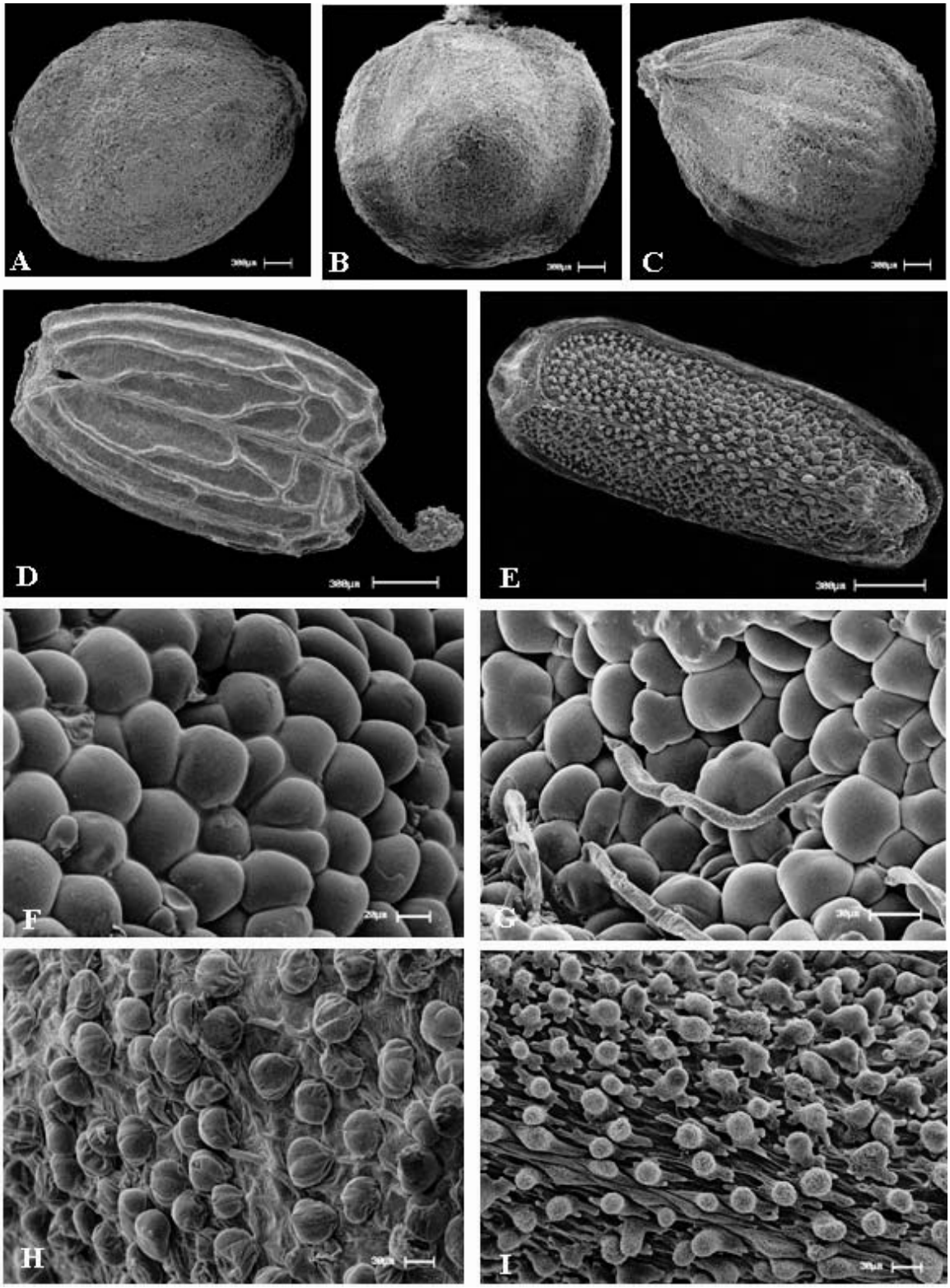

Fig.7 . SEM micrograph of fruit and their surface ornamentation in *Vitex* spp. and *Vrebena officinalis*. A & F, *V. agnus-castus*; B, *V. pseudo-negundo*; C & G, *V. negundo*; H, *V. trifolia*; E, I, *Verbena officinalis*. Scale bar: A-E=300 μm; F=20 μm; G-I=30 μm

Parenchyma cells are in different sizes and shapes with a thin wall, fill the inner space. Petiole is winged with accessory bundles enclosed within the median strand in an approximately medullary position. However in *V. pseudo-negundo* both winged and simple forms are observed. A deep crescentic median vascular strand is observed. The arrangement and shape of inner vascular bundles are characteristic in different species. In *V. agnus-castus* they are irregular at the margin and separated, 4, 5, 6, 9 segmented bundles with a semicircular shape (Fig. 8, A). In *V. trifolia* regular, connected to separate bundles arranged in two parts (Fig. 8, C). In *V. negundo* irregular separate bundles arranged in two parts (Fig. 8, B). In *V. pseudo-negundo* irregular separate 3, 6, 10, 11 segmented bundles arranged in two or separate more or less spiral group (Fig. 9, A-D). The anatomical structure of petiolule is similar to petiole (Fig. 8, D).

In *Verbena officinalis* 2-3 layers of collenchyma, a shallow crescentic median vascular strand was observed. The lack of double vascular bundles in medullary position is characteristic in this species. In the sections from the second leaf node the parenchyma cells penetrate into the vascular bundles and dissected bundles can be observed (Fig. 8, E).

Palynological observations

The pollen grains in *Vitex* spp. are tricolpate, isopolar and circular in polar view. Pollen shape is oblate-spherioidal (P/E=0.91-0.95 µm), Fig.10. The polar length varies between 27.3 (in *V. negundo*) to 36.3 (in *V. trifolia*). The equatorial width also varies between 28.4 (in *V. negundo*) to 38.5 (in *V. trifolia*). Referring to these measurements *V. negundo* has the smallest pollen size and *V. trifolia* has the largest pollen (Tabel 2).

The wall thickness varies between 2-2.5 µ. The species can be divided in two groups by their pollen wall thickness: in the first group the wall thickness is 2.4-2.5 µm (*V. agnus-castus, V. negundo*) in the second group the wall thickness is 2-2.1 µm (*V. trifolia, V. pseudo-negundo*), tabel 2.

The exine ornamentation is a characteristic for each species. The following types have been observed: perforate-fenestrate (*V. agnus-castus*); rugulate-suprareticulate (*V. trifolia*); tectate-perforate (*V. negundo*) and reticulate-perforate (*V. pseudo-negundo*). The perforation size usually is larger in mesocolpium than apocolpium (Figs. 11 & 12).

In *Verbena officinalis* pollen is three colporate, triangular in polar view. Pollen shape is suboblate (P/E= 0.83-0.88 µm). The mean wall thickness is 2.2 µm. Exine is tectate-regulate with a few perforations near colpus membrane (Fig. 11).

Phytochemical observations

In the oil of *V. agnus-castus* 24 compounds were identified (Tabel 3) which comprises 94.4% of the total compounds. Monoterpenoids were the major constituents (82.8%), of the oil, among them 1,8-cineole (18.5%), α-pinene (17.8%), limonene (15.7%) and sabinene (14.9%) were the major monoterpenoids. β-caryophyllene and bicyclogermacrene were the sesquiterpenoids with more than 2% concentration. Furthermore two diterpenoids (epi-13-manool and methyl linoleate) were identified.

Because of morphological variation in *V. pseudo-negundo* three different specimens were examined for comparison (Tabel 1).

Mono- and sesquiterpenoids have more or less equal concentration in the oil (Lorestan, a: 42.7, 50.7; Kermanshah, b: 46.64, 46.2; Kermanshah, c: 48.4, 39) α-pinene (10.3, 22.9, 24.5%), and limonene (4.4, 12.8, 10.4%) are the major monoterpenoids. The major sesquiterpenoids in each of these three specimens were different, α-guaiene (14.2%), germacrene D (11.6%), α-cadinol, (10 %) are present each in one of the three specimens (Tabel 3).

In *V. negundo*, monoteroenoids were the major constituent of the oil, α-pinene (18.8%), sabinene (7.6%) and limonene (6.9%) the major monoterpenoids. α-guaiene (10.5%) is the major sesquiterpenoid (Tabel 3). In the oil of *V. trifolia* monoterpenoids are the major constituent and α-pinene (23.9%), sabinene (24.6%) are the major compounds. Among the sesquiterpenoids β-caryophyllene has the highest concentration (10.5%).

Comparing the terpenoid characters, reveals that the characteristic compounds in *Vitex* are α-pinene, sabinene and limonene which are present in all examined specimens with high concentration (Tabel 3).

In *Verbena officinalis* as reported (Chalchat & Garry, 1996) the main constituents of the oil were sesquiterpenoids, spathulenol (10.80%), caryphyllene oxide, (7.30%), ar-curcumeme (6.0%) are predominant. The characteristic monoterpenoids are limonene (7.50%), 1,8-cineole (7.5%), thymol (1.51%) and carvacrol, (1.18%), neral. (2.50%), alpha terpineol (2.17%), geranial (3. 34%).

Morphological observations

Vitex is a shrub with stems branched, square in transverse sections, covered with different types of trichomes as described above. The leaves are opposite, petiolate, compound, the leaflets are in 3-5 and rarely 7. Inflorescence is an axillary cyme or terminal panicle. Flowers are blue-violet. Calyx is campanulate, persistence in fruit, reticulately veined, with five more or less equal to unequal teeth. Corolla is bilabiate, the

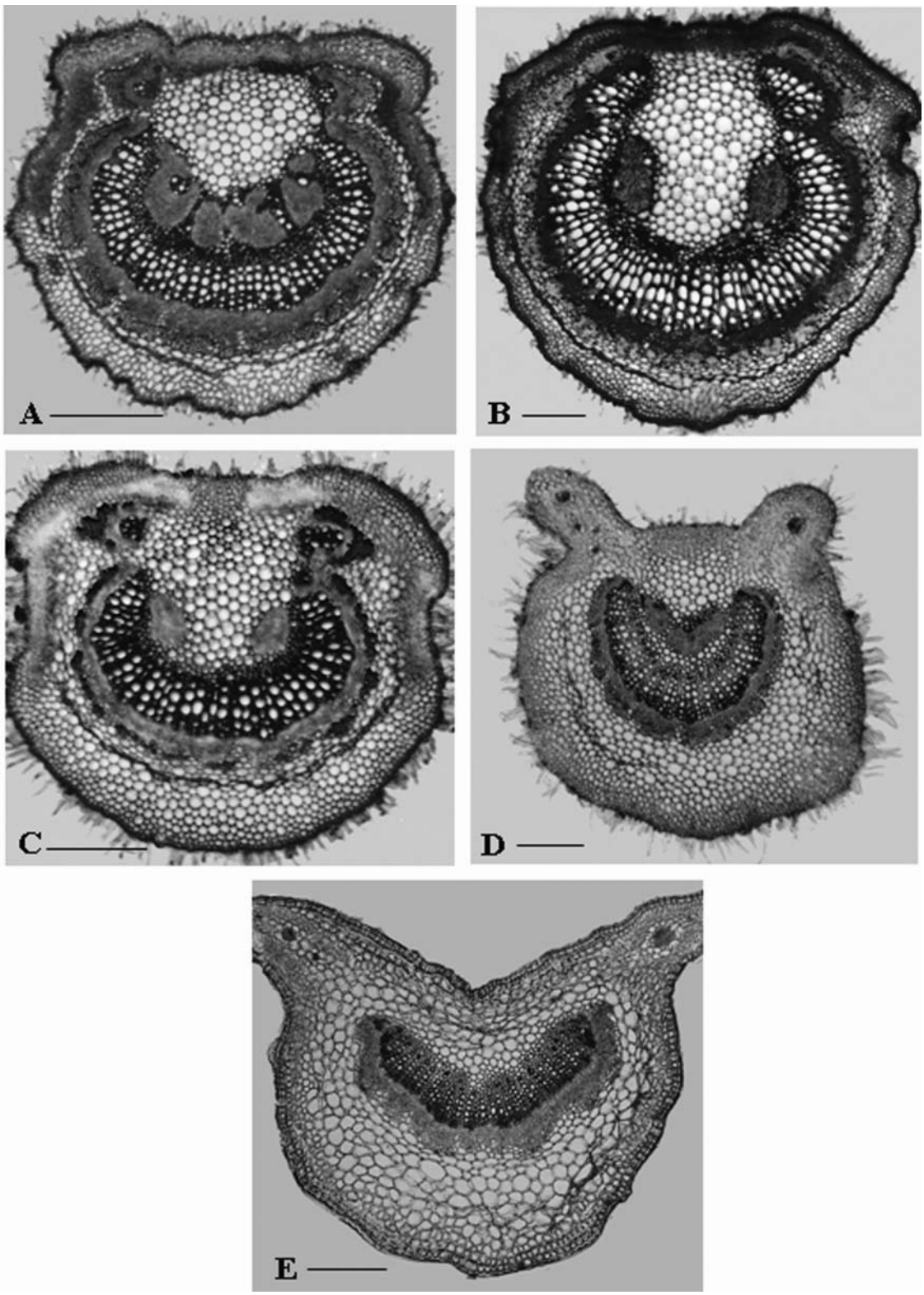

Fig. 8. Petiole of *Vitex* spp. in TS: A, *V. agnus-castus*; B, *V. negundo*; C, *V. trifolia*; D, *V. pseudo-negundo*; E, *Verbena officinalis*. Scale bar=0.3 mm.

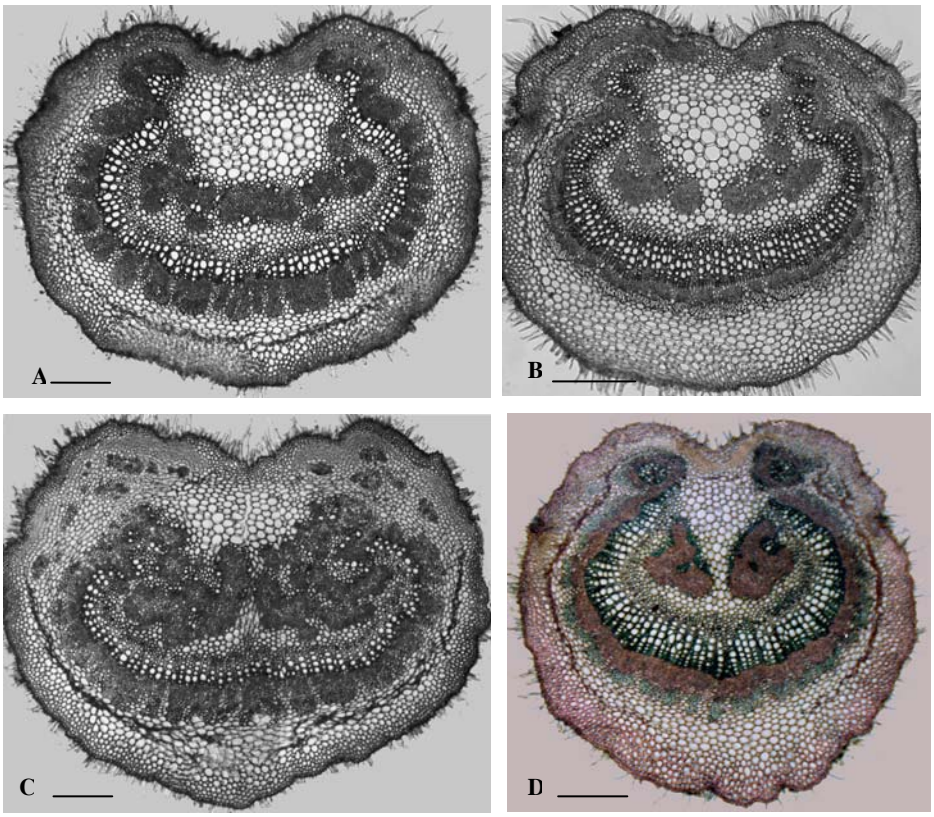

Fig. 9: Different anatomical structure in petiole of *V. pseudo-negundo*. Scale bar= 0.3 mm.

Table 2: Pollen data for the species of *Vitex*. Colum 1: Species; Colum 2: Polar length; Colum 3: Equatorial width; Colum 4: Shap (P/E); Colum 5: Mean wall thickness.

1	2			3			4	5
Vitex agnus-castus	(29.3-)	30.7	(-34.3)	(27.7-)	32	(-34.3)	0.95	2.4
Vitex negundo	(26.5-)	27.3	(-29.5)	(27.0-)	28.4	(-31.0)	0.95	2.5
Vitex trifolia	(31.0-)	36.3	(-42.0)	(32.5-)	38.5	(-44.5)	0.94	2.3
Vitex pseudo-negundo	(27.0-)	28.2	(-29.6)	(27.5-)	30.1	(-32.3)	0.93	2.2
Verbena officinalis	(29.0-)	30.4	(-32.0)	(28.5-)	35.3	(-37.0)	0.85	2.3

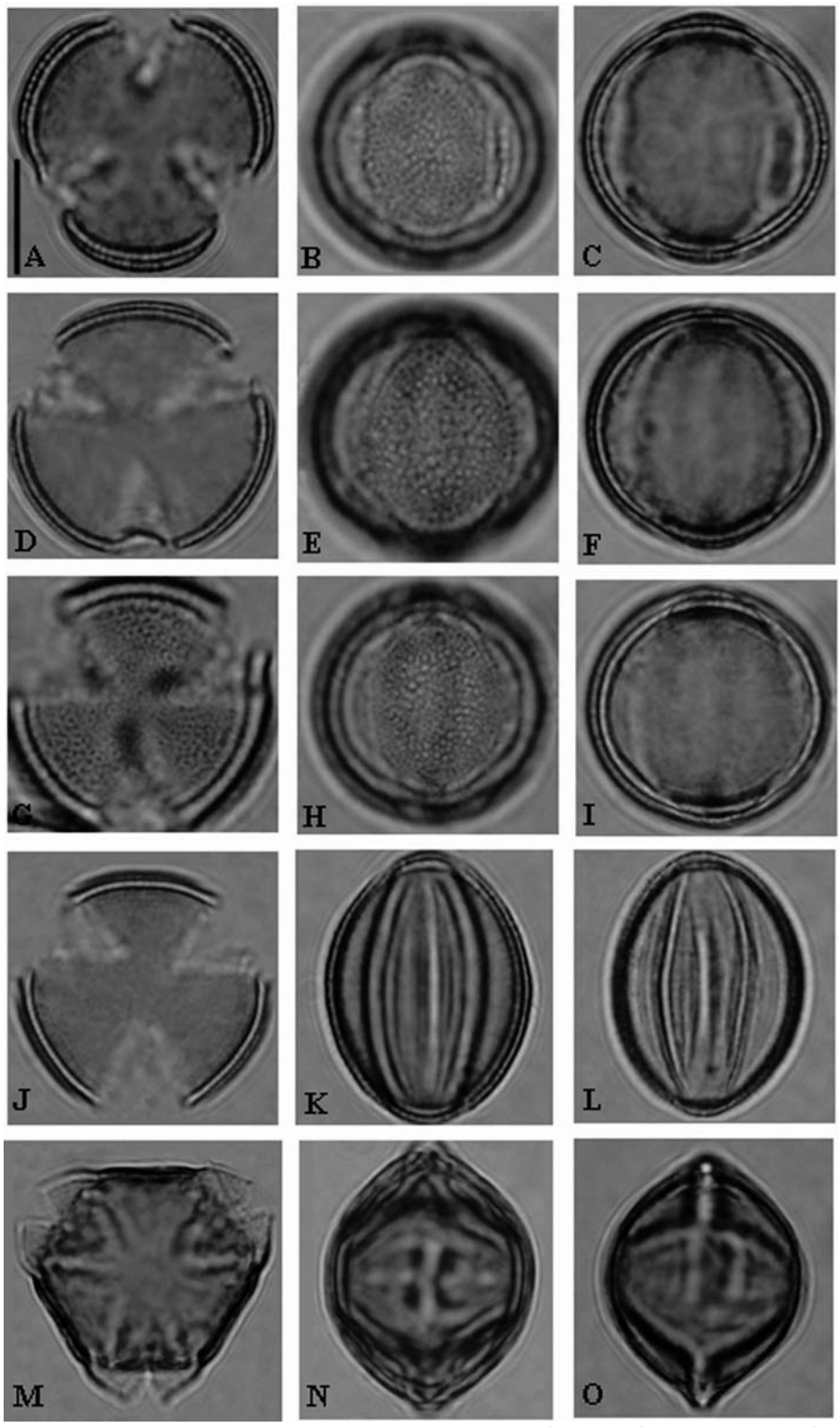

Fig. 10. Pollen morphology, LM. A-C, *V. agnus-castus*; D-F, *V. pseudo-negundo*; G-I, *V. negundo*; J-L, *V. trifolia*; M-O, *Verbena officinalis*; A, D, G, J, M, Pollar view (G high focus; A, D, J, M, low-mid focus); B, C, E, F, H, I, K, L ,N, O, equtorial view (B, E, H, high focus; K, N, C, F, I, L, O, Low- mid focus). Scale bar=0.01 mm

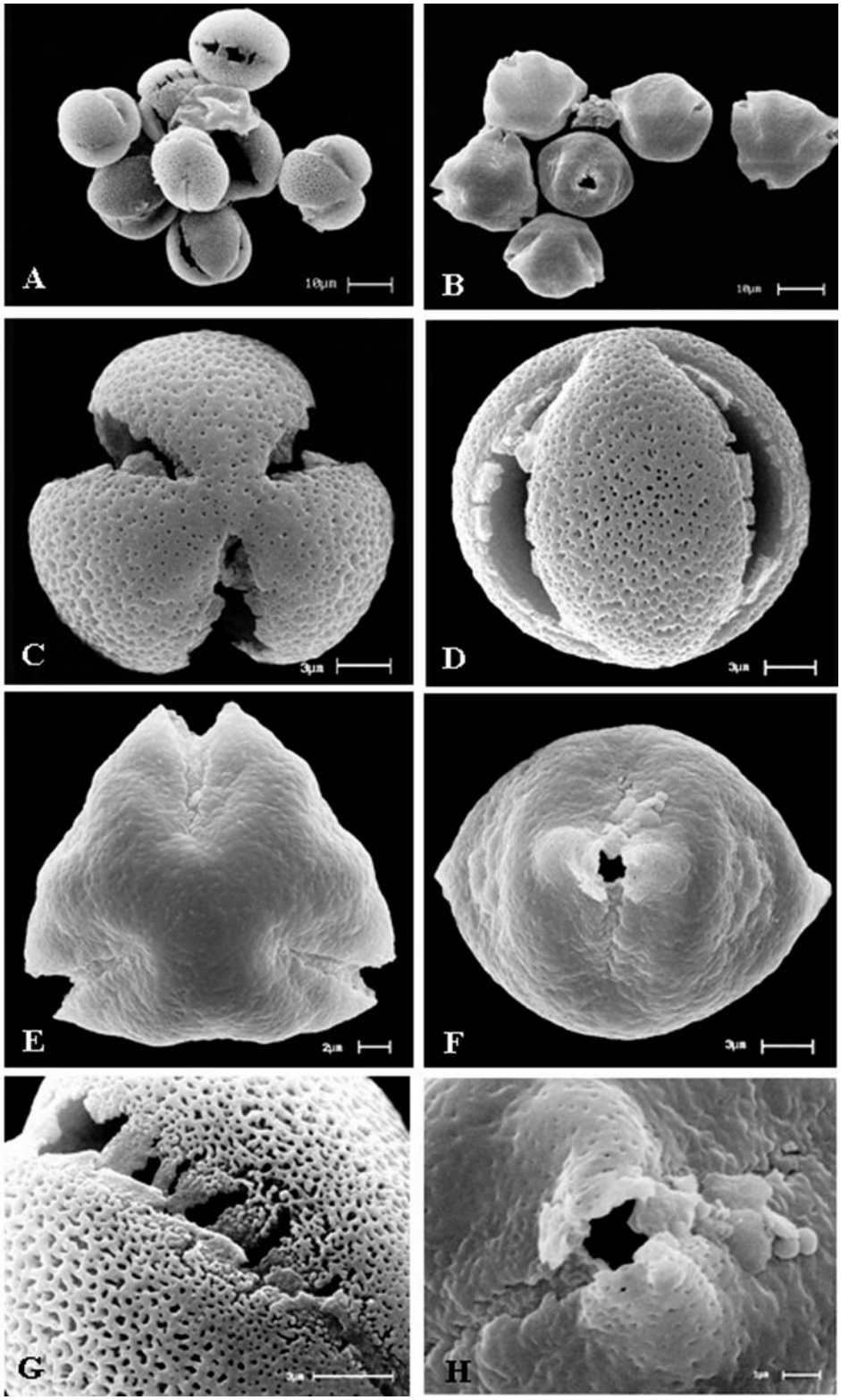

Fig. 11. SEM micrograph of the pollen of *V. agnus-castus*; and *Verbena officinalis*. A, C, D, G, *V. agnus-castus*; B, E, F, H, *Verbena officinalis*; A & B, Pollen group; C & E, Polar view; D & F, equatorial view; G, colpus membrane; Scale bar: A&B=10 µm; C, D, F, G=3 µm; E= 2 µm; H=1 µm.

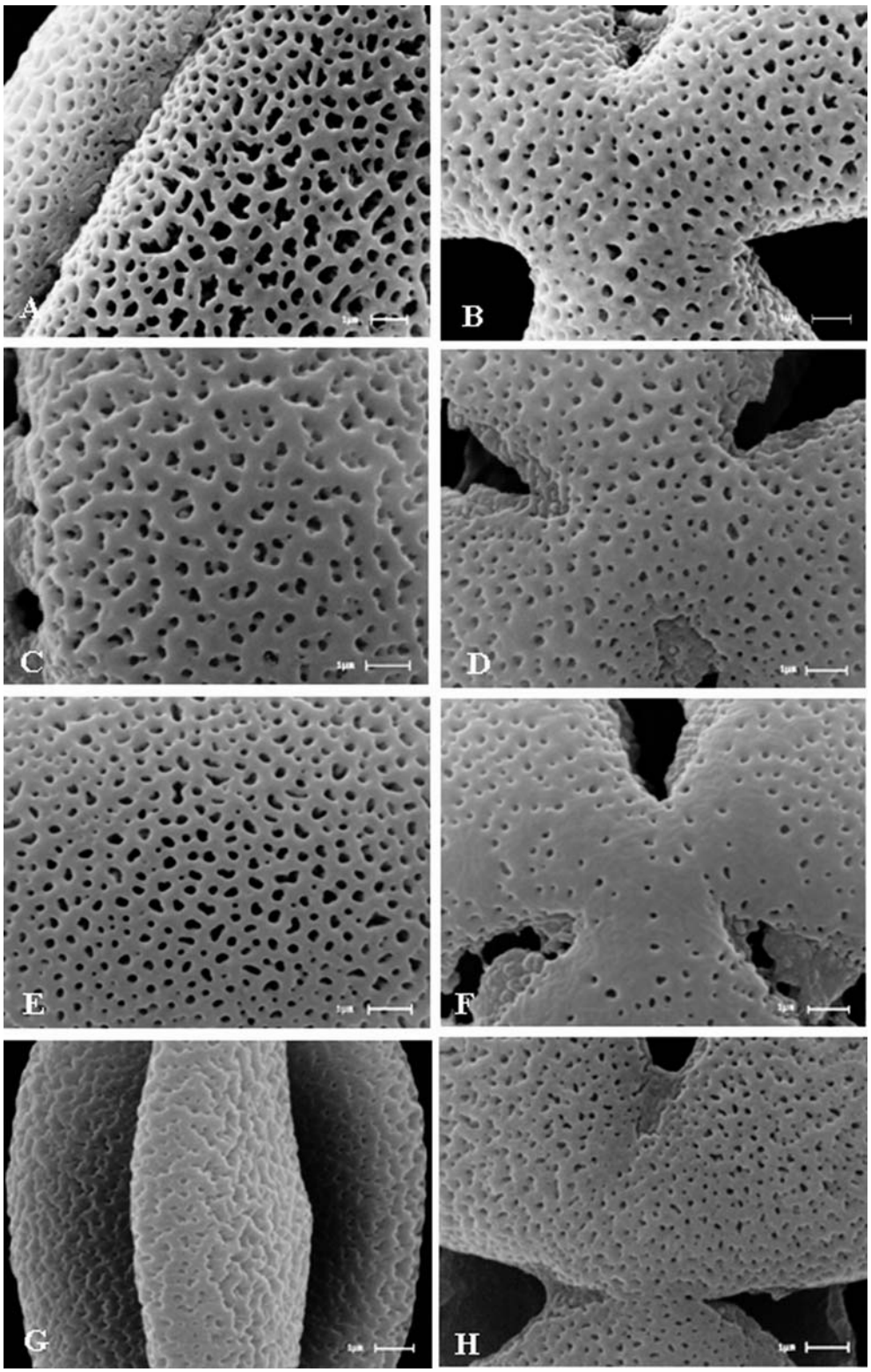

Fig. 12. The exine ornamentation in *Vitex* spp. A & B, *V. agnus-castus*; C & D, *V. pseudo-negundo*; E & F , *V. negundo*; G & H, *V. trifolia*; A, C, E, G, mesocolpium; B, D, F, H, apocolpium. Scale bar: A-H=1 µm

Table 3. Chemical composition of the essential oil of *Vitex* spp. (1- *V. agnus-castus*, 2- *V. negundo*, 3- *V. trifolia*, 4- *V. pseudo-negundo*).

Compound name	KI (DB-5)	KI (DB-1)	1 (%)	2 (%)	3 (%)	4a (%)	4b (%)	4c (%)
α -thujene	926	927	0.3	0.8	0.6	-	-	-
α -pinene	939	939	17.8	18.8	23.9	10.3	22.9	24.5
sabinene	976	974	14.9	7.6	24.6	2.3	1.8	2.2
β -pinene	980	979	0.8	0.8	-	-	0.4	0.5
myrcene	998	984	1.5	1.2	0.8	-	1.4	1.1
α -phellandrene	1003	1001	0.5	-	-	-	0.5	-
α -terpinene	1017	1014	-	-	0.8	-	-	-
P-cymene	1025	1026	-	1.1	1.1	1.0	0.9	1.2
limonene	1031	1027	15.7	6.9	7.7	4.4	12.8	10.4
1,8-cineole	1033	1029	18.5	20.8	-	10.9	-	1.0
(E)- β -ocimene	1050	1040	0.4	-	-	-	-	-
γ - terpinene	1060	1052	0.6	-	1.8	-	-	-
terpinolene	1089	1084	0.3	-	-	-	0.3	-
linalool	1098	1086	0.3	0.3	-	0.9	0.6	0.5
trans- pinocorveol	1139	-	-	-	-	0.6	-	0.4
delta- terpineol	1166	1153	0.3	-	-	-	-	-
terpinen-4-ol	1177	1170	1.6	1.5	3.4	2.5	-	0.7
α -terpineol	1189	1179	0.8	4.6	-	4.3	-	-
bornyl acetate	1284	-	-	-	-	-	0.5	0.7
lavandulyl acetate	1286	1272	-	-	1.3	-	-	-
thymol	1290	1281	-	-	1.7	-	-	-
α -terpinyl acetate	1350	1342	8.4	0.3	-	4.3	1.8	2.5
citronellyl acetate	1354	1333	-	0.5	-	1.2	2.3	2.7
β –bourbonene	1384	-	-	-	-	1.2	0.7	-
α -gurjunene	1409	-	-	-	-	-	0.7	-
β -caryophyllene	1418	1431	2.5	3.9	10.5	1.1	6.4	4.5
γ - elemene	1433	-	-	2.9	-	4.7	-	-
α -guaiene	1439	-	-	10.5	-	14.2	-	-
(Z)- β -Farnesene	1443	1451	1.9	-	5.7	-	-	-
α -himachalene	1447	-	-	-	-	-	1.0	9.1
allo-aromadendrene	1460	1470	0.4	-	-	-	1.6	1.3
germacrene D	1483	1487	0.3	0.6	-	1.4	11.6	1.4
β - selinene	1490	1495	-	-	1.5	-	0.7	0.5
α - selinene	1498	1504	-	-	2.2	-	-	-
bicyclogermacrene	1500	1506	4.6	-	-	-	9.9	1.0
(Z)-γ -bisabolene	1515	-	-	-	-	2.0	-	-
β-sesquiphellandrene	1524	-	-	1.4	-	-	-	-
elemol	1550	1545	-	-	3.5	-	-	-
germacrene B	1556	1555	-	0.5	-	-	5.3	0.7
trans-nerolidol	1564	-	-	-	-	-	-	0.8
spathulenol	1576	1579	1.1	-	-	-	3.0	7.0
caryophyllene oxide	1581	1586	-	-	2.2	5.8	-	-
globulol	1583	-	-	-	-	-	1.4	3.3
torreyol	-	1639	-	-	1.1	-	-	-
β -eudesmol	1649	1651	-	4.9	1.8	4.5	-	-
α -eudesmol	1652	1652	-	-	0.7	-	5.6	5.3
α -cadinol	1653	-	-	-	-	10.0	-	-
α -bisabolol	1671	1672	-	3.3	-	5.8	-	0.7
β -bisabolol	1683	-	-	-	-	-	-	3.7
epi- 13-manool	2057	1964	0.3	-	-	-	-	-
methyl linoleate	2096	2090	0.6	-	-	-	-	-
α –guaiene	1439	-	-	10.5	-	14.2	-	-

Table 3. (count.)

(Z)- β -Farnesene	1443	1451	1.9	-	5.7	-	-	-
α -himachalene	1447	-	-	-	-	-	1.0	9.1
allo-aromadendrene	1460	1470	0.4	-	-	-	1.6	1.3
germacrene D	1483	1487	0.3	0.6	-	1.4	11.6	1.4
β - selinene	1490	1495	-	-	1.5	-	0.7	0.5
α - selinene	1498	1504	-	-	2.2	-	-	-
bicyclogermacrene	1500	1506	4.6	-	-	-	9.9	1.0
(Z)-γ -bisabolene	1515	-	-	-	-	2.0	-	-
β-sesquiphellandrene	1524	-	-	1.4	-	-	-	-
elemol	1550	1545	-	-	3.5	-	-	-
germacrene B	1556	1555	-	0.5	-	-	5.3	0.7
trans-nerolidol	1564	-	-	-	-	-	-	0.8
spathulenol	1576	1579	1.1	-	-	-	3.0	7.0
caryophyllene oxide	1581	1586	-	-	2.2	5.8	-	-
globulol	1583	-	-	-	-	-	1.4	3.3
torreyol	-	1639	-	-	1.1	-	-	-
β -eudesmol	1649	1651	-	4.9	1.8	4.5	-	-
α -eudesmol	1652	1652	-	-	0.7	-	5.6	5.3
α -cadinol	1653	-	-	-	-	10.0	-	-
α -bisabolol	1671	1672	-	3.3	-	5.8	-	0.7
β -bisabolol	1683	-	-	-	-	-	-	3.7
epi- 13-manool	2057	1964	0.3	-	-	-	-	-
methyl linoleate	2096	2090	0.6	-	-	-	-	-
bicyclogermacrene	1500	1506	4.6	-	-	-	9.9	1.0
(Z)-γ -bisabolene	1515	-	-	-	-	2.0	-	-
β-sesquiphellandrene	1524	-	-	1.4	-	-	-	-
elemol	1550	1545	-	-	3.5	-	-	-
germacrene B	1556	1555	-	0.5	-	-	5.3	0.7
trans-nerolidol	1564	-	-	-	-	-	-	0.8
spathulenol	1576	1579	1.1	-	-	-	3.0	7.0
caryophyllene oxide	1581	1586	-	-	2.2	5.8	-	-
globulol	1583	-	-	-	-	-	1.4	3.3
torreyol	-	1639	-	-	1.1	-	-	-
β -eudesmol	1649	1651	-	4.9	1.8	4.5	-	-
α -eudesmol	1652	1652	-	-	0.7	-	5.6	5.3
α -cadinol	1653	-	-	-	-	10.0	-	-
α -bisabolol	1671	1672	-	3.3	-	5.8	-	0.7
β -bisabolol	1683	-	-	-	-	-	-	3.7
epi- 13-manool	2057	1964	0.3	-	-	-	-	-
methyl linoleate	2096	2090	0.6	-	-	-	-	-

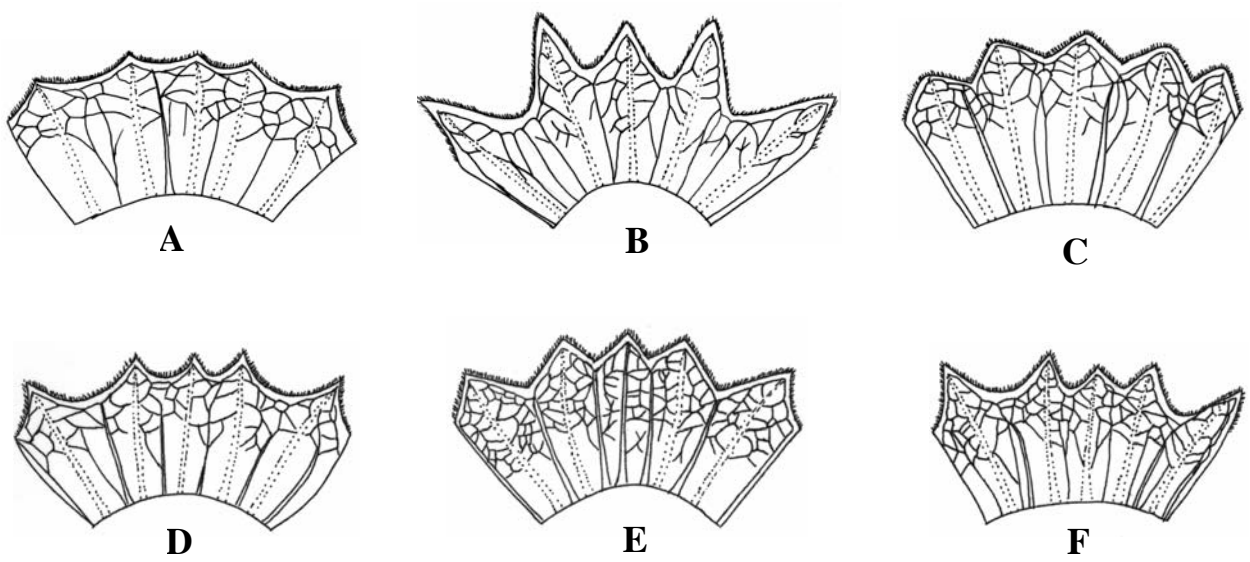

Fig. 13. The calyx shape of Vitex spp. A, *V. agnus-castus*; B, *V. negundo*; C, *V. trifolia*; D-F, *V. pseudo-negundo*. magnification: A=10x; B & C=11x; D= 11.8x; E=10x; F=5x

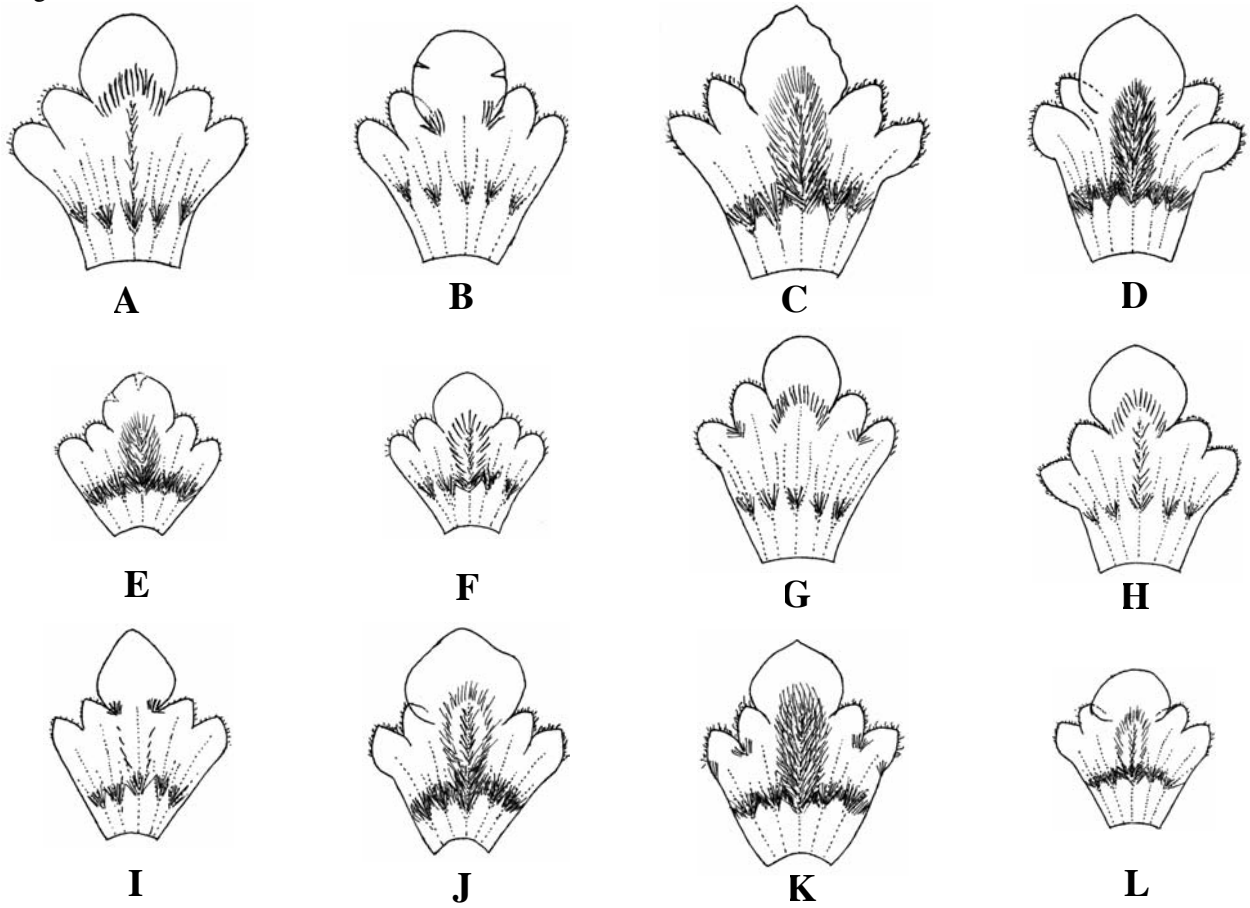

Fig. 14. The shape and hairiness of middle lobe of the lower lip in *Vitex* spp. A & B, *V. agnus-castus*; C & D, *V. negundo*; E & F, *V. trifolia*; G-L, *V. pseudo-negundo*. magnification: A, C, J, K=4.4x; B & D= 4.2x; E, F, G, H, L=5x; I=4x

Table 4: Comparisom of morphological and anatomical characters in *Vitex* spp.

Species	Leaf	Calyx		Corolla			Vascular bundle
		teeth shape	teeth to tube ratio	middle lobe shape	hairiness	Length	
V. agnus-castus	3-5 rarely 7	irregular	± 1/6	rounded to semicircular shape	tuft of hair at the base or a tuft of hair on the angles between the lobes	9-11	irregular separate, 4, 5, 6, 9 segmented bundles in a semicircular outline
V. negundo	5 petiolate	regular	± 1/2	ovate-triangular, acute with undulate margin	with a dense to lax tuft of hairs at the base, connected to the annulus of the corolla tube	9-10	irregular separate bundles arranged in two groups
V. trifolia	3-5 often 3 petiolate	regular	± 1/4	circular with entire to undulate margin,	with a dense to lax tuft of hair at the base, connected to the annulus of the corolla tube	7-8	regular, separate to connected bundles arranged in two groups
V. pseudo-negundo	3-5 rarely 7	irregular	1/3-1/4	varies from almost circular to ovate-triangular and rarely ovate-lanceolate	high variation of hairiness	6.5-11	irregular separate, 3, 6, 10, 11 segmented bundles arranged in two or separate more or less spiral group

upper lip two lobed and the lower lip, three lobed, with a ring of hairs or interrupted tufts of hairs inside the corolla tube and a tuft of hair with different density on the middle lobe of the lower lip. The stamens are 4, didynamous, all fertile. Ovary is superior four locular with one ovule in each locule. Fruit is a drupe with woody endocarp. The surface of fruits are covered with dense peltate glands intermixed with dense or lax simple one to four cellular hairs. The density of these hairs is different in different species.

Leaves, trichomes, corolla and calyx are diagnostic characters for identification of species. In *V. trifolia* the upper leaves of stem are all trifoliate but there are 3-5 leaflets in the leaves of lower parts of the stems. In the other species all leaves are 3-5, rarely 7 foliolate. The shape and size of calyx varies among species, in *V. negundo* and *V. trifolia* the calyx teeth are ± equal and the ratio of teeth length to calyx length is about 1/2 in the first but the ratio is about 1/4. In *V. pseudo-negundo* and *V. agnus-castus* the calyx teeth are unequal, calyx is distinctly bilabiate, the ratio of teeth length to calyx length is 1/3-1/4 in the first and 1/6 in the second species. On the other hand the longest calyx teeth belong to *V. negundo* and the shortest belong to *V. agnus-castus* (Fig. 13, A-F).

There are differences in the shape and hairiness of middle lobe of the lower lips in different species (Fig.14 & table 4). In *V. agnus-castus* the middle lobe of the lower lip is rounded with a lax semicircular shape tuft of hairs at the base or a tuft of hair on the angles between the lobes; the corolla tube with interrupted tufts of hairs inside(Fig. 14 A & B). In *V. negundo* the middle lobe of the lower lip is ovate-triangular, acute with undulate margin and with a dense to lax tuft of hairs at the base, connected to the annulus of the corolla tube (Fig. 14 C & D). In *V. trifolia* the middle lobe of the lower lip is circular with entire to undulate margin, with a dense to lax tuft of hair at the base, connected to the annulus of the corolla tube. In *V. pseudo-negundo* a high variation of shape and hairiness of the middle lobe of the lower lip was observed. It varies from almost circular to ovate-triangular, and rarely ovate-lanceolate. The hairiness is also different and all patterns observed in above mentioned species could be detected in this species (Fig. 14, A-L).

In *Verbena officinalis* leaves are simple, oblong, oblong-lanceolate, with serrate, lobed or pinnatisect margins. The trichomes of leaf surface are stiff and with swollen base. Calyx tubular with five short teeth. Corolla with five lobes and with a ring of hairs in throat. Nutlets with reticulate surface on dorsal side and papillate on ventral side.

Discussion

Reviewing the results of anatomical studies, shows that there are clear differences among *Vitex* spp. (*Lamiaceae*) and *Verbena officinalis* (*Verbenaceae*) Tabel 4 . The pollen grains of *Vitex* spp. are tricolpate

which is similar to Lamiaceae genera of subfamily Lamioidae.

The essential oils of the examined *Vitex* spp. are characterized by having α-pinene, sabinene and limonene, these characteristics appear in some *Lamiaceae*, *Nepetoidae* genera. *Vitex agnus-castus* and *V. negundo* are closely related by having the characteristic compounds of the genus plus high concentration of 1,8-cineole.

In *Verbena officinalis* spathulenol, limonene, 1,8-cineole ar-curcumene and geranial are the major and characteristic compounds (Chalchat & Garry, 1996). The reports from other *Verbenaceae* genera like *Lantana camara* L. shows other characteristic compounds (β-caryophyllene, bicyclogermacrene, and 1,8-cineole, Sefidkon, 2002). In *Lippia citriodora* Kunth, 1,8-cineole, citral A. citral B and geraniol are characteristic compounds (Fujita, 1965; Velasco-Negueruela & Perez-Alonso, 1993; De Assis Brasil e Silva et al., 1979). Different patterns of terpenoid contents in the oil of this genus have been defined which can be used for taxonomic treatment of it (Terblanché & Kornelius, 1996).

The flower morphology which has been used as diagnostic character in previous taxonomic treatments of the genus shows high variation, in *V. pseudo-negundo.* This is also congruent with other variable characters of this species i.e. anatomy and phytochemistry and can be inferred as the occurrence of possible hybrids between this species and *V. agnus-castus* or an adaptive radiation resulting from ecological and geographical factors.

Comparing the anatomical, phytochemical, palynological characteristics of the examined *Vitex* spp. reveals the close relationships of *V. agnus-castus* and *V.negundo* (Pollen shape and wall thickness; the essential oil components). Also the results of this work show that *V. agnus-castus*, *V.negundo* and *V.trifolia* can be recognized as independent species but *V. pseudo-negundo* may be considered as a hybrid plant as was recognized by Moldenke (1941).

In some previous taxonomic treatments of the genus in Asia (Nasir, 1974) subspecific taxa have been recognized for *V. agnus-castus* and *V. pseudo-negundo* is considered as a variety of it. However morphological, anatomical and phytochemical data help to infer the hybrid origin of *V. pseudo-negundo*.

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