

AYURVEDIC PROCESSINGS OF NUX VOMICA: QUALITATIVE AND QUANTITATIVE DETERMINATION OF TOTAL ALKALOIDAL CONTENTS AND RELATIVE TOXICITY

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The seeds of Strychnos nuxvomica Linn. (Loganiaceae) were processed as prescribed in the Ayurvedic classics. The seeds were soaked in Kanji (3 days; Sample A); boiled in cow milk (3 h; Sample B); soaked in cow urine (7 days), boiled in cow milk (3 h) and roasted in cow ghee (till swollen; Sample C) and a sample roasted in cow ghee (till swollen; Sample D). The processed nux vomica were compared with an untreated sample (Sample E) to assess the effect of processing. This study evaluated the percentage change in total alkaloidal content (TAC). The chromatographic finger printing was carried out using HPTLC and LC-MS. The chromatograms obtained exhibit the different modifications in the alkaloidal constituents of the processed and the unprocessed nux vomica. Sample B and C exhibited a relatively low percentage of TAC when compared with the TAC of unprocessed seeds. Among the two processed samples, TAC of Sample B showed development of some distinct new peaks with a different R_f/R_t, when compared with other samples. Also, both the samples were found to be relatively less toxic towards orally dosed albino mice.

Keywords: Ayurvedic processings, LC-MS, Detoxification, Loganiaceae, Strychnos nuxvomica, HPTLC

INTRODUCTION

Nux vomica, commonly known as *Kucila* in Northern part of India, are ripe seeds obtained from the plant *Strychnos nuxvomica* (Loganiaceae). The tree is native of tropical regions and monsoonal forests and is a very promising drug in the alternative systems of medicine. The fruit is bitter and poisonous. It has been used as a tonic, aphrodisiac, diuretic, emmenagogue and to cure pain of the joints (Kirtikar and Basu 1935). The plant in Ayurvedic classics (Bhavmisra 2002) has been reported to possess various properties such as analgesic, appetite enhancing, and stimulant. More than 60 formulations for nux vomica have been reported in the literatures of the Indian system of medicine, of which 30 formulations are

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used in the disorders of *vāta dosha* (Katiyar 1984). The plant in Ayurvedic texts is classified in the *up-visha* category (sub-toxin) and is suggested to be used after detoxification (*shodhan*). Among the methods that are quoted in the Ayurvedic literatures, some are time consuming and some require merely a few hours (Sharma 1979). Processing method such as treatment of the seeds firstly with cow urine (7 days), then boiling in cow milk (3 h) and finally frying the seeds in cow ghee, has been described as an official method (Ayurvedic Pharmacopoeia Committee 2000). Alkaloids are the major component of the seed and are responsible for the toxic effects. Many researchers have explored different methods for analysis and quantification of nux vomica alkaloids especially strychnine and brucine. But these alkaloids have been primarily determined in biological samples, since the drug has been very often used for poisoning. Techniques like liquid chromatography electrospray mass spectroscopy (Choi *et al.* 2004; Van Eenoo *et al.* 2006), HPLC (Jiang, Yang and Gong 2002), RP-HPLC (Xu *et al.* 2003; Wang *et al.* 2004), gas chromatography (Gao, Sun and Sha 1990) and other sophisticated techniques have been used.

Bhanu and Vasudevan (1989) used UV spectrophotometer to demonstrate strychnine and brucine contents in the processed nux vomica. The methods of processing were different from those considered under present study. However, the extent to which the processing methods affect the alkaloidal contents qualitatively as well as quantitatively in the seeds was not known. The purpose of this study was to evaluate the quality (phytochemical profile) of the seeds when treated with different methods.

METHODS

Plant Material

The dried seeds of *S. nuxvomica* were supplied by Phytoconcentrates, Ahmedabad, India. The authenticated specimen has been deposited in the department.

Materials for Processing

Cow urine and cow milk were collected from the regular supplier, at Gujarat Ayurved University, Jamnagar, India. Cow ghee (clarified butter) was freshly prepared. All the collections were done in the early morning hours.

Chemicals and Reagents

The chemicals used were acetonitrile (HPLC grade; Rankem), ammonia (Rankem), chloroform (Rankem), diethylamine (Central Drug House, New Delhi, India), ethyl acetate (Rankem), methanol (Rankem), n-hexane (Rankem), hexane (Rankem), sodium hydroxide (Rankem), and sulphuric acid (Central Drug House (P) Ltd., New Delhi). Precoated thin layer chromatographic plates were purchased from Merck, India. Deionised water was prepared using Millipore water purification system. All solvents and solutions were filtered once through a Millipore filter (0.2 µm) before use. The markers were isolated from the unprocessed seeds and verified with ¹H and ¹³C NMR.

Ayurvedic Processings (*Shodhan*)

The processing of seeds was carried out on laboratory scale by using the same batch of raw *S. nuxvomica* seeds.

Sample A

The seeds (4 kg) were processed by dipping in *Kanji* (fermented preparation made of rice) (pH: 3; 6 L) for three days and put in the sun for the whole day. The media was changed every 24 h. On the fourth day, the seed coat was removed, embryo separated and the cotyledons were dried and powdered (Sharma 1979).

Sample B

The seeds were tied in a muslin cloth. The cloth containing the seeds was hanged in an open vessel (*dolāyantra*), containing boiling cow milk q.s. to properly immerse the seeds, for 3 h. Fresh milk (near to pH 7) was added

intermittently to maintain the volume of the milk. The seeds were then peeled and the cotyledons were used further (Sharma 1979).

Sample C

The seeds were soaked in cow urine for seven days (~7 L) and kept under the sun. The next day the urine was replaced with the same volume of fresh urine. The process was repeated for seven nights. On the eighth day, the seeds were properly washed. They were then tied in a muslin cloth and hanged in a vessel filled with boiling milk, quantity sufficient to properly immerse the seeds, in an open vessel for 3 h. Milk was added intermittently to maintain the volume of the milk during boiling. The seeds were washed, peeled, embryo removed, dried and fried in cow ghee till they turned brownish-red. They were then powdered (Ayurvedic Pharmacopoeia Committee 2000; Bhavmisra 2002).

Sample D

The washed seeds were fried in cow ghee till brownish-red in colour and swelled. The seeds were immediately peeled and cotyledons powdered (Sharma 1979; Shashtri 1999).

Sample E

The seeds of *S. nuxvomica* were steamed to facilitate the removal of the seed coat and the embryo. The cotyledons so obtained were used as reference for further experiments.

Extraction of Total Alkaloidal Content (TAC)

An accurately weighed amount (10 g) of pulverised coarse powder of seed cotyledons of the samples was extracted. The powder was first basified with 20 mL of 10% aqueous ammonia solution and kept for 3 h. The mixture was sonicated for 5 min in a sonicator and extracted thrice with 40 mL of chloroform. The marc was again moistened with fresh aqueous ammonia solution (20 mL; 10%) and kept overnight. The mixture was again extracted thrice with 40 mL of chloroform. The chloroform solution containing the alkaloids was further extracted with 1% sulphuric

acid solution; aqueous layer was separated, basified slowly with 1% sodium hydroxide solution and re-extracted thrice with 40 mL of chloroform.

Animals Used

Swiss albino mice (20–25 g) of either sex were used in the experiments. The animals were procured from the Animal Division Laboratory of Central Drug Research Institute, Lucknow, India. They were housed in groups of 5 per cage and were acclimatised to laboratory conditions with free access to standard diet and water *ad libitum* for at least 1 week on a 12 h light/12 h dark cycle (from 08:00 to 20:00 h) prior to pharmacological studies. Experimental protocols were designed to meet the 'Guidelines of Animal Experimentation' approved by the Commission of Ethics in Animal Experimentation of the Institute. All animals were fasted overnight before the test and water was supplied *ad libitum*. The ambient temperature was 25±1°C. Behavioral observations took place in the forenoon and each animal was used only once.

Exploratory Toxic Dose Study

Single toxic dose was determined in albino mice (n=10) by administering seeds suspension orally at two dose levels (200 and 500 mg/kg body weight). The animals were observed for 14 days for change in behaviour and mortality.

Analytical Chromatographic Conditions

The TAC obtained above was analysed on HPTLC, loaded on a C18 column and analysed on LC-MS to analyse any phytochemical change in the processed seeds of *S. nuxvomica*. The details of the analytical conditions have been described previously.

Instruments

HPTLC

HPTLC analysis of the alkaloid fraction was performed on Merck pre-coated TLC plates (silicagel 60F254) using a Camag (Muttensz, Switzerland) HPTLC system equipped with a Linomat IV applicator, twintrough chamber, a model 3 scanner and Cats integration software (version 4.03).

HPLC-MS

The HPLC-MS analysis of alkaloid fraction was performed using a Waters HPLC-MS system equipped with model 2525 pump and a PDA detector. The gradient of ACN:buffer (ammonium acetate buffer (pH 4.5)) with a flow-rate of 1 mL/min provided the separation of the studied components on a C18 column (250 × 4.6mm, 5micron, Phenomenex). Mass spectra were acquired using a Waters 3100 Mass Detector equipped with an APCI source. Data acquisition and processing were performed with Empower™ software. All solvents used were of HPLC grade. Details of the gradient and mass spectrometer conditions are shown in Tables 2 and 3.

Solvent systems

HPTLC

The fingerprinting of the TAC was carried out on Silica gel GF254 on aluminium sheet (pre-coated) using solvent systems (a) benzene:ethyl acetate:diethyl amine (7:2:1 v/v) [Fig. 1 and 2], (b) n-hexane:ethyl acetate:methanol:diethyl amine (8:6:0.4:1.5 v/v) [Fig. 3 and 4], (c) ethyl acetate:methanol (20:0.7 v/v) [Fig. 5 and 6] and (d) methanol:water (8:2 v/v) [Fig. 7 and 8]. The different solvent systems were chosen considering the varied nature of the alkaloids present in the TAC. The TLC chambers of the different solvent systems were saturated for 8–10 min, 8–10 min, 12–14 min and 8–10 min, respectively for (a), (b), (c) and (d). The analysis was done in background of brucine (Track 1) and strychnine (Track 7) as standards.

RESULTS AND DISCUSSION

Shodhan is one of the important procedures described in the ancient literatures of Ayurveda. *Shodhan* or detoxification/processing (although the process is beyond mere detoxification) is a term, which describes a technique, advised by the divine scholars in their respective texts to reduce or modify the poisonous effects of any toxic drug and render it suitable to be used therapeutically. The methods were in practice since the time of *Caraka Samhita* (1000 B.C. to 500 B.C.). According to the text, *Agnivesh* while elaborating the term *samskara* mentions certain fundamental pharmaceutical procedures to detoxicate/alter the properties of the drug. The frequency of use of the methods for *shodhan* and the time required for completion has been the two important factors taken into consideration while selection of methods. The present manuscript has been designed to report the qualitative changes in the phytoconstitution (total alkaloidal) after processing the seeds with different methods.

More than one method has been proposed for the extraction, keeping in view the basic property of alkaloids (Bruneton 1998). Some of these methods require application of heat during extraction, making the process susceptible to degradation of alkaloids or to loss of solvents during heating. Another drawback of these methods is that the separation of pure alkaloids from the extract is very cumbersome. Keeping in view the effect of heating and the availability of sonicators for extraction, maceration was preferred. Vacuum was applied so as to lower the temperature required for solvent recovery. Also the previous study done on the extraction process of the Asian species of *nux vomica* revealed that chlorobromomethane and dichloromethane present as trace impurity in chloroform, tend to react with strychnine and brucine to form dichloromethobromides in the solution. Thus, care was taken to minimise the exposure time of the alkaloids with the chloroform, to avoid any transformations in the solution (Bisset and Phillipson 1976).

Table 1 shows the percentage of TAC extracted from the cotyledons (end product of processing) of *nux vomica* before and after processing it with the said methods. A significant difference in the total alkaloids was observed in cotyledons (medicinally important portion of the seed).

From Table 1 it is evident that, only two methods, *viz.* (i) treatment of seeds with cow milk and (ii) the official method, have significantly affected the alkaloidal contents. Nux vomica treated with cow milk (Sample B) exhibited about 51% decrease in the alkaloidal contents whereas treatment of seeds with the official method (Sample C) decreased the alkaloidal contents by 97% when compared with the TAC of crude nux vomica.

Table 1: Percentage of total alkaloidal content (TAC) from the unprocessed and processed (with different methods) nux vomica.

S. No.	Sample	Quantity of crude drug (g)	Weight of TAC in g (dried at 37°C in <i>vacuo</i>)*	% TAC (w/w)	% decrease in TAC w.r.t. Sample E
1	A	10.00	0.173±0.001	1.722	2.99
2	B	10.00	0.087±0.001	0.870	50.99
3	C	10.00	0.058±0.001	0.059	96.68
4	D	10.00	0.144±0.001	1.430	19.43
5	E (unprocessed)	10.00	0.173±0.000	1.775	–

Note: *Mean ± SE; n=3

The TAC of all the differently processed seeds were chromatographed on precoated silica gel plate with different solvent systems and compared for the presence or absence of any spot. The plates were screened densitometrically using TLC Scanner (Camag UV Scanner; 254 nm). Standard brucine and strychnine were run as reference markers. For better detection and comparison, the figures were resolved into 2-dimensional (2-D) and 3-dimensional (3-D) view at 254 nm. These figures revealed the presence of some new compounds that appear in the tracks of the TAC from the processed nux vomica, which have a different R_f and are in very minute quantity. Some minor peaks were observed at R_f (0.05, 0.41 and 0.43) in TAC of Sample B when monitored on TLC using solvent system EtOAc:MeOH (20:0.7 v/v). Besides these three peaks, a very prominent peak (R_f of 0.78, λ_{max} 200 nm in benzene: ethyl acetate:diethyl amine (7:2:1 v/v) was observed in the same sample (Sample B) which was absent in others (Fig. 1 and 2). This particular compound was noticed in different solvent systems used (Fig. 5 and 7). Encircled peak on the HPTL chromatograms (Fig. 1 and 3) that has

emerged very distinctly may be a modification of the major alkaloids such as strychnine or brucine. This can be confirmed by characterisation of the compound and comparison after isolation.

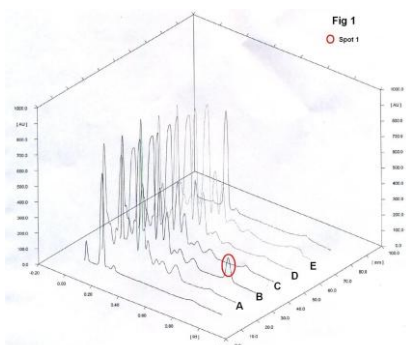


Fig. 1: 3-dimensional view of the chromatogram in solvent system (a).

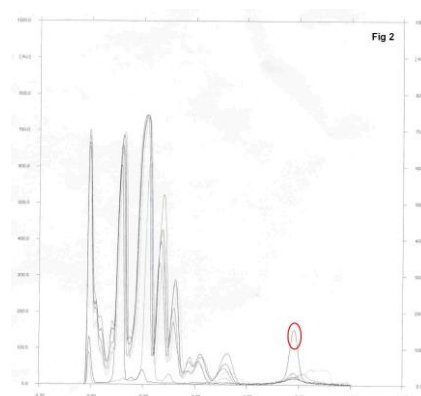


Fig. 2: 2-dimensional view of the chromatogram in solvent system (a).

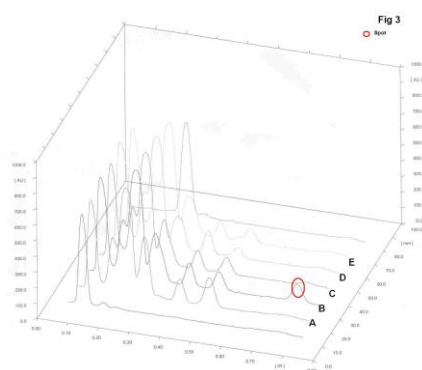


Fig. 3: 3-dimensional view of the chromatogram in solvent system (b).

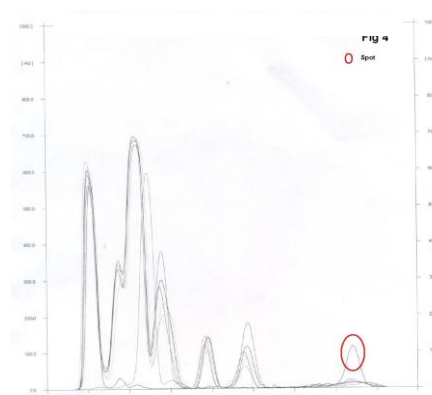


Fig. 4: 2-dimensional view of the chromatogram in solvent system (b).

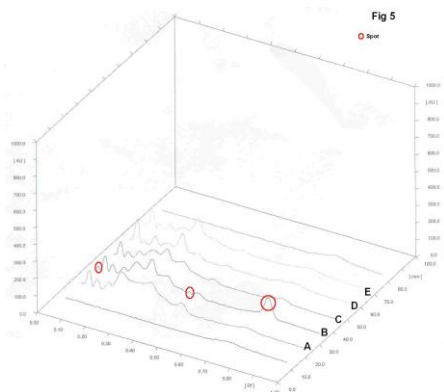


Fig. 5: 3-dimensional view of the chromatogram in solvent system (c).

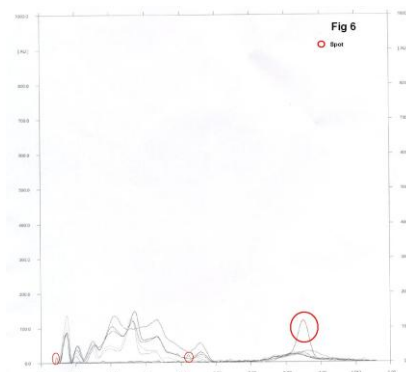


Fig. 6: 2-dimensional view of the chromatogram in solvent system (c).

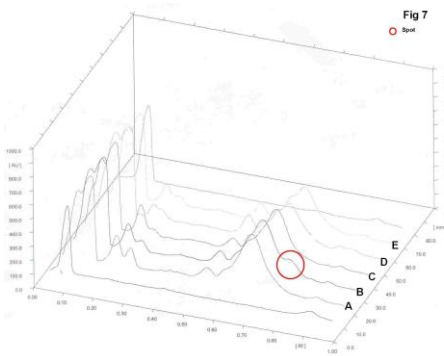


Fig. 7: 3-dimensional view of the chromatogram in solvent system (d).

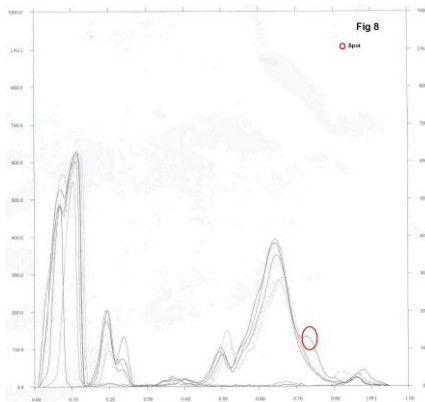


Fig. 8: 2-dimensional view of the chromatogram in solvent system (d).

Table 2: Details of the rate of change of solvents during a 50 min gradient run through the column.

S. No.	Flow rate (mL/min)	Time (min)	Acetonitrile (%)	Buffer (%)
1	2.0	0	10	90
2	2.0	20	35	65
3	2.0	23	35	65
4	2.0	26	60	40
5	2.0	27	10	90
6	2.0	32	10	90

Table 3: Mass spectrometer conditions during the qualitative evaluation of the TAC.

Instrument Parameters		
Polarity	- ES+	
Calibration	- Dynamic 1	
Capillary (kV)	- 3.50	3.50
Cone (V)	- 20.00	21.61
Extractor (V)	- 1.00	1.34
RF lens (V)	- 0.5	0.5
Source temp (°C)	- 80	80
Desolvation temperature	- 320	318
Cone gas flow (l/h)	- 50	49
Desolvation gas flow (l/h)	- 650	646
LM 1 resolution	- 15.0	
HM 1 resolution	- 15.0	
Ion energy 1	- 0.5	
Entrance	- 50	-50
Collision	- 2	-3
Exit	- 50	-50
LM 2 resolution	- 15.0	
HM 2 resolution	- 15.0	
Ion energy 2	- 0.5	
Multiplier (V)	- 500-499	
Syringe pump flow (μ l/min)	- 10.0	
Gas cell Pirami Pressure (mbar)	- < 1e-4 mbars	

These observations were further explored using the LC-MS. The TAC was loaded on a column (reverse phase) and analysed using LC-MS to acquire some more data in support of this qualitative study. The use of volatile buffers limit the use of ammonium acetate buffer for the resolution on LC-MS. Considering this limitation, different pH ranges and polarity, during the run time, resulted in the chromatograms. The TAC of Sample B was considered for the study, since it showed the most prominent qualitative change in the TLC analysis. Figures 9 and 10 show the resolution of TAC of Sample B and Sample E respectively, on the LC-MS. The LC-MS spectra of TAC of Sample B showed peak at masses (ES+) 454 (t_R 18.11 min), 341 (t_R 21.20 min) and 382 (t_R 36.75 min), which were not evident in the LC-MS spectra of the TAC of the unprocessed cotyledons of nux vomica (reference sample, Fig. 10). This analysis confirmed the presence of some new adducts (as expected from the spectras and chromatograms run under similar conditions) and transformations that might have occurred during the processing and added some qualitative details to the study.

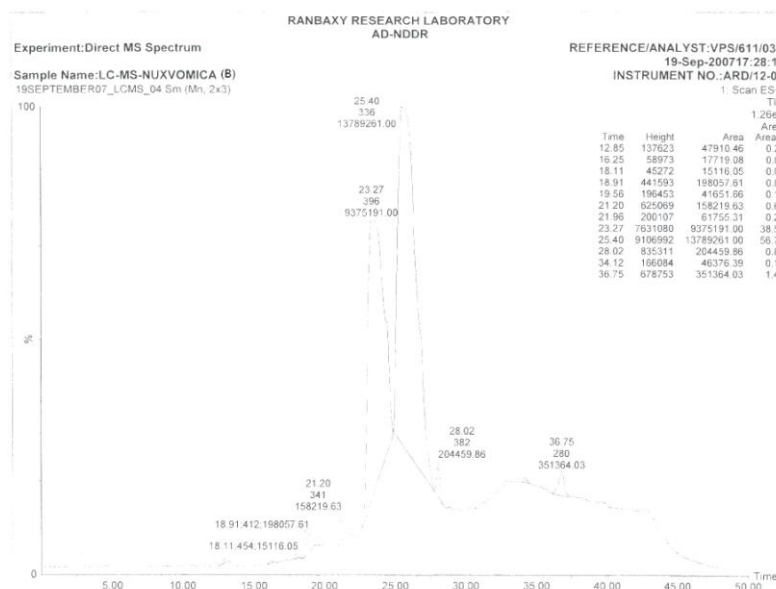


Fig. 9: LC-MS chromatogram of the TAC obtained from Sample B. The chromatogram shows the peak at t_R of 21.20 min (MW 341) and 28 min (MW 382) which was not found in the Sample E (unprocessed sample).

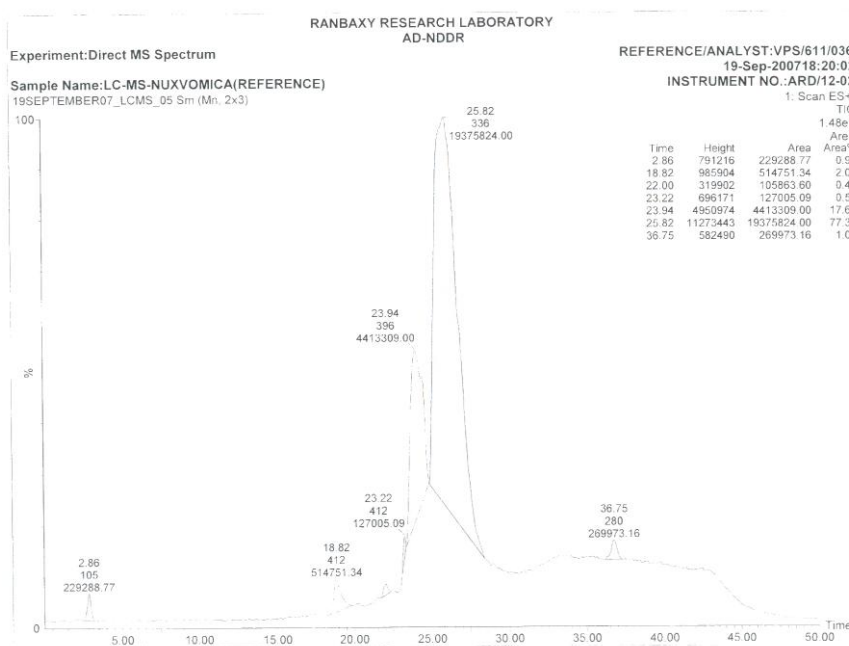


Fig. 10: LC-MS chromatogram of the TAC obtained from Sample E (reference).

The above chromatographic analysis and decreased percentage of total alkaloids may justify the pharmacological and exploratory toxic dose study (Kumar *et al.* 2006a). The exploratory toxic dose study was performed in order to determine any effect of processing and change in the toxicity of the *nux vomica*.

The study was done using a dose of 200 mg/kg body weight. The group of animals treated with Sample B, C and D exhibited no mortality and groups treated with Sample A and E exhibited 50% mortality at this dose level. No mortality or any signs of toxicity were observed during the next 14 days in the animals that survived. Samples B, C and D were further administered at 500 mg/kg body weight dose level. At this dose level Sample D-treated animals showed 100% mortality and the other two i.e. Sample B and C-treated animals showed 50% mortality. Those survived were further observed for 14 days. Our study shows that the concept of *shodhan*/detoxification as suggested by the divine scholars brings about a change in the toxic dose of the seeds and the toxic dose varies depending on the method used for processing. The study was

performed in order to explore the change in toxic dose, as nux vomica is already a widely studied and well established drug.

An interesting fact about the processings which gets highlighted with this study is a two-way safety. Samples B and C showed almost similar acute safety dose levels. This revealed that both the methods, producing Samples B and C, are equally effective in reducing the toxicity. Reduction in toxicity may be expected to be due to qualitative change in Sample B and decreased TAC in Sample C. The presence of some minor but newer compounds (adducts) might be contributing towards the safety of Sample B whereas reduction in TACs is likely to be the key factor behind safety of Sample C. Sample C, despite having decreased alkaloidal content (97%), also shows better pharmacological potency than the unprocessed seeds (Kumar *et al.* 2006a, b).

CONCLUSION

Among all the four methods used for processing, the seeds processed with the official method and those processed with milk proved to be equally safe compared to the unprocessed seeds, in rodents. Further study is required to determine relative safety for humans. Supplementary studies are being done to quantify the individual alkaloidal contents. Attempts are being made to isolate the newer compounds as evident from the TLC fingerprinting profile.

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