

DETOXIFICATION OF *NERIUM INDICUM* ROOTS BASED ON INDIAN SYSTEM OF MEDICINE: PHYTOCHEMICAL AND TOXICITY EVALUATIONSARYAMITRA A. BANERJEE^{1*}, KAMALA K. VASU², HARIT PANCHOL², MANDAPATI RAJANI²
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Abstract: Indian system of medicine describes the usage of certain very toxic plant based drugs after performing a detoxification process (*Shodhana samskara*). *Nerium indicum* is traditionally used as a medicine though known to cause severe allergic symptoms, tachycardia and gastrointestinal effects leading to fatalities. In this study, the detoxification (*shodhana*) for *Nerium indicum* was scientifically validated based on phytochemical and toxicity profiles. *Shodhana* was performed according to traditional literature. HPTLC densitometric studies were performed for the pre- and post-*shodhana* powders followed by sub-acute toxicity evaluation in rats. Preparative TLC and LC-MS showed the reduction of oleandrin peak in the post-*shodhana* sample. Prominent features of cardiotoxicity including tachycardia were noted in the pre-*shodhana* *Nerium* treated animals along with mortality. However, no such toxicity was encountered in the post-*shodhana* *Nerium* treated animals. Hence, using the recommended detoxification (*shodhana*), the toxicity of an important medicinal plant was significantly nullified. Such studies provide a scientific support towards our traditional medicinal practices using modern analytical and experimental methodologies and may prove to be very useful in establishing standard scientific procedures for routine and safe use of traditional medicines.

Keywords: *Nerium indicum*, toxicity, phytochemistry, detoxification

Ayurveda, the ancient Indian system of medicine mainly deals with herbal and herbo-mineral formulations to cure the pathological symptoms as well as to maintain good health. Some of the most important herbal drugs are toxic in nature and hence Ayurveda has classified such drugs in the *Visha-Upavisha Varga* (poisonous and semi poisonous drugs) and mentions the *Shodhana samskara* (process of detoxification) for the same to reduce their toxicity and potentiate their medicinal properties (1, 2).

Nerium indicum (family: Apocynaceae) has been traditionally attributed with several medicinal properties like kushtha or leprosy, valipalita or aging (Charak), upadamsha or syphilis (Sushrita); kandu or pruritis, indralupta or alopecia (Vagbaht), vrana or wound, paamaa or dermatitis and netra kopa or conjunctivitis (Chakara dutta) (3). Several pharmacological properties have also been studied including anti stress, anti-inflammatory, antifungal, car-

diotonic, neuroprotective and anticancer activities (4–9).

Modern techniques have confirmed the major constituents of *Nerium indicum* to be inclusive of oleandrin, nerideinone A, 12 β -D-hydroxypregna-4,6,16-trien-3,20-dione, adynerin, ursolic acid, daneric acid and neriu coumaric acid (10).

However, all parts of the plant have been reported to demonstrate toxic effects including both gastrointestinal and cardiac toxicity. The gastrointestinal complications include nausea and vomiting, excessive salivation and abdominal pain with mild to severe diarrhoea (11). Cardiac reactions consist of irregular heart rate, sometimes characterized by a racing heart at first that then slows to below normal, further along in the reaction. The heart may also beat erratically with no sign of a specific rhythm (12).

Hence, the current study was designed with the following objectives: (1) to evaluate the significant differences in the phytochemical profile of the toxic

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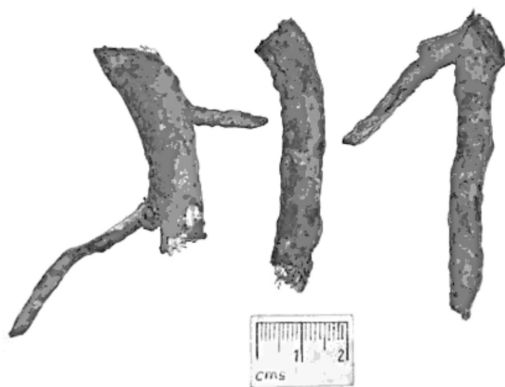


Figure 1. Roots of *Nerium indicum* used for the study

herbal drug *Nerium indicum* before and after shodhana process by using spectral and chromatographic methods (2) to separate, isolate and identify the compounds removed or modified due to shodhana and (3) to carry out toxicity studies for the drug before and after shodhana process.

EXPERIMENTAL

Collection and preparation of plant material

Roots of *Nerium indicum* were collected, dried and stored after authentication by a taxonomist at NBRI – Lucknow (Fig. 1; herbarium specimen # PERD/PP/0706/17). The plant material was weighed and divided in two parts. One part was used for pre-shodhana and the other for post-shodhana studies. Each part was further subdivided into two parts for either phytochemical evaluation or for toxicity studies.

Procedure for shodhana

This treatment was performed according to the traditional methods detailed in the Sarangdhar Samhita, Madhyam Khand, Adhyaya 12, 300. Briefly, 200 g of roots of *Nerium indicum* were cleaned with dry dust free cloth. A 60 × 60 cm muslin cloth was taken, washed with water, dried and further washed with cow milk. The cloth was then folded in four folds. The cleaned roots were placed on the cloth and the four edges of the cloth were brought together forming a pouch, which was knotted using a thread. The other end of the thread was tied to a clamp fitted to a stand.

Further, 800 mL of fresh cow milk was taken in a 3L capacity vessel. The above vessel was placed on a heating mantle. The pouch containing

the roots was carefully dipped into the cow milk such that the pouch was completely dipped, however, did not touch the bottom of the vessel. The milk was then heated gently at low flame for 3 h. The change in temperature and color of the milk was noted every half an hour. Continuous stirring was performed throughout the heating process. After 3 h the pouch was taken out of the milk and opened. The milk was collected separately and the roots were washed with reverse osmosis water. The water washed portion was also collected separately for analysis. The roots were spread on butter paper, dried, powdered and stored in a labeled glass bottle till further use.

Phytochemical analysis

Methanolic extract of *Nerium indicum* roots

One gram of *N. indicum* root powder was taken and 10 mL of methanol was added, the mixture was warmed and stirred continuously on water bath. The extract was filtered and the residue was again extracted twice with 10 mL of methanol each time. The filtrates were combined, mixed and concentrated to 10 mL. The same procedure was used to prepare the methanolic extract of post-shodhana *N. indicum* root powder. The extracts were packed in air tight glass vials, labeled and stored till further use. These extracts were checked for TLC and HPTLC profile and compared.

Comparative TLC of methanolic extract of pre- and post-shodhana *N. indicum* root powder

Both the pre- and post-shodhana methanolic extracts were compared in an HPTLC system using precoated silica G plate as stationary phase, toluene : ethyl acetate : methanol (1.7 : 0.2 : 0.1, v/v/v) as mobile phase and detection using short (254 nm) and long wave (366 nm) UV light. The plates were also observed after derivatization with Kedde's reagent to detect the presence of cardiac glycosides (13).

Preparation of extract of shodhana media

Plain milk, shodhana media (milk) and water washed part were extracted with ethyl acetate, and the ethyl acetate extracts were dried under reduced pressure. All the three extracts were washed with petroleum ether (three times) and the remaining portion was reconstituted again in ethyl acetate and concentrated. The shodhana media were analyzed in a TLC system using a precoated silica G plate as stationary phase, toluene : ethyl acetate : methanol (1.7 : 0.2 : 0.1, v/v/v) as the mobile phase using short and long wave UV detection system.

Quantitative estimation of total cardenolides in pre- and post-shodhana *Nerium indicum*

Powdered drug of each of pre- and post-shodhana *Nerium* roots (0.25 g of #80 powder) was extracted for one hour with 50 mL of water with intermittent shaking at room temperature. Five milliliters of 15% w/v solution of lead acetate was added to both samples and shaken. After a few minutes 7.5 mL of 4% w/v solution of disodium hydrogen orthophosphate was added and filtered. Five milliliters of 15% HCl was added to 50 mL of this filtrate, heated under reflux on water bath for one hour and transferred to a separating funnel followed by rinsing of the flask with two 5 mL water washes. This concentrate was extracted with chloroform (3 × 25 mL), dried over anhydrous sodium sulfate and then diluted to 100 mL with chloroform. Forty milliliters of chloroform extract were evaporated and to each residue of pre- and post-shodhana extract 7 mL of 50% ethanol, 2 mL of 3,5- dinitrobenzoic acid solution (2% in 96% ethanol) and 1 mL of 1 M NaOH were added. The absorbance of the resulting solution was measured at 540 nm at different intervals during the first 12 min until the maximum was reached, using a mixture of 7 mL of ethanol (50%), 2 mL of 3,5- dinitrobenzoic acid solution (2% in 96% ethanol) and 1 mL of 1 M NaOH solution in the reference cell. The cardenolide content was calculated as compared to digoxin standard, which was prepared by dissolving 50 mg of digoxin in 30 mL of double distilled water and 3 mL of 15% w/v HCl.

Preparative TLC of methanolic extract of pre- and post-shodhana *N. indicum*

A stationary phase consisting of precoated silica G plate and a mobile phase including toluene : ethyl acetate : methanol (1.7 : 0.2 : 0.1, v/v/v) was used to carry out the preparative TLC of the methanolic extract of pre- and post-shodhana *N. indicum* roots. CAMAG Linomat V Automatic Sample Spotter with a 100 µL Hamilton syringe (Bonaduz, Switzerland), a glass twin trough TLC chamber (20 × 10 × 4 cm; Camag) and TLC densitometric scanner 3 linked to WINCATS software (Camag) were used for the analysis. Short and long wave UV detection was employed.

Toxicity studies

Animals

Wistar male rats (n = 24) weighing 156 ± 17 g and female rats (n = 24) weighing 148 ± 16 g bred at PERD Centre animal house from the original breed obtained from National Centre for Laboratory Animal Science, Hyderabad were used for the study.

The animals were housed 3 per cage in polypropylene cages with autoclaved rice husk as bedding material and the general environmental conditions were strictly controlled in the colony, such as 10% air exhaust in the air conditioning unit, relative humidity of 60 ± 5%, temperature 20 ± 3°C and a 14 : 10 h light:dark:cycle. Amrut certified rodent diet (Maharashtra Chakan Oil Mills Ltd.) was given 40 g per animal per day and tap water (boiled and cooled to room temperature) was provided *ad libitum*.

Animal handling techniques were performed in accordance with Good Laboratory Practice (GLP). Animal house is registered with Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Social Justice and Empowerment, Government of India, vide registration no. 116/1999/CPCSEA, dated 19/5/1999.

Study design

The animals were acclimatized to the laboratory conditions one week before the study and the health status was examined by a veterinarian before the start of the study. Clinical signs and symptoms were recorded a day before the study initiation. Total randomization was done and the following groups (n = 6 males and 6 females/group) were divided: (1) Normal control (0.2% agar); (2) Pre-shodhana low (4.17 mg/kg/day of pre-shodhana powdered *N. indicum* roots); (3) Pre-shodhana high (41.7 mg/kg/day of pre-shodhana powdered *N. indicum* roots); (4) Post-shodhana low (4.17 mg/kg/day of post-shodhana powdered *N. indicum* roots) (5) and Post-shodhana high (41.7 mg/kg/day of post-shodhana powdered *N. indicum* roots).

These rats were administered pre- and post-shodhana powder of *Nerium indicum* as a single oral dose every day for 42 days during the morning hours. Any discomfort resulting after the dosing and any other clinical signs were recorded daily during the entire study period.

Drug

Both pre- and post-shodhana powdered *Nerium indicum* roots were used for the current study. Drugs were weighed daily and a suspension was made in freshly prepared 0.2% agar prior to dosing. The dosage was calculated everyday according to the weight of the animal on that particular day.

Assessment procedure

Body weight and food consumption

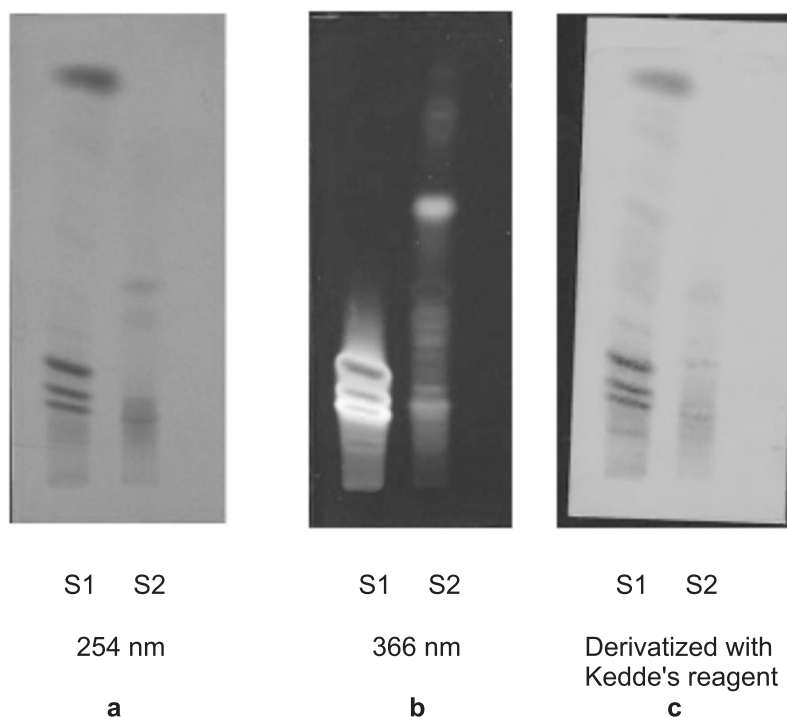
All the animals were weighed individually on every day of the study and food consumption was also monitored daily.

Table 1. Total cardenolide content of pre and post shodhana *N. indicum* roots (represented as optical density or O.D.).

No.	Sample	O.D.	Blank	O.D.-Blank
1.	Digoxin standard	0.696	0.045	0.651
2.	Pre Nerium test	0.476	0.045	0.431
3.	Post Nerium test	0.303	0.045	0.258

Table 2. Average beats per minute obtained in pre and post shodhana treatment groups (*p < 0.05 when compared to control).

Group		Average BPM	
		Male	Female
CONTROL 0.0 mg/kg		389.92 ± 7.51	398.59 ± 8.07
Pre-shodhana	LOW 4.17 mg/kg	340.79 ± 23.74	382.08 ± 9.29
	HIGH 41.7 mg/kg	461.99 ± 10.27*	444.85 ± 9.52*
Post-shodhana	LOW 4.17 mg/kg	346.78 ± 54.69	382.26 ± 61.34
	HIGH 41.7 mg/kg	388.93 ± 25.74	395.35 ± 79.90

Figure 2. Comparative TLC of methanolic extract of pre- and post-shodhana *N. indicum* root powder (S1 – Pre-shodhana sample, S2 – Post-shodhana sample)

Clinical signs and mortality

The external appearance and behavior of the animals were recorded daily.

Blood and serum biochemistry analysis

At the end of the treatment period, the animals were fasted overnight and blood samples were collected by retro orbital sinus bleeding into heparinized (for hematology) and non-heparinized (for serum collection, for clinical biochemistry) tubes and the following parameters were assessed in the whole blood: red blood cells, white blood cells, hematocrit, hemoglobin and differential leukocyte count. Hematology was performed using a KX 21 automated hematology analyzer (Sysmax, Japan).

The serum biochemistry was performed using diagnostic kits supplied by Transasia India Ltd. on a Smartlab automated clinical biochemistry analyzer (ERBA, Germany) and the following parameters were evaluated: glucose, serum glutamyl pyruvate transaminase, serum glutamyl oxaloacetate transaminase, creatinine, urea, cholesterol, triglyceride, total protein and albumin.

Electrocardiogram

At the end of the treatment period, electrocardiogram was taken for all the animals using a MP 35, BIOPAC physiological data monitoring system (Biopac System Inc. CA, USA).

Pathological anatomy

All the animals in the study were subjected to a full autopsy and the following organs were weighed: lungs, liver, heart, kidney, spleen, adrenals, testis and epididymis (in males), ovary and uterus (in females).

Statistical analysis

All results have been presented as the mean \pm SEM. Statistical analysis has been performed using one way ANOVA followed by Dunnett's test and $p < 0.05$ has been considered significant.

RESULTS AND DISCUSSION

Outcome of phytochemical analysis

Figure 2 demonstrates the comparative TLC of methanolic extract of pre- and post-shodhana *N. indicum* root powder wherein S1 lane represents the pre-shodhana sample and S2 represents the post-shodhana sample. At 254 nm (UV) some of the bands present in the pre methanolic extract are not visible in S2 (Fig. 2a). In Figure 2b we see one distinct fluorescent band at higher Rf in S2, which is

however not seen in S1. In the derivatized plate (Fig. 2c) S1 shows many bands which are not observable in S2 lane.

Figure 3 shows the densitometric profile of the milk used for shodhana process as compared to plain milk. It can be observed that the peaks seen in the shodhana media differ from the profile of plain milk. Hence, it is clear that some components of the *Nerium* root powder has been removed or diluted in the media due to the shodhana process.

The total cardenolide content of pre- and post-shodhana root extract was estimated (Table 1) and the cardenolide present in pre-shodhana sample was found to be more than that of post *Nerium* powdered drug, which were 31.61 %w/w and 24 % w/w, respectively. There was a decrease in the cardenolide content of the shodhit *Nerium*.

Preparative TLC of methanolic extract of pre- and post-shodhana drugs showed many peaks. One of the peaks prominently seen in the pre-shodhana samples was considerably reduced in the shodhit sample as seen in lane S8 (Fig. 4). This peak was separated and its mass was determined by using LC-MS (Perkin Elmer, API-165, USA). The mass obtained was 556 (M+1) which corresponded to the mass of oleandrin (mol. w. 555).

Thus, we could demonstrate that the process of shodhana results into the reduction, loss or modification of few components from the original root powder. This may be due to their extraction into the shodhana medium or any structural change in these compounds. Hence, the removal of these compounds may cause a reduction in their toxic effects.

Outcome of toxicity studies

All animals in both low and high dose groups in pre- and post-shodhana drug treatments survived till the end of the treatment regimen. No gross abnormalities were detected in any animals in both low and high dose groups in pre- and post-shodhana drug treatments. There was no effect on body weight, food consumption and no major clinical signs were observed during the entire study period. Clinical biochemistry and hematological parameters did not show any significant variation from the normal range (data not presented). However, the animals in high dose group in pre-shodhana drug treatment showed tachycardia, which is a known toxicity of the drug. This was characterized by a significant rise in heart rate (beats per minute; $p < 0.05$) in both male and female pre-shodhana high dose groups (Table 2).

Necropsy conducted on all the animals did not detect any abnormalities in any animals in both low

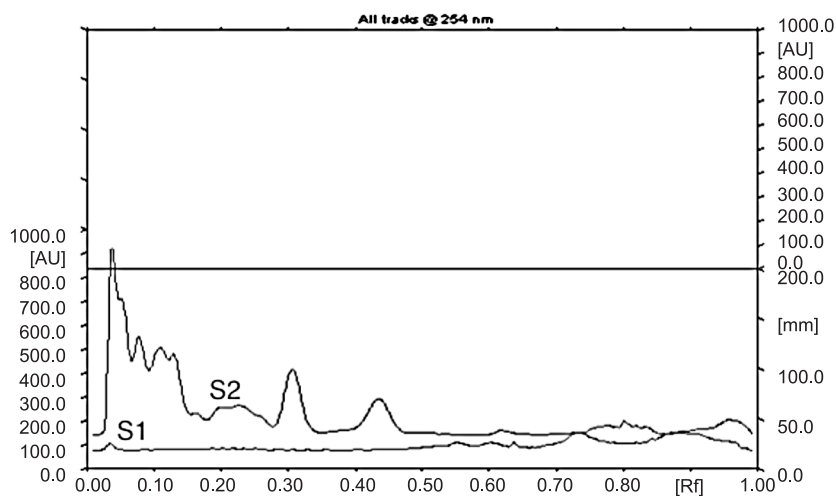


Figure 3. Profile of shodhana media (milk) as compared to plain milk (S1 – ethyl acetate fraction of plain milk; S2 – ethyl acetate fraction of shodhana media)

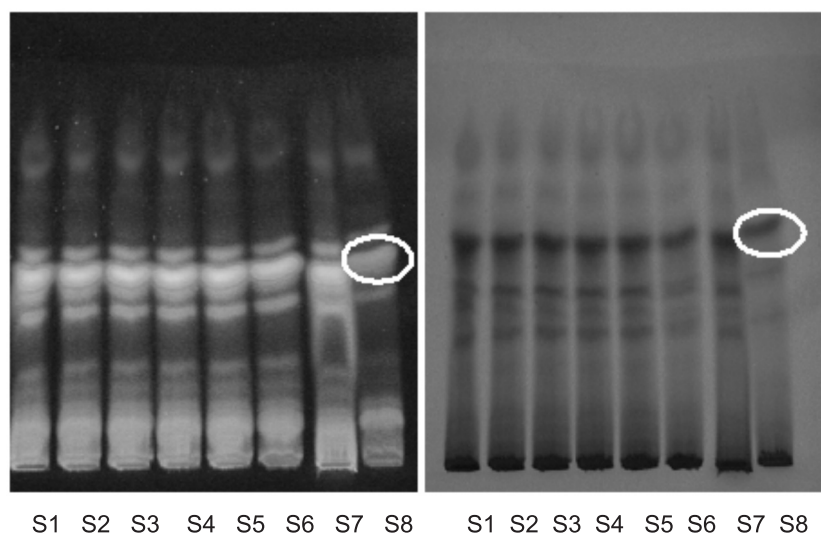


Figure 4. Preparative TLC of methanolic extract of *N. indicum* roots (S1 to S7: Pre-shodhana methanolic extract; S8: Post-shodhana methanolic extract) at 366 and 254 nm

and high dose groups in pre- and post-shodhana drug treatments and the average weights of the major target organs were comparable in all groups (data not presented). The gastrointestinal toxicity, which is commonly encountered in humans, could not however be mimicked in the rodents.

It is hence concluded that pre-shodhana powdered *Nerium indicum* roots did produce the common toxic symptom of cardiotoxicity in rats, which

was however not encountered when the drug was used after the shodhana process.

CONCLUSION

Hence, the shodhana procedure as cited in our traditional literature does indeed cause a reduction in the cardiac glycosides in the drug and thus leads to a reduction in the cardiac toxicity symptoms. This

study also scientifically validates the procedure of shodhana for this particular drug based on directives from the Ayurvedic system. Further, studies directed towards understanding the mechanism of detoxification as well as efficacy studies for the post-shodhana drug need to be undertaken.

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