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# *In Vitro* Anti-inflammatory property and phytochemical content of methanol extract of *Strychnos nux-vomica* L (Seeds)

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**Abstract**
The use of plants as medicine is an ancient practice common to all societies especially the Indian society. This practice continues to exist in the developing nations. It is on this basis that researchers keep on working with medicinal plants in order to produce the best medicines for physiological uses. The aim of present study was to isolate and identify bioactive compounds from the methanol extracts of *Strychnos nux-vomica* seeds using HPTLC profiling and GC-MS. HPTLC fingerprints scanned at 254 & 366 nm revealed the presence of phytoconstituents of highest concentration 39.64% & 65.35% respectively. Five constituents were eluted by GC-MS a major compound eluted at RT 19.119 mins was identified as 1-cyclohexylethyl ester of trifluoroacetic acid which accounted for 100%. The compounds were identified and confirmed by comparing their mass spectrum with the original spectrum obtained from the inbuilt libraries NIST-11. The methanol extract of *S nux- vomica* seeds showed significant anti-inflammatory activity.

**Keywords:** GC-MS, HPTLC, antiinflammatory, *S nux- vomica*

## Introduction
In recent years, traditional Indian Ayurvedic system of medicine is gaining increasing popularity worldwide. Analysis of components in the herbs is essential for the discovery and development of new drugs of natural origin as well as for herbal standardization, toxicological and biological investigations [1]. Fingerprinting (marker compound analysis) by chemical and validated chromatographic and spectroscopic techniques are gaining importance for standardization in the herbal medicinal formulations. The evaluation of an herbal product by metabolomic fingerprinting can be accomplished by appropriate methods, including GC-MS, HPTLC, FT-IR, NMR or a combination of these techniques [2, 3]. Such techniques also provide useful information about qualitative and quantitative composition of herbal medicines and their pattern recognition by chemometry [4].
*Strychnos nux-vomica* L. commonly known as snake-wood or nux-vomica tree is grown extensively in southern Asian countries and widely used in Chinese folk medicine [5, 6]. It has been effectively used in Chinese folk medicine for the treatment of liver cancer and associated pathological abnormalities for a long history [7]. Phytochemically the plant has been reported to contain alkaloids like strychnine, brucine, and strychnine and glycosides like loganin, coffee tannic acid and also traces of copper [8]. In pharmacology only few activities such as analgesic, apoptotic effect, antidepressant antidote for snake poisoning, antitumor has been proved [9-16]. Hence the present study was undertaken to evaluate its potential against inhibition of inflammation.

## Material and Methods
**Collection and processing of plant material:** The ripened fruits of *Strychnos nux-vomica* were collected from the western ghats of Karnataka and authenticated from the institutional Botanist, Department of botany, St. Aloysius College (Autonomous), Mangalore. Seeds were collected from ripened fruits of *S. nux- vomica*, the seeds were thoroughly washed with tap water twice and dried under shade for 7 days and ground into fine powder. After sieving they were transferred to air tight polyethylene zipper bags, labeled and stored till further use.

**Preparation of sample Extraction:** The powdered seeds were subjected for chloroform extraction for 48hrs at room temperature with continuous stirring and residue was collected by filtration. Then residue was subjected for methanol extraction for 48 hrs at room temperature. After extraction supernatant was collected by filtration and the solvent was evaporated by rotary evaporator for obtaining the methanol extracted compounds.

**GC – MS analysis:** GC-MS technique was used in this study to identify the phyto components present in the extracts. In the GC-MS-5975C (AGILENT) analysis, 1microlitre of sample was injected in GC-MS equipped with a split injector. The MS was operated in the E1 mode (70 eV). Helium was employed as the carrier gas and its flow rate was adjusted to 1.5ml/min. The analytical column length 30-60m×0.25mm with 0.25µm film thickness. The column head pressure was adjusted to 196.6kPa. Column temperature programmed from 70 ºC to 300 ºC at 10 ºC/min and from 200-300 ºC at 15 ºC/min withhold time 3 and 35 min respectively. The injector temperature was set at 250 ºC. The GC-MS interface was maintained at 240 ºC. The MS was operated in the acquisition mode scanning from m/z 40 to 700.0. In the full scan mode, electron ionization (EI) mass spectra in the range of 40-700(m/z) were recorded at electron energy of 70 eV. Compounds were identified by comparing mass spectra with library of the National Institute of Standard and technology (NIST-11).

**HPTLC:** 100µl of the sample was diluted 1ml with 900µl of methanol. 8µl of the above sample was applied on a pre-coated silica gel $F_{254}$ on aluminum plates to a band width of 7 mm using Linomat 5 TLC applicator. The plate was developed in Chloroform: Methanol (6.0:1.0). The developed plates were visualized in UV 254, 366 nm and then derivatised with vanillin sulphuric acid reagent and scanned under UV 254 and 366 nm. $R_f$, color of the spots and densitometric scan were recorded.

**HRBC Membrane Stabilisation Method**

**Heat induced haemolysis:** Methods of Sadique *et al*., 1989; Saket *et al*., (2010) were used to study HRBC by heat induced method [18, 19]. Aspirin was used as a reference drug. The reaction mixture (2ml) consisted of 1ml test sample at different concentrations and 1ml of RBC's suspension. Only saline was added to the control. All the centrifuge tubes containing reaction mixture were incubated in water bath at 56 ˚C for 30 minutes at the end of incubation the tubes were cooled under running tap water. The reaction mixture was centrifuged at 2500 rpm for 5mins and the absorbance of the supernatants was taken at 560nm. Experiment was performed in triplicates for all test samples. Percentage inhibition of haemolysis was calculated using the formula % inhibition of haemolysis = (A control – A test/ A control) X 100.

**Anti-lipoxygenase activity:** Anti Lipoxygenase activity was studied using linoleic acid as substrate and lipoxidase as enzyme (Shinde *et al*., 1999). To different concentration of extract, 1ml of lipoxydase enzyme was added and then incubated at 25 ˚C for 5mins.After which, 1ml of linoleic acid solution (0.6 mM) was added, and then mixed well and absorbance was measured at 234nm. Aspirin was used as a standard reference drug. Experiment was performed in triplicates for all test samples. Percentage inhibition of Anti-lipoxygenase activity was calculated using the formula % of inhibition = (A control – A test/ A control) X 100.

**Protein Denaturation method:** Methods of Mizushima and Kobayashi (1968) and Saket *et al*. (2010) followed with minor modifications [20]. The reaction mixture was consisting of 1% aqueous solution of bovine albumin fraction and 250µl of different concentration of extract. The sample extracts were incubated at room temperature 37 ˚C for 30minutes. Then it was heated at 57 ˚C and cooled. After cooling the turbidity was measured spectrophotometrically at 600nm. Experiment was performed in triplicates for all test samples. Percentage inhibition was calculated using the formula % of inhibition = (A control – A test/ A control) X 100.

**Results and discussion**

**GC-MS analysis**

The analysis and extraction of plant material play an important role in the herbal formulations. Hence the present study was aimed to find out the bioactive compounds present in the methanol extract of *S. nux-vomica* (SNV) seeds by using GC-MS. The active compounds with their peak number, concentration (peak area %) and retention time (RT) are presented in Table 1 and Fig. 1. The analysis showed the presence of five constituents were eluted by GCMS from the methanol extract of SNV. Out of five constituents 1 was not identified and as its mass fragmentation showed similarity below 60%. A major compounds eluted at RT 19.119 and 31.418 mins were identified as 1-cyclohexylethyl ester of Trifluoroacetic acid and Strychnine which accounted for 100.00% and 82.648% respectively. Out of 5 constituents 1 was unidentified and 4 were identified.

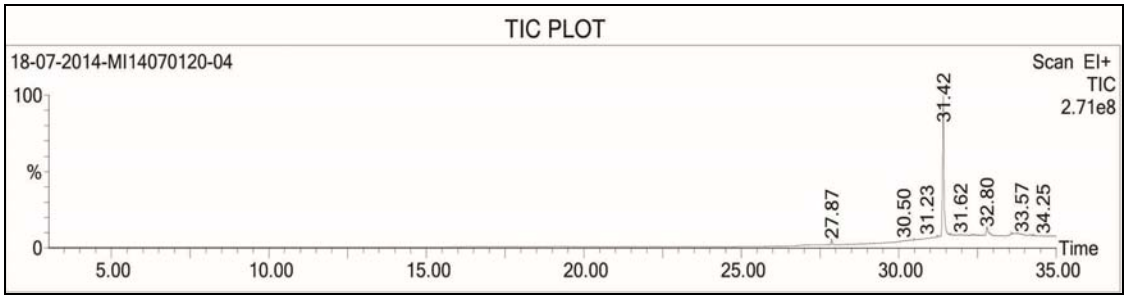


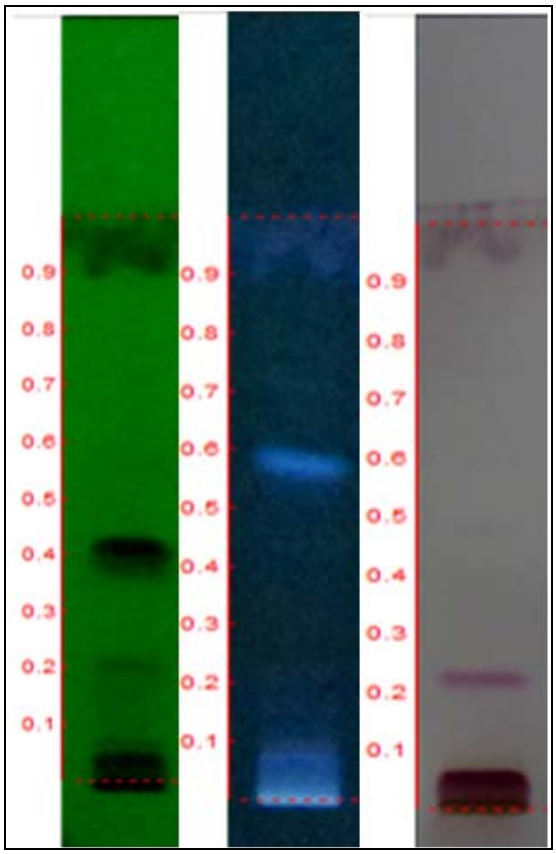

**Fig 1:** GLC Chromatogram of Methanol extract of *S Nux vomica* seeds.

**Table 1:** GC-MS identification of Methanol extract of *S Nux vomica* seeds.

| Number | RT | Match | Name | % Area |
|---|---|---|---|---|
| 1 | 19.119 | 65.0 | 1-cyclohexylethyl ester of Trifluoroacetic acid | 100.00 |
| 2 | 27.870 | 71.2 | 2,6-dimethyl-1,5-Heptadiene | 1.872 |
| 3 | 31.418 | 81.5 | Strychnine | 82.648 |
| 4 | 32.800 | 69.8 | Urs-12-en-24-oic acid, 3-oxo-, methyl ester, (+)- | 7.141 |
| 5 | 33.721 | 53.8 | -- | 8.338 |

**HPTLC fingerprint**

The results from HPTLC finger print scanned at wavelength 254nm for methanol extract of *S nux-vomica* seeds showed 10 polyvalent phytoconstituents. The corresponding ascending order of Rf values start from 0.01 to 0.60 in which highest concentration of the phytoconstituents was found to be 39.64% and its corresponding Rf value was found to be 0.37 Rf and was recorded in table 3 and corresponding HPTLC is presented in Fig 2.




**Solvent system -** Chloroform: Methanol (6.0:1.0)

**Fig 2:** TLC photo documentation of Methanol extract of *S Nux vomica* seeds.

**Table 2:** $R_f$ values of methanol extract of *S nux -vomica* seeds.

| At 254nm | At 366nm | Post derivatization |
|---|---|---|
| SNV | SNV | SNV |
| - | - | 0.05 (D. red) |
| 0.08 (L. green) | 0.08 (F. blue) | - |
| - | - | - |
| 0.17 (L. green) | - | - |
| - | - | - |
| 0.21 (D. green) | - | - |
| - | - | 0.23 (D. pink) |
| - | - | - |
| - | - | - |
| - | - | - |
| 0.42 (D. green) | - | - |
| - | - | - |
| - | - | 0.49 (L. purple) |
| - | 0.58 (F. blue) | - |
| - | - | - |
| - | 0.72 (FL. blue) | - |
| - | 0.75 (FL. blue) | - |
| - | - | - |
| - | - | - |

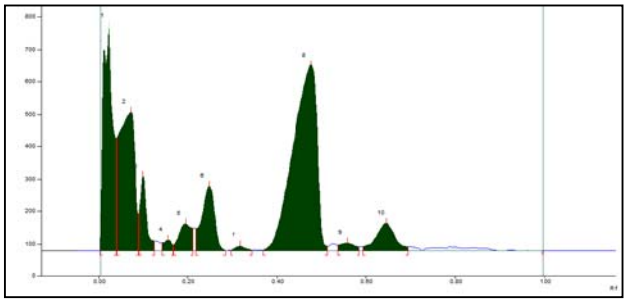


**Fig 3:** HPTLC Densitometric scan at 254nm

**Table 3:** Rf Values for methanol extract of *S nux- vomica* seeds at 254 nm.

| Peak | Start Position | Start Height | Max Position | Max Height | Max % | End Position | End Height | Area | Area % |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 0.00 Rf | 0.0 AU | 0.02 Rf | 689.2 AU | 29.41 % | 0.04 Rf | 41.6 AU | 11634.6 AU | 20.87 % |
| 2 | 0.04 Rf | 344.8 AU | 0.07 Rf | 425.8 AU | 18.17 % | 0.09 Rf | 96.9 AU | 10393.5 AU | 18.64 % |
| 3 | 0.09 Rf | 115.4 AU | 0.10 Rf | 228.3 AU | 9.74 % | 0.13 Rf | 28.7 AU | 2330.0 AU | 4.18 % |
| 4 | 0.14 Rf | 23.8 AU | 0.15 Rf | 31.8 AU | 1.36 % | 0.17 Rf | 17.4 AU | 476.0 AU | 0.85 % |
| 5 | 0.17 Rf | 17.6 AU | 0.20 Rf | 80.7 AU | 3.45 % | 0.21 Rf | 67.5 AU | 1614.5 AU | 2.90 % |
| 6 | 0.22 Rf | 67.1 AU | 0.25 Rf | 198.2 AU | 8.46 % | 0.29 Rf | 0.2 AU | 3931.7 AU | 7.05 % |
| 7 | 0.30 Rf | 1.7 AU | 0.32 Rf | 13.6 AU | 0.58 % | 0.35 Rf | 1.1 AU | 232.2 AU | 0.42 % |
| 8 | 0.37 Rf | 2.2 AU | 0.48 Rf | 569.5 AU | 24.30 % | 0.51 Rf | 13.2 AU | 22096.7 AU | 39.64 % |
| 9 | 0.54 Rf | 16.9 AU | 0.56 Rf | 23.5 AU | 1.00 % | 0.59 Rf | 11.6 AU | 570.0 AU | 1.02 % |
| 10 | 0.60 Rf | 12.4 AU | 0.65 Rf | 82.7 AU | 3.53 % | 0.69 Rf | 11.7 AU | 2469.8 AU | 4.43 % |

The results from HPTLC finger print scanned at wavelength 366 nm for methanol extract of *S Nux vomica* seeds. There are 3 polyvalent phytoconstituents and corresponding ascending order of Rf values start from 0.01 to 0.56 in which highest concentration of the phytoconstituents was found to be 65.35% and its corresponding Rf value was found to be 0.01 Rf and was recorded in table 4 and corresponding HPTLC is presented in Fig 4.

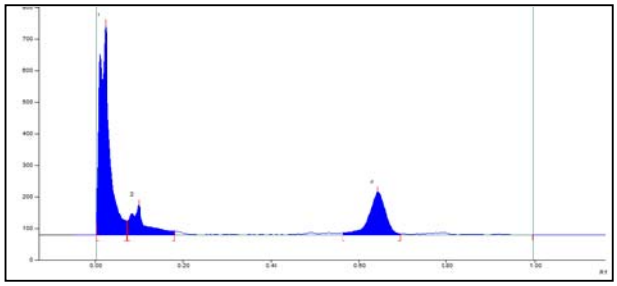


**Fig 4:** HPTLC Densitometric scan at 366nm

**Table 4:** Rf Values for methanol extract of *S nux-vomica* seeds at 366 nm.

| Peak | Start Position | Start Height | Max Position | Max Height | Max % | End Position | End Height | Area | Area % |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 0.00 Rf | 32.1 AU | 0.02 Rf | 662.0 AU | 74.33 % | 0.07 Rf | 42.9 AU | 11270.2 AU | 65.35 % |
| 2 | 0.07 Rf | 43.2 AU | 0.10 Rf | 94.0 AU | 10.56 % | 0.18 Rf | 11.7 AU | 2275.7 AU | 13.19 % |
| 3 | 0.56 Rf | 6.2 AU | 0.65 Rf | 134.6 AU | 15.11 % | 0.70 Rf | 3.3 AU | 3701.2 AU | 21.46 % |

Abid kamal, *et al* (2012) developed a method for the simultaneous quantification of Strychnine and brucine in the seeds of *S nux-vomica* by simple, sensitive, specific, very economic, and a laboratory friendly validated high performance thin layer chromatography (HPTLC). The methods so far reported for the analysis of strychnine and brucine include their estimation using circular chromatography, nonaqueous capillary electrophoresis, UV spectrophotometry, thin layer chromatography, column liquid chromatography showed low resolution owing to poor reproducibility.

**Anti-inflammatory property**

In the present investigation carried out to evaluate the anti-inflammatory potential of Methanol extract of *S. nux-vomica* through *in vitro* methods. Extract was found to have significant anti-inflammatory activity at 1000 µg/ml during *in vitro* anti-inflammatory assay. The results are reported in table 5.

Lipoxygenase are the key enzymes in the biosynthesis of leukotrienes that play an important role in several inflammatory diseases such as arthritis, asthma, cancer and allergic disease. Methanol extract of *S. nux-vomica* showed significant lipoxygenase inhibitory activity.

Stabilization of RBCs membrane was studied for further establishes the mechanism of anti-inflammatory action of methanol extract of *S nux-vomica*. This showed effective inhibition of heat induced haemolysis and provide evidence for membrane stabilization. The results are reported in table 5.

Denaturation of proteins is a well-documented cause of inflammation. As part of the investigation on the mechanism of the anti-inflammation activity, ability of an extract on protein denaturation was studied. During inflammation condition, protein of the cell gets denatured, thus here albumin protein is used as a model whose protection in denaturation by plant extract was studied. The 1000 µg/ml dose extract was found to be effective in inhibiting heat induced protein denaturation (88.92±0.6344) in compared with standard Aspirin which showed 86.95±0.6310% inhibition in protein denaturation at 100 µg/ml.

**Table 5:** Percent inhibition of Lipoxygenase, Protein Denaturation and Heat induced haemolysis.
(Mean ±SEM, N=4, ***=Significant at *P*<0.05)

| | **Aspirin (100 µg/ml)** | ***S nux -vomica* (1000 µg/ml)** | **P value** |
|---|---|---|---|
| Lipoxygenase | 70.98±0.5778 | 91.81±0.9822 | *P*<0.0001, ***, t=18.28,df=6 |
| Protein Denaturation | 86.95±0.6310 | 88.92±0.6344 | *P*=0.0707, NS, t=2.193,df=6 |
| Heat induced haemolysis | 68.76±0.4394 | 80.48±0.310 | *P*<0.0001,***, t=21.78,df=6 |

***=Significant at *P*<0.05)

*In vitro* anti-inflammatory studies of *S nux-vomica* showed significant suppression of inflammation. From the preliminary phytochemical screening study it showed the presence of alkaloids, phenols and flavonoids [17]. Antiinflammatory activity of flavonoids has been recognized long back in rodents and reviewed exhaustively. Some examples include quercetin Silymarin, genistein etc.

**Conclusion**

The results of the present study identified novel phytochemicals in the methanol extract of *S nux -vomica* seeds. HPTLC fingerprint was performed using optimized solvent system. The fingerprint can be used for the identification and authentication of the extracts. The gas chromatogram shows the relative concentration of various compounds getting eluted as a function of retention time. These mass spectra are fingerprint of the compound which can be identified from the NIST-11 data library.

The methanolic extract of *S nux -vomica* seeds shows the notable anti-inflammatory activity. Hence can be used to prevent consequences of inflammation. Further investigations are anticipated to identify the active components and lead to their further clinical use.

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