HEPATOPROTECTIVE ACTIVITY OF DETOXIFIED SEEDS OF NUX-VOMICA AGAINST CCl_4 INDUCED HEPATIC INJURY IN ALBINO RATS

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Summary

The present study was conducted to assess the effect of detoxification (shodhana) on the phytochemical, hepatoprotective and tissue antioxidant activity of seeds of *Strychnos nux-vomica* L.

The seeds were subjected to shodhana process as prescribed in ancient texts of Ayurveda and a native Ayurvedic practitioner with slight modification. In the ancient process the seeds were detoxified by immersing seeds in cow's urine for 7 nights, then swedana (boiled with cow's milk for 3 hours) and finally fried with cow's ghee. In the 2nd process instead of swedana, the seeds were immersed in cow's milk for 3 days and remaining procedure was described as per ancient literature. Three samples (unprocessed and processed by two procedures were subjected to screen for phytochemical and hepatoprotective properties. CCl_4 induced hepatotoxicity in rats was used as animal model and biochemical parameters like serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT), alkaline phosphatase (ALP), serum bilirubin, serum cholesterol and liver tissue lipid peroxidation (LPO) and tissue reduced glutathione (GSH) levels.

The content of strychnine and brucine were reduced upon subjecting it to shodhana process. Similarly it was observed that the LD_{50} of unprocessed and processed seeds were 260 mg/kg and 2600mg/kg respectively. This indicated that toxicity of the seeds was reduced. Treatment with CCl_4 enhanced all the above mentioned biomarkers of hepatic injury. Pre-treatment with processed (detoxified)

and unprocessed seeds at 520mg/kg and 52mg/kg doses reduced the levels of biomarkers. Treatments with detoxified seeds (both traditional process and modified process) were found to be better than the unprocessed seeds.

From the present study it may be concluded that the shodhana (detoxification) process reduced the concentration of active principles and improved the therapeutic efficacy. Therefore subjecting seeds of *strychnos nux-vomica* to shodhana process before being used in clinically is justifiable.

Keywords: *Strychnos nux-vomica* L., CCl_4, shodhana, detoxification, hepatoprotective activity, tissue antioxidant activity.

Introduction

Ayurveda is an ancient system of medicine mainly utilises natural substances like herbs, minerals, etc., for treating human ailments. However, it lists out some of the plants as toxic e.g. aconite, nux-vomica, croton, kavera etc. Ayurveda adopts such poisonous substances as drugs after subjecting them to various shodhana (detoxification) processes (1).

The Rasa-shasthra describes that shodhana is not only the process of detoxification but also enhances the efficacy. Nux-vomica is one such herb listed as toxic drug and treating various ailments as single therapeutic agent or incorporated in poly-herbal formulations, after properly subjecting it to shodhana process.

Strychnos nux-vomica L. (Loganiaceae) known in Sanskrit, as kupilu, vishamusti. The plant is used in Ayurveda, Unani and Siddha medicines as bitter stomachic, in gastric and peptic ulcers, nerve tonic, cathartic, aphrodiasic, anti-obese, analgesic, anti-inflammatory, liver diseases, juandice and it is used one of the important ingredient in various ayurvedic poly-herbal formulations. But the seeds of this plant are used only after subjecting it to the process of shodhana. There are reports in Ayurveda that the unprocessed seed powder shows more toxicity than the processed one (1, 2 and 3). However in the modern literature there are reports that the plant possesses strychnine, brucine, pseudostrychnine, N-oxides of strychnine,brucine, isostrychnine and isobrucine, novacine and other indole alkaloids along with loganin glycosides, fixed oil and proteins(4). The plant is reported to have analgesic, anti-inflammatory (5), anti-tumour (6), anti-snake venom (7), anti-diarrhoeal activities (8). In Chinese system of medicine, the seeds of this plant are used after subjecting them to detoxification as prescribed in their ancient texts. In modern literature the reports are indicating that the toxicity of the seeds is reduced after subjecting it to Chinese method of detoxification.

There is a report that involves the application of Chinese method of detoxification which reduces the strychnine and brucine content after detoxification of nux-vomica seeds (9, 10). There is one report that the toxicity of seeds was reduced by 10 times with shodhana (detoxification), as indicated by increased LD_{50} values by 10 folds (11). However there are no scientific reports regarding the influence of shodhana on the phytochemicals, pharmacological profiles of the plant. Keeping this in view the present investigation was carried out to give scientific basis for the ancient knowledge of detoxification.

Several Ayurvedic practitioners are detoxifying the seeds as per the methods described in the ancient texts. But some native practitioners are using slightly modified methods of shodhana. In the present study the validity of both the process of detoxification processes i.e 1. Ancient method of shodhana and 2. Modified method of shodhana and their influence on the hepatoprotective profile of the seeds of *Strychnos nux-vomica* is studied.

Materials and methods

Plant material

The seeds of *Strychnos nux-vomica* L. were collected from Yucca enterprises, Mumbai, India. The authenticity of the plant was confirmed by one of the authors. A voucher specimen (SCSCOP/P.COG/12/04-05) has been deposited at the Herbarium of Medicinal Plant in the department of Pharmacognosy, S C S College of Pharmacy, Harapanahalli-583131, Karnataka, India.

Selection of seeds

The dry seeds were first dropped in a beaker containing water. The seeds which float on the surface of water or found broken, black in colour are rejected and the seeds which are settled at the bottom of the beaker are selected for purification after drying in air.

Detoxification (Shodhana) process

Shodhana (detoxification) of nux-vomica seeds is performed as per the method described in Ayurvedic Rasa-Shastra. The Rasa-shastra describes stepwise procedure for detoxification (shodhana) of nux vomica and it was adopted in the present study (1).

***Step 1*:** The clean and dried seeds (1kg) are kept in cow's urine for 7 nights. The urine is changed every night.
Step 2: The seeds after first step are collected and subjected to swedana (swedana process was done by keeping the seeds after first step in muslin cloth with banana leaf and tied. It is completely dipped in cow's milk and boiled on low fire) for 3 hours.
***Step 3*:** The above seeds were collected and washed with water. The seed coat and embryo removed. The seeds were roasted with cow's ghee on low flame on iron pan. The seeds become dark brown and crispy. Then the seeds were immediately powdered.

Detoxification (Shodhana) by modified method

This method is adopted by the local native practitioner of Shimoga, Karnataka, India. The same procedure is adopted here as explained by him. In this process step -1 and step – 3 are same but in step – 2 instead of swedana, the seeds after 1st step are immersed in the cow milk for 3 days. The milk is changed every day. The step – 3 is continued after modified step -2 as usual.

Strychnos nux-vomica seed powder unprocessed form UNV, seeds powder detoxified by ancient method i.e. step 1, 2 & 3 and seed powder detoxified by modified method are named as PNV1 and PNV2 respectively.

Preliminary phytochemical screening

The UNV, PNV1 & PNV2 samples were extracted with 70% alcohol and screened for the phytoconstituents (12).

Identification of major alkaloids

The major alkaloids strychnine and brucine were identified by TLC. All the samples were defatted with petroleum ether. The defatted (2g) samples were mixed with 10% ammonia and extracted with 25 ml methanol for 1 hour. The methanol extract was concentrated to 5 ml and used as test sample. Silica gel G is used as stationary phase while toluene, ethyl acetate and diethylamine (7:2:1) were used as mobile phase. All the extracts along with pure markers (1% strychnine in methanol and 1% brucine in methanol) were run in thin layer chromatography chamber for 30 minutes. The developed chromatogram is dried and first detected at UV-254nm in UV chamber. Then they were detected by spraying with Dragendorff's reagent. The Rf values of pure markers and test samples were compared for the presence and intensity of major strychnine and brucine alkaloids in the test samples (13).

Isolation and estimation of Strychnine and Brucine

The seed powder (200g) were mixed with 200ml suspension of 10% calcium hydroxide in water and left overnight at room temperature. Air dried and extracted with chloroform in a soxhlet extractor for 3 hr. The chloroform solution was then extracted 5 times with 25 ml of 5% sulphuric acid solution, and subsequently basified with 10% aqueous sodium hydroxide. After cooling, the crystals are filtered.1.5 volume of 50% ethanol was added, and the mixture is refluxed until most of the solid have been dissolved. After addition of a little activated charcoal, the solution is filtered hot and left overnight. The crystals of strychnine were filtered and washed with a little 50% ethanol. The mother liquor and washings were kept for the isolation of brucine.

Preparation of strychnine sulphate

The crude strychnine was dissolved in 9 volumes of boiling water, and 15% sulphuric acid solution added slowly, with stirring, until the reaction is slightly acid to Congo red. Activated charcoal was added and the solution refluxed for 1hr and filtered hot. The strychnine sulphate crystals obtained after cooling. The resulted crystals filtered and washed with cold water.

Purification and estimation of strychnine

Strychnine sulphate was dissolved in 15 volumes of water at 80˚C and neutralized with 10% aqueous sodium carbonate; after addition of charcoal, the solution is filtered hot. Strychnine precipitated on addition of aqueous sodium carbonate and by cooling. The precipitate was filtered on a Buchner funnel and washed with cold water. The precipitate was recrystallized from ethanol, which yields pure strychnine. The strychnine is estimated at 254nm by comparing with standard marker by spectroscopy.

Purification and estimation of Brucine

The mother liquor remained after strychnine separation was concentrated *in vacuo* on a water bath until most of the alcohol has been removed. The residue was acidified to pH 6 with dilute sulphuric acid and then concentrated to a volume of 5 ml. After standing in a refrigerator overnight the product was filtered and washed with cold water. Brucine sulphate was purified by dissolving in 4.5 volumes of hot distilled water and boiled with a little charcoal for 1 hr. It was filtered hot and left in a refrigerator for several days. Brucine was recovered from the sulphate in a manner analogous to that outlined for strychnine. The brucine was recrystallized from aqueous acetone.

The brucine was estimated at 264 nm by comparing with standard brucine by spectroscopy (14).

Animals

Adult, healthy Wister rats (150-200 g) of either sex were used for the study, obtained from Venkateshwara Enterprises, Bangalore, Karnataka. After one week of acclimatization the animals were used for further experiments. They were housed in well ventilated room under standard husbandry conditions, fed with standard rodent pellet diet (Lipton India Ltd., Mumbai, India) and with tap water *ad libitum*. All the animal protocols were approved by institutional animal ethical committee (Reg. no. 157/1999/CPCSEA) as per the Indian CPCSEA guidelines.

Hepatoprotective activity

The hepatoprotective activity of study material was assessed by using the method of Suja *et al.* (15). The albino rats of either sex were divided into six groups of 6 animals each and were fasted for 24 hr with water *ad libitum*. The animals of group I and II received 1 ml of saline, whereas animals of group III received silymarin (100mg/kg, p o), IV group received UNV (52mg/kg, p o), V and VI received PNV1 and PNV2 in the dose of 520mg/kg, p o for 5 days. Animals of group I received 1ml/kg of liquid paraffin on 2^{nd} and 3^{rd} day, whereas animals of group II, III, IV, V and VI received 2ml CCl_4:paraffin (1:1) subcutaneously on 2^{nd} and 3^{rd} day, 30 min after treatment with vehicle,silymarin,UNV,PNV1 & PNV2. The blood samples were collected by cardiac puncture under mild ether anesthesia. All the animals were sacrificed by cervical dislocation and liver tissues were collected.

Assessment of liver function

Blood samples collected were used for the estimation of biochemical markers of hepatic injury viz. Serum glutamate pyruvate transaminase (SGPT) (16), serum glutamate oxaloacetate transaminase (SGOT) (17), alkaline phosphatase (ALP) (18), serum bilirubin(19), cholesterol (20). Liver tissues were collected from the above groups of animals were subjected to the determination of tissue glutathione (GSH) level and lipid peroxidation. Tissue GSH levels were determined by using the method of Ellamn and modified by Aykae *et al* (21, 22). Similarly liver tissue lipid peroxidation was assessed by the method of Buege and Steven (23).

Statistical analysis

Results were expressed as mean ± SE. Statistical analysis was performed with one way analysis of variance (ANOVA) followed by Turkey-Kramer multiple comparisons test. *P* value less than 0.05 was considered to be statistically significant when compared to control.

Results and Discussion

The preliminary phytochemical investigation showed the presence of strychnine, brucine alkaloids, tannins, carbohydrates, proteins, fixed oils in 70% alcohol extracts of processed and unprocessed samples. The loganin glycoside is present in unprocessed extract but absent in processed samples. The presence of strychnine and brucine was confirmed by comparing with standard markers of strychnine and brucine by Rf values by TLC. In the earlier report there is a reduction in the toxicity by ten folds upon detoxification of seeds (11). So this is well correlated with the fact that the alkaloid content reduces with detoxification, which further influences the toxicity. In the present study $1/5^{th}$ of the reported LD_{50} values were selected for assessing antihepatotoxicity property.

In the control group (+ve control) CCl_4 significantly enhanced the biochemical markers of liver injury like SGPT, SGOT, ALP, Bilirubin, and Cholesterol. Pre-treatment with UNV (52mg/kg), PNV1 & PNV2 (520mg/kg) significantly reduced the elevated levels of all the above mentioned biochemical indicators (Table 2). In addition, CCl_4 depleted the levels of liver tissue GSH and enhanced the tissue lipid peroxidation. This is in conformity with the earlier report (24). Pre-treatment with UNV and PNV1 & PNV2 prevented the depletion of tissue GSH levels and reduced lipid peroxidation (Table 3). Pre-treatment with PNV1 & PNV 2 significantly reduced the elevated levels of biochemical markers to near normal and significantly prevented the depletion of tissue GSH and reduced lipid peroxidation (Table 1 & 2). There is a significant protection showed by pretreatment with PNV2 when compared with PNV1 (Table 2 & 3). Evidently, the hepatoprotective effects of PNV1 & PNV 2 were near to that of standard i.e. Silymarin. Both the PNV1 & PNV2 detoxified samples used in the study showed significant protective property than un processed (UNV) group. However the un processed UNV was found to be less potent than that of standard drug. However PNV1and PNV2 was found to be better than the UNV.

The tissue glutathione was found to be depleted upon CCl_4 intoxication, indicate that the tissue damage is due to over powering the inbuilt free radical

scavenger mechanisms. This tissue GSH depletion was inhibited by the pre-treatment with test samples. Similarly Lipid peroxidation induced by CCl_4 treatment was reversed by test sample. The results are compiled in the table 3.
The mechanism of hepatic damage by CCl_4 is well documented. CCl_4 is metabolized by Cytochrome P-450 enzyme system to highly reactive trichloromethyl radical (CCl_3•). This in turn reacts with molecular oxygen and gets converted to trichloromethyl peroxide radical ($OOCCl_3$). This radical forms covalent bonds with sulfhydryl group of several membrane molecules like glutathione, which is considered as the initial step in the chain of events leading to lipid peroxidation and hepatic tissue destruction (23, 25 & 26). PNV1, PNV2 & UNV has significantly reduced the elevated serum biochemical markers of hepatic injury (Table 1). Detoxified samples showed more significant reduction of serum biochemical markers, tissue LPO and increase in tissue GSH levels compared with un processed sample. It is apparent from the present results that the detoxification process and antioxidant principles of processed nux-vomica preparations prevented the formation of trichloromethyl peroxy radical thereby reducing tissue damage when compared with unprocessed preparation. The UNV group also showed significant decrease in elevated serum markers when compared with carbon tetrachloride group animals. This is further confirmed by the fact that PNV1&2 has shown significant restoration of GSH and reduced lipid peroxidation. Therefore the hepatoprotective activity of PNV1 and PNV2 may be due to its antioxidant potential.

In the present study the PNV1 & PNV2 possesses better hepatoprotective activity and this may be attributed to the antioxidant principles that are present in the plant. During detoxification process the alkaloids like strychnine and brucine are diffused from the seeds to the cow urine in which the resulted cow urine is replaced every night for 7 nights. During this detoxification process, the toxic alkaloids strychnine and brucine from seeds have been removed by diffusion process in to cow urine. Further the seeds are boiled or kept in cow milk in which the alkaloids strychnine and brucine also diffused from the seeds into cow milk. It is also confirmed by TLC and chemical tests. The toxic alkaloids like strychnine and brucine concentration were decreased and loganin absent in detoxified seeds.

According to the chinese system of medicine the studies demonstrated that the traditional detoxification process may be significantly reduce toxic side effects of Semen Strychni (27, 28). It was reported that the chinese traditional detoxification method for Semen Strychni seeds decreased the concentration of strychnine and brucine but also changed the intrinsic alkaloids such as brucine and strychnine into their N-oxidative derivatives with less toxic or non- toxic and effective pharmacological activity.

The studies reported that the content of the of isostrychnine, isobrucine, strychnine N-oxide, and brucine N-oxide after the thermal treatments(28). However no such reports are available regarding the influence of Shodhana on the phytochemical profile of the plant. From our studies it is clear that the concentration of prinicple alkaloids (Strychnine & Brucine) is reduced to the extent of about1/3rd in PNV1 or the strychnine content is reduced to 1/5th and the brucine content is reduced to 1/3rd in PNV2 when compared with unprocessed nux-vomica (of their original concentration) while toxicity is reduced to the extent of 1/10th of their original toxicity upon subjecting it to shodhana process. This may be due to the fact that some quantity of these alkaloids are diffused out in urine and milk and some amount might have been converted into its derivatives (i.e. N-oxides) with lesser toxicity and higher potency. Further studies are needed not only to confirm but also to identify the phytochemicals responsible enhanced potency and reduced toxicity.

Conclusions

It may be concluded from the studies that subjecting to shodhana process enhanced the potency (hepatoprotective property) and it is due to reduced strychnine and brucine concentrations and eliminating the toxic loganin glycoside. This finding is in conformity with the claims of ancient scriptures that detoxification (shodhana) enhances the efficacy and reduces the toxicity. Therefore the Shodhana process should be undertaken for the detoxification of nux-vomica is clinically justifiable.

Acknowledgements

The authors are very much thankful to Shri Sha. Bra. Chandramouleshwara Swamiji, President and Shri T.M. Chandrashekharaiah, Secretary, TMAE Society, Harapanahalli for their encouragement in carrying out this work. We are also very much thankful to Dr. Niketan Dept. of Pathology, JJ Medical College, Davanagere, Karnataka.

Table 1: Effect of detoxification (Shodhana) process of seeds of nux-vomica on strychnine and brucine content

Sample	Strychnine (%w/w)	Brucine (%w/w)
UNV	**1.5**	**0.85**
PNV1	**0.41**	**0.30**
PNV2	**0.3**	**0.24**

Table 2: Effect of detoxification (Shodhana) process of seeds of *Strychnos nux-vomica* against CCl_4 induced hepatotoxicity in albino rats.

Treatment	SGOT (U/L)	SGPT (U/L)	ALP(U/L)	Total Bilirubin (mg/dl)	Direct Bilirubin (mg/dl)	Cholesterol (mg/dl)
Control (saline 0.5ml po, 5days)	53.53 ± 2.39	56.38 ± 1.43	117.4 ± 3.13	0.88 ± 0.07	0.38 ± 0.06	113.33 ± 4.85
CCl_4(2ml/kg)	305 ± 3.84	321.01 ± 6.63	279.46± 11.07	4.52 ± 0.27	1.48 ± 0.16	174.98 ± 2.63
Silymarin (100mg/kg,po 5 days) + CCl_4	70.58± 3.51*** (76.85)	66.28± 2.62*** (79.35)	90.88± 2.37*** (67.48)	1.06± 0.05*** (76.54)	0.255± 0.01*** ((82.77)	118.81± 4.03*** (32.10)
UNV (52mg/kg,po) + CCl_4	106.58± 6.14*** (65.24)	112.83± 8.35*** (65.10)	110.45± 7.63*** (60.06)	1.21± 0.06*** (73.23)	0.32± 0.02*** ((78.37)	150.33± 3.98*** ((14.08)
PNV1 (520mg/kg, po)+ CCl_4	99.53± 6.62*** (67.36)	101.6± 6.67*** (68.53)	102.31± 7.83*** (63.39)	1.21± 0.07*** (73.23)	0.29± 0.02*** ((80.40)	126.88± 3.62*** (27.64)
PNV2 (520mg/kg, po)+ CCl_4	69.61± 3.62*** (77.17)	67.9± 3.63*** (78.84)	91.98± 1.67*** (67.08)	1.14± 0.12*** (74.77)	0.27± 0.02*** ((81.75)	122.03± 2.80*** ((30.26)

Values are mean ± SE from 6 animals in each treatment ***P <0.001 Vs CCl_4 group. Figures in parentheses are the percentage of protection

Table 3: Effect of Detoxification (Shodhana) process on hepatic tissue levels of GSH and lipid peroxidation in the CCl_4 induced liver toxicity in rats

Treatment	Tissue levels of GSH	Tissue lipid peroxidation
Control (saline 0.5ml po, 5days)	0.266 ± 0.012	0.0813 ± 0.005
CCl_4 (2ml/kg sc)	0.0695 ± 0.0132	0.236 ± 0.021
Silymarin (100mg/kg, po 5 days)	0.250 ± 0.0256*** (72.2)	0.0821 ± 0.004*** (65.21)
UNV (52mg/kg, po) + CCl_4	0.169 ± 0.017*** (58.87)	0.122 ± 0.015*** (48.30)
PNV1 (520mg/kg, po)+ CCl_4	0.209 ± 0.007*** (66.74)	0.0906 ± 0.01*** (61.61)
PNV2 (520mg/kg, po)+ CCl_4	0.246 ± 0.015*** (71.74)	0.083 ± 0.004*** (64.83)

Values are mean ± SE from 6 animals in each treatment ***P <0.001 Vs CCl_4 group. Figures in parentheses are the percentage of protection.

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