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Epicuticular Waxes from *Solanum nigrum* Complex: Chemotaxonomic Implications

A. MOHY-UD-DIN*, Z. KHAN†, M. AHMAD‡, M.A. KASHMIRI‡, S. YASMIN‡, M.N. ASGHAR‡ and S.R. AHMAD‡
Department of Chemistry, Centre for Plant Product Research, G.C. University, Katchery Road, Lahore-54000, Pakistan
Tel: (92)(426)624323; E-mail: ayeshamdin@hotmail.com

Chemotaxonomic studies of five locally available plant taxa of *Solanum nigrum* complex were carried out in order to resolve the international taxonomic controversy about these plants. GC-MS analysis of the *n*-hexane-extracted epicuticular waxes of *S. americanum*, *S. chenopodioides*, *S. retroflexum*, *S. nigrum* and *S. villosum* showed the presence of squalene, phytol, palmitic acid, linolenic acid, ester of palmitoleic acid along with a variety of hydrocarbons as the chemical constituents of these waxes. The presence of the hydrocarbons, alcohols, some of the esters, acids, aldehyde and ketone in the epicuticular wax of *S. nigrum* had never been reported in the literature. Multivariate analysis was employed for grouping of these five taxa on the basis of constituents of the epicuticular waxes. The significant distance found between *S. chenopodioides* and *S. villosum* as well as in *americanum* and *S. nigrum*, in their respective clusters, indicated them as distinct species. But *S. retroflexum* did not show such a marked difference and hence might be regarded as a variety or subspecies of *S. nigrum*.

Key Words: Chemotaxonomy, Epicuticular waxes, *Solanum nigrum* complex, Multivariate analysis.

INTRODUCTION

Solanum is one of the most important and largest genera of the family Solanaceae which comprises of about 84 genera and 3000 species[1]. *Solanum nigrum* is the largest and most variable species of the genus *Solanum*. It is now named as *Solanum nigrum* complex because it is composed of a large number (about 30) of morphologically distinct taxa[2]. The plants of *S. nigrum* complex has been traditionally used as an analgesic, antispasmodic, for disorders of neuro-vegetative system, antiseptic, antidysentric, antinarcotic, emollient, diuretic, tonic, soporific, laxative, anticancer, antiulcer, *etc.*[3-6]. But each taxa of this complex had been found to be having its own specific medicinal and nutritional value[7]. *S. nigrum* was first delimited in four taxa

†Department of Botany, G.C. University, Katchery Road, Lahore-54000, Pakistan.
‡Department of Chemistry, G.C. University, Katchery Road, Lahore-54000, Pakistan.

with polynomials by Dillenius. Linnaeus subsequently modified Dillenius's work, describing these in six varieties under the binomial *S. nigrum*[7]. Since then, the plants morphologically related to *S. nigrum* have been reclassified many times. The boundaries between many of the species are still ill-defined, with many of the 'new' taxa proving to be no more than slight morphological variants of those already described. The situation is further complicated by the researchers who either treated different members of the section as varieties of *S. nigrum* or considered them as different species on the basis of morphological differences[2,7-9].

Three taxa belonging to *S. nigrum* complex *viz. S. americanum* Mill., *S. nigrum* L. and *S. villosum* Mill. had been reported in Pakistan[2]. *S. chenopodioides* Lam. and *S. retroflexum* Dunal are two other species that were found growing wild in and around botanic garden, GC University, Lahore. Morphologically *S. nigrum* is different from *S. villosum* in the respect that the former has black matured berries with peduncles longer than pedicels while latter has orange/orange-red matured berries and peduncles shorter than or equal to the pedicels. Classification of *S. nigrum* and *S. villosum* as varieties or distinct species started taxonomic controversy between Linnaeus and Miller[7]. Though *S. americanum* Mill., *S. chenopodioides* Lam. and *S. retroflexum* Dunal have morphological resemblance with *S. nigrum*, yet no chemotaxonomic relationship has so far been established due to lack of a comprehensive study of their chemical composition.

The epicuticular waxes and fatty acid composition had often been considered as a potential character for chemotaxonomy of the plants in general[10-12]. Hanna *et al.*[13], although, had reported the presence of some fatty acids such as palmitic acid, palmitoleic acid, stearic acid, linolenic acid and squalene without specifying the taxon of *S. nigrum* complex. Yet no detailed chemotaxonomic study based upon chemical constituents of epicuticular waxes of the complex has been undertaken. The objectives of the present study were: (a) to study the composition of epicuticular wax of five locally available taxa of *S. nigrum* complex and (b) to mark the boundaries among these five taxa using the chemotaxonomic data to help resolve the international taxonomic controversy regarding *S. nigrum* complex.

EXPERIMENTAL

Plant samples of five morphologically different plant taxa (*ca.* 5 Kg each) of *S. nigrum* complex were grown under controlled conditions in botanical garden of GC University, Lahore, Pakistan, each in specified area. Third accession of each at flowering-seeding stage was collected for analysis. Voucher specimens were authenticated by Khan and deposited in Herbarium, GC University, Lahore, Pakistan.

Epicuticular wax extraction: *n*-Hexane (GC-grade), purchased from Merck, Germany, was further distilled twice and microfiltered before use. Epicuticular waxes were extracted by immersing the plant samples in cold *n*-hexane for 90 s. This procedure extracts only surface *n*-hexane-soluble compounds without disturbing the inner chemical make-up[14]. Extracts were filtered and concentrated by rotary

evaporation. Complete dryness of residue was achieved by evaporation at room temperature in a ventilated fumehood. Dry extracts were weighed to calculate percentage yield. A measured amount of residue was redissolved in *n*-hexane and microfiltered to prepare diluted samples (10 µg/250 mL).

GC-MS analysis: The composition of the waxes was established using GC-MS technique. GC-MS analyses were performed on a Shimadzu GCMS-QP2010A system in EI mode (70 eV) equipped with a split/splitless injector (250 ºC), at a split ratio of 50/50 using DB-5MS column (30 m × 0.25 mm i.d., film thickness: 0.25 µm, J and W scientific, Fulsom, CA, USA. The composition of the waxes was established using GC-MS. Samples were injected at 250 ºC with a split ratio of 50/50. Injection volume was 1 µL and electronic pressure programming was used to maintain a constant flow (0.67 mL/min) of the helium carrier gas. The oven temperature was programmed from 150 ºC (4 min) to 320 ºC at a rate of 2 ºC/min and held at this temperature for 2 min. The mass spectrometer was set to scan the mass range 40-600 amu with ion source temperature 200 ºC and interface temperature 250 ºC. Analyses were performed in triplicate with a blank run after every analysis. The resulting data was processed using Shimadzu Lab Solution GCMS Postrun analysis software. The relative apparent percentage of each compound was determined by area normalization method. Compounds were identified by comparing the mass fragmentation pattern of the reported data and NIST 147 and NIST 27 libraries. Statistical analysis of the 34 compounds identified was carried out by multivariate cluster analysis using minitab 3.2 statistical software.

RESULTS AND DISCUSSION

Species delimitation: The difficulty of distinguishing genetically-controlled characteristics from phenotypic plasticity has long been known to impede species level taxonomy in *Solanum*[7]. The accessions of five morphologically different plant taxa of *S. nigrum* complex were taken at flowering-seeding stage for chemotaxonomic investigation and voucher specimens were deposited in Herbarium of GC University, Lahore, Pakistan (voucher numbers are given in supporting information file 1). Morphological comparison of the taxa is given in Table-1. All the accessions were homogeneous and did not suffer from any pest or disease. Epicuticular wax from each of the five taxa was extracted using *n*-hexane.

The wax yield of the plant samples varied from 0.022-0.97 %. Different classes of compounds were found by applying GC-MS analysis (Table-2, see supporting information file 2 for GC spectra). Waxes had consistently been found to contain hydrocarbons, alcohols, fatty acids, esters, aldehydes and ketones[15-18]. In addition to the four compounds which had been reported previously[13], 30 more compounds were detected in quantifiable amounts in the five plant species analyzed (Table-3). To identify possible chemotaxonomic features, the results obtained were compared with respect to morphology and taxonomy of the taxa.

TABLE-1
MORPHOLOGICAL COMPARISON OF FIVE TAXA OF *Solanum nigrum* COMPLEX

Character	SA[a]	SC	SN	SR	SV
Leaf shape	Ovate-lanceolate to lanceolate	Lanceolate	Ovate-lanceolate, ovate-rhombic	Rhomboidal to ovate-lanceolate	Rhombic to ovate-lanceolate
Leaf margin	Entire to sinuate rarely sinuate-dentate	Entire to sinuate	Sinuate or irregularly dentate	Deeply lobed, 3-5 (7)[b]	Entire to sinuate-dentate
Leaf length[c]	2.5-3.2 (4.0)	2.7-5.4	2.5-7	4-4.8	2.0-7.0 (10)
Leaf breadth	1.1-4.0 (4.6)	(1.8) 2.2-5.4	2.0-4.5	3-4.2	1.5-4.0 (6.0)
lnflorescence type	Umbellate cymes	Umbellate cymes	Extended cymes	Simple to Umbellate cymes	Umbellate to solitary cymes
Calyx length[d]	1.1-2.0 (2.4)	2.4-3.5	1.2-2.5	2-3	1.2-2.2
Corolla colour	White, occasionally purple	White	White	White with distinctive purple stripes on each petal	White
Style length[d]	1.2-3.5 (4.5)	4.0-5.5 (6.5)	2.8-3.5 (4.5)	2-4.5	2.9-5.0 (6.0)
Stylar exsertion	Usually exserted beyond anthers	Exserted up to 2 mm beyond anthers	Not exserted beyond anthers	Exserted up to 2 mm beyond anthers	Rarely exserted beyond anthers
Berry diameter[d]	4-7 (8)	6.3-8.5	6-10	7-9	6-10
Berry shape	Globose	Globose to broadly ovoid	Broadly ovoid	Spherical	Occasionally globose
Berry colour	Black, rarely dark green	Purple	Dull purple	Purple	Red, orange
Cuticle	Shiny opaque	Dull opaque	Shiny opaque	Dull opaque	Translucent
Seed length[d]	0.8-l.5	1.0-1.8	1.7-2.4	1.5-2.3	1.6-2.2

[a]Abbreviation of species, SA: *S .americanum*, SC: *S. chenopodioides*, SN: *S. nigrum*, SR: *S. retroflexum*, SV: *S. villosum*. [b]Figures in parentheses refer to infrequent values below or above the regular range. [c]Values are given in cm. [d]Values are given in mm.

Alkanes and alkenes: The epicuticular wax of many Solanaceous plants like tobacco leaves, tomato leaves/fruit, bell peppers, aubergines, potato leaves have been reported to contain alkanes[19-23]. In the present study, *n*-alkanes ranged from 15.06-42.19 % of the total wax samples except *S. americanum* in which free alkanes were not detected (Table-2). Unlike the other plant species analyzed, 1-chloroalkanes of C21, C25, C27 and aromatic hydrocarbons were also found in *S. americanum* (Table-3). The individual *n*-alkanes were composed of C14 to C28. The alkanes C15, C16, C18, C19, C20, C21, C23, C24 C25, C27 and C28 were found common in all the samples in varying amounts. But the alkanes C14 and C17 showed selective occurrence. The taxa can be distinguished from one another by the distribution of the *n*-alkanes, taking into account the two main alkanes[24], *e.g*. by quoting one main alkane outside and the second main alkane inside the parenthesis as: *S. chenopodioides*-C17 (C16); *S. nigrum*-C27 (C25); *S. retroflexum*-C20 (C18); *S. villosum*-C27 (C18). The occurrence of the individual alkane was almost the same in *S. chenopodioides*

TABLE-2
WAX YIELD AND THE DISTRIBUTION[a] OF DIFFERENT CLASSES OF COMPOUNDS

Parameter	Percentage in species (code)[b]				
	SA	SC	SN	SR	SV
Wax Yield	0.022	0.42	0.076	0.29	0.97
Alkanes	–	42.19	15.480	23.58	15.06
Chloroalkanes	1.450	–	–	–	–
Aromatic hydrocarbons	2.070	–	–	–	–
Alkenes	24.860	1.76	13.520	12.10	0.83
Alcohols	–	19.24	7.040	12.85	2.72
Free fatty acids	1.180	6.10	4.240	5.15	67.37
Esters	53.910	30.70	59.720	46.34	11.25
Chloroesters	2.450	–	–	–	–
Aldehydes	–	–	–	–	2.78
Ketones	14.070	–	–	–	–

[a]Expressed as relative apparent percentages: combined areas of peaks for compounds of given class ÷ combined areas of all identified peaks for all classes × 100. [b]For abbreviation of species, see Table-1.

and *S. villosum* qualitatively and matched closely with the alkanes of *S. nigrum* and *S. retroflexum*, but there were marked quantitative variations. The highest percentage of alkanes was observed in *S. chenopodioides* (42.19 %) followed by *S. retroflexum* (23.58 %), *S. nigrum* (15.48 %) and *S. villosum* (15.06 %). The absence of *n*-alkanes and presence of 1-chloroalkanes and 2,6-diisopropylnaphthalene positioned *S. americanum* unique among the *Solanum* species under study. Grossi and Raphel[25] reported 1-chloro alkanes in the leaf waxes of chenopodioides plants and showed that these compounds had potential value as chemotaxonomic and/or environmental markers.

Squalene was detected in all the five taxa in variable quantity and could be regarded as a characteristic compound of genus *Solanum*. The variation in quantity could be attributed to differentiation at species level. It was the major component in *S. americanum* (24.86 %). A small quantity of another alkene, 10-methyl-1-octadecene, was also detected only in *S. retroflexum* (Table-3).

Alcohols, aldehydes and ketones: Free alcohols are widespread components of plant waxes[26]. Homologous series of primary and secondary alcohols were found in *S. tuberosum*[23]. In the present study, variable amounts of alcohols ranging from 2.72-19.24 % were found in all the taxa except *S. americanum* (Table-2). There was not much variety in long chain alcohol types. Phytol was detected in the four taxa and it accounted for 19.24 % of the total wax in *S. chenopodioides*. Two more unsaturated alcohols, 3,13-octadecadiene-1-ol and 9-eicosen-1-ol, were found in *S. villosum* in minute quantities. In addition to 9-eicosen-1-ol, *S. retroflexum* also showed the presence of *n*-cetyl alcohol and an aldehyde that were not found in other taxa. Alcohols were not detected in *S. americanum*, instead 3-hydroxyspirost-8-en-11-one (14.07 %) was detected (Table-3).

TABLE-3
COMPOSITION OF EPICUTICULAR WAX EXTRACTED
FROM *Solanum nigrum* COMPLEX

Compound	M^+, Base peak	Percentage in species (code)[a]				
n-Tetradecane [14C][b]	198, 57	–	2.45	tr[c]	–	0.60
n-Pentadecane [15C]	212, 57	–	3.43	tr	tr	1.03
n-Hexadecane [16C]	226, 57	–	4.13	tr	1.14	1.19
n-Heptadecane [17C]	240, 57	–	4.45	–	2.41	1.43
n-Octadecane [18C]	254, 57	–	3.89	tr	2.96	1.44
n-Nonadecane [19C]	268, 57	–	3.51	0.95	2.83	1.41
n-Eicosane [20C]	282, 57	–	3.24	1.73	3.13	1.23
n-Heneicosane [21C]	296, 57	–	2.93	1.63	2.46	1.27
n-Tricosane [23C]	324, 57	–	4.05	1.09	1.4	0.63
n-Tetracosane [24C]	338, 57	–	2.48	1.96	2.55	0.90
n-Pentacosane [25C]	352, 57	–	1.77	1.99	1.13	0.89
n-Heptacosane [27C]	380, 57	–	3.81	4.29	1.58	2.22
n-Octacosane [28C]	394, 57	–	2.05	1.84	1.99	0.82
1-Chloroheneicosane	340, 57	tr	–	–	–	–
1-Chloropentacosane	386, 57	tr	–	–	–	–
1-Chloroheptacosane	414, 57	1.45	–	–	–	–
Octadecyl chloroacetate	346, 57	2.45	–	–	–	–
2,6-Diisopropylnaphthalene	212, 197	2.07	–	–	–	–
10-Methyl-1-octadecene	266, 57	–	–	–	1.23	–
Squalene	410, 69	24.86	1.76	13.52	10.87	0.83
n-Cetyl alcohol	242, 55	–	–	–	1.01	–
9-Eicosen-1-ol	296, 55	–	–	–	1.08	1.02
3,13-Octadecadiene-1-ol	308, 43	–	–	–	–	0.75
Phytol	296, 71	–	19.24	7.04	10.76	0.95
7,10,13-Hexadecatrienal	234, 79	–	–	–	–	2.78
3-Hydroxyspirost-8-en-11-one	428,314	14.03	–	–	–	–
Palmitic acid	256, 43	1.18	4.68	2.94	4.2	24.04
Stearic acid	284, 43	–	1.42	1.3	0.95	3.84
α-Linolenic acid	278, 41	–	–	tr	–	19.81
γ-Linolenic acid r	306, 41	–	–	–	–	19.68
Palmitoleic acid methyl ester	236, 55	53.91	30.7	59.72	46.34	9.15
Palmitic acid, methyl ester	270, 74	–	–	–	–	0.63
Palmitic acid, ethyl ester	284, 88	–	–	–	–	0.84
Linolenic acid,methyl ester	292, 79	–	–	–	–	0.63
Total	–	99.9	99.9	100	100	100

[a]For abbreviation of species, see Table-1. [b]Alkane Carbon number. [c]tr: trace (0.01-0.1 % detectable).

Free fatty acids and esters: Free aliphatic fatty acids are common components of leaf waxes, but are usually present in low concentrations[26]. Small amounts of free fatty acids were found in waxes of all the taxa except *S. villosum* where they constituted 67.37 % of wax (Table-2). Stearic acid, detected in small quantity in the four taxa, matched in quantity in *S. chenopodioides*, *S. nigrum* and *S. retroflexum* but was more than double in *S. villosum*. α-Linolenic acid was also detected in reasonable amount (9.81 %) in *S. villosum* and in a trace amount in *S. nigrum* but not found in detectable amount in other three taxa. Its methyl ester (0.63 %) was

also detected in *S. villosum*. γ-Linolenic acid (19.68 %) was present in *S. villosum* only. Palmitic acid was previously reported in *S. nigrum*[13] and S. tuberosum[23] and seems to be a characteristic acid of genus *Solanum*. Present findings were also in line with the already reported data. Concentration of palmitic acid was relatively high in *S. villosum* (24.04 %) and here its methyl and ethyl esters were also detected though in small amounts. *S. americanum* showed the unique presence of chlorinated ester of acetic acid in small quantity (Table-3).

Palmitoleic acid methyl ester was detected as the major constituent (except for *S. villosum*) of the waxes, *S. americanum* (53.91 %), *S. chenopodioides* (30.70 %), *S. nigrum* (59.72 %) and *S. retroflexum* (46.34 %). Palmitoleic acid had been reported from *S. nigrum* by Hanna *et al*.[13].

Multivariate analysis: Statistical analysis of the data presented in Table-3 was carried out using minitab 3.2 statistical software. By cluster analysis (Fig. 1), the two samples *S. nigrum* and *S. retroflexum* were first to segregate. They showed many similarities in the distribution of epicuticular wax constituents except some minor differences like presence of 10-methyl-1-octadecene, *n*-cetyl alcohol and 9-eicosen-1-ol in *S. retroflexum* that were not detected in *S. nigrum*. Also tetradecane and palmitic acid were present in trace amounts in *S. nigrum* but not detected in *S. retroflexum*. *S. americanum* deviated from the average distribution pattern of wax components due to unique occurrence of a ketone, aromatic hydrocarbon, chlorinated hydrocarbon and chlorinated ester and the absence of free hydrocarbons, some fatty acids and some esters occurring in other taxa. These features aligned *S. americanum* more distantly with above mentioned cluster as shown in Fig. 1. *S. chenopodioides* and *S. villosum*, although not much closely related to one another as compared to

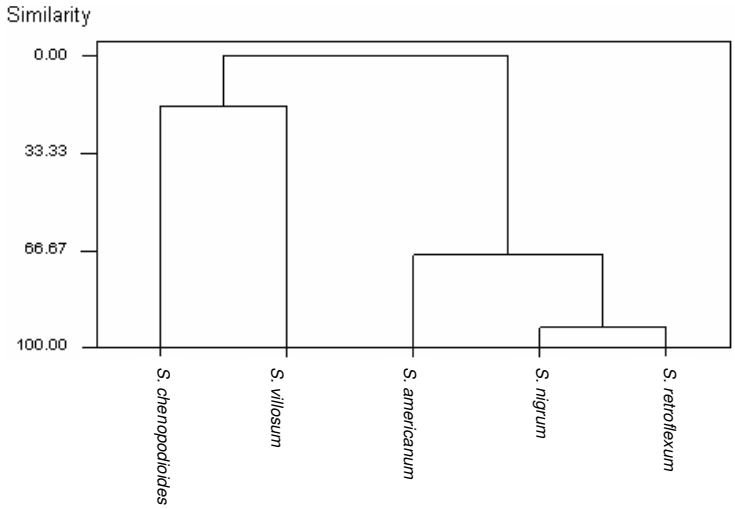

Fig. 1. Affinity relationships between different taxa of *Solanum nigrum* complex based on the distribution of epicuticular wax components and determined by similarity and multivarial cluster analysis

the previously discussed cluster, made another cluster. Their epicuticular wax showed all the hydrocarbons, squalene, phytol, palmitic acid, stearic acid and ester of palmitoleic acid with different amounts. However, eight such compounds were present in *S. villosum* that were not detected in *S. chenopodioides*.

Conclusion

GC-MS analyses of epicuticular waxes from five taxa of *S. nigrum* complex demonstrated that each of five taxa had its own specific set of chemical constituents. But still there was a close relationship among all with respect to some compounds like squalene, palmitic acid and ester of palmitoleic acid. The reason behind this may be ascribed to having a common genus *Solanum*. *S. americanum* was found to contain very small number of compounds. Most of these compounds (*e.g.* chlorinated compounds and one each of aromatic hydrocarbons and ketones) were found unique to *S. americanum*. *S. villosum* was also found different for having greatest variety of compounds detected (26 out of 34). Some of them such as some alcohols, esters, an aldehyde and an acid were not detected in other taxa. The qualitative chemical composition of epicuticular waxes of *S. americanum*, *S. chenopodioides*, *S. nigrum* and *S. villosum* suggested that they had significant differences and might be treated as separate species and not the varieties/subspecies of *S. nigrum*. In case of *S. retroflexum*, many similarities in chemical constituents particularly in hydrocarbon composition were observed. So it could be regarded as the variety/subspecies of *S. nigrum*. Some minor differences could be attributed to differentiation at variety/subspecies level. Because of the taxonomic misunderstanding surrounding the component species and the tendency to refer to all members as '*S. nigrum*', it is advisable that the information found in literature may be reinterpreted in the light of above chemotaxonomic suggestion.

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