A reverse phased high-pressure liquid chromatographic method for the estimation of a poisonous matter in *Strychnos nux-vomica*

**Abstract**

Seeds of *Strychnos nux-vomica* were subjected to preliminary phytochemical tests and its presence was confirmed by thin layer chromatography (TLC) method. The TLC profile of the methanolic extract of seeds of *S. nux-vomica* was developed using the solvent system toluene:chloroform:methanol in the ratio 8:2:1. The plate was observed in visible light after spraying with Dragendorff’s reagent (specific method). High-performance liquid chromatography (HPLC) profile of the methanolic extracts of *S. nux-vomica* was developed, and the amount of strychnine seems to be 0.36% (w/w) in the seeds. The TLC and HPLC profiles developed are very valuable for the identification of the original drug from their adulterants. The TLC profile identifies the presence of strychnine in the plant material. The quantification method for the strychnine in the seeds can be used for the quality standardization of the raw drug because the strychnine is reported to have some toxicity.

**Key words:** High-performance liquid chromatography, strychnine, *Strychnos nux-vomica* seeds, thin layer chromatography

**INTRODUCTION**

Some plants contain toxic substances in their roots, leaves, fruits or seeds. These toxic substances are the secondary metabolites or alkaloids and are prepared by the plants to protect them from herbivorous animals and insects. Poisonous plants are also dangerous to man, as they can be eaten by children or mistaken for edible plants by adults. Particularly dangerous to children that are plants with attractive fruits or seeds, such as the deadly nightshade (*Atropa belladonna*) and *Abras precatorius* seeds while hemlock is easily mistaken for parsley or other edible umbelliferous herbs. In the past, epidemics due to plant toxins have occurred, including St. Anthony’s fire caused by ergot (fungus) infection of rye (used for making bread) and milk sickness due to *Eupatorium rugosum* – White Snakeroot (*Ageratina altissima*).[1] *Strychnos nux-vomica* L. (Loganiacea) seen in almost all parts of India is classified as a spinal poison [Figure 1].[2] The main active principles of *S. nux-vomica* L. are known to be alkaloids, such as strychnine, brucine and loganin. The active principles are in the highest concentration in seeds. *Nux-vomica* seeds usually contain about 3% total alkaloids of which about one half is strychnine.[3] Pharmacologically brucine is much less active than strychnine being only 1/10th as toxic; the pharmacopeial assay of *nux-vomica* is a determination, not of total alkaloids but of strychnine only.[3]

In the present study, preliminary phytochemical tests were done to find out various chemical constituents in methanolic extract of *S. nux-vomica* and presence of strychnine was confirmed by thin layer chromatography (TLC) method. High-performance liquid chromatography (HPLC) profile of *S. nux-vomica* was developed and standardized. The amount of strychnine in the sample was calculated by considering the area under the peak for the standard strychnine and the strychnine peak in the sample profile. The study was conducted on 2007.
Aim and objectives
• To perform the preliminary phytochemical screening to identify the major chemical groups present in the seeds of S. nux-vomica by carrying out specific chemical tests
• To detect the presence of strychnine in the methanolic extracts of S. nux-vomica by carrying out TLC analysis
• To develop HPLC profile for methanolic extract of S. nux-vomica and to quantify the strychnine in the same.

MATERIALS AND METHODS

Collection of seeds
Fresh seeds of S. nux-vomica were collected from Herb garden, Arya Vaidya Sala, Kottakkal and authenticated by the Botany Division of Center for Medicinal Plants Research, Kottakkal, India. The collected raw drug was then dried in the shade and powdered.

Preparation of extracts
Five gram of dried powdered seeds were extracted by refluxing in methanol for 8 h. The extracts were filtered and concentrated, and these were used for the phytochemical screening, TLC profiling and HPLC profiling.

The methanolic extract of the seeds of S. nux-vomica was subjected to preliminary phytochemical screening for the detection of major class of compounds. The details of the tests are shown in Table 1.

Thin layer chromatography

Materials
Precoated silica gel plates 60 F254 with layer thickness 0.25 mm (E. Merck), micro syringe (25 µl, Hamilton = 802) and a UV chamber.

Chemicals
Toluene, ethyl acetate, chloroform, methanol, ethanol, anisaldehyde, glacial acetic acid, H₂SO₄, antimony trichloride (GR grade, E. Merck, India).

Method
Sample preparations: One milliliter of each concentrated extract was diluted to 10 ml in respective solvents (pet, ether, chloroform and methanol) and was used for the analysis.

Chromatographic condition
• Stationary phase – TLC precoated silica Gel F254 aluminum sheet
• Solvent system – Toluene:chloroform:methanol (8:2:1) (methanol extract)
• Detection – By visualization under visible light
• Spray reagent: Dragendorff reagent
• Development distance: 8 cm.

The chromatogram of plant extracts was eluted under the chromatographic conditions mentioned above, and the analytic detection was done under daylight. A corresponding band in the sample solution is observed to the standard strychnine.⁶

Assay/analytical method
High-performance liquid chromatographic estimation of strychnine in S. nux-vomica.

Chromatographic system
Shimadzu® High Performance Liquid Chromatographic system equipped with LC-10ATVP pump, a rheodyne 7725i injector with 20 µl loop volume, A Shim-Pack CLC, ODS (C-18), (250 × 4.6 mm i.d., 5 µ) was used for the separation. SPD M10AVP photodiode array detector in combination with CLASS-VP 6.12 SP5 integration software. The mobile phase was composed of mixture of methanol and water (55:45 v/v). It was filtered through a 0.45 µ membrane filter and degassed for 10 mins. Detection was carried out at 254 nm.

Chromatographic conditions
• Mobile phase: Methanol: Water (55:45)

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**Table 1: Preliminary phytochemical screening of plant extract**

| Tested for   | Test performed                                                                                     |
|--------------|---------------------------------------------------------------------------------------------------|
| Phenols      | Phosphomolybdic acid test - The extract was spotted on the filter paper. A drop of phosphomolybdic acid reagent was added to the e spot and was exposed to ammonia vapors |
| Flavonoids   | Lead acetate test - To 5 ml of extract, 10% alcoholic ferric chloride solution was added           |
| Alkaloids    | Dragendorff’s test - A drop of extract was spotted on a small piece of precoated TLC plate, and the plate was spray with modified Dragendorff’s reagent |
| Anthraquinones | Borntrager’s test - About 50 mg of extract was heated with 10% ferric chloride solution and 1 ml of concentrated HCl. The extract was cooled, filtered and the filtrate was shaken with diethyl ether. The ether extract was further extracted with strong ammonia |
| Glycosides   | Legal’s test - 0.1 g of the extract was dissolved in pyridine (2 ml); 2 ml nitroprusside solution was added and made alkaline with NaOH solution |
| Carbohydrates | Fehling’s test - A small portion of extract was dissolved in water and treated with Fehling’s solution |
| Steroids     | Liebermann-Burchard’s test - To 1 ml of extracts of drug, 1 ml of chloroform, 2–3 ml of acetic anhydride and 1–2 drops of concentrated H₂SO₄ were added |
| Saponins     | Foam test - 1 ml of extract was diluted separately with distilled water to 20 ml and shaken in a graduated cylinder for 15 min |
| Amino acid   | Ninhydrin test - A small quantity of the extract was dissolved in few ml of water and 1 ml of ninhydrin reagent was added |

TLC: Thin layer chromatography
Five gram of each powdered drug were weighed accurately and extracted in a reflux apparatus with (150 × 3) ml of methanol for about 8 h. The filtrate was filtered and evaporated to remove methanol. The residue was dissolved in 25 ml methanol. One drop of concentrated HCl was added to liberate the alkaloids, and the same was filtered through 0.45 μm membrane filter and used for HPLC analysis.

**Standard solution**
Ten milligram of strychnine was dissolved in 10 ml methanol.

**Procedure**
Twenty microliter of suitably diluted sample solution/standard solution was injected, and the peak responses recorded. Retention time of strychnine was 3.7 min. The UV spectrums of the corresponding times were also compared to confirm the identity of the peaks. The percentages of the marker compound in the test solution were calculated by comparing the peak areas of the standard compounds with those of the corresponding peaks present in the chromatogram of the test solution.[6]

### RESULT

Dried and powdered seeds of *S. nux-vomica* were extracted by refluxing with methanol and subjected to preliminary phytochemical screening for the presence of different chemical groups [Table 2]. Tannins, alkaloids, and glycosides were found to be the major groups present, along with phenol, steroids and flavonoids in smaller quantities [Table 3].

The TLC profile of the methanolic extract of seeds of *S. nux-vomica* was developed using the solvent system toluene:chloroform:methanol in the ratio 8:2:1. The plate was observed in visible light [Figure 3] after spraying with Dragendorff's reagent (specific method). A corresponding band in the sample solution was observed in the standard strychnine [Figure 2].

High-performance liquid chromatography profile of the methanolic extracts of *S. nux-vomica* is given in Table 4. In our study, the amount of strychnine in the sample was calculated by considering the area under the peak for the standard strychnine (0.2–14.0 μg/ml) and the strychnine peak in the sample profile [Figure 3].

### DISCUSSION

Now-a-day HPLC is being used a major tool for fingerprinting and routine quality control analysis. HPLC chromatogram of standard strychnine was also developed under the same chromatographic conditions. By comparing the chromatogram of standard and sample, the purity of the compound can be known. If additional peaks are obtained, impurities are present. Patel *et al.* studied the phytochemical constituents of *S. nux-vomica* and found
that alkaloid, carbohydrate, tannin, steroid, triterpenoid, and glycoside are the major contents when compared to flavonoid and phenol. The poisonous matters presented in S. nux-vomica are strychos alkaloids, mainly monomeric tertiary indole alkaloids; chief constituent being strychnine and brucine. The TLC identity was developed to confirm the presence of strychnine in the plant. For selection of TLC plate we choose precoated plate over handmade plate as precoated plates with different support material and with different sorbent layers are available in different format and thickness by various manufactures. Usually, plates with sorbent thickness of 100–250 mm are used for qualitative and quantitative analysis. Proper sample preparation is an important process for the success of the TLC separation. For sample, preparation 1 ml of each concentrated extract of S. nux-vomica was diluted to 10 ml in respective solvents (pet, ether, chloroform and methanol). Sharma suggested that for detecting colorless spots, techniques like nonspecific and specific method can be used. Gaitonde and Joshi studied the TLC-spectrophotometric analysis of strychnine and brucine from the ayurvedic pills of S. nux-vomica and developed a chromatogram for the same.

High-performance liquid chromatography fingerprint profile of S. nux-vomica was developed and standardized. In this study, the amount of strychnine seems to be 0.36% (w/w) in the seeds. Choi et al. compared the content of strychnine in unprocessed and detoxified S. nux-vomica seeds and found that unprocessed seed contains 0.411 ± 0.028 mg/g by HPLC-ESI/MS method.

**CONCLUSION**

From the present investigation, the methanol extract of the seeds S. nux-vomica contains tannins, alkaloids and glycosides in higher amounts along with phenols, steroids and flavonoids in smaller quantities.

The TLC and HPLC profiles developed are very valuable for the identification of the original drug from their adulterants.

**Figure 1:** Chemical structure of strychnine (R=H)

**Figure 2:** Thin layer chromatography identity test

**Figure 3:** HPLC chromatogram of the methanolic extract of Strychnos nux-vomica. HPLC: High-performance liquid chromatography
Table 4: HPLC profile of the methanolic extract of *Strychnos nux-vomica*

| Detector A-254 nm | Pk number | Retention time | Area | Area (%) | Height | Height (%) | Start time | Stop time |
|------------------|-----------|----------------|------|----------|--------|------------|------------|-----------|
|                  | 1         | 1.90           | 4919 | 0.01     | 259    | 0.01       | 1.38       | 2.08      |
|                  | 2         | 3.70           | 26,600,487 | 45.17 | 1,414,589 | 53.33     | 2.08       | 3.98      |
|                  | 3         | 4.17           | 12,931,596 | 21.96 | 711,161   | 26.81     | 3.98       | 4.58      |
|                  | 4         | 5.12           | 9,404,842  | 15.97 | 257,024   | 9.69      | 4.58       | 5.54      |
|                  | 5         | 5.60           | 1,896,887  | 3.22  | 81,082    | 3.06      | 5.54       | 6.19      |
|                  | 6         | 6.25           | 1,082,422  | 1.84  | 26,460    | 1.00      | 6.19       | 7.02      |
|                  | 7         | 7.48           | 1,242,133  | 2.11  | 33,345    | 1.26      | 7.02       | 7.91      |
|                  | 8         | 8.16           | 676,536    | 1.15  | 19,932    | 0.75      | 7.91       | 8.62      |
|                  | 9         | 8.92           | 416,519    | 0.71  | 12,938    | 0.49      | 8.62       | 9.23      |
|                  | 10        | 9.61           | 696,554    | 1.18  | 22,306    | 0.84      | 9.23       | 9.93      |
|                  | 11        | 10.41          | 1,534,335  | 2.61  | 26,641    | 1.00      | 9.93       | 12.07     |
|                  | 12        | 12.66          | 940,843    | 1.60  | 16,107    | 0.61      | 12.07      | 13.95     |
|                  | 13        | 14.12          | 156,123    | 0.27  | 3597      | 0.14      | 13.95      | 14.71     |
|                  | 14        | 15.42          | 370,642    | 0.63  | 5742      | 0.22      | 14.71      | 16.31     |
|                  | 15        | 16.68          | 120,191    | 0.20  | 2914      | 0.11      | 16.31      | 17.06     |
|                  | 16        | 17.51          | 322,489    | 0.55  | 9259      | 0.35      | 17.06      | 18.20     |
|                  | 17        | 18.62          | 214,751    | 0.36  | 2216      | 0.08      | 18.20      | 19.20     |
|                  | 18        | 20.20          | 6907       | 0.01  | 1092      | 0.04      | 20.19      | 20.30     |
|                  | 19        | 20.33          | 31,489     | 0.05  | 1073      | 0.04      | 20.30      | 20.83     |
|                  | 20        | 21.45          | 95,656     | 0.16  | 1308      | 0.05      | 20.83      | 22.60     |
|                  | 21        | 22.63          | 9186       | 0.02  | 440       | 0.02      | 22.60      | 23.01     |
|                  | 22        | 23.58          | 28,652     | 0.05  | 502       | 0.02      | 23.01      | 24.49     |
|                  | 23        | 24.50          | 258        | 0.00  | 57        | 0.00      | 24.49      | 24.60     |
|                  | 24        | 24.64          | 330        | 0.00  | 43        | 0.00      | 24.60      | 24.79     |
|                  | 25        | 26.13          | 31,670     | 0.05  | 877       | 0.03      | 25.14      | 26.21     |
|                  | 26        | 26.24          | 46,902     | 0.08  | 871       | 0.03      | 26.21      | 27.44     |
|                  | 27        | 27.79          | 5050       | 0.01  | 196       | 0.01      | 27.74      | 28.29     |
|                  | 28        | 28.80          | 17,069     | 0.03  | 378       | 0.01      | 28.29      | 29.62     |
|                  | 29        | 29.72          | 147        | 0.00  | 37        | 0.00      | 29.62      | 29.79     |
|                  | 30        | 29.90          | 56         | 0.00  | 12        | 0.00      | 29.85      | 29.97     |
| Totals           |           | 58,885,641     | 100.00     |       | 2,652,458 | 100.00    |           |           |

HPLC: High-performance liquid chromatography

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