

## CHEMOTAXONOMIC VALUE OF ALKALOIDS IN *SOLANUM NIGRUM* COMPLEX

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### Abstract

The comparison of alkaloidal profile of delimited species in the 5 locally available taxa of *S. nigrum* complex were used to establish the boundaries among close taxonomic groups. Several glycoalkaloids (Solanosine,  $\alpha$ -Solanagine,  $\beta$ -Solanagine and  $\alpha$ -Solanine) and their aglycones (Solasodine and Solanidine) were analysed that were shown to be a valuable tool to resolve the international taxonomic controversy based on morphological characters. HPLC and GC-MS were used for the first time for the analysis of alkaloids in *S. nigrum* complex. Qualitative and quantitative comparison by cluster analysis demonstrated significant distances among *S. chenopodioides* and *S. villosum* as well as in *S. 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*.

### Introduction

*Solanum* is one of the most important and largest genera of the family Solanaceae comprising of about 84 genera and 3000 species (Yasin, 1985). *Solanum nigrum* is the largest and the most variable species of the genus *Solanum*. Any taxon belonging to the section *Solanum* has invariably been identified as species, especially in many of the older floras. This, together with the fact that many earlier workers failed to recognize the cytomorphological characteristics associated with the taxon, has led to much of the taxonomic confusion surrounding this species. It has reported to be having about 30 morphologically distinct taxa and is named as *Solanum nigrum* complex (Schilling & Andersen, 1990). Dilleniis delimited *S. nigrum* in four taxa while Linnaeus designed six varieties in it. The situation is 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. Five taxa belonging to *S. nigrum* complex viz.: *S. americanum* Mill., *S. chenopodioides* Lam., *S. nigrum* L., *S. retroflexum* Dunal and *S. villosum* Mill., were found as growing wild in Pakistan, although only two have been reported in Flora of Pakistan as varieties of *S. nigrum* viz: *S. nigrum* var. *nigrum* and *S. nigrum* var. *villosum* (Yasin, 1985). 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 (Stebbins & Paddock, 1949; Symon, 1970; Schilling & Andersen, 1990; Edmonds & Chweya, 1997). *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.

### Key to the investigated taxa of *S. nigrum* complex

***S. americanum* Mill.:** Plants glabrescent to moderately pilose with appressed eglandular hairs; flowers small, fruiting pedicels usually erecto-patent; berries spherical, black and usually shiny when mature; seeds 1-1.5 mm long.

***S. chenopodioides* Lam.:** Plants somewhat tomentose; umbellate cyme inflorescence with 4,6 sometimes 8 flowers; fruiting peduncles strongly deflexed from the base; berries globose to ovoid, purple with dull opaque cuticle.

***S. nigrum* L.:** Plants sub-glabrous to pubescent usually with appressed, eglandular-headed multicellular hairs; berries black.

***S. retroflexum* Dunal:** Plants pubescent with appressed, eglandular-headed multicellular hairs; flowers white with distinct purple vein to outer surface of petals; berries usually spherical, purple with opaque cuticles.

***S. villosum* Mill.:** Plants villous, covered with glandular headed and often patent multicellular hairs; stems usually terete, with smooth ridges; berries red, orange or yellow.

The plants of *S. nigrum* complex has been traditionally used as an analgesic, antispasmodic, antiseptic, antidyseptic, antinarcotic, emollient, diuretic, tonic, soporific, laxative, anticancer, antiulcer and for disorders of neuro-vegetative system etc. (Saijo *et al.*, 1982; Akhtar & Muhammad, 1989; Schilling *et al.*, 1992; Edmonds & Chweya, 1997; Manoko *et al.*, 2007). This medicinal value is mainly attributed to the alkaloidal contents of the plants. The *Solanum* glycoalkaloids have been intensively studied during recent decades and as a result of substantial research efforts, thousands of articles concerning various aspects of glycoalkaloids have been published. *S. nigrum* is especially known for its toxicity because it contains solanine, a neurotoxic glycoalkaloid (Abbas, 1998). Alkaloids are said to be excellent taxonomic markers by a number of researchers (Izaddoost, 1975; Tetenyi, 1987; Greinwald *et al.*, 1995; Jamil *et al.*, 2007; Dinchev *et al.*, 2008; Loaiza *et al.*, 2008). GC-MS technique is cited (Suau *et al.*, 2002) for the easy determination and identification of alkaloids and the application of the technique to chemotaxonomic studies. Taxonomic studies for various plant taxa by using different parameters have been successfully carried out in Pakistan including that of cereals (Ashraf *et al.*, 2003), legumes (Ahmad *et al.*, 2007) and maize (Nawaz & Ashraf, 2007). In the present study, variation was investigated in qualitative and quantitative glycoalkaloid aglycone contents in all the five taxa belonging to *S. nigrum* complex.

### Materials and Methods

**Material:** The alkaloid standards were purchased from Sigma Co., St. Louis, MO. The glycoalkaloids were analysed using HPLC apparatus consisting of Shimadzu LC-10A system equipped with a model LC-10AT pump, an SPD-10A variable wavelength detector, a CBM-10A interface module with class LC-10 HPLC software using a Merck C-18 column (250×4.6, i.d., 5 µm particle size). The SGAA derivatives were determined using Shimadzu GCMS-QP2010A system on an NB-54 fused-silica capillary column (15 m, 0.20 mm i.d., Nordion, Finland).

Plant samples of five morphologically different plant taxa of *S. nigrum* complex were grown under controlled conditions in Botanic Garden of GC University Lahore, Pakistan, each in specified area. Third accession of each (approx. 5Kg each) at flowering-seeding stage was collected for analysis. Voucher specimens were authenticated and deposited in Dr. Sultan Ahmad Herbarium, GC University Lahore, Pakistan. The dried plant powder of each sample was dipped in *n*-hexane for 45 sec to ensure the removal of surface fats and epicuticular wax without disturbing the interior chemical make-up so that they may not interfere in alkaloid analysis (Medina *et al.*, 2006).

**Estimation of total glycoalkaloid content:** Total Glyco-Alkaloid (TGA) contents of the defatted samples were determined by titrometric method (Fitzpatrick & Osman 1974). A sample (20 g) of each taxon was extracted with 100 mL of methanol-chloroform (2:1), filtered and the extract was mixed with 100 mL of 0.8% Na<sub>2</sub>SO<sub>4</sub>. The upper chloroform layer was separated, dried and the residue was dissolved in 15 mL of 2 N H<sub>2</sub>SO<sub>4</sub>. The solution was then heated for 2 h and made basic with 10 mL of 4 N NaOH. The glycoalkaloids were extracted with benzene and after evaporating the benzene, the residue was taken up in 5 mL of methanol. Samples were titrated with a solution of 0.067% bromophenol blue and 10% phenol in absolute methanol, against a blank of methanol. The TGA were calculated by using a standard curve prepared with known concentration of Solasodine and  $\alpha$ -Solanine in methanol.

**HPLC analysis of steroidal glycoalkaloids:** Steroidal Glycol-Alkaloids (SGA) for compositional analyses were extracted by a method based on a modification of the technique of Dao & Friedman (1996). Sample of 15 g of each powdered plant material were extracted thrice with 200 mL of 5% aqueous acetic acid, vacuum filtered and its pH was adjusted to 11 with ammonium hydroxide. The alkaline extract was partitioned with water saturated butanol, evaporated to dryness, purified and the residue was weighed, dissolved in methanol and analyzed. HPLC conditions were set as described by Sotelo & Serrano (2000) for *S. tuberosum* except that the buffer used was Ammonium dihydrogen Phosphate with pH 6.1 min and the UV absorbance detector was set at 205 nm. Solasonine,  $\alpha$ -Solanamine,  $\beta$ -Solanamine and  $\alpha$ -Solanine were used as internal standards. The mean recoveries obtained from triplicate samples were 99.2  $\pm$  0.47 to 99.6  $\pm$  0.54%. The results of the validity study showed that the method used was efficient and useful for this glycoalkaloid analysis.

**GC-MS analysis of steroidal glycoalkaloids aglycones derivatives:** Steroidal glycoalkaloid aglycones (SGAA) were obtained by dissolving dried plant materials (10 g) and standards (20  $\mu$ g each) separately in 2 mL of 1 M HCl in methanol and heated for 3 h at 70 °C. The free aglycones were liberated from the hydrolysate by adding 2 mL of 25% ammonia and extracted with 2 mL of dichloromethane after a few minutes. After vigorous mixing and 5 min., of centrifugation, the dichloromethane layer was removed with a pipette. The aglycon extracts were then evaporated to dryness. For derivatization, 20  $\mu$ L of Trimethylsilylimidazole (TMS) and dry acetonitrile (50  $\mu$ L) were added to each sample and standard (solasodine and Solanidine). The mixtures were heated at 60 °C for 15 min, cooled and 1  $\mu$ L of each solution was injected into the GC-MS system. The SGAA derivatives were determined under the analytical conditions recommended by Laurila *et al.*, (1999) for *Solanum* species operating at an ionization voltage of 70 eV (EI mode) with ion source temperature of 180°C using split sampling mode and an oven

temperature of 180 to 285°C heated at 7.5°C min<sup>-1</sup>. Injector and detector temperatures were 285°C. Helium was used as the carrier gas (flow rate = 0.5 mL min<sup>-1</sup>). Identification of the aglycones in the plant materials was based on the GC/MS spectra of TMS derivatives of authentic standards and on reports of GC and MS glycoalkaloid aglycon data (Laurila *et al.*, 1996; Van Gelder *et al.*, 1989).

**Statistical analysis:** Statistical Analysis of the compounds identified was carried out by Multivariate Cluster analysis using Minitab 13.1 Statistical software.

## Results and Discussion

**HPLC analysis of SGA:** All the five taxa showed much similar SGA profile on HPLC. This is due to the fact that these taxa belong to the genus *Solanum* which is very well-known for the presence of SGA. So to make a chemotaxonomic comparison the quantitative analysis was required. The glycoalkaloids are particularly difficult to separate due to their similarity in structure  $\alpha$ - and  $\beta$ -Solamargine have identical sugar constituents, but different attachment pattern with aglycones i.e., solasodine and solanidine, respectively. Similarly, solasonine and solanine contain the same sugar moieties, but have the solasodine and solanidine aglycone backbones, respectively.

There had been many reports on the SGA of different species of genus *Solanum* but not a single one on *S. nigrum*. Therefore, different reported conditions that can affect selectivity of SGA of *Solanum* appreciably were applied. The best results were obtained by the method of Sotelo & Serrano (2000) so it is discussed here. Based on the relative areas obtained in the chromatograms, greater signal intensities were seen for standard analytes at 205 nm so it was selected for analysis. For this study, Solasonine,  $\alpha$ -Solamargine,  $\beta$ -Solamargine and  $\alpha$ -Solanine are the SGA of interest to our studies. The SGA were, therefore, further analysed qualitatively and quantitatively by the HPLC of the alkaloids extracted using the standard compounds. Four of the peaks were tentatively identified on the basis of retention times of spiked standards. The Solasonine,  $\alpha$ -Solamargine,  $\beta$ -Solamargine and  $\alpha$ -Solanine contents of each taxa is given in Table 1. According to our study,  $\beta$ -Solamargine levels varied among different taxa of *S. nigrum* complex (1.69-9.8 mg g<sup>-1</sup>). Its concentration in *S. villosum* was distinctly higher than other taxa especially in contrast to *S. americanum* in which it was not detected. The  $\alpha$ -Solamargine was detected at lower levels than the  $\beta$ -Solamargine. In fact it had not been detected in *S. chenopodioides* but its level in *S. nigrum* was slightly higher than other samples. Solasonine and  $\alpha$ -Solanine were detected in all taxa with less concentration variations.

The chemical profile for each subfamily, as expressed by occurrence of the major categories of secondary metabolites (indole alkaloids, iridoids, triterpenes and anthraquinones) is remarkably distinctive (Young *et al.*, 1996). So far, secondary metabolites profile can contribute to the taxonomic position of some tribes, which remain with a morphological controversy (Cardoso *et al.*, 2008). SGA determined in this study corroborated the evolutionary taxonomic distribution made by Miller, Dunal and Lamarck, who recommended these taxa as distinct species contrary to that proposed by Linnaeus, who classified these as the varieties of *S. nigrum*. Comparison of the taxa by cluster analysis (Fig. 1) segregated *S. americanum* and *S. chenopodioides* more early than others but with a less similarity index. Then *S. nigrum* and *S. retroflexum* join this group at almost similar position. But *S. villosum* was unique and depicted very low similarity with rest of the taxa.

**Table 1.** Concentration of SGA in five taxa of *S. nigrum* complex as determined by HPLC.

No.	Compound	Concentration (mg/g) in species (Code)				
		SA	SC	SN	SR	SV
1.	$\beta$ -Solamargine	nd <sup>a</sup>	2.53	1.69	4.78	9.8
2.	$\alpha$ -Solamargine	1.96	nd	5.03	1.45	1.17
3.	Solasonine	3.5	4.4	5.8	2.9	2.01
4.	$\alpha$ -Solanine	3.29	3.08	4.7	5.7	1.5

<sup>a</sup> nd: Not detected.

**Table 2.** SGAA concentration in five taxa of *S. nigrum* complex as determined by GC-MS.

Sample	Retention time <sup>a</sup> (min)		Percentage of aglycones <sup>b</sup> (%)	
	Solanidine	Solasodine	Solanidine <sup>b</sup>	Solasodine <sup>c</sup>
<i>S. americanum</i>	24.91	26.64	8.85	85.67
<i>S. chenopodioides</i>	25.17	26.74	11.08	66.94
<i>S. nigrum</i>	25.19	26.84	16.03	75.89
<i>S. retroflexum</i>	24.98	24.66	20.31	71.76
<i>S. villosum</i>	25.33	26.67	10.15	74.71

<sup>a</sup>Results were presented as mean (n=3)

<sup>b</sup>Determined by area normalization method

**GC-MS analysis of SGAA derivatives:** The steroidal glycoalkaloid aglycones of interest to this work will be referred to as SGAs. Only two SGAs were important to our work, namely, solanidine and solasodine. The lack of GC-MS reports, on the alkaloids of *S. nigrum* complex prompted to quantify the aglycones by this useful technique. Solanidine produced mono-TMS derivatives with molecular ion peaks at m/z 469. In its GC-MS spectrum, solasodine showed the di-TMS derivative after silylation with base peak at m/z 125 and at m/z 559 [M++2H<sup>+</sup>]. According to the literature the tetrahydrofuran ring opens, after which the formed hydroxyl group has been attached to the TMS group. Moreover, it has been stated that such a phenomenon can be related to the presence of the nitrogen ring, for example the silylation of diosgenin containing oxygen instead of nitrogen gave a mono-TMS derivative only (Laurila *et al.*, 1999). Quantification of aglycones was carried out using an external standard calibration method. The principal glycoalkaloid present in all taxa was solasodine with a percentage range of 66.94-85.67%. Solanidine concentration was much lower ranging from 8.85-20.31%. Solanidine is reported to be toxic so care must be taken in using *S. americanum* in herbal medicine and as food. Calibration was performed by injecting standard mixtures of solasodine and solanidine at levels ranging from 4 to 200 mg L<sup>-1</sup>. Good linearity of response was found for solanidine and solasodine this concentration range belonging to cited interval, with correlation coefficients greater than 0.995.

Cluster analysis (Fig. 2) separated the taxa into three main groups. *S. nigrum* and *S. retroflexum* formed a much closely related group. *S. chenopodioides* and *S. villosum* constituted another group but with slight less similarity index. However *S. americanum* showed a characteristic behavior of its own with highest percentage of solasodine and lowest of solanidine, so it aligned distantly with the above two groups.

This grouping pattern of these five taxa was much similar to that obtained while comparing their epicuticular wax composition and flavonoid profiles (Mohy-ud-din *et al.*, 2009).

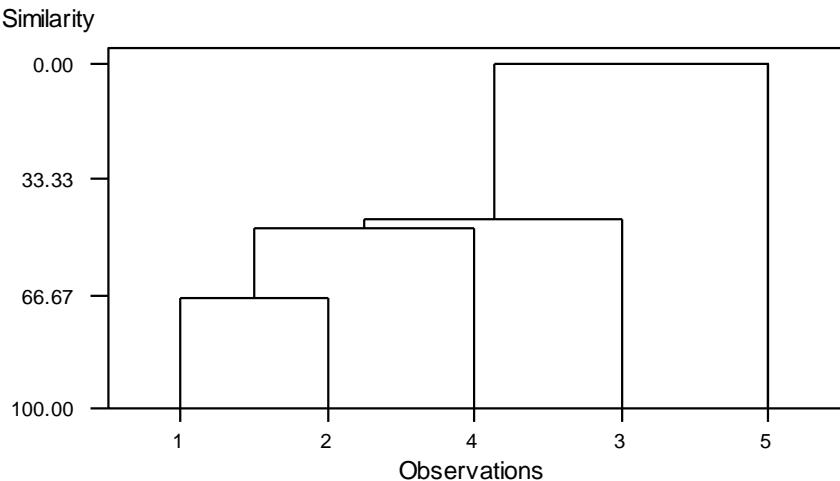


Fig. 1. Affinity relationships among different taxa of *Solanum nigrum* complex based on the distribution of SGA and determined by similarity and Multivariable cluster analysis. Species, 1: *S. americanum*, 2: *S. chenopodioides*, 3: *S. nigrum*, 4: *S. retroflexum*, 5: *S. villosum*.

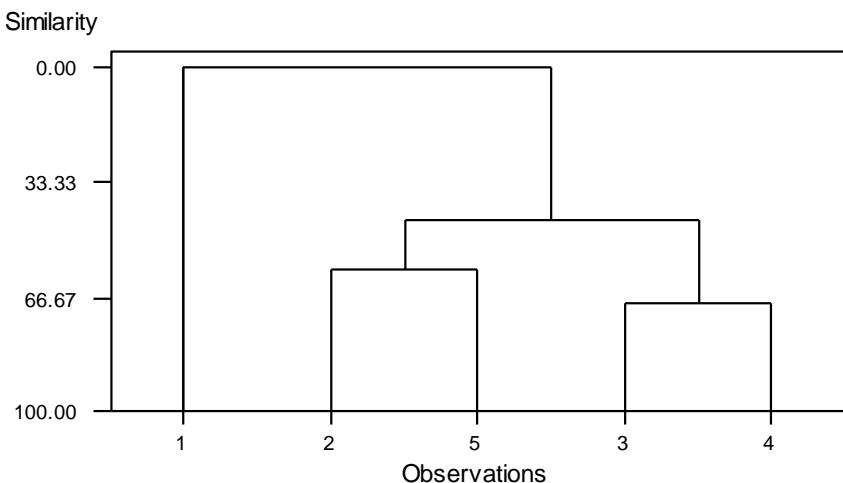


Fig. 2. Affinity relationships among different taxa of *Solanum nigrum* complex based on the distribution of SGAA and determined by similarity and Multivariable cluster analysis. Species, 1: *S. americanum*, 2: *S. chenopodioides*, 3: *S. nigrum*, 4: *S. retroflexum*, 5: *S. villosum*.

## Conclusion

Analyses of alkaloids from five taxa of *S. nigrum* complex as glycones and aglycones demonstrated that each of five taxa is unique regarding the concentrations of the chemical constituents. But still there was a close relationship among all with respect to occurrence of compounds like Solasonine and  $\alpha$ -Solanine. The reason behind this may be ascribed as belonging to a common genus *Solanum*. *S. americanum* was found to be slightly related to *S. chenopodioides* in its SGA profile however the SGAA marked it

unique to other taxa. Similarly *S. villosum* was also found different in SGA concentration for showing presence of all the compounds analysed but it had matching in SGAA content with *S. chenopodioides*. In case of *S. retroflexum*, many similarities in qualitative and quantitative chemical composition of alkaloids (glycones and aglycones) with *S. nigrum* 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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