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Original Research Article

A new steroidal derivative from the skin of Indian Toad (*Bufo melanostictus*) as a hypolipidemic and CYP 3A inhibitor

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Abstract

Purpose: *To investigate the inhibitory effect of a novel steroidal derivative (NSD) on CYP3A in Wistar rats.*
Methods: *Column chromatography and thin-layer chromatography (TLC) were used for the isolation and identification of NSD, while its structure was elucidated using, Infrared (IR) ^{13}Carbon Nuclear Magnetic Resonance (^{13}C NMR), Proton nuclear magnetic resonance (^{1}H-NMR), and liquid chromatography with tandem mass spectrometry (LC-MS). Toxicity studies were conducted in female Wistar rats according to Organisation for Economic Co-operation and Development (OECD) 423 guidelines. Hyperlipidemia was induced in the rats with high-fat diet (HFD). In vitro cytochrome P-450 (CYP) 3A studies were carried out by erythromycin demethylation assay (EMD), while pharmacokinetic studies were undertaken after treatment for eight days with NSD. Plasma drug concentrations were assessed using high-performance liquid chromatography (HPLC), while pharmacokinetic parameters were computed using WinNonlin 8.2 software. Lipid profile was evaluated by Cholestech LDX analyzer; furthermore, hematoxylin-and eosin-stained histological sections of the arch of the aorta were examined by microscopy.*
Results: *The lethal dose-50 (LD_{50}) was 200 mg/kg. In vitro studies shown CYP activity in liver microsomes (551.41 ± 107.70 to 136.11 ± 2.978) and in intestine microsomes (496.71 ± 20.23 to 146.20 ± 23.7), compared to control ($p < 0.001$). Pharmacokinetic studies C_{max}, increased from 55.26 ± 5.16 to 387.8 ± 40.95 ng/mL; and area under the curve (AUC) from 547.588 ± 20.150 to 2730.548 ± 27.19. The volume of distribution (Vd), mean residual time (MRT), time to maximum concentration (T_{max}) were decreased. NSD significantly reduced serum cholesterol (SC) from 254.5 ± 27.5 to 88.6 ± 8.8 mg/dL, and other lipids as well.*
Conclusion: *NSD inhibits CYP3A-mediated ATV drug metabolism and is also a potent hypolipidemic agent in vitro and in vivo studies. Co-administration of ATV and NSD may expedite oral bioavailability.*

Keywords: *Atherosclerosis, Atorvastatin, Bioavailability, Novel Steroidal Derivative, High-fat diet*

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INTRODUCTION

Natural products derived from plants, animals, microbial, and marine sources are often used to treat various ailments for centuries because of the abundance of biodiversity in natural sources, new prospects [1]. Toads are known to contain a variety of potent compounds that have a wide range of pharmaceutical applications. There are approximately 300 species of toads that have been reported to secrete acrid venom known as bufotoxin and bufotenin from their skin and parotoid glands. Many bioactive molecules from amphibians have been reported because amphibians have a unique defense system against predators, and microorganisms are directly related to skin glands that produce a variety of toxins. Toads produce a variety of biologically active compounds, including bufotenine, bufagenins, bufotoxins, cinobufalin, and others [2]. These substances are found in all Bufo species, but the chemistry of each substance produced by different toads varies [3]. Toad skin extracts have been reported to be antimicrobial, anti-inflammatory and anti-cancer agents [4]. Orally administered drug molecules are subjected to extensive first-pass metabolism or pre-systemic metabolism, which results in significant bioavailability loss due to Cytochrome P-450 (CYP-450) present at intestine segments liver [5]. As co-administrators, CYP inhibitors are required to circumvent this pre-systemic effect [6]. CYP enzymes play an essential role in drug metabolism, and CYP 3A is responsible for the metabolism of more than half of all drugs. The liver and small intestine have the highest expression and activity of CYP3A [7], and CYP inhibition is vital for increasing drug bioavailability [8].

EXPERIMENTAL

Materials

All chemicals are analytical grade, and they were obtained from the sources listed. Erythromycin (substrate for CYP3A) - (Vasudha - Pharma Chemicals Ltd, Hyderabad), Ketoconazole - (Sigma–Aldrich -USA), Sucrose- (SD fine chemicals Mumbai), Tris HCL Buffer - (SD fine chemicals Mumbai), $CaCl_2$- (SD fine chemicals Mumbai), K2HPO4- (SD fine chemicals Mumbai), KH2PO4-(SD fine chemicals Mumbai), Glycerol - (SD Fine Chemicals, Mumbai), Histidine Sucrose Buffer (HSB) -(SD fine chemicals Mumbai), Formaldehyde and Trichloroacetic acid- (SD fine chemicals Mumbai) NADPH- (SRL chemicals Hyd).

Sample preparation and isolation of compound

From April through September, adult live toads weighing 45 to 50 g were gathered from Warangal and the University's surroundings. The skins were carefully isolated after collecting the toads and cleaning them well with a dry brush. After thorough drying, the skins were shade dried at room temperature (27^0 C), and approximately 140 g of dry skins were soaked in methanol for 30 days in an amber-colored bottle; the supernatant was started to gather, and the dark brown solid mass methanol extract (4.5g) was recovered using a rota evaporator (Manika *et al*, 1996). Toad Skin Extract (TSE-M) was the term given to this methanolic extract, and the percentage yield of TSE was found to be 3.21 percent. TSE (4.5g) is placed on a cylindrical glass column containing a stationary phase of a silica gel, and elution is done slowly by a fluid (mobile phase) solvent from above, which flows down the column using gravity or external pressure. Acetate ethyl: Hexane: Water used for elution (75:15:10), and various fractions had been gathered [9]. All fractions were subjected to silica gel (230 - 400 mesh Merck) column chromatography (Meyers, 2001). For TLC, a pre-coated 0.2 mm thick aluminum plate was used [10].

Spectral characterization

A spectral analysis was carried out. The identification of components was done using a mass spectral library in LC-MS analysis 2.6.1. ^{13}C NMR spectra samples were created by dissolving extract in 500 µl of DMSO with 1 µl of DMF as an internal standard, and ^{13}C NMR spectral reports were made by comparing observed chemical shift values to published values. The ^{1}H spectra were acquired at 300 K using a 5-mm inverted probe with a z-shielded gradient on a spectrometer running at 600.13 MHz (14.1T). To discover functional groups, an IR spectrophotometer (Alpha-II) is employed. The structure was evaluated using Perkin Elmer's Chem-Draw pro 8.0.

Toxicity studies

Acute oral toxicity studies are carried out in accordance with the OECD 423 guidelines. Fifteen female rats were used in this study; they were obtained from Vyas animal suppliers-Hyd. They were divided into five categories (n = 3). The first group received normal saline, the second group received 5 mg/kg of NSD, the third group received 50 mg/kg of NSD, the fourth group received 300 mg/kg of NSD, and the fourth group received 2000 mg/kg of NSD. Bodyweight, food

consumption, and water consumption, locomotor activity, and behavioral changes were all recorded over the course of 14 days. The mortality rate was taken into consideration when determining the LD_{50} [11].

Induction of atherosclerosis

Rats to induce hyperlipidemia and atherosclerosis through a special high fat diet for eight weeks which the National Institution of Nutrition supplied-Hyd, given to every rat except control group which are fed with standard *ad libitum* [12].

Determination of *in vitro* CYP3A activity

In vitro CYP3A study carried out in accordance with the previously reported methods with slight modification [13]. The study relies on principles while erythromycin is converted by the CYP3A enzyme; N-demethyl erythromycin and formaldehyde are produced. Nash reagent generated a yellow colour. Preparation of liver microsomes was performed according to the previously stated method. A minor modification of previously published procedures was used to make the intestinal microsomes [14].

Erythromycin-N-demethylation (EMD) assay

At 37 ^{o}C, a mixture of microsomal suspension (0.1 ml with 25%), erythromycin (0.1 ml with 10 mM), and potassium phosphate (0.6 ml with 100 mM, pH 7.4) were incubated at 37^{0}C with NSD of 50, 300, 2000 mg/ml. and ketoconazole (10 mM) (positive control). The interaction of these agents began with the addition of NADPH (0.1 mL with 10 mM) and was terminated after 10 minutes of ice-cold trichloroacetic acid addition (0.5 mL, 12.5 percent w/v) solution. To get rid of proteins, it was centrifuged (2000 g; 10 min). 1 mL of Nash Reagent (2 M ammonium acetate, 0.05 M glacial acetic acid, 0.02 M acetylacetone) was added to 1 mL of this supernatant and heated in a water bath at 50^{0}C for 30 min. After cooling, the absorbance at 412 nm was measured with a UV spectrophotometer. The activity was calculated using standard (1-100 M formaldehyde) prepared by replacing the measure with a standard solution that was run in parallel with the sample. The CYP3A4 activity was measured in µM of formaldehyde obtained. CYP activity (C) was calculated based on the equation mentioned as in Eq 1 [15].

$$C = A/(1/25 * 1/10) \quad \ldots\ldots\ldots\ldots (1)$$

where A = amount of CHO produced *(n/mol)*, B = 1/25 of protein, and D = 1/10 min.

Pharmacokinetic studies

The rats were separated into groups consisting of six animals (n=6) each group, group-1 was treated with ATV 20 mg/kg.p.o.) for 10 days and group-2 was treated with NSD (100 mg/kg.p.o.) and ATV (20 mg/kg for 10 days and group-3 treated with NSD (200 mg/kg.p.o) and ATV (20 mg/kg for 10days) and group-4 treated with ATV (20mg/kg for 10days;p.o) and KET (100 mg/kg.p.o), blood samples (approximately 0.20 mL) were collected from the tail vein into heparinised microcentrifuge tubes prior to dose and at 0.5, 1, 1.5, 2, 3, 6, 9, 12 and 24 h post-dose by tail vein of rats (Frank et al., 1991). The blood samples were centrifuged for 15 minutes at 15000 rpm, until the HPLC analysis, the plasma samples were kept at -20°C. The non-compartmental model computed pharmacokinetic parameters using Phoenix WinNonlin version 6.2 software (Certara-Pharsight Corporation, USA).

Analysis of plasma samples

ATV concentration was measured by a modified HPLC method, and lovastatin was used as an internal standard. The mobile phase consisted of sodium dihydrogen phosphate buffer-acetonitrile (60:40 with v/v) adjusted at pH 5.5 and was used at a flow rate of 1.0 ml/min, UV detection was at 245 nm while retention time (R.T) of ATV 2.9 min and internal standard R.T 4.8min [16].

Evaluation of the hypolipidemic activity of NSD

The rats were segregated into 5 groups (n=6), first group have been treated with DMSO, second group has been treated ATV 20 mg/kg.p.o., Third group was treated with NSD -100 mg/kg.p.o, with ATV 20 mg/kg.p.o, fourth group treated with NSD -200 mg/kg.p.o and ATV 20 mg/kg.p.o for 10days and fifth group treated with ATV -20 mg/kg.p.o and KET-100 mg/kg.p.o for 10 days, blood samples analysed for estimation lipid profiles [17].

Histopathological studies of arch of aorta

Animals were sacrificed at the end of the study, and the aorta was dissected out, washed, and 5 µm thin section slides were developed and stained with hemotoxylin - eosin, subsequently observed using light microscopy.

Statistical analysis

The results of the study were expressed as mean, and SD (n=6), and a two-way ANOVA

with (*Bonferroni post-hoc test*) was employed, with a p-value < 0.05 considered significant. Pharmacokinetic parameters were calculated using WinNonlin 8.3 version software, and pharmacokinetic data were examined using GraphPad Prism, version 8.4.2.

RESULTS

Structure of NSD

LC-MS spectra

Mass spectral peaks were found at 203.1179, 301.1410, 530.2742, 629.4152, and 703.5762, and found m/z 528.4162 $[M+H]^+$, 529.6 for the compound.

^{13}C-NMR spectra

^{13}C NMR spectra showed the presence of 30 carbons.

^{1}H-NMR spectra

^{1}H-NMR Spectra exhibited thirty methylene multiplets, four singlets, and four doublets identified at different positions.

IR spectra

IR spectra showed absorption at 3551.22 cm^{-1}, 3434.32 cm^{-1}, 3411.36 cm^{-1}, and other near peaks, which are assigned for O-H Stretching vibrations and N-H/C-H/OH stretching of amines and 2306.45 cm^{-1} and 2054.94 cm^{-1} assigned for C-N bonding. 1629.14 cm^{-1}, 1363.48 cm^{-1} which were assigned to C-O functional groups. Based on the spectral analysis using LC-MS, IR spectra, ^{13}C NMR, and ^{1}H NMR δ values, the structure of NID is elucidated (Figure 1).

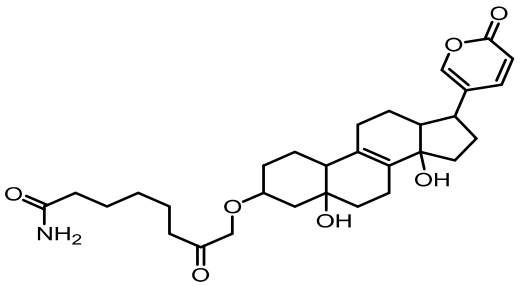

Figure 1: Structure of NSD

IUPAC name

8-((5,14-dihydroxy-17-(2-oxo-2*H*-pyran-5-yl)-2.3.4.5.6.7.10,11,12,13,14,15,16,17-tetradecahydro-1*H*- cyclopenta (a) phenanthren-3-yl)oxy)-7-oxooctanamide.

Elemental data

The isolated compound's chemical formula was identified to be C_{30} H_{44} NO_7, and determined the fundamental elemental analysis of all those elements present in it (Table 1).

Table 1: Elemental analysis of NSD

Element	Percent
C	68.29
H	7.83
N	2.65
0	21.23

Acute toxicity

In three animals, mortality was found at 300, and 2000 mg/kg NSD treated groups, according to OECD-423 guidelines, comes under category-3 (LD_{50} cut-off dose 200 mg/kg) [1]. The animals survived after NIA administration at 5mg/kg and 50mg/kg. Bodyweight was slightly lower in NSD 5mg/kg and 50mg/kg when compared to the control. Food and water intakes were slightly lower in NSD 5mg/kg and 50mg/kg compared to control, respectively, and locomotor activity was unaffected (Fig 2A-2D), with 200 mg/kg considered the maximum tolerated dose of NSD.

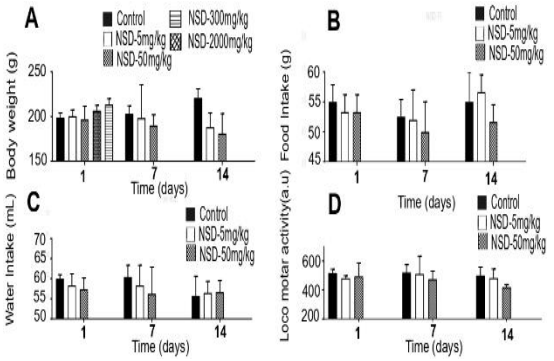

Figure 2: NID toxicity observational study in female Wistar rats for 14 days. A) Bodyweight, B) Water intake, C) Food intake, D) Locomotor activity changes. NSD-1: Novel Steroidal Derivative 5mg/kg, NSD-2: Novel Steroidal Derivative 50mg/kg, NSD-3: Novel Steroidal Derivative 300mg/kg, NSD-4: Novel Steroidal Derivative 2000mg/kg. LMA: Locomotor activity

In vitro CYP 3A activity

In vitro studies have shown NSD suppressed CYP activity nM/mg/protein/min in normal liver, intestine microsomes, and atherogenic liver and intestine microsomes dose-dependently (Fig 3). NSD-50, 300, 2000 mg/mL significantly inhibited CYP3A activity (p <0.05 and 0.01). NSD inhibition ranged from 551.41 ± 107.70 to 136.11 ± 2.97 in normal liver microsomes and from

317.21 ± 2.220 to 111.761 ± 4.73 in atherogenic liver microsomes. CYP3A inhibited from 496.71 ± 20.23 to 146.20±23.7 in the intestine and from 509.73 ± 11.39 to 109.50 ± 6.67 in the atherogenic intestine microsomes.

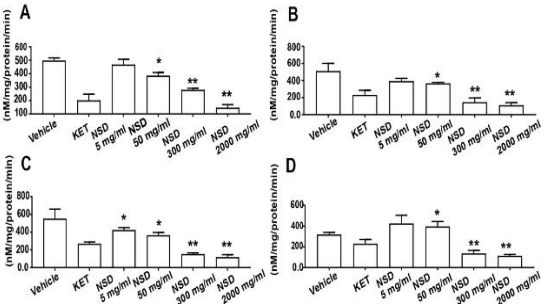

Figure 3: *In vitro* studies- Inhibitory effect of NSD on CYP 3A mediated metabolism of erythromycin. ***Note:*** ATV: Atorvastatin, KET: Ketoconazole, NSD: Novel steroidal derivative: A-Liver microsomes, B-Intestine microsomes, C-Atherogenic liver microsomes, D-Atherogenic intestine microsomes

Hypolipidemic activity of NSD

NSD seemed to have a considerable hypolipidemic effect by lowering serum levels of biochemical markers such SC, TD, LDL, VLDL, and increasing HDL levels, decreased SC from 254.5 ± 27.5 to 88.60 ± 8.76 mg/dL, increased HDL from 20.76 ±1.10 to 52.10 ± 7.92, TGs from 181.0 ± 15.52 to 100.83 ± 4.01, LDL from 145.93 ± 7.5 to 74.06 ± 9.05, and VLDL from 45.15 ± 1.50 to 22.36 ± 0.04 (Table 2).

Effect of NSD on plasma drug concentration of ATV

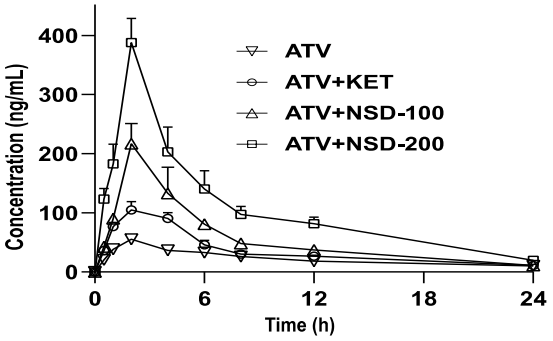

Figure 4: NSD impact on atorvastatin pharmacokinetics in Wistar rats. ***Note:*** ATV:Atorvastatin, KET: Ketaconazole, Data represents Mean ± SD values (n = 6). Plasma ATV concentrations increased 1.8-fold compared to standard inhibitor (KET) treatment, 3.9 folds in NSD-100 mg/kg, and seven folds in NSD-200 mg/kg. The oral bioavailability of ATV was greatly enhanced by NSD 100 and 200 mg/kg dose-dependently

Table 2: Effect of NSD on pharmacokinetics of ATV

Parameter	ATV- 20 mg/kg	ATV+ KET-100 mg/kg	ATV+ NSD-100 mg/kg	ATV+ NSD -200 mg/kg
C_{max} (ng/mL)	55.26 ± 5.16	104.81 ± 4.11*	216.86 ± 8.16**	387.80±10.95**
T ½ (h)	2.00 ± 0.00	2.0 ± 0.00	2.00 ± 0.00	2.00 ± 0.00
T_{max} (h)	12.51 ± 1.56	8.93 ± 1.53	7.36 ± 0.09	6.35 ± 0.26*
AUC (ng/mL/h)	547.58 ± 20.15	875.90 ± 14.27*	1330.81 ± 17.30**	2730.54 ± 27.19**
AUMC (ng/mL/h)	4758.979 ± 242.43	6525.79 ± 132.90	8926.08 ± 59.12**	18571.71 ± 122.29**
CL/F (L/h/kg)	27.28 ± 1.25	19.67 ± 0.81*	13.81 ± 0.161*	6.86 ± 0.66*
K_{el} (h^{-1})	0.083 ± 59.12	0.080 ± 0.015	0.094 ± 0.000	0.109 ± 0.005
Vd (L/kg)	4912.79 ± 51.31	2520.54 ± 34.34*	1467.93 ± 18.65*	629.27 ± 2.43**
MRT (h)	8.687 ± 0.142	7.451 ± 0.118*	6.802 ± 0.04*	6.708 ± 0.046*

ATV: Atorvastin, KET: Ketaconazole NSD: Novel steroidal derivative. Data is a representation of mean ± SD; for statistical analysis, two-way ANOVA followed by *Bonferroni post-hoc* test has been employed, the significant difference between the control and KET treated, $p < 0.05$, and NSD 100 and NSD 200 mg/kg treated, $p < 0.01$

Table 3: Lipid profiles of rats

Group	SC (mg/dl)	HDL (mg/dl)	TG (mg/dl)	LDL (mg/dl)	VLDL(mg/dl)
Atherogenic-control	254.5±27.5	20.76±1.10	181.0±15.52	145.93±7.5	45.15±1.50
ATV	121.19±20.3	43.30±7.40	139.0±8.76	99.21±7.06	39.76±1.19
ATV+RIF	110.33±6.34	45.50±9.30*	143.33±13.8	80.73±6.06	27.93±0.63*
NSD-100	92.60±8.40	50.26 ±8.04	111.33±2.50*	83.70±10.6*	24.20±0.52*
NSD-200	88.60±8.76**	52.10±7.92*	100.83±4.01**	74.06±9.05**	22.36±0.04**

ATV: Atorvastatin KET: Ketoconazole NSD-100: Novel steroidal derivative 100 mg/kg NSD-100 : Novel steroidal derivative 100 mg/kg, SC: Serum cholesterol, HDL: High-density lipids, TG: Tryglyceroids, LDL: Low-density lipids, VLDL: Very low-density lipids Data represents Mean ± SD values, one way ANOVA was used for statistical analysis. Significant difference found to be $P < 0.05$, $P < 0.001$, in comparison with the ATV treated

Histopathological features of arch of the aorta

The aorta of atherogenic diet-fed animals reveals substantial atheromatous thickening and intima damage in histopathological segments. NSD-treated groups absent atheromatous inflammatory abnormalities and atherogenic plaques (Figure 5).

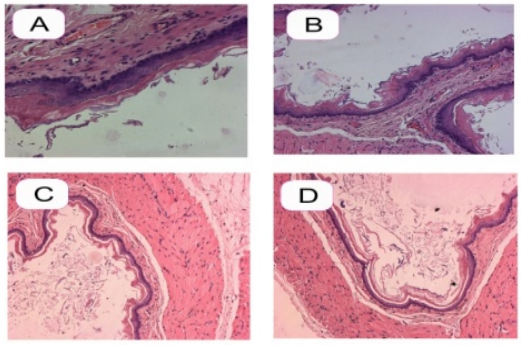

Figure 5: A: Histopathological studies of the arch of aorta A: Aortic intima layer damage after induction of atherosclerosis by HFD; B: Formation of atherosclerotic plaques after induction of atherosclerosis by HFD; C: Aortic intima layer repair after NSD treatment; D: Atherosclerotic plaques disappeared after NSD treatment

DISCUSSION

The characterized compound is confirmed as having a steroidal structure in this study, which had not previously been reported. The structure of NSD is similar to that of arenobufagin, which has been found in argentine toads (*Bufo arenarum*) and American toad (*Rhinella arenarum*). Toxicity analysis revealed the compound's potency, which reflects its affinity and efficacy towards the biological system. Many bufadienolides have been tested in animals for potency and toxicity. The isolated compound has evidenced a similar effect [18]. Atherogenic microsomes demonstrated an increased inhibitory capacity towards CYP3A and were used to perform this investigation for the first time by using atherogenic liver and intestinal microsomes. Numerous herbal extracts and plant components have been investigated, and CYP-3A suppression has been demonstrated [19].

NSD has shown a similar effect. In pharmacokinetic investigations, further CYP3A inhibition of NSD was confirmed. AUC and C_{max} are generally used for measuring the extent of bioavailability; AUC, AUMC, and C_{max} were increased in NSD treated groups and considerably increased (Table 2). The pharmacodynamic investigations in atherogenic Wistar rats validated these results. TC, VLDL, LDL, TGs, and good cholesterol as HDL were decreased substantially in this study. In different stages of development, TC, VLDL, LDL have been risking factors and play a key function [20], and all these levels were decreased with NSD (Table 3). All the results are correlated to some of the hypolipidemic studies reported earlier [21].

Ateromatous thickening was significant, damaging the intima in the histological parts of the aortic arch of the atherogenic diet-feeding animals. In those treated with NSD, atheromatous inflammatory alterations and atherogenic plaques were reduced (Figure 5). Reduced lipid profiles can help prevent and treat cardiovascular disorders caused by atherosclerosis, and plant extracts can be used as an alternative to synthetic drugs [22-23]. NSD may be developed as a new anti-hyperlipidemic drug in the future.

CONCLUSION

This work demonstrates that NSD from Indian toad skin extract suppresses CYP3A and has hypolipidemic and antiatherosclerotic properties. The isolated molecule from Indian toad skin is a new steroidal derivative that has not been reported previously, to the best of our knowledge, it inhibits CYP3A-mediated drug metabolism considerably. More research is needed to develop this NID as a substantial P-gp inhibitor that may be used to treat a variety of ailments.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article, and all liabilities pertaining to claim relating to the content of this article will be borne by the authors. All the authors contributed equally contributed to this work.

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