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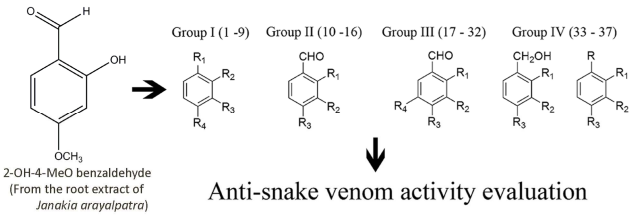

Molecular modeling and snake venom phospholipase A_2 inhibition by phenolic compounds: structure-activity relationship

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ABSTRACT

In our earlier study, we have reported that a phenolic compound 2-hydroxy-4-methoxybenzaldehyde from *Janakia arayalpatra* root extract was active against Viper and Cobra envenomations. Based on the structure of this natural product, libraries of synthetic structurally variant phenolic compounds were studied through molecular docking on the venom protein. To validate the activity of eight selected compounds, we have tested them in *in vivo* and *in vitro* models. The compound **21 (**2-hydroxy-3-methoxy benzaldehyde), **22** (2-hydroxy-4-methoxybenzaldehyde) and **35** (2-hydroxy-3-methoxybenzylalcohol) were found to be active against venom-induced pathophysiological changes. The compounds **20**, **15** and **35** displayed maximum anti-hemorrhagic, anti-lethal and PLA_2 inhibitory activity respectively. In terms of SAR, the presence of a formyl group in conjunction with a phenolic group was seen as a significant contributor towards increasing the antivenom activity. The above observations confirmed the anti-venom activity of the phenolic compounds which needs to be further investigated for the development of new anti-snake venom leads.

Key words: Antisnake venom activity; PLA_2 inhibitors; Phenolic compounds; 2-hydroxy-3-methoxybenzyl alcohol; SAR.

Abbreviations used: ASP, Aspartate; $CDCl_3$, Deuterated chloroform; DMSO, Dimethyl sulfoxide; FT-IR, Fourier transform-Infrared spectroscopy; GLY, Glycine; LD_{50}, Lethal Dose 50 (dose required to kill 50 percent of a population of test animals); MCDP/S,Minimum clotting dose of plasma/serum; MDD, Minimum defibrinogenating dose; MHD, Minimum hemorrhagic dose; MHz, Megahertz; NMR, Nuclear magnetic resonance; PLA_2, Phospholipase A_2; ppm, part per million; SAR, Structure-activity relationship; TLC, Thin layer chromatography; TMS, Tetramethylsilane.

1. Introduction

Snake envenomation is one of the neglected problems of the world. Antiserum still remains the only therapeutic agent available for snakebite treatment. In the remote and rural areas of many developing countries, antiserum is generally not easily available. The antiserum sometimes does not produce enough protection against venom induced hemorrhage, necrosis, nephrotoxicity and hypersensitive reactions [1-3]. Further development of antiserum in animal is time consuming, costly and requires strict cold chain conditions. To overcome these factors search for natural/synthetic molecules as an alternate source of antidote is an urgent requirement. The development of stable and effective venom neutralizing drug/antidote is being ventured throughout the World. One of the strategies could be to develop a molecule from the natural/ synthetic sources to combat the effect of venom partially or completely which may help in improving the chances of survival of snake bite victims. Venom neutralizing drugs may prove to be more effective life saver [4]. Development of such molecules is vital to address the cases when identification of the snake is difficult, specific treatment is unavailable and the health centre is far away.

Many plants and plant products have been used in folk medicines as antidote against snakebite [5-8]. Preliminary investigations have suggested that several classes of constituents such as steroids, terpenoids, alkaloids and glycosides from plant origin are capable of neutralizing snake venom induced pathophysiological changes [9-16]. More than 800 plant species have been screened for the anti-snake venom activity [15], however, very few plant extracts have shown significant protection against snake-venom and none of the pure isolates has displayed equivalent activity [4,17,14,18,19]. Several workers have also reported natural as well as synthetic compounds as active inhibitors of snake venom phospholipase A_2 (PLA_2) [20-22].

Phenolic compounds such as phenolic acids (hydroxy benzoic acid or benzaldehydes, cinnamic acid), flavonoids, isoflavonoids, coumarins, pterocarpans, phenolic glycosides and tocopherols have been reported to display protection against variety of snake venoms [9,17,23]. In our earlier study, a phenolic compound 2-hydroxy-4-methoxy-benzoic acid was shown to possess anti-venom and antioxidant activity [4,17]. Through *in silico* approach we identified a natural compound 2-hydroxy-4-methoxy-benzaldehyde (**21**) as snake venom PLA_2 inhibitors [IOXL] [24]. The natural product (**21)** was isolated from the root extracts of *Janakia arayalpathra* which is used in folk medicine in Western India as an antidote of snake bite. It was also reported in the literature as an immunomodulator [5,6] and potent inhibitor of tyrosinase, which is essentially indicative of its binding capabilities to specific receptor proteins [25]. In the present study, we examined several analogs of phenolic compounds against Viper and Cobra venom induced pathophysiological changes which were found to be effective both in wet lab and in *in silico* models.

2. Materials and Methods

2.1.Materials

Unless otherwise stated, all reagents for chemical synthesis were purchased from Sigma-Aldrich and used without further purification. The solvents used in reactions were distilled and dried before use. All the material either natural or synthetic was in pure form. The purity of the natural as well as synthetic compounds was found to be more than 95%. All reactions were monitored by TLC on 0.25 mm silica gel 60 F254 plates coated on Aluminum sheet (E. Merck). ^{1}H NMR and ^{13}C NMR spectra were recorded on Brucker Avance DPX-200 instrument at 200 MHz and 500 MHz respectively using $CDCl_3$ as solvent with TMS as internal standard. Chemical shift is expressed in δ (ppm) and coupling constant in Hertz.

2.2.Chemistry

The melting point of the compounds are uncorrected. Reagents, chemicals and solvents were purchased from Sigma Aldrich and E Merck. TLCs were run on 0.25 mm silica gel 60 F_{254} plates (E. Merck) using UV light or Ceric ammonium sulfate solution for detection/visualization of the spots. The purification of the products was carried out by column chromatography over silica gel (60-120 mesh). NMR spectra were run on Bruker Avance DPX-200 at 200MHz, IR on Bruker 270-30 FT-IR and Mass spectra on JEOL MSD 300. Elemental analysis was performed on ElementarVario EL-III. Anisaldehyde, 2-hydroxy-3-methoxybenzaldehyde, 3-methoxy-4-hydroxy-benzaldehyde, 3-hydroxy-4-methoxy-benzaldehyde, 1,2-dihydroxybenzene, 3,4-dihydroxybenzaldehyde, 2-hydroxybenzaldehyde, 3-ethoxy-4-hydroxy-benzaldehyde were purchased from Sigma-Aldrich.

2.2.1. Isolation and characterization of natural and synthetic compounds

The plant material of *Janakia arayalpatra* (root) was obtained from local market and was identified in the Department of Taxonomy, IIIM, Jammu.

2.2.1.1. Isolation of 2-hydroxy–4-methoxybenzaldehyde

Crushed dry roots of *Janakia arayalpatra* (500g) was steam distilled for 6 h to get a pale yellowish liquid (1.4 ml, 0.28%) which on cooling afforded a solid mp 40-41°C (Lit mp 40-42 °C). IR (KBr pellet): 3260, 3030, 2944, 1644, 1630, 1576, 1504, 144, 1366, 1336, 1296, 1222, 1204, 1164, 1136, 1020 and 798 cm-1. ^{1}H NMR (200 MHz, $CDCl_3$): δ 3.82 (3H, s, Ar-OMe), 6.30 (1H,d, J=2.2 Hz, Ar-H) 6.34 (1H, dd, J=8Hz & 2.2 Hz. Ar-H) 7.26(1H,d, J=8Hz, Ar-H), 9.72 (1H,s,-CHO), 11.33 (1H,s,OH). MS M^+ m/z (%) 152(11), 137(33), 123(27), 121(34), 82(10), 53(24).

2.2.1.2. Preparation of 2-hydroxy–4-ethoxybenzaldehyde

Anhydrous potassium carbonate (2g) and ethyl iodide (0.5 ml) were added to 2,4-dihydroxy benzaldehyde (0.7g, 5mmol) acetone (30 ml) and the contents stirred for 12h at room temperature. After the completion of the reaction as indicated by TLC, the mixture concentrated in vacuo and re-dissolved in benzene (3×10 ml). The organic layer concentrated and the residue chromatography on silica gel (60-120 mesh) using n-hexane: ethyl acetate mixture (46:4) as eluent to give JA-2 a crystallized solid (0.75g, 45%) melting point 38-39 °C analyzed $C_9H_{10}O_3$. IR (KBr pellet): 3340, 3284, 1674, 1604, 1566, 1444, 1408, 1328, 1252, 1230, 1216, 1180, 1086, 1026, 916 and 846 cm^{-1}. ^1H NMR ($CDCl_3$): δ1.45(3H,t, J=6.8Hz, CH_3-CH_2O), 4.17 (2H,q, J= 6.8 Hz, CH_3-CH_2O), 6.71 (1H,s, Ar-H),7.03 (1H,d, J=8.5Hz, Ar-H),7.41(1H,dd, J=8.5 Hz & 2.2Hz, Ar-H),9.8 (1H,s, Ar-CHO). MS M^+ m/z (%) at 166(100), 149(5), 137(9), 109(14), 108(13), 80(40), 79(31), 63(44).

2.2.1.3. Preparation of 2-hydroxy–4-propyloxybenzaldehyde

To 2, 4-dihydroxy benzaldehyde (0.7g, 5mmol) in acetone (30 ml) was added anhydrous potassium carbonate (2g) and n-propyl bromide (0.5 ml) and the contents stirred for 12 h at room temperature. The contents concentrated in vacuo and re-dissolved in benzene (3×10 ml). The organic layer concentrated and the residue chromatography on silica gel (60-120 mesh) using n-hexane: ethyl acetate mixture (46:4) as eluent to give an oily compound (0.46g, 52%) analyzed for $C_{10}H_{12}O_3$. IR (KBr pellet): 3384, 2932, 1668, 1634, 1578, 1498, 1454, 1428, 1374, 1336, 1292, 1260, 1216, 1170, 1114, 998, 826 cm^{-1}. ^1H NMR (200 MHz,$CDCl_3$): δ 1.0 (3H,t,J=7.4 Hz, CH_3-CH_2),1.77-1.88 (2H,m,OCH_2-CH_2-CH_3), 3.97 (2H,t, J=6.53Hz ,OCH_2-CH_2), 6.41 (1H,d, J=2.02Hz, Ar-H),6.5 (1H,dd, J=8.6 & 2.0 Hz, Ar-H) 7.41 (1H,d, J=8.6Hz, Ar-H), 9.75

(1H,s,CHO).MS M^+ m/z (%) 180(100), 138(98), 120(16), 110(38), 109(15), 92(18), 82(12), 81(50), 69(35), 65(50).

2.2.1.4. Preparation of 2-hydroxy–4-butyloxybenzaldehyde

To 2, 4-dihydroxy benzaldehyde (0.7g, 5mmol) in acetone (30 ml) was added anhydrous potassium carbonate (2g) and n-butyl bromide (0.5 ml) and the contents stirred for 12 h at room temperature. The contents concentrated in vacuo and re-dissolved in benzene (3×10 ml). The organic layer concentrated and the residue chromatographed on silica gel (60-120 mesh) using n-hexane: ethyl acetate mixture (46:4) as eluent to give an oily compound (0.53g, 55%) analyzed for $C_{11}H_{14}O_3$. IR (KBr pellet): 3344, 2957, 1681, 1644, 1573, 1478, 1216, 1178, 1080, 914, 832 cm-1. ^{1}H NMR (200 MHz,CDCl3) : δ 0.98 (3H,t, J=7.28 Hz, CH_3-CH_2) 1.43-150 (2H, m, CH_2-CH_2), 1.71-1,85 (2H, m, OCH_2-CH_2-CH_2), 4.0 (2H,t, J=6.46Hz, OCH_2-CH_2), 6.41 (1H,d,J=2.1Hz,Ar-H), 6.65 (1H,dd, J=2.1 & 8.6 Hz, Ar-H), 7.42 (1H, d, J=8.6Hz, Ar-H), 9.7 (1H,s, CHO). MS M^+ m/z (%), 194(52), 151(2.6), 139(16), 138(100), 137(98), 110(13), 92(5), 81(16), 69(11), 65(19).

2.2.1.5. Preparation of 2-hydroxy–4-heptyloxybenzaldehyde

To 2, 4-dihydroxy benzaldehyde (0.7g, 5mmol) in acetone (30 ml) was added anhydrous potassium carbonate (2g) and n-heptyl bromide (0.5 ml) and the contents stirred for 12 h at room temperature. The contents concentrated in vacuo and redissolved in benzene (3×10 ml). The organic layer concentrated and the residue chromatography on silica gel (60-120 mesh) using n-hexane: ethyl acetate mixture (46:4) as eluent to give an oily compound (0.58g, 50%) analyzed for $C_{14}H_{20}O_3$. IR (KBr pellet): 3330, 3240, 2955, 1677, 1634, 1573, 1322, 1244, 1180, 1084, 1026, 914, 846 cm^{-1}. ^{1}H NMR (200 MHz,$CDCl_3$): δ0.81 (3H,t, J=7.3Hz, CH_3-CH_2), 1.21-1.34 (8H, m, 4×CH2), 1.70-1.84 (2H,m, OCH_2-CH_2),3.92 (2H,t, J=6.5Hz, OCH_2-CH_2),6.32 (1H,d,

J=2.1Hz, Ar-H),6.55 (1H,dd, J=8.65 &2.1 Hz , Ar-H),7.33 (1H,d, J=8.65 Hz, Ar-H), 9.62 (1H,s, CHO). MS M^+ m/z (%) 236(37), 151(2), 139(100), 110(10), 95(2), 81(9), 69(12), 65(16).

2.2.1.6. Preparation of 4-allyloxy-2-hydroxybenzaldehyde

The title compound was prepared from 2,4-dihydroxy benzaldehyde (2.0g, 15mmol) and allyl bromide (1.4ml, 17mmol) by the method as described for 2-hydroxy–4-ethoxybenzaldehyde to furnish a semisolid (1.12g, 42%) analyzed for $C_{10}H_{10}O_3$. IR (KBr Pellet) 2932, 1668, 1634, 1578, 1498, 1454, 1428, 1374, 1336, 1292, 1260, 1216, 1170, 1114, 998, 926cm^{-1}. ^1H NMR (200 MHz,$CDCl_3$): δ 4.13 (2H, d, J=6.0 Hz,-OCH_2-CH=), 4.75-5.10 (2H, m,-CH=CH_2), 5.22-5.43 (1H, m, -CH=CH_2), 6.03(1H, dd, J=8.5 & 2.0Hz, Ar-H), 6.83 (1H,dd,J=8.5& 2.0 Hz, Ar-H),9.0 (1H, s, Ar-CHO).MS M^+ m/z (%) 178(18), 163(8), 149(5), 137(9), 69(5), 54(8), 43(100).

2.2.1.7. Preparation of 2-hydroxy-3-methoxybenzaldehyde

The title compound was prepared from 2,3-dihydroxy benzaldehyde (1.0g, >7mmol) and methyl iodide (0.5ml, 7mmol) by the method as described for 2-hydroxy–4-ethoxy-benzaldehyde to furnish a semisolid (0.48g, 46%) analyzed for $C_8H_8O_3$. IR (KBr Pellet): 3184, 2932, 1668, 1634, 1578, 1498, 1454, 1428, 1374, 1336, 1292, 1260, 1216, 1170, 1114, 998, 926cm^{-1}. ^1H NMR (200MHz, $CDCl_3$): δ3.87 (3H,s,OMe), 6.92(1H,dd,J=7.92 & 7.88Hz,Ar-H),7.08(1H,d, J=7.92 Hz, Ar-H),7.14 (1H,d,J=7.83Hz,Ar-H),9.87(1H, s, Ar-CHO), 11.07(1H,s,OH). MS M^+ m/z (%) 152(18), 137(8), 121(15), 81(8), 53(100).

2.2.1.8. Preparation of 2,4-dimethoxybenzaldehyde

The compound prepared from 2,4-dihydroxy-benzaldehyde (1.1g, 8mmol) and iodomethane (1.9ml, 30mmol) in acetone (50ml) containing Potassium carbonate (3.5g). The contents refluxed (2.5h), concentrated and diluted with water (200 ml), pH adjusted to 6.0 and then

extracted with solvent ether (3×30ml). The organic layer washed with water (2×10ml) dried over anhydrous sodium sulfate and concentrated to give 2, 4-dimethoxy benzaldehyde (1.25g, 94%) mp 70 °C (mp 71 °C) 1 analyzed for C9H10O3 [C 65.14 H 6.10 $C_9H_{10}O_3$ requires C 65.04 H 6.06 %] IR (KBr pellet): 2984, 1672, 1602, 1582, 1502, 1466, 1408, 1264, 1214, 1110, 1012 and 814 cm^{-1}. ^1H NMR (200MHz, Acetone-d6): δ 3.96 (6H, s, 2×Ar-OMe) 6.4 (1H,d, J=8.5 Hz, Ar-H) 6.5 (1H, s, Ar-H) 7.56 (1H,d, J=8.5 Hz, Ar-H) and 10.1(1H,s, Ar-H-CHO). MS M^+ m/z (%) at 166(100), 149(60), 135(31), 121(28), 106(27), 77(30) and 63(41).

2.2.1.9. Preparation of 1-nitro-3, 4-methylenedioxybenzene

To a solution of 1,3-benzodioxol (10mmol) in acetic acid (8ml) at -5 °C under stirring conditions added nitrating mixture (1ml) prepared from nitric acid: sulfuric acid (4:1 eq.). The reaction worked up by dilution with ice water and extraction with DCM, the organic layer washed with brine solution and dried over $CaCl_2$ and concentrated to give crude product which on column chromatography over silica gel (60-120 mesh) and elution with hexane: ethyl acetate (19:1) afforded a yellow compound (1.51g, 90%) mp 150 °C IR (KBr pellet): 2984, 1605, 1582, 1543, 1502, 1496, 1440, 1365, 1311, 1264, 1214, 1110, 1044 and 814cm^{-1}. ^1H NMR (200MHz, $CDCl_3$): δ 6.14 (2H,s, -OCH_2O-), 6.77(1H,d, J=8.6Hz, Ar-H), 7.67 (1H,d, J=2.2Hz, Ar-H) and 7.92 (1H, dd, J=8.6 & 2.2 Hz, Ar-H). MS M^+ m/z (%) at 167(100), 139(56), 123(36), 111(29), 93(44).

2.2.1.10. Preparation of 2,4-diacetoxybenzaldehyde

The title compound prepared from 2,4-dihydroxy benzaldehyde (2g, 15 mmol) acetic anhydride (4 ml) and pyridine (0.8 ml) by stirring the contents at room temperature for 15 h. The reaction worked up is dilution with ice cold water (50 ml), extraction with chloroform (3×20ml), and washing of organic layer with 0.1M HCl solution followed by water. Drying of the organic

layer over anhydrous sodium sulfate and crystallization of the product with n-hexane: ethyl acetate (9:1) to afford 2,4-diacetoxybenzaldehyde (3g, 90%) mp 68 °C (Lit mp 69 °C). IR (KBr pellet): 2960, 1770, 1692, 1660, 1610, 1448, 1428, 1372, 1318, 1188, 1144, 1000 cm^{-1}. ^1H NMR (200MHz, $CDCl_3$): δ 2.16 (6H,s,2×$OCOCH_3$), 6.5 (2H,bs,Ar-H) 7.26 (1H,d, J=8.5Hz, Ar-H) and 9.4(1H, s, Ar-CHO). MS M^+ m/z (%) 222(3), 180(18), 138(20), 81(4), 54(5), 45(100).

2.2.1.11. Preparation of 2,4-dimethoxybenzaldoxime

To a stirring ethanolic solution (20ml) of 2,4-dimethoxybenzaldehyde (2g, 12mmol) added aqueous solution (3 ml) of hydroxylamine hydrochloride (1.6g, 20mmol) followed by aqueous solution of sodium hydroxide (5M, 12ml) and stirred the contents for 90 minutes (TLC monitored). The contents worked up by dilution with water , pH adjusted to 7, extracted with ethyl acetate (4×25 ml) washed with water and concentrated after drying over sodium sulfate to give a solid (2.05g, 94% yield) mp 104 °C. (lit mp 106 °C) analyzed for $C_9H_{11}NO_3$ (C 60.01 H 6.19 $C_9H_{11}NO_3$ requires C 59.66 H 6.11 N 7.70%). IR (KBr pellet) 2944, 1610, 1504, 1466, 1414, 1270, 1206, 1112, 1026, 922 and 832 cm^{-1}. ^1H NMR (200MHz, $CDCl_3$): δ 3.6 (6H,s,2xAr-OMe) 6.06(1H,d, J=8.5Hz, Ar-H) 6.03 (1H,s,Ar-H) 7.0(1H,d, J= 8.5 Hz Ar-H), 7.73 (1H,s,Ar-CH=N.OH). MS M^+ m/z (%) 181(58), 163(53), 149(100), 134(36), 121(73), 77(45), and 46(78).

2.2.1.12. Preparation of 2,4-dimethoxybenzonitrile

To 2,4-dimethoxybenzaldoxime (0.9g, 5mmol) was added acetic anhydride (2ml) and the contents heated on an oil bath at 150 °C for 1h, the contents cooled and poured into ice-cold water (100 ml) and the resulting solid filtered washed with water (3×10ml) and dried in vacuo to give a colorless solid mp 90 °C (lit mp 91 °C), analyzed for $C_9H_9NO_2$ (C 66.36 H 5.59 N 8.6%) $C_9H_9NO_2$ requires C 66.24 H 5.55 N 8.56). IR (KBr) 2948, 2220, 1604, 1502, 1476, 1438, 1422,

1328, 1286, 1214, 1172 and 1006 cm^{-1}. ^1H NMR (200MHz, CDCl3): δ 3.6(6H,s, 2×Ar-OMe) 6.96 (1H,s,Ar-H) 6.03 (1H,d, J=8.5 Hz, Ar-H) 6.86 (1H,d, J=8.5Hz Ar-H). MS M^+ (m/z)(%) 177(21), 162(100), 135(38), 115(24).

2.2.1.13. Preparation of 2,4-diacetoxybenzaldoxime

The title compound was prepared from 2,4-diacetoxy benzaldehyde (0.9g, 4mmol) and hydroxylamine hydrochloride (0.6g, ~7mmol) and sodium hydroxide as for the procedure described for the preparation of 2,4-dimethoxy benzaldoxime to give the benzaldoxime product (058g, 60%). IR (KBr) 3376,1702,1626,1602,1522,1490,1448,1364,1258,1210,1168,1116 and 974 cm^{-1}. ^1H NMR (200MHz, $CDCl_3$): δ 2.3 & 2.36(3H each, s, 2×OCOCH3) 7.13(1H,d, J= 8.5Hz, Ar-H) 7.2 (2H,s, Ar-H and CH=N.OH),7.7 (1H,d, J= 8.5 Hz, Ar-H). MS M^+ (m/z) (%) 237(12), 220(8), 195(34), 145(42), 117(100), 100(82).

2.3.Pharmacology

2.3.1. Snake venom antiserum

The lyophilized snake venoms of *Daboia russelii* and *Naja kaouthia* were obtained from Calcutta snake park, (Kolkata, India) and preserved in a desiccator at 4 °C for further use. It was dissolved in 0.9% saline and centrifuged at 2000 rpm for 10 min. The supernatant was used as venom and kept at 4 °C until further use. Venom concentration was expressed in terms of dry weight. Lyophilized polyvalent snake venom antiserum (as reference serum) was obtained from Haffkine Institute Mumbai, Maharastra and antiserum (mg ml^{-1}) was dissolved in 0.9% saline prior to its use.

2.4. Animals

Male Swiss albino mice (20±2 g) and Wistar rats (150-200 g) of both sexes and of same age were used in this study after acclimatization to the laboratory condition. All mice/rats were housed five/cages and fed standard laboratory diet and water *ad libitum* with 12-h dark/light cycles at constant temperature of 25±2 $^{\circ}$C. The animals were allowed 1 week to adapt to their environment. All animals were cared for and handled in accordance with the guideline of the Insitutional Ethical Committee. All animal experiments were performed following 'Principles of laboratory animal care' (NIH publication No. 85-23, revised in 1985) as well as Indian laws on 'Protection of Animals' under the provision of authorized investigators.

2.5. Sample Preparation

The test compound was dissolved in 5% DMSO-water and mixed with the venom solution.

2.5.1. Venom inhibiting activity

The pure compound (mg ml^{-1}) was dissolved in 0.9% normal saline (warm) and mixed with the venom solution. The following pharmacological experiments were performed.

2.5.1.1.Neutralization of lethal venom activity

The toxicity of snake venom was assessed by injection of different concentrations of venom in 0.2 mL physiological saline into tail vein of mice [26] (Table 1). To assess the *in vitro* antagonism, various doses of venom (1-15 LD_{50}) were mixed with a fixed amount of compound. The mixture incubated at 37 $^{\circ}$C for 1h, and centrifuged at 2000 rpm for 10 min. The supernatant was injected (*i.v*) in mice, ten mice per dose. The median lethal dose (LD_{50}) was calculated 24 h after injection of the venom-compound mixture. Lethal toxicity was also assessed by sub-cutaneous (*s.c*) injection of various doses of venom. The neutralizing potency of each compound was assessed by injecting (*s.c*) 1-5 LD_{50} of venom into group of 10 mice followed immediately

afterwards by the fixed doses of compound (*i.v*). In time kinetics study, where *D. russelii* venom was injected (MLD) followed by the compound (*i.v*) at 0 min, 30 min and 60 min into groups of six mice and then assessed the mortality rate after 24 h venom injection.

2.5.1.2.Neutralization of venom hemorrhagic activity

The minimum hemorrhagic dose (MHD) of venom defined as the least amount of venom which when injected intradermaly (*i.d*) into mice resulted in a hemorrhagic lesion of 10 mm diameter 24 h later [26] (Table 1). Neutralization of the hemorrhagic activity was estimated by mixing a fixed amount of compound (100 mmol) with different amounts of venom (1-20 MHD). The compound-venom mixture was incubated at 37 °C for 1h, centrifuged at 2000 rpm for 10 min, and 0.1 mL of supernatant injected intradermally (*i.d*). The hemorrhagic lesion was estimated after 24 h (*in vitro*). To assess the anti-hemorrhagic activity of venom *in vivo*, various amount of venom (1-5 MHD) were injected (*i.d*) followed immediately by the compounds and the hemorrhagic lesion measured after 24 h.

2.5.1.3.Neutralization of venom defibrinogenating activity

The minimum defibrinogenating dose (MDD) of venom (*D. russelii)* is defined as the minimum amount of venom which when injected (*i.v*) into mice caused in coagulable blood 1h later [26] (Table 1). Neutralization of this activity was estimated by mixing different amount of venom (1-20 MDD) with fixed amounts of compound (100 mM), incubating at 37 $^{\circ}$C for 1h, and centrifugation. The supernatant was injected (*i.v*) into albino mice (20±2 g) as described above (*in vitro*). For *in vivo* studies, the MDD of venom was injected (*i.v*) followed by the compound (*i.v)* and the nature of the blood observed after 1h.

2.5.1.4.Neutralization of coagulant activity

The minimum clotting dose of plasma (MCDP) was determined as described by Theakston and Reid [26] (Table 1). Neutralization of this activity was estimated by mixing different amount of venom, with a fixed amount of compound, incubating for 1h at 37 °C. Different concentration of incubate were added to the experimental tube in place of 0.1ml physiological saline, and the clotting time was recorded.

2.5.1.5.Neutralization of serum inhibitory activity

To examine whether the decreased clotting of *D. russelii* venom on goat plasma was due to venom inhibition in serum, goat serum was collected without anticoagulant, incubated in glass tubes for 2h at 37 °C and then centrifuged at 2000 rpm for 15 min. Pooled sera were kept frozen at –20 °C. Equal volumes of Tyrode – Hepes buffer (THB) (137 mM NaCl; 2.7 mM KCl; 12 mM $NaHCO_3$; 0.42 mM NaH_2PO_4, 10 mM Hepes; pH 7.4) or goat serums were incubated with venom solution of different concentrations for 5 min at 37 °C. Aliquots of 50 µl were then added to 200 µl of goat plasma (maintained for 2 min at 37 °C) and the clotting time was recorded. MCDS were then calculated (Table 1). Neutralization of serum inhibitory activity was estimated by mixing different amount of venom with fixed amount of compound, incubating for 1h at 37 °C and centrifuged. Supernatant was then added to the experimental group and the clotting time was observed.

2.5.1.6.Neutralization of phospholipase A_2 (PLA_2) activity

For PLA_2 activity experiments, egg yolks (obtained from commercially available eggs), 0.5% EDTA, 50 mM Tris-HCl buffer (pH 7.5), 2% sodium chloride, 1% calcium chloride and 0.9% sodium chloride were prepared and used.

For carrying out the experiment, 2 ml of egg yolk suspension and 0.2 ml of test material (venom only or venom-compound mix) were mixed in the test tube and incubated at 37 °C for 1

h. After incubation the time required for coagulation of egg yolk was recorded by placing the test tube containing test materials (venom or venom-compound mix) on the boiling water bath. A blank was run with normal saline instead of test material. One unit enzyme activity was defined as the amount of venom, which increased the coagulation time of the egg yolk control by one minute [16].

Neutralization of the enzyme activity was estimated on different amount of compound mixed with the different amount of viper venom. The venom-compound mixture was incubated at 37 °C for 1 h. Centrifuged at 2000 rpm for 10 minutes; supernatant was tested in a total of 0.2 ml for the enzyme neutralization activity.

2.6. Molecular docking studies

Molecular modeling studies have been carried out using Glide (Grid-based Ligand Docking with Energetics), software using Schrodinger Maestro 10.1, Glide, running on Linux 64 operating system. In this direction, we docked the ability of the compounds to inhibit the enzymatic activity of venom PLA_2 (PDB code: 1OXL). The molecular docking studies mainly involve selection and preparation of appropriate protein, grid generation, ligand preparation followed by docking and its analysis. The docking score, binding free energy and hydrogen bonds interaction formed with the surrounding amino acids are used to predict their binding affinities and proper alignment of these compounds at the active site of the PLA_2 enzyme.

3. Results and Discussion

Phenolic compounds or polyphenols constitute one of the most numerous and ubiquitously distributed group of plant secondary metabolites, with more than 8000 phenolic structures currently known. The natural phenol ranges from simple molecules (phenolic acids, flavonoids) to highly polymerized compounds (lignin, melanins, tannins). Based on our previous report,

anti-snake venom activity of phenolic compound 2-hydroxy-4-methoxy benzaldehyde is one of the major components of the snake venom PLA_2 inhibitors [24]. In continuation to our previous study, we envisaged to investigate the sensitivity response of *Daboia russelii* and *Naja kaouthia* venoms towards the venom-neutralizing activity of simple phenols, mono-, di- and tri-substituted phenolic compounds by *in vivo*/*in vitro* experiments. The neutralizing effect of lethal, hemorrhagic, coagulant, anticoagulant and PLA_2 inhibiting activities of *Daboia russelii* and lethal activity neutralization against *Naja kaouthia* venoms were studied. The experiments were carried out on four small libraries based on their structures. The first library comprised of simple phenols and their methyl ethers (compounds **1-9**, Fig. 1A) and the second library contained phenols bearing formyl groups and formyl group substituted alkylated phenolic compounds (compounds **10-16**, Fig. 1B). The third library comprised of formyl group bearing dihydroxy benzenes or their alkyloxy/acyl derivatives (compounds 1**7-32**, Fig. 1C), and the fourth library comprised of hydroxymethyl substituted phenols, phenols with aldoximes ornitrile groups (compounds **33-37**, Fig. 1D). The compounds **38** and **39** are known and reported as anti-snake venom activity and are taken for comparison with our present study. All the four compound libraries were screened for anti-venom activity.

Compounds **1-8** displayed anti-hemorrhagic activity with compound **2** found to be potent (5-fold and 3 fold protection in *in-vitro* and *in-vivo* experiments) against *Daboia russelii* venom but displayed no protection against lethal activity (Tables 2 and 3). Compounds **10-16** exhibited anti-venom activities in experimental animal and **15** showed 11-fold protection against lethal, 3-fold against hemorrhagic and 10-fold against defibrinogenating activity of *Daboia russelii* venom. Notably, substituted alkylated phenols bearing formyl group displayed much higher anti-lethal activity than formyl group substituted non-alkylated phenols (displaying higher

hemorrhagic activity) in both the protocols, i.e. in animals with administration of venom and the drug combination injections (Table 2) or venom injection immediately followed by drug injection (Table 3). In *in vivo* experiments, the maximum protection was found in case of compounds **15** (lethal) and 1**2** (hemorrhagic) in mice. While 3-formylated phenol **12** (3-hydroxybenzaldehyde) showed maximum anti-hemorrhagic activity, 3-formylated anisole **15** (3-methoxybenzaldehyde) displayed maximum anti-lethal activity in *in vitro* and *in vivo* study. With respect to lethal activity, higher degree of protection was found against *Daboia russelii* venom and lesser for *Naja kaouthia* venom (Tables 2 and 3). Among compounds **(17-31)**, **17** (8-fold)**, 18** (10-fold)**, 20** (12-fold)**, 21** (10-fold)**, 22** (10-fold)**, 25** (10-fold) and **28** (8-fold) exhibited protection against viper venoms induced hemorrhagic activity and the lethal activity showing maximum fold of protection in compounds **21, 22, 25, 30** and **35** (Table 2). Three fold of protection was seen against *Naja kaouthia* venom-induced lethal activity neutralization by all the compounds. In *in vitro* study, among phenolic compounds with formyl- and alkyloxy substituent, maximum lethal protection of 11-fold against Viper, 3-fold against Cobra and 10-fold against Viper venom-induced hemorrhagic activity was observed. For venom neutralization activity, when the venom was administered (*s.c*) for lethal and intradermally (*i.d*) for hemorrhagic activity followed by intravenous (*i.v*) injection of the phenolic molecule, the degree of protection was dramatically reduced. In general, the degree of protection was always found to be higher in case of *Daboia russelii* venom compared to *Naja kaouthia* venom both in *in vitro* and *in vivo* experiments.

Substantial fall in the inhibitory activity profile of the compounds was observed in *in vivo* studies when the venom was injected subcutaneously followed by the drug given by intravenous route separately (Table 3). The compound **18** showed only 2 fold lethal protection and 1-fold

hemorrhagic protections (*in vivo*) against 2-fold and 10-fold protection in *in vitro* studies. In compounds of group-IV (Fig. 1D) where aldehydic group was replaced either by a primary alcohol group or by an aldoximic or a nitrile group, the activity increased dramatically both *in vitro* and *in vivo* (Tables 2 and 3). These changes in the functional group of the compounds resulted in drastic change in its venom inhibitory upshot. It was indicative of the importance of formyl and aldehydic groups in these compounds.

From the above observations, it became apparent that many of these molecules possess potent antisnake venom activity and therefore, few of them were considered for further studies. The short-listed compounds **18**, **21**, **22**, **25**, **28**, **34**, **35**, and **36** were taken for viper venom neutralization effect of lethal, hemorrhagic, coagulant, defibrinogenating and PLA_2 activities. In time kinetic experiments, when the viper and cobra venom was injected via *s.c* route followed by the administration of drug by *i.v* route at 0 min, 30 min and 60 min (after venom injection), significant lethal protection was observed at 0 min for majority of the test compounds. The activity got reduced after 30 min and after 60 min only 20% protection of venoms was observed in mice. In these experiments, mono-alkylated mono-hydroxy benzaldehyde and aldoximic derivatives (**21**, **22, 25, 30, 35** and **36**) exhibited protections at 0 min, 40-50% after 30 min and 10-30% after 60 min (**18, 21, 22, 35** and **36**). It was also observed that the degree of protection against viper venom (*D. russelii*) was always higher than the cobra venom (*N. kaouthia*), having no protection after 30 and 60 min of compounds introduction (Fig. 2). The selected compounds effectively antagonized the Viper venom induced defibrinogenating activity. In *in vitro* studies, the phenolic compounds (100 mmol) showed protection upto 10 fold of minimum defibrinogenating dose (MDD) in mice. In *in vivo* studies, venom induced defibrinogenating activity was antagonized by all the compounds except **18** and **34**. The protection was always higher in *in vitro* studies than *in vivo* (Table 4).

The effects of *Daboia russelii* venom on coagulant activity and the neutralization effect of the selected compounds was also studied by plasma recalcification time. The normal value (0.9% saline + calcium-induced) of minimum clotting dose of plasma (MCDP) / serum (MCDS) was found to be 25±0.17 sec and venom + calcium-induced was found to be 15±0.20 sec. In the above experiments the selected molecules (20 mmol) were incubated with different amounts of venom (1-100 µg) and tested for coagulant activity. The compounds antagonized the venom-induced coagulant activity was neutralized effectively as evident by plasma recalcification time. Compound **35** exhibited maximum protection (Fig. 3A). Phospholipase A_2 activity of *Daboia russelii* venom was assessed by egg yolk coagulation method. One unit of *Daboia russelii* venom activity was found to be 2 µg which increased the coagulation time by one minute (control 0.9% saline, coagulation time was found to be 45 ± 1.16 sec). The compounds **18, 21, 22, 25, 28, 34, 35**, and **36** were incubated with different amounts of viper venom (2-100 µg) and tested for phospholipase A_2 activity. The venom PLA_2 was found to be effectively neutralizing the compounds. The compound **35** (20 mmol) significantly neutralized the venom (60 µg) induced PLA_2 activity. The degree of protection was found to be higher in compound **35** (Fig. 3B).

Molecular Docking

PLA_2 enzyme is one of the major and most toxic proteins found in snake venom. In our present study, we tried to search for PLA_2 enzyme inhibitors that may be useful to design antivenom compound(s) to treat snake envenomations. In order to suggest a mode of action of the compounds, we performed molecular docking study. The docking scores and binding energy of the said compounds and co-crystal ligand with the active site of PLA_2 is summarized in Table

S1 of Supplementary material. The crystal structure of PLA_2 was prepared for docking with the Protein Preparation Wizard workflow of Maestro that allows addition of hydrogen atoms which were subsequently minimized with OPLS-2005 force field to optimize the protonation state. The receptor grid was generated by applying a Van der Waals radii of non-polar atoms, which decreases penalties for close contacts (scaling factor = 1.00 and partial charge cut off = 0.25). Before docking calculation, all the compounds were subjected to ligand preparation with the LigPrep tool. Finally, the ligand docking was done using receptor grid file and LigPrep out file in the Glide tool of Application view. The docking results revealed that two amino acids ASP 49 and GLY 30 located in the binding pocket of enzyme played vital roles in designing the snake anti-venom compound(s). Among all the titled compounds for PLA_2, compound **35** was found to be most potent with a high docking score. The compound **35** assumes favorable orientation within the binding site. The binding mode of compound **35** is exactly same as the co-crystal ligand (IDA: 501). The docked pose (Fig. 4A) of the compound **35** showed hydrogen bond interactions with amino acid ASP 49 (H....O=C, 1.90 Å, Acceptor angle 152.75, Donor angle 146.72) and GLY 30 (H.... O=C, 2.21 Å, Acceptor angle 150.38, Donor angle 111.11) and HIP 48 (O....HN, 2.07 Å, Donor angle 96.99). The CYS 45 (H....O=C, 2.73 Å, Acceptor angle 127.32, Donor angle 124.24) makes an additional hydrogen bonding whereas co-crystal ligand IDA: 501 forms hydrogen bond with ASP 49 (H....O=C, 2.04 Å, Acceptor angle 142.50, Donor angle 98.75) and GLY 30 (H.... O=C, 1.95 Å, Acceptor angle 155.65, Donor angle 178.22) and HIP 48 (O....HN, 2.13 Å, Donor angle 113.42) shown in Fig. 4B. The hydrophobic enclosure visualization pose of Compound **35** was shown in Fig. 4C. The aligned pose of all the compounds were also shown in Fig. 4D. In addition, the enzyme surface model was showed in Fig. 5, which revealed that the molecule **35** occupies the binding pocket of PLA2.

Binding Energy Estimation

Binding energy estimation can be used to calculate ligand binding energies and ligand strain energies for a set of ligand and a single receptor PLA_2 using Prime molecular mechanics-generalized born surface area (MM-GBSA) method. All the water molecules were deleted from the designed complexes prior to dG binding free energy calculation using solvation model VSGB by Prime MM-GBSA, Maestro 10.1. All the compounds are well fitted in the active sites of PLA2. The binding poses for each compound were analyzed by examining their free energy scores. The dG binding energy of compounds was found in the range of -26.587 to -78.857 Kcal/PLA2 (Table S1, Supplementary material). All the compounds displayed a good binding free energy (dG bind) for PLA_2 which indicates that compounds may have better selectivity and affinity towards enzyme.

Structure-activity relationship (SAR)

Phenolic compounds are commonly found in both edible and inedible plants, and they are known to have multiple biological effects including antioxidant, anti-inflammatory and cardio-protective. Phenolic compounds are widely accepted and recommended for their antioxidant properties as the chemical structure of phenolic compound imparts them the ability to serve as free radical scavengers. A phenolic compound consists of a benzene ring and an alcoholic hydroxyl group, which are the defining features of its acidic nature. In the present investigation it became evident that the substituted phenolic analogs of low molecular weight have the potential to neutralize snake venom and may also possesses potent antioxidants activity like, aspirin, paracetamol, coramine et cetera [27,28]. The phenolic compounds with a reactive formyl group may easily interact with the macromolecule toxins (enzymes/proteins) present in the snake venom through chelation or chemical modification. Furthermore, there are a number of mechanisms by which phenolic compounds may act as anti-snake venom: via free radical

scavenging, hydrogen donation, singlet oxygen quenching, metal-ion chelation, or as substrates for attack by superoxide [29]. The other possibility of the potent anti-snake venom effect of phenolic compounds might be due to blocking of one or more of active sites of the venom toxins or blocking the receptors which are structurally prone to chemical attack which is also evident from our docking studies. In this study, we have shown that molecular docking is an important tool in evaluating the interaction between PLA_2 and polyphenols. Our results demonstrate that the snake venom PLA_2 binds strongly with compounds **21, 22, 25, 28, 35,** and **36** by molecular docking (Table S1, Supplementary material). The docking results revealed that two amino acids ASP 49 and GLY 30 located in the binding pocket of enzyme played vital role in the designing anti-snake venom activity. The compound **35** assumes favorable orientation within the binding site, which is exactly same as the co-crystal ligand (IDA: 501). In addition, the enzyme surface model also revealed that the molecule **35** occupies the binding pocket of PLA_2. All the active compounds fit well in the active sites of PLA_2. The binding poses for each compound were analyzed by examining their free energy scores. The dG binding energy of compounds was found in the range of -26.587 to -78.857 Kcal for enzyme PLA_2 which indicates that compounds may have better selectivity and affinity towards enzyme PLA_2.

It is evident from the above results that the potent anti-snake venom activity is exhibited by the substituted benzaldehydes. It is also reported that 2-hydroxy-4-methoxybenzaldehyde possesses potent tyrosinase inhibition activity, strong chelating action, and potent antioxidant property [25]. In our earlier studies we observed that the phenolic compound 2-hydroxy-4-methoxy benzoic acid possessed potent anti-snake venom and antioxidant property. It also showed that the mechanism of anti-venom activity is through venom induced free radical

inhibition. The relationship between chemical structure of phenolic and their anti-snake venom activity has been of considerable interest. Apart from the general observations made above, more specific effects have been noted. The antisnake venom activity of phenols increases when additional hydroxyl group is added and when the hydroxyl or methoxyl group is present at 3-position of the benzene ring. Rice-Evans *et al*. [30] showed that ortho-substitution with electron-donating such as methoxyl group increases the stability of the aryloxyl radical and hence its antioxidant potential. It is also reported that the antioxidant activity of phenolic compound depends on the structure and substitution pattern of hydroxyl groups [31]. The essential requirement for effective free radical scavenging is the 3,4-orthodihydroxy configuration in ring B and 4-carboxyl group in ring C. The presence of 3-OH group or 3-and 5-OH groups, giving a catechol like structure in ring C, was also beneficial for the antioxidant activity of the phenolic compounds [29,32,33]. It also showed a structurally important role of 3-OH group of the chromane ring responsible for the enhancement of antioxidant activity. In our present study, the phenolic compounds having hydroxyl and methoxyl group present at 3-position have showed potent anti-hemorrhagic and anti-lethal effect respectively against snake envenomations. The presence of a neighbouring hydroxyl or a methoxyl group further helped in stabilizing the substrate-protein complex which might be forming a Schiff's base by interaction of CHO group of the phenolic compound (substrate) with snake venom toxins (PLA_2 enzyme) that might leads to the increase in activity of phenolic compounds [30]. The protection against venom induced pathophysiological changes by the phenolic compounds may also be due to the formation of a complex between a venom protein and the compound, reducing the toxic effects of the venom. In variance to separate the administration of the two (compound injected *i.v.* followed by venom *s.c*) as in case of *in vivo* experiments where the activities of the compounds were drastically diminished. Briefly, the results obtained herein re-enforce the idea that the polyphenolic

compounds present a significant inhibiting capacity of the snake-venom PLA_2 activity. Possibly, the phenolic hydroxyls are bound to ASP 49 and GLY 30 that may influence the capacity of these amino acids to participate in the coordination of calcium atom that is essential to catalytic enzyme. Apparently, the number of hydroxyls is not a limiting factor for the efficiency in capacity of PLA_2 inhibition. The presence of methoxyl group in the benzene ring also helps to enhance the activity of the phenolic compound (compound **35**, for instance) and it might be bound with ASP 49 and GLY 30 of PLA_2 much strongly that may increases the inhibitory efficiency of these compounds.

4. Conclusion

In conclusion, several substituted phenols have been shown effective against snake venom with induction of pathophysiological changes both *in silico* and in wet lab experiments. In this study, molecular docking of the phenolic compounds having hydroxyl and methoxyl groups in their benzene ring showed maximum inhibitory potency as they bound to active site of (ASP 49 and GLY 30) snake venom PLA_2. The results showed here led us to the opening of a window of opportunity for screening more potent molecules that may be developed through *in silico* chemistry which may lead to manifold anti-snake venom activity. Further work is intended to establish the mechanism of actions of the phenolic compound(s) against venom-induced pathophysiological changes and to better understand the action of phenolic compounds on snake-venom PLA_2 activities.

Supplementary material

Docking scores and binding free energy of compounds at the active sites of PLA_2 (Table S1) are represented in Supplementary Material available online.

Conflict of Interest

The manuscript was written through contributions of all authors. This manuscript has not been published and is not under consideration for publication elsewhere and we have no conflicts of interest to disclose.

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Figure legends

Fig. 1. **A**. The first group of compounds (**1-9**) comprised of simple phenols and their methyl ethers. **B**. The second group of compounds (**10-16**) comprised of formylated phenols or their alkyl derivatives. **C.** The third group of compounds (**17-32**) was formylated dihydroxy benzenes or their alkyl/acyl derivatives. **D.** The fourth group of compounds (**33-37**) comprised of substituted benzyl alcohols and benzaldoximes / benzonitriles.

Fig. 2. Lethal action neutralization of (A) *Daboia russelii* and (B) *Naja kaouthia* venom by the compounds in male albino mice (Time kinetics). Results are expressed as mean of ten observations ± SD of 3 replications. MLD, minimum lethal dose = 70 µg (*Daboia russelii* venom); 8 µg (*Naja kaouthia* venom). Deaths time: *Daboia russelii* venom (1 Minimum lethal dose) = 13.24± 0.30: *Naja kaouthia* venom (1 Minimum lethal dose) = 10.68 ± 0; * = No protection.

Fig. 3. Anticoagulant and Phospholipase A_2 activities. (A) Coagulant activity neutralization of *Daboia russelii* venom by the compounds. Results are expressed as mean of 10 observations. MCDP, minimum clotting dose plasma; MCDS, minimum clotting dose serum. (B) Phospholipase A_2 activity neutralization of *Daboia russelii* venom by the compounds. Results are expressed as mean of ten observations. 1 unit= one unit of enzyme activity of *Daboia russelii*. One unit PLA2 activity of the Venom was defined as the amount of venom which increased the coagulation time of egg yolk by one minute. 2 µg of venom = 1 unit of PLA2 activity.

Fig. 4. Molecular docking: (A) Docked Pose of Compound 35 represented as ball and stick in the binding site of PLA-2 showing hydrogen bond interaction (red dash lines) with amino acids

ASP 49, GLY 30, CYS 45 and HIP 48 (labeled with purple colour). The interacting amino acids are shown as green colour stick. The red in the ball and white in the sticks represent oxygen and hydrogen atoms respectively, **(B) Docked Pose of Co-crystal ligand (IDA 501)** represented as ball and stick in the binding site of PLA-2 showing hydrogen bond interaction with ASP 49, GLY 30 and HIP 48.The red dash lines denote the hydrogen bonds and the amino acids are represented as thin tube, **(C) Hydrophobic enclosure visualization pose of Compound 35,** hydrophobic atoms on protein that enclose hydrophobic group in the ligand are displayed as gray in CPC representation. Hydrophobic atoms in compound **35** are displayed as ball and stick (green color) representation. The hydrophobic amino acids enclosures of PLA_2 are TYR 22, PHE 106, ILE 9 and PHE 5 (labeled with pink colour), **(D) All the compounds aligned properly with co-crystal ligand,** represented as ball and stick in the binding site of PLA_2 showing hydrogen bond interaction with different amino acids such as ASP 49, GLY 30, GLY 32, TYR 22, ALA 18, LEU 2 and HIP 48. The hydrogen bonds are shown as red dash lines. The interacting amino acids are represented as green colour stick.

Fig. 5. Enzyme surface model of compound 35 in binding cavity of PLA_2. The hydrogen bonding interaction with amino acids are shown.

Table 1. Properties of snake venoms in male Swiss albino mice

Venom	LD_{50} (iv) / 20 g mice (μg)	LD_{50} (sc) / 20 g mice (μg)	Minimum hemorrhagic dose (μg)	Minimum defibrinogenating dose (μg)	Minimum clotting (μg ml^{-1})
Daboia russelii	2.24	40	5	2.5	1
	(1.4–3.4)	(26.5–60.2)			
Naja kaouthia	2.28	4.88	NR	NR	NR
	(1.7–4.4)	(2.9–6.8)			

Results are expressed as mean of six observations. Figures in parentheses are the fiducial limits. NR, not required; $LD_{50,}$ lethal dose 50.

Table 2. Snake venom induced lethal and hemorrhagic action neutralizations by the compounds in male albino mice *in vitro.*

Compounds No.	mmol	*Daboia russellii* μ g (LD_{50})	*Naja kaouthia* μ g (LD_{50})	*Daboia russellii* μ g (MHD)	log P
Group I					
1. 1-hydroxy benzene (Phenol)	100	NP	NP	10(2)	1.76
2. 1,2- dihydroxy benzene (Catechol)	100	NP	NP	25(5)	1.48
3. 1, 3- dihydroxy benzene (Resorcinol)	100	NP	NP	10(2)	1.48
4. 1, 4- dihydroxy benzene (Quinol)	100	NP	NP	20(4)	1.48
5. 1-methoxy benzene(anisole)	100	NP	NP	10(2)	1.44
6. 1, 2-dimethoxy benzene(veratrole)	100	NP	NP	10(2)	1.44
7. 1, 3- dimethoxy benzene	100	NP	NP	10(2)	1.44
8. 1 , 4- dimethoxy benzene	100	NP	NP	10(2)	1.47
9. 3,4-methylenedioxynitrobenzene	100	NP	NP	NP	1.47
Group-II					
10. Benzaldehyde	100	NP	NP	NP	1.47
11. 2- hydroxy benzaldehyde	100	NP	NP	NP	1.47
12. 3- hydroxy benzaldehyde	100	6(2.6)	3(1.0)	30(6)	1.15
13. 4 -hydroxy benzaldehyde	100	6(2.6)	3(1.0)	15(3)	1.15
14. 2-methoxy benzaldehyde	100	18(8.0)	6(2.1)	10(2)	1.15
15. 3-methoxy benzaldehyde	100	25(11.2)	9(3.2)	15(3)	1.15
16. 4-methoxy benzaldehyde	100	NP	NP	15(3)	1.19
Group-III					
17. 2, 3-dihydroxy benzaldehyde	100	5(2.1)	3(1.0)	40(8)	1.19
18. 2, 4- dihydroxy benzaldehyde	100	6(2.6)	3(1.0)	50(10)	1.19
19. 2, 5- dihydroxy benzaldehyde	100	5(2.1)	3(1.0)	35(7)	1.53
20. 3, 4- dihydroxy benzaldehyde	100	6(2.6)	3(1.0)	60(12)	2.0
21. 2- hydroxy-3-methoxy benzaldehyde	100	25(11.2)	9(3.2)	50(10)	2.39
22. 2-hydroxy-4-methoxy benzaldehyde	100	22(9.8)	9(3.2)	50(10)	3.19
23. 2- hydroxy-5-methoxy benzaldehyd	100	11(4.9)	6(2.1)	20(4)	1.93
24. 2- hydroxy-4-acetoxy benzaldehyde	100	NP	NP	NP	0.94
25. 2- hydroxy-4-allyloxy benzaldehyde	100	22(9.8)	9(3.2)	50(10)	1.22
26. 2- hydroxy-4-ethoxy benzaldehyde	100	NP	NP	5(1)	1.19
27. 2- hydroxy-4-propyloxy benzaldehyde	100	NP	NP	5(1)	1.53
28. 2- hydroxy-4-butyloxy benzaldehyde	100	18(8.0)	6(2.1)	140(8)	0.94
29. 2- hydroxy-4-heptyloxy benzaldehyde	100	6(2.6)	3(1.0)	5(1)	0.97
30. 2,4-dimethoxy benzaldehyde	100	22(9.8)	9(3.2)	25(5)	0.97
31. 3- hydroxy-4-methoxy benzaldehyde	100	NP	NP	10(2)	0.97
32. 4- hydroxy-3-methoxy benzaldehyde	100	22(9.8)	9(3.2)	20(4)	1.87
Group IV					
33. 4- hydroxy-3-ethoxy benzaldehyde	100	11(4.9)	3(1.0)	15(3)	1.81
34. 2, 3- dihydroxy benzyl alcohol	100	18(8.0)	6(2.1)	15(3)	---
35. 2-hydroxy-3-methoxy benzyl alcohol	100	25(11.2)	9(3.2)	40(8)	---
36. 2-hydroxy-4- methoxy benzyl alcohol	100	22(9.8)	9(3.2)	30(6)	---
37. 2, 4- dimethoxy benzaldoxime	100	6(2.6)	3(1.0)	5(1)	1.87
38. [+]2-hydroxy-4-methoxy benzoic acid	6.0	22(9.8)	9(3.2)	20(4)	1.21
39. [+]Aspirin	12	6.6(3)	3(1)	100(20)	1.24
Commercial antiserum	2mg	4.5(2)	5.6(2)	10(2)	----

Venom-compound mixture incubated at 37 °C/ 60 min, injected *i.v* (lethal) and *i.d* (hemorrhage) activity. The values in parentheses are the number of LD_{50}/MHD. Results are expressed as mean of ten observations. NP= No Protection; LD_{50} = Lethal dose 50 = 2.28 μ g of *Daboia russelii* venom and 2.82 μ g of *Naja kaouthia* venom; MHD= Minimum hemorrhagic dose = 5 μg; **(**[+] Alam and Gomes, 1994 & 1998)

Table 3. Snake venom induced lethal and hemorrhagic action neutralizations by the compounds in male albino mice *in vivo*.

Compounds	mmol	*Daboia russelii* μg (LD_{50})	*Naja kaouthia* μg (LD_{50})	*Daboia russellii* μg (MHD)
Venom injected *s.c* (lethal) and *i.d* (hemorrhage) followed immediately by the compound *i.v.*				
Group I				
Entry (compound No.)				
1	100	NP	NP	5(1)
2	100	NP	NP	15(3)
3	100	NP	NP	10(2)
4	100	NP	NP	10(2)
5	100	NP	NP	5(1)
6	100	NP	NP	5(1)
7	100	NP	NP	5(1)
8	100	NP	NP	5(1)
9	100	NP	NP	NP
Group-II				
10	100	NP	NP	NP
11	100	50(1.25)	NP	10(2)
12	100	50(1.25)	NP	15(3)
13	100	50(1.25)	NP	10(2)
14	100	100(2.5)	NP	5(1)
15	100	100(2.5)	NP	5(1)
16	100	NP	NP	5(1)
Group-III				
17	100	NP	NP	10(2)
18	**100**	**100(2.5)**	**8(1.6)**	**5(1)**
19	100	50(1.25)	NP	15(3)
20	100	50(1.25)	NP	20(4)
21	**100**	**150(3.75)**	**10(2.0)**	**10(2)**
22	100	150(3.75)	10(2.0)	10(2)
23	100	100(2.5)	8(1.6)	10(2)
24	100	NP	NP	NP
25	**100**	**150(3.75)**	**10(2.0)**	**10(2)**
26	100	NP	NP	NP
27	100	NP	NP	NP
28	100	150(3.75)	10(2.0)	10(2)
29	100	NP	NP	NP
30	100	100(2.5)	8(1.6)	10(2)
31	100	NP	NP	10(2)
32	**100**	**150(3.75)**	**10(2.0)**	**5(1)**
Group IV				
33	100	**100(2.5)**	**10(2.0)**	**10(2)**
34	**100**	**100(2.5)**	**10(2.0)**	**10(2)**
35	**100**	**150(3.75)**	**10(2.0)**	**10(2)**
36	**100**	**150(3.75)**	**10(2.0)**	**5(1)**
37	100	50(1.25)	NP	NP
38.2-hydroxy-4-methoxy benzoic acid	6.0	150(3.75)	10(2)	10(2)
39.Aspirin	12	50(1.25)	NP	10(2)
Commercial antiserum	2mg	4.5(2)	5.6(2)	10(2)

The values in parentheses are the number of LD_{50} / MHD. Results are expressed as mean of ten observations. NP= No Protection; LD_{50} = Lethal dose 50 = 2.28 μg of *Daboia russelii* venom and 2.82 μ g of *Naja kaouthia* venom; MHD= Minimum hemorrhagic dose = 5 μg

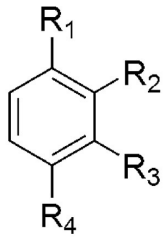
Table 4. Defibrinogenating activity neutralization of *Daboia russelii* venom by the compound in male albino mice.

Compound (100 mmol)	Venom (μg)	Fold of neutralization (In terms of MDD)	Nature of blood
Venom – compound incubated 37^0 C/ 60 mins and injected *i.v*			
18	2.5	NP	Non Clot
21	25.0	10	Clot
22	22.0	8.8	Clot
25	5.0	2.0	Clot
28	6.0	2.4	Clot
34	2.5	NP	Non Clot
35	25.0	10	Clot
36	25.0	10	Clot
Venom injected *i.v* followed immediately by the compound *i.v*			
18	2.5	NP	Non Clot
21	3.5	1.5	Clot
22	3.5	1.5	Clot
25	3.5	1.5	Clot
28	3.5	1.5	Clot
34	2.5	NP	Non Clot
35	3.5	1.5	Clot
36	3.5	1.5	Clot

Results are expressed as mean of 10 observations. MDD, minimum defibrinogenating dose = 2.5 μg; NP= No protection.

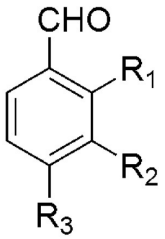
Fig. 1

A



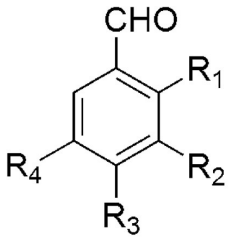
1. R_1 = OH, R_2 = H, R_3 = H, R_4 = H
2. R_1 = OH, R_2 = OH, R_3 = H, R_4 = H
3. R_1 = OH, R_2 = H, R_3 = OH, R_4 = H
4. R_1 = OH, R_2 = H, R_3 = H, R_4 = OH
5. R_1 = OMe, R_2 = H, R_3 = H, R_4 = H
5. R_1 = OMe, R_2 = H, R_3 = H, R_4 = H
6. R_1 = OMe, R_2 = OMe, R_3 = H, R_4 = H
7. R_1 = OMe, R_2 = H, R_3 = OMe, R_4 = H
8. R_1 = OH, R_2 = H, R_3 = H, R_4 = OMe
9. R_1 = NO_2, R_2 = H, $R_3 + R_4$ = OCH_2O

B



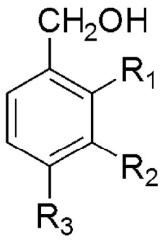
10. R_1 = H, R_2 = H, R_3 = H
11. R_1 = OH, R_2 = H, R_3 = H
12. R_1 = H, R_2 = OH, R_3 = H
13. R_1 = H, R_2 = H, R_3 = OH
14. R_1 = OMe, R_2 = H, R_3 = H
15. R_1 = H, R_2 = OMe, R_3 = H
16. R_1 = H, R_2 = H, R_3 = OMe

C

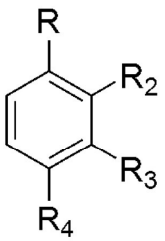


17. R_1 = OH, R_2 = OH, R_3 = H, R_4 = H
18. R_1 = OH, R_2 = H, R_3 = OH, R_4 = H
19. R_1 = OH, R_2 = H, R_3 = H, R_4 = OH
20. R_1 = H, R_2 = OH, R_3 = OH, R_4 = H
21. R_1 = OH, R_2 = OMe, R_3 = H, R_4 = H
22. R_1 = OH, R_2 = H, R_3 = OMe, R_4 = H
23. R_1 = OH, R_2 = H, R_3 = H, R_4 = OMe
24. R_1 = OH, R_2 = H, R_3 = OAc, R_4 = H
25. R_1 = OH, R_2 = H, R_3 = OC_3H_5, R_4 = H
26. R_1 = OH, R_2 = H, R_3 = OC_3H_7, R_4 = H
27. R_1 = OH, R_2 = H, R_3 = OC_4H_9, R_4 = H
28. R_1 = OH, R_2 = H, R_3 = OC_7H_{15}, R_4 = H
29. R_1 = OMe, R_2 = H, R_3 = OMe, R_4 = H
30. R_1 = H, R_2 = OH, R_3 = OMe, R_4 = H
31. R_1 = H, R_2 = OMe, R_3 = OH, R_4 = H
32. R_1 = H, R_2 = OEt, R_3 = OH, R_4 = H

D



33. R_1 = OH, R_2 = OMe, R_3 = H
34. R_1 = OH, R_2 = H, R_3 = OMe



35. R = CH=NOH, R_1 = OEe, R_2 = H, R_3 = OEe
36. R = CH=NOH, R_1 = OH, R_2 = H, R_3 = OH
37. R = CN, R_1 = OMe, R_2 = OH, R_3 = OMe

Fig. 2

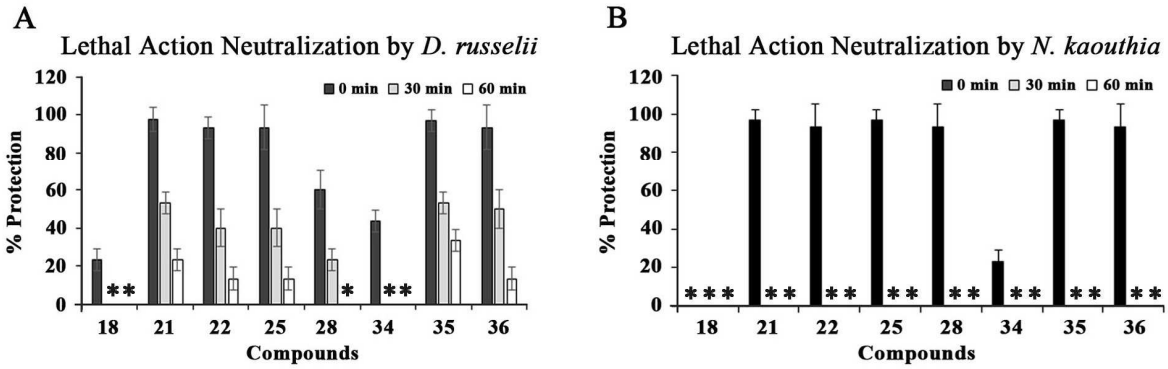


Fig. 3

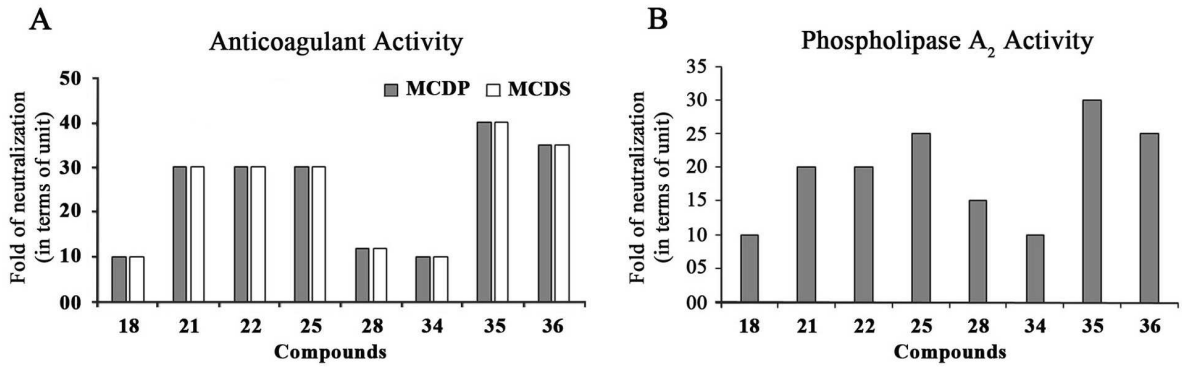


Fig. 4

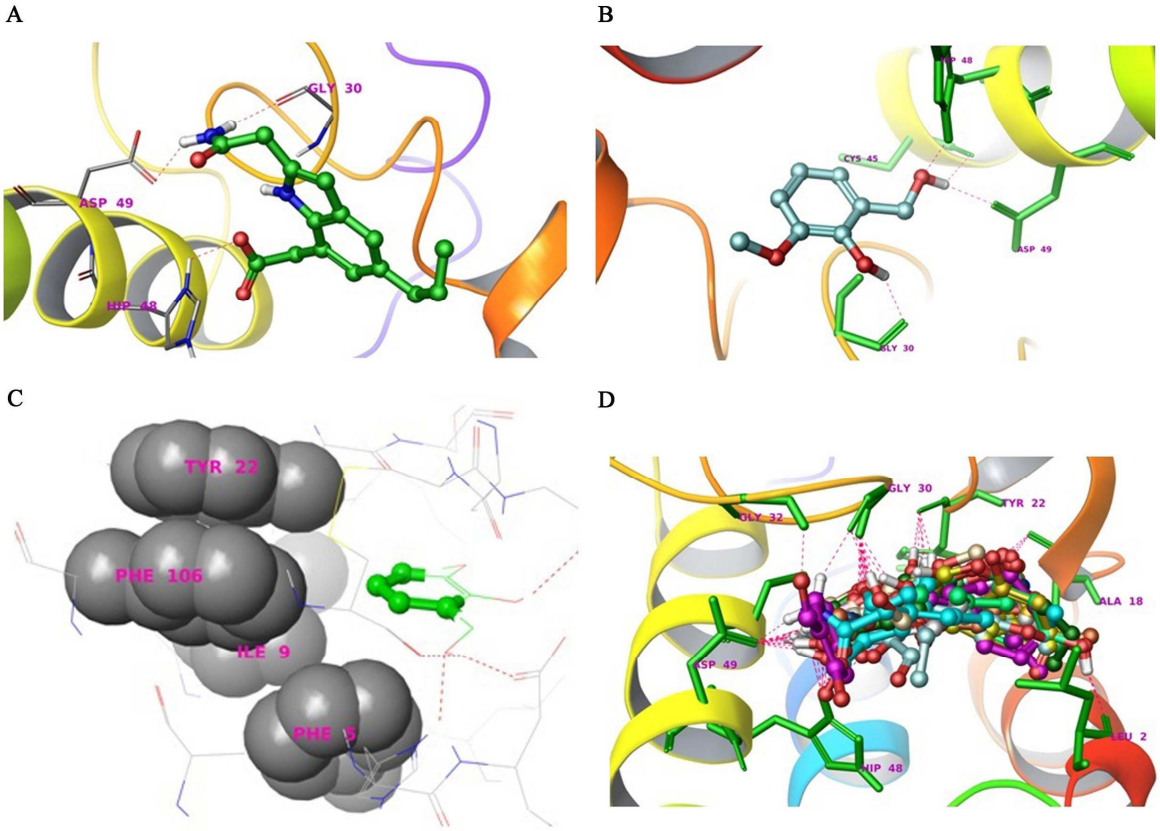
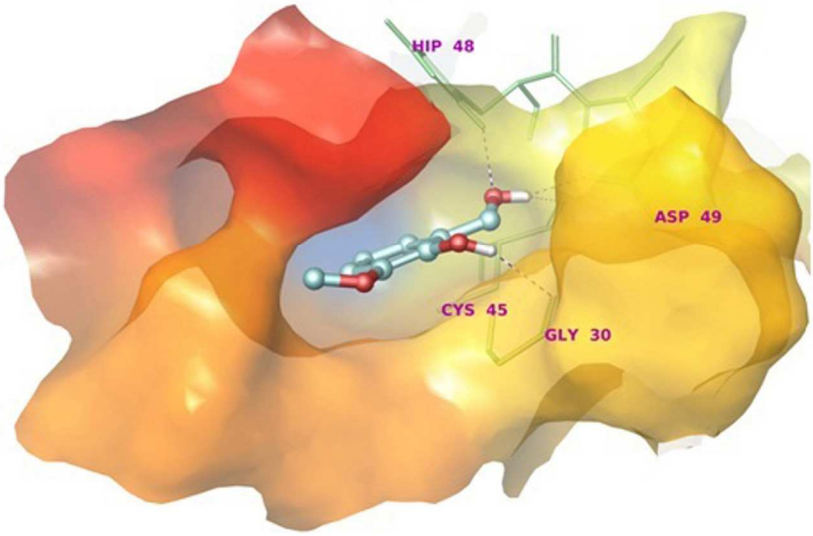


Fig. 5



1. Many Indian medicinal plants are used in folk medicine as an antidote against snake bite.

2. 2-hydroxy-4-methoxybenzaldehyde from *Janakia arayalpatra* and its analogues were found active against Viper and Cobra venoms. .

3. A formyl group in conjunction with a phenolic group was found to be a significant contributor towards anti-venom activity.

4. Structurally variant phenolic compounds were studied via molecular docking on the venom protein.

5. Identified potent molecules that may be developed through *in silico* chemistry which may lead to manifold anti-snake venom activity.