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Effect of Shodhana on chemical and toxicological profile of a potent Indian medicinal plant *Nerium indicum*

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Abstract
Ayurveda emphasizes the need of Shodhana (purification / detoxification) for poisonous herbal, animal and mineral drugs. The drugs which are poisonous should be administered to the patients after proper Shodhana process. This process enhances the therapeutic efficacy and quality of the crude drugs and makes them potent and less toxic. The knowledge of traditional purification/detoxification methods is mentioned in various Ayurvedic literatures and text. Here the roots of *Nerium indicum* was detoxified using Shodhana process, the crude roots and the detoxified roots of *Nerium indicum* was tested for chemical and toxicological profile (Acute oral toxicity evaluation in mice). The results showed that the toxicity of *Nerium indicum* roots was reduced after Shodhana process. The Methanolic extract of roots of *Nerium indicum* was investigated for presence of bioactive constituents using column chromatography technique. Two chemical constituents were isolated from the species and were analyzed using ^{1}H NMR, ^{13}C NMR, FTIR and mass spectroscopy. The isolated compounds were identified as Lupeol and 5-hydroxyflavone.

Keywords: *Nerium indicum,* Shodhana, Acute oral toxicity, Lupeol and 5-hydroxyflavone.

Introduction
Ayurveda, is one of the ancient science of life, practiced for attaining complete health. Charaka identified the necessity of complete knowledge on both poisonous and non-poisonous herbs and their utility in therapeutics. It is claimed in the ancient manuscripts of Ayurveda that the 'Visha' becomes 'Amrita' after logical use and the physicians of Ayurveda successfully employed these drug in a number of diseases after proper purification using some of the specific media. Acharya Charaka has elaborately mentioned various poisonous drugs with their adverse effects and provided its management to reduce its toxicity. Many poisonous drugs are used as a single drug or a compound formulation in Ayurveda after proper Shodhana (Purification/detoxification) process. *Nerium indicum* (Karaveera in Ayurveda) is mentioned as one of the poisonous drug/plant in the group of Upavisha (moderately poisonous drugs) in Ayurvedic texts. Apart from these, screening of natural sources such as plant extracts and fermentation products in search of new pharmacologically active compounds led to the discovery of useful drugs that play key role in the treatment of human diseases.

Literature Review

Table 1: List of compounds isolated from *Nerium indicum*

S. No	Compound name	Reference
1	Oleanderol	1
2	Oleanolic acid	2
3	Kanerin	3
4	3α-hydroxy-urs-18,20-dien-28-oic acid (Kanerocin)	4
5	Gentiobiosyl nerigoside	5
6	Cis-Karenin, Trans-Karenin	6
7	3β-hydroxy-5β-carda-8,14,16,20(22) tetraenolide	7
8	Oleanderic acid, Oleanderolide	8
9	20β,28-epoxy-28α-methoxytaraxaster-21en-3β-ol	9
10	20β,28-epoxy-28α-methoxytaraxasteran-3β-ol	10
11	4-oxooctyl-2-hydroxyl-undecanoate	11
12	Odoroside, uzarigenin	12
13	Trans5-O-caffeoylquinic acid	13
14	D^{16}-Dehydroadynerigenin	14

Pharmacological Review

Administration of chloroform extract of *Nerium indicum* to diabetic rats significantly increased the body weight and decreased blood glucose level. This effect could conclude the anti-diabetic activity [15] of *Nerium indicum*. The flower extract of *Nerium indicum* showed a significant reduction in ulcer index which conclude to possess anti-ulcer activity [16] of *Nerium indicum.*

The Methanol extract of leaves and Methanol extract of flowers of *Nerium indicum* was analyzed for Antioxidant activity (AOA) in terms of DPPH free radicals, indicated that the methanol extracts of *Nerium indicum* flowers have more potent antioxidant activity [17] than leaves. Extracts of flowers of *Nerium indicum* was treated orally in albino mice at the dose level of 400 mg/kg body weight for Central Nervous System activity, delay in the onset of Pentylenetetrazole and Maximal electroshock induced seizures as well as decrease in the severity. This effect could conclude the CNS activity [18] of *Nerium indicum.*

It was reported, the aqueous extract of *Nerium indicum* bark is an effective insecticide against Blatta, orientalis, which exhibited molluscicidal activity [19].

Objective

From the literature review, it was observed that the *Nerium indicum* roots was found to possess toxic principles like Oleandrin and Oleandrigenin, which makes the roots poisonous. Though it is poisonous, most known active effects of *Nerium indicum* is due to compounds like Neriodorin, Odorin, and Oleandrin when administered in a proper form and proper dosage. The fundamental goal and objective of this research work was to detoxify the roots of *Nerium indicum* using Shodhana process, the crude roots and the detoxified roots of *Nerium indicum* was tested for chemical and toxicological profile and to isolate bioactive constituents from the Methanol extract of dried roots of *Nerium indicum.*

Materials and Methods

Collection of Plant Material

The roots *of Nerium indicum* were collected, dried and authenticated by a taxonomist at NBRI Lucknow in the month of November 2017. The dried roots were weighed and divided into two parts. One part was used as unshodith (pre-shodhana) and other was used as shodith (post-shodhana procedures). Both unshodith and shodith samples were extracted by hot extraction using Soxhlet apparatus and cold extraction using magnetic stirrer.

Procedure for shodhana

200 g of roots of *Nerium indicum* was weighed and cleaned with dry dust free cloth. A 60 × 60 cm muslin cloth was taken and washed with water twice. The cleaned roots of *Nerium indicum* was placed on the muslin cloth and four edges of the cloth was brought together forming a pouch, which was knotted using a thread. The ends 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 3hrs. The change in temperature and color of the milk was noted every half an hour. Continuous stirring was performed throughout the heating process. After 3hrs the pouch was taken out of the milk and opened. The roots were washed with reverse osmosis water. The root were spread on butter paper, shade dried, powdered and stored in a glass bottle for further use and labelled as “shodith roots” of *Nerium indicum*[20].

Extraction Process

Hot extraction using soxhlet apparatus

The fine powder of *Nerium indicum* roots (5g each unshodith and shodith root powder) was successively extracted by soxhlet extractor using Hexane, Ethyl acetate, Methanol, and Aqueous methanol and also extracted directly with methanol at 40-60 °C for 12h. The resulting extracts was then evaporated under reduced pressure using a rotary evaporator. All the extracts were packed in air tight vials and subjected for preliminary phytochemical screening, LC-MS study. The yield and percentage yield (%) of above extracts obtained from both unshodith and shodith root powder of *Nerium indicum* is mentioned in the table: 4.

Cold Extraction

The fine powder of *Nerium indicum* roots (1g each unshodith and shodith root powder) was extracted with methanol (50ml) by magnetic stirrer at 1600rpm for 6hrs without application of heat. The solution obtained was then subjected to vacuum filtration and the extract was concentrated using Rota evaporator. The marc obtained was further extracted with aqueous methanol (1:1). The solution obtained was then subjected to vacuum filtration and the extract obtained was concentrated using Rota evaporator. Both the extracts was packed in air tight vials and subjected for preliminary phytochemical screening, LC-MS study. The yield and percentage yield (%) of above extracts obtained from both unshodith and shodith root powder of *Nerium indicum* is mentioned in the table: 5.

Preliminary phytochemical screening of extracts of *Nerium Indicum*

1) Tests for alkaloids

In this 3ml of given extract was treated with 3ml of HCl and stirred on water bath. From the above solution 1ml each, taken into three test tubes for Dragendorff’s test, Wager’s test and Hager’s test.

A) Dragendorff’s Test

The acid layer of extract when treated with few drops of Dragendorff’s reagent (Potassium bismuth iodide), formation of red/orange precipitate indicates the presence of alkaloids.

B) Wagner’s Test

The acid layer of extract when treated with few drops of Wagner’s reagent (solution of iodine in potassium iodide) gives reddish brown precipitate indicates the presence of alkaloids.

C) Hager’s Test

The acid layer of extract when mixed with few drops of Hager’s reagent (Saturated solution of picric acid), formation of yellow colour precipitate indicates the presence of alkaloids.

2) Tests for glycosides

A) Molisch’s Test

The extract solution when treated with few drops of molisch's reagent (α-naphthol) and 2ml of conc.H_2SO_4 is added slowly from the sides of the test tube, formation of purple ring at the junction of two liquids indicates the presence of glycosides.

B) Keller-Kiliani Test
The chloroform solution of extract when treated with few drops of glacial acetic acid in 2ml of $FeCl_3$ solution and few drops of conc.H_2SO_4 is added from the sides of test tube results in separation of two layers, lower layer shows reddish brown and upper layer shows bluish green colour, indicates the presence of glycosides.

3) Tests for carbohydrates
A) Molisch's Test
The extract when treated with Molisch's reagent (α-naphthol) and conc.H_2SO_4 along the sides of the test tube, a violet ring indicates the presence of carbohydrates.

A) Fehling's Test
The extract when treated with Fehling's A and B solutions gives an orange red precipitate showing the presence of reducing sugar.

4) Tests for terpenoids
A) Liebermann Burchard's Test
The chloroform solution of the extract (2ml) treated with few drops of acetic anhydride and mixed well, 2ml of conc.H_2SO_4 is added from the sides of test tube, formation of red ring indicates the presence of terpenoids.

B) Salkowski Test
A few drops of concentrated H_2SO_4 is added to the chloroform extract, shaken and allow it to stand for few minute, a reddish brown ring at the interface indicates the presence of terpenoids.

5) Tests for steroids
A) Salkowski Test
A few drops of conc.H_2SO_4 is added to the $CHCl_3$ (2ml) extract, shake and allowed it to stand for few minutes, formation of brown ring indicates the presence of steroids.

B) Liebermann Burchard's Test
To the chloroform solution of the extract (2ml), add few drops of acetic anhydride are and mixed well. 1 ml of conc.H_2SO_4 is added from the sides of test tube, a bluish green ring is formed at the junction of two layers indicates the presence of steroids.

6) Tests for flavonoids
A) Shinoda Test
Methanolic solution of the extract (2ml) was treated with few fragments of magnesium ribbon and conc.H_2SO_4 (1ml), formation of magenta or crimson pink colour indicates the presence of flavonoids.

B) Alkaline Test
A few drops of sodium hydroxide solution was added to little quantity of alcoholic extract. An intense yellow colour is formed which turns colourless upon addition of dilute HCl, indicates the presence of flavonoids.

7) Test for phenols
A) Lead Acetate Test
A few drops of lead acetate solution (10%) was added to the alcoholic solution of the extract (2ml), formation of white precipitate indicates the presence of phenols.

8) Tests for tannins
A) Ferric chloride Test
2ml of extract was dissolved in 2ml of distilled water and treated with 1%ferric chloride solution, the extract gives blue, green or brownish colour indicating the presence of tannins.

9) Test for Amino acids
A) Ninhydrin Test
To 2ml of extract, 5 drops of 0.25%w/v of ninhydrin reagent was added and boiled for few minutes. Formation of blue colour indicates the presence of amino acids.

LC-MS study of Extracts
All the extracts obtained from hot soxhlet extraction and cold maceration was submitted to LC-MS study profile.
Column: Luna C_8 (250×4.6mm, 5.0u)
Mobile phase: Gradient
Injection volume: 10µl
Flow rate: 1.0ml/min

Preparation of column
The unshodith macerated methanol extract of roots of *Nerium indicum* was subjected to column chromatography. A cotton plug was placed at the bottom of pre-cleaned and dried column. Silica gel (100-200 mesh) was taken and mixed with hexane to form a rapidly pourable mixture (slurry). It was poured into the column and allowed to set.

Preparation of sample
The methanol extract (15g) was dissolved in acetone (200 ml) and silica gel was added (45g). The acetone was removed under vacuum on Rota evaporator and the powder obtained was transferred to a column of silica gel (100-200 mesh) set in hexane and a cotton plug was placed on it.

Gradient elution technique
In order to isolate the compounds in a pure state from methanol extract depending on its solubility, gradient elution technique was used. The column was eluted successively with Hexane first, Hexane: Ethyl acetate (95:5), Hexane: Ethyl acetate (85:15). Fractions of 100 ml were collected. All the fractions was monitored by TLC. The TLC spots were visualized by various visualizing agents like 5%H_2SO_4, Anisaldehyde reagent, UV chamber, Iodine chamber etc. The fractions were pooled according to their TLC patterns as follows:

Table 2: Fractionation of unshodith macerated methanol extract of roots of *Nerium indicum*

Eluent	Fraction No	Group No	Compound
Hexane: Ethyl acetate 95:5	1-3	I	A
Hexane: Ethyl acetate 85:15	3-5	II	B

Acute oral toxicity study of unshodith and shodith methanol extracts of roots of *Nerium indicum* in mice
Principle of the study
The acute oral toxicity study was conducted as per OECD guidelines by fixed dose method adopted by OECD (420). The study involves a preliminary sighting study in small number of animals in order to derive the dose effect relation for toxicity and mortality and to provide information on dose selection for the main study. In the preliminary sighting study, the effect of various dosed administered to single animals of each sex was investigated in a sequential manner. The sighting study generally yields information on the dose –

toxicity relationship including an estimate of the minimum lethal dose and maximum tolerated dose. In the main study, the test sample was administered to groups of 5 males and 5 female animals at one of the fixed doses (5, 50,300 and 2000mg/Kg). Animal ethics committee approval was obtained from the institute for animal usage.

Procedure

Experimental Animals

Balb/c mice (20-25 g) were randomly selected and housed in poly- propylene cages at room temperature (22 ± 2°C) with proper ventilation. Prior to the experiments, animals were fed with standard diet for one week in order to adapt laboratory conditions. They were fasted but allowed free access to water 16 – 18 h prior to administration of the test sample.

Sighting Study

In the sighting study, the effect of various doses was investigated in single animal of single sex. Dosing was sequential allowing at least 24hrs before dosing the next animal. All animals were carefully observed for signs and symptoms of toxicity continuously up to 24hrs and later up to 7 days. The sighting study was conducted with sequential doses of 5, 50, 300 and 2000 mg/kg of the test sample. If the initial dose chosen did not produce severe toxicity, the next higher dose was selected. In this sighting study, the dose that produced evident toxicity but not death was identified. Dose escalation was continued up to 2000mg/kg.

Main Study

At least 5 animals (5 females) in each group was used for the dose level in this study. Animals were fasted overnight and the test extracts was administered as 2% gum acacia suspension by oral route. The dose used in this study was selected from one of the four levels 5mg/kg, 50mg/kg, 500mg/kg and 2000 mg/kg i.e. the dose that produced evident toxicity but not mortality from sighting study. The animals were observed for signs and symptoms of toxicity apart from the cage side observations which include changes in skin and fur, eyes, mucous membrane, respiratory, circulatory, and autonomic and central nervous systems. The animals were also observed for mortality during the study period.

V. Results and Discussion

Table 3: Weight of the dried roots before and after shodhana process

Weight of the dried roots before shodhana	Shodhana process using milk (800ml) Shade dried for 14 days	Weight of the dried roots after shodhana
200g		194g

Table 4: Yield and Percentage yield of successive extracts of unshodith and shodith root powder of *Nerium indicum* using hot extraction

S. No	Solvents	Weight of the sample	Unshodith extract Yield (g)	Unshodith extract %yield	Shodith extract Yield (g)	Shodith extract %yield
1	Methanol	5g	0.8391	16.782	0.7997	15.994
Successive solvent extract						
2	Hexane	5g	0.2251	4.502	0.2129	4.258
3	Ethyl acetate	-	0.1106	2.212	0.1337	2.674
4	Methanol	-	0.6683	13.366	0.6117	12.234
5	Aq. Methanol	-	0.2069	4.1381	0.2196	4.392

Table 5: Yield and Percentage yield of unshodith and shodith root powder of *Nerium indicum* using cold extraction

S. No	Solvents	Weight of the sample	Unshodith extract yield (g)	Unshodith extract %yield	Shodith extract yield (g)	Shodith extract %yield
1	Methanol	1g	0.1371	13.71	0.1133	11.33
2	Aq. Methanol	-	0.0253	2.53	0.0336	3.36

Table 6: Preliminary phytochemical screening of extracts (unshodith) of *Nerium indicum* roots

S.NO	Phytochemical Test	Maceration		Soxhlation				
		Methanol	Aqueous Methanol	Direct Methanol	Hexane	Ethyl Acetate	Methanol	Aqueous Methanol
1	**Alkaloids**							
	a) Dragendroff's test	-	-	-	-	-	-	-
	b) Hager's test	-	-	-	-	-	-	-
	c) Wagner's test	-	-	-	-	-	-	-
2	**Glycosides**							
	a) Molisch test	+	+	++	-	+	+	++
	b) Keller-killani test	+++	+++	+	-	+	+	+++
3	**Carbohydrates**							
	a) Molisch test	+	+	+	-	+	+	+
	b) Fehling's A&B test	+	+	+	-	-	+	+
4	**Terpenoids**							
	a) Liebermann test	+	+	+	++	+	+	+
	b) salkowski test	+	-	+	+	+	+	-
5	**Phytosterols**							
	a)Salkowski test	+	-	+	+	+	+	-
	b)Liebermann test	-	-	-	+	-	-	-
6	**Flavonoids**							
	a) Alkaline test	+	+	-	-	-	+	+
	b) Shinoda test	+	-	-	-	-	+	-

7	Phenols							
	Lead acetate test	+	-	+	-	-	+	-
8	Amino Acids							
	Ninhydrin test	-	-	-	-	-	-	-
9	Tannins							
	Ferric chloride test	++	-	+	-	-	+	-

(+) indicates positive test and (-) indicates negative test

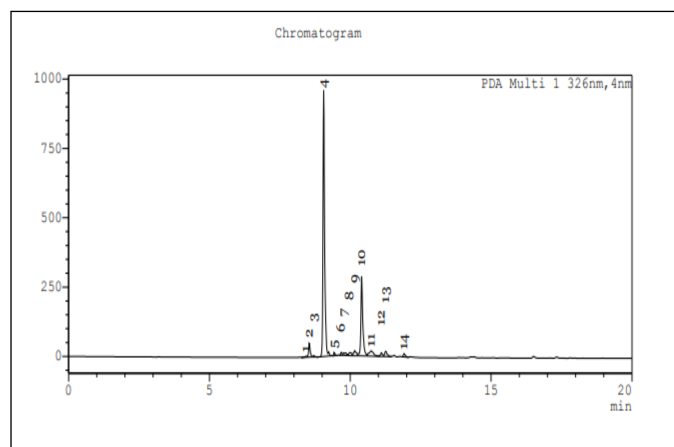
Table 7: Preliminary phytochemical screening of extracts (shodith) of *Nerium indicum* roots

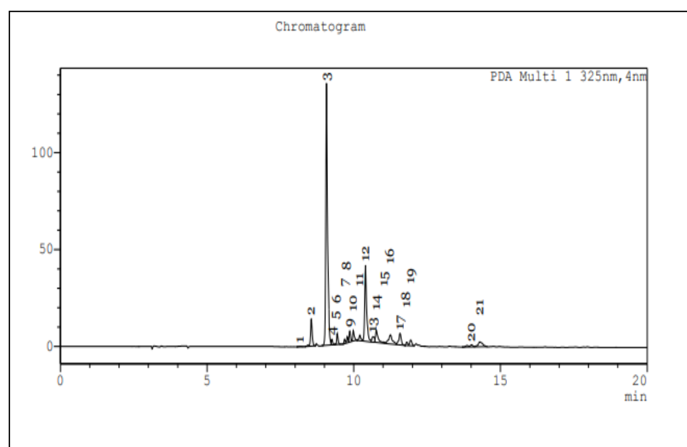
S.NO	Phytochemical Test	Maceration		Soxhletion				
		Methanol	Aqueous Methanol	Direct Methanol	Hexane	Ethyl acetate	Methanol	Aqueous Methanol
1	Alkaloids							
	a) Dragendorff test	-	-	-	-	-	-	-
	b) Hager test	-	-	-	-	-	-	-
	c) Wagner's test	-	-	-	-	-	-	-
2	Glycosides							
	a) Molisch test	+	+	+	-	+	+	+
	b) Keller-killani test	+	+	+	+	+	+	+
3	Carbohydrates							
	a) Molisch test	+	+	+	+	+	+	+
	b) Fehling's A&B test	+	-	+	-	-	+	+
4	Terpenoids							
	a) Liebermann test	+	+	+	+	+	+	+
	b) salkowski test	+	+	+	+	+	+	+
5	Phytosterols							
	a)Salkowski test	-	-	-	+	-	-	-
	b)Liebermann test	-	-	-	+	-	-	-
6	Flavonoid							
	a) Alkaline test	-	-	-	-	-	-	-
	b) Shinoda test	-	-	-	-	-	-	-
7	Phenols							
	Lead acetate test	+	-	+	-	-	-	-
8	Amino Acids							
	Ninhydrin test	-	-	-	-	-	-	-
9	Tannins							
	Ferric chloride test	+	-	-	-	-	-	-

The root powder of *Nerium indicum* (unshodith and shodith) extracted by hot extraction using soxhlet apparatus and cold extraction using magnetic stirrer, the extracts were submitted for phytochemical investigation. The results showed reduction in glycoside content (Oleandrin is a glycoside, a toxic principle of *Nerium indicum*) after performing shodhana process. This was confirmed by the difference in the intensity of color in keller-killani test between unshodith and shodith macerated methanol extract.

LC-MS Report of Extracts

From all the extracts the major difference in the L-MS profile was observed between unshodith macerated methanol and shodith macerated methanol extract. Thus the unshodith macerated methanol and shodith macerated methanol extract was submitted for acute oral toxicity evaluation in mice in order to determine the reduction in toxicity by shodhana process.





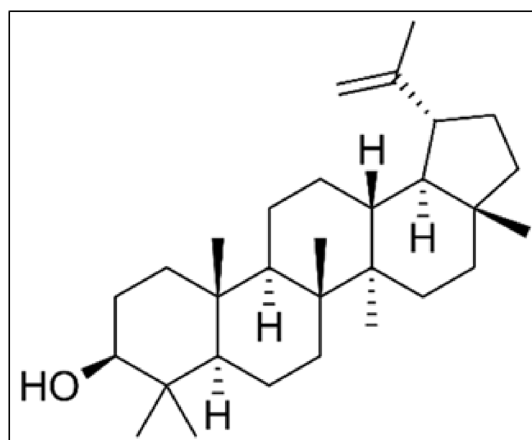
1a 1b

Fig 1: LC-MS data of unshodith macerated methanol (1a) and shodith macerated methanol (1b) extracts of roots of *Nerium indicum.*

Structural elucidation and characterization

Structure of compound A

Compound A was isolated as white solid, M.P: 212-214°C, $[\alpha]_D$: +27.2° at 20° C/D (c = 4.8 in chloroform) analysed for $C_{30}H_{50}O$ and revealed a molecular ion peak at *m/z* 426 $[M]^+$. The IR spectrum of compound **A** showed very intense broad band at 3311 cm^{-1} and moderately intense band at 1189 and 679 cm^{-1} was observed for the O-H bond vibrations of hydroxyl group. C-H vibrations due to unsaturated part was observed at 821 cm^{-1}. The corresponding C=C vibrations was shown around 1659 cm^{-1} as weakly intense band. The stretching and bending vibrations of methyl group was noticed as the intense band at 2923 cm^{-1}. Vibration due to methylenic part was shown by the band at 2855 cm^{-1}. The ^1H NMR spectra (recorded in $CDCl_3$, 300 MHz)showed seven tertiary methyl singlet's at δ 0.76, 0.78, 0.82, 0.91, 0.97, 1.02, 1.68; and one secondary hydroxyl group as doublet of doublets at δ 3.20. It also showed two olefinic protons at δ 4.56 and 4.70 representing the exocyclic double bond. data it was identified as Lupeol. Its ^1H and ^{13}C NMR data are summarized in table 8.



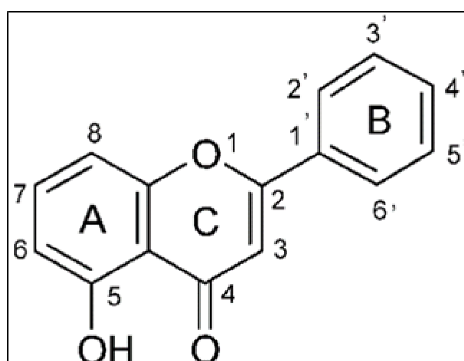
Lupeol

Table 7: ^1H NMR and ^{13}C NMR spectral data of lupeol

Position	^1H NMR(300 MHz, $CDCl_3$)δ(ppm)	^{13}c NMR (75 MHz $CDCL_3$)δ(ppm)			
		Position	δ (ppm)	Position	δ (ppm)
H-2A	1.61(s, 3H,H)	1	38.0	16	35.6
H-2B	1.54(s, 3H,H)	2	25.3	17	43.2
H-3	3.20 (1H, dd, J = 5.4, 10.6 Hz)	3	78.4	18	48.2
H-18	0.91(t, 1H,H)	4	38.6	19	47.8
H-21	0.76(s, 3H,H)	5	55.1	20	151.6
H-23	1.02(s, 1H,H)	6	18.1	21	30.0
H-25	0.82(s, 3H,H)	7	34.1	22	40.2
H-27	0.97(s, 3H,H)	8	41.2	23	28.2
H-28	0.78(s, 3H,H)	9	49.7	24	16.0
H-29a	4.56(s, 1H,H)	10	37.3	25	16.8
H-29b	4.70(s, 1H,H)	11	21.1	26	16.4
H-30	1.68(s, 3H,H)	12	27.5	27	15.1
		13	39.2	28	18.0
		14	42.6	29	108.6
		15	27.6	30	19.5

Structure of Compound B

Compound B was isolated as yellow crystal solid, M.P: 178-180°C and analysed for $C_{15}H_{10}O_3$, revealed a molecular ion peak at *m/z* 239 $[M+H]^+$. The IR spectrum shown an intense broad band between 2,600 and 3,200 cm^{-1} due to presence of OH group. A sharp and intense band present in this region at 3,059 cm^{-1} corresponds to the stretching vibration (C-H). The C=O vibration of compound B generates two intense bands placed at 1,654 and 1,615 cm^{-1}. The ^1H NMR spectra (recorded in $CDCl_3$, 300MZH) indicates the presence of OH at δ_H 12.67. The ^{13}C NMR spectra showed 13 signals. Comparison of above physical, chemical and spectral data of compound B with its reported data it was identified as 5-hydroxyflavone (Primuletin). Its ^1H and ^{13}C NMR data are summarized in table 9. The ^{13}C NMR of the compound showed 30 signals for the terpenoid of lupane skeleton which includes a carbon bonded to the hydroxyl group at C-3 position appeared at δ 78.4, while the olefinic carbons of the exocyclic double bond appeared at δ 151.6 and 108.6. Comparison of above physical, Chemical and spectral data of compound **A** with its reported



5-hydroxyflavone

Table 8: ^1H NMR and ^{13}C NMR spectral data of 5-hydroxyflavone

Position	^1H NMR(300 MHz, $CDCl_3$)δ(ppm)	^{13}c NMR 75 MHz $CDCl_3$	
		Position	δ (ppm)
H-3	7.12 (s)	2	164.7
H-6	6.8 (d, J = 8.3)	3	103.1
H-7	7.64 (t, J = 8.1)	4	182.2
H-8	7.26 (d, J=8.1)	5	159.8
H-3′-4′-5′	7.62(m)	6	111.0
H-2′-6′	8.1 (dd, J=7.8, 1.7)	7	135.9
OH	12.67(s)	8	107.4
		9	154.9
		10	110.1
		C-1′	130.53
		C-2′ C-6′	126.6
		C-3′ C-5′	129.2
		C-4′	132.7

Acute oral toxicity study of extracts of *Nerium indicum* roots in mice

Table 10: It represents the physical differences observed after administration of various doses of the given extracts of *Nerium indicum*

Dose	Extract 1A Mouse (1F)	Extract 1B Mouse (1F)
5 mg/kg	Normal	Normal
50 mg/kg	Normal	Normal
300 mg/kg	Mild convulsions were observed Died After 24hrs	Normal up to 14 days period No mortality was observed
2000 mg/kg	Continues Tremors, jerk movements, followed by severe convulsions were observed within 5 min period. Died after 15 min time period	Intermittent Tremors, jerk movements, followed by severe moderate convulsions were observed. Onset of action was delayed by 30 min. Animal Died after 3 hours of administration
1150 mg/kg	Continues Tremors, jerk movements, followed by severe convulsions (Clonic & Tonic). Response was observed after 15 min Died after 60 min of oral administration	Intermittent Tremors, jerk movements, followed by moderate Clonic and tonic convulsions were observed. Onset of action was delayed by 40 min. Died after 5 hours of oral administration
725 mg/kg	Intermittent Tremors, jerk movements, followed by moderate convulsions (Clonic Tonic). Response was observed after 20 minutes Died after 2 hours of oral administration	Intermittent Tremors, jerk movements, followed by moderate convulsions (Tonic). Onset of action was delayed by 60 minutes Died after 6 hrs. of oral administration
175 mg/kg	Normal	---

From the sighting study it was revealed that both the extracts 1A&1B were found to be toxic at 2000, 1150 mg/kg, and 725 mg/kg doses followed by mortality. Delayed onset of tonic convulsions and mortality were observed in 1B at 725, 1150, 2000 mg/kg which indicates the moderate safety of compound when compared to 1A. The extracts were further evaluated at low dose levels (300 mg/kg, 175 mg/kg) and found that 1A showed mild convulsions with delayed mortality but 1B did not show any convulsions and found to be safe at 300 mg/kg. 1A was tested again at intermediate dose 175 mg/kg and observed for 14 days period and it has not shown any toxic symptoms and mortality and animals were found to be safe.

Main Study

Table 11: The body weights on different time intervals for both the extracts (1A & 1B). (+) indicates the percentage increase in body weight compared to basal weights, n=5

Sample Code	Mouse No	Initial Body weight	Final Body weight	Percentage Change
1A 175 mg/kg	F1	21.10	23.40	10.90 (+)
	F2	20.80	21.60	3.85 (+)
	F3	22.10	23.40	5.88 (+)
	F4	21.50	22.70	5.58 (+)
	F5	21.90	22.70	3.65 (+)
Mean ± S.E.M		21.48 ± 0.24	22.76 ± 0.32	5.97 ± 1.31
1B 300 mg/kg	F1	22.00	22.90	4.09 (+)
	F2	23.10	24.50	6.06 (+)
	F3	22.60	23.50	3.98 (+)
	F4	23.50	24.10	2.55 (+)
	F5	20.90	21.90	4.78 (+)
Mean ± S.E.M		22.42 ± 0.45	23.38 ± 0.45	4.29 ± 1.31

Table 12: It represents the Maximum Tolerated Dose of both 1A and 1B extracts of *Nerium indicum* that do not show any toxicity symptoms

Species	Dose	1A (175 mg/kg)	1B (300 mg/kg)
Balb/c Mice N = 5	175mg/kg (unshodith-1A) 300 mg/kg (shodith-1B)	Normal No toxicity symptoms were observed during the study	Normal No toxicity symptoms were observed during the study

The main study was conducted for both extracts by choosing the one of the safe dose (175mg/kg (1A) and 300 mg/kg (1B)) from sighting study observations. They have not shown any toxic symptoms, mortality and observed there is an increase in body weight of animals(Table:12) for both the sample 1A and 1B in main study when compared to basal values at the doses 175mg/kg and 300 mg/kg respectively up to 14 days. From the above data obtained the Maximum Tolerated Dose of both 1A and 1B macerated Methanolic root extracts was found to be 175mg/kg and 300 mg/kg respectively (Table: 12).

Summary and Conclusion

All the extracts obtained from hot soxhlet extraction and cold maceration was submitted to LC-MS study. From all the extracts, the major difference in the L-MS profile was observed between unshodith macerated methanol and shodith macerated methanol extract. The results obtained from preliminary phytochemical screening indicates reduction in toxicity after performing Shodhana process, which was confirmed by the difference in the intensity of color in keller-killani test (Oleandrin, a toxic principle which is a potent cardiac glycoside causing tachycardia) between unshodith and shodith macerated methanol extract. Reduction in toxicity was further confirmed from acute oral toxicity evaluation in mice, which ensures safety and to determine Maximum Tolerated Dose (MTD) of plant extracts, as per the OECD guidelines 420. The results from sighting and main study confirmed that the extract 1A was found to be toxic at 2000, 1150 mg/kg, and 725 mg/kg doses followed by mortality, but delayed onset of tonic convulsions and mortality were observed in 1B at 725, 1150, 2000 mg/kg which indicated the moderate safety of compound when compared to 1A. Both the extracts 1A and 1B was tested at intermediate dose levels 175mg/kg and 300mg/kg. Extracts 1A and 1B have not shown any toxic symptoms and mortality at the doses 175mg/kg and 300mg/kg respectively up to 14 days. Thus Maximum Tolerated Dose of 1A (unshodith macerated methanol extracts of roots of *Nerium indicum*) was found to be 175mg/kg and Maximum Tolerated Dose of 1B (shodith macerated methanol extracts of roots of *Nerium indicum*) was found to be 300 mg/kg respectively. Thus Shodhana procedure mentioned in the Ayurvedic text and literature caused reduction in toxicity. Thus this study validates the process of shodhana based on chemical and toxicological profile of roots of *Nerium indicum*.

The present study shows detailed description, structural elucidation and characterization of the isolated compounds, Lupeol and 5-hydroxyflavone from the unshodith macerated methanol extract of roots of *Nerium indicum.*

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