NOVEL 3-(2-AMINOETHYL)-5-METHOXY-1H-INDOL-6-OL FROM INDIAN TOAD PARATOID GLANDULAR SECRETION AS PROTECTIVE AGENT BY INHIBITING IL-6 AND TNF-A

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ABSTRACT

The discoveries of potential anti-inflammatory agents are still needed to mitigate the inflammation mediated cardiovascular and neurodegenerative disorders. Many anti-inflammatory compounds were discovered from plant sources, but very little research is done based on animal sources. This study discovered a Novel Bufotenin Variant (NBV), which was not reported earlier, from paratoid gland venom of Indian Toad with potential anti-inflammatory activities. NBV was characterized by IR, ^{1}H NMR, ^{13}C NMR, LC-MS, P-XRD, COSY, HSQC and HMBC, and *in silico* molecular docking demonstrated that, proteins for PDB-2L3Y (IL-6) and 2AZ5 (TNF-α) with a stronger binding affinity. Due to limited information on investigation of protective activities with different inducers such as λ - carrageenan induced paw edema and air pouch mouse models, Bisphenol-A is used for kidney and hepato toxicity and colitis induced by Dextran Sodium Sulphate (DSS) for *in vivo* studies and NBV treated groups significantly inhibited TNF-α and IL-6, and further confirmed by histological and immunohistochemical studies. Paw edema, liver, kidney and colon tissue, serum levels of IL-6 and TNF-α significantly decreased with high dose of NBV (20mg/kg).This study expedited that NBV as a potent protective agent with the mechanism of action by inhibition of both TNF-α and IL-6. Further NBV will become a new chemical entity with protective properties can attract the researchers to discover better anti-inflammatory drugs and it may help in the new drug discovery for the treatment of many inflammations mediated diseases. Further systematic studies are needed to get the potential therapeutic benefits of NBV.

Key words: Indian Toad, Venomous secretion, Novel Bufotenin Variant (NBV).

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Introduction:

Amphibians contains two types of cutaneous glands throughout the body, the first are mucous glands, responsible for maintaining humidity and cutaneous respiration (Hutchinson and Savitzky, 2004), and the second aregranular glands, responsible for chemical defense against predators (Takai et al.). Chemical defence systems are essential in amphibians, such like anti-predatory and antibacterial strategies provided by dermal glands that produce and store toxic substances. The parotoid are the most common macro glands in Bufonidae toads, and they are spread across the skin and/or aggregated in multiglandular complexes. Isolation of bioactive compounds from venoms represents from Indian toad is an extremely valuable pharmacological approach for the development of animal toxin-based drugs with high specificity and potency towards their molecular targets mainly located in the cardiovascular and nervous systems (Perera Córdova et al., 2016). These glands produce proteins, peptides, biogenic amines, poisonous steroidal bufodienolide, toxicsamandarine alkaloids, and indole pseudophenylamine alkaloids are among the other chemical substances, depending up on the species. Bufotoxins in bufonid paratoid macro glands, which are well known for their poisonous secretions, include bufotoxins, indole amine and the toxin reported as a potent local anesthetic and anti-inflammatory agent (Zheng et al., 2020).

Indolealkylamines (IAAs) are 5-hydroxytryptamine (5-HT) derivatives, altering central nervous system functions, so fararound 14 IAAs were identified including 5-methoxy-N, N-dimethyl tryptamine (5-MeO-DMT) (Shen et al., 2010). Toad species skin and epidermal glands contains the IAAs like bufotenin, bufotenidine, and cinobufotenine. Bufotenin isa common metabolite of tryptamine alkaloid, found in living cell, skin, glandular secretions from toads. The bufotenine derivatives from Senso toad venom of Japan origin and Chansu from China origin were using since long time in the treatment of cardiovascular problems, anti-inflammatory and psychotropic activities (Emanuele et al., 2010). Dimethoxy Phenylisopropylamine, lysergic acid diethylamide (LSD) and bufotenine interacts with 5-HT2A receptor, and bufotenine derivatives are found to have psychotropic activities and anti-inflammatory properties (Chen et al., 2020).

BPA, also known as 2,2-bis (4-hydroxyphenyl) propane, is a chemical that pollutes the environment and is frequently used in the production of epoxy resins and polycarbonate plastics (Eid et al., 2015). BPA changed mouse hepatocyte viability and altered liver weights. BPA is a nephrotoxic substance because the kidneys can't get rid of its toxic by-products, which build up over time (Moon et al., 2012). Additionally, BPA stimulates the production of reactive oxygen species (ROS), which harms the liver, kidneys, and other organs' tissue (Sangai et al., 2014).

Dextran sodium sulphate (DSS), a chemical colitogen with anticoagulant characteristics, is likely the most frequently used animal model of colitis. DSS is a negatively charged, water-soluble sulfated polysaccharide with a molecular weight range of 5 to 1400 kDa. The delivery of 40-50kDa DSS in drinking water causes the most severe murine colitis, which most closely matches human UC (Okayasu et al., 1990). Although the exact method by which DSS causes intestinal inflammation is unknown, it is most likely the result of damage to the large intestine's epithelial monolayer, which allows proinflammatory intestinal contents, such as bacteria and their products, to spread into underlying tissue. In IBD research, the DSS colitis model is widely used because of its speed, simplicity, repeatability, and controllability (Chassaing et al., 2014).

Inflammation is mediated through immune reaction, harmful stimuli, pathogenic interactions, damaged cellular actions, poisonous chemicals, and radiation. The natural defence against these reactions is operated by eradicating damaging stimuli and starts the healing process (Ferrero-Miliani et al., 2007; Medzhitov, 2010). So, inflammation is an essential defence mechanism for maintaining good health (Nathan and Ding, 2010). The acute inflammation is reduced as a result of this mitigation mechanism, which also helps to restore tissue homeostasis. However, unchecked acute inflammation can turn chronic and contribute to a number of chronic inflammatory disorders (Zhou et al., 2016).When an infection or damage occurs, the body's immunological, vascular, and inflammatory cells respond locally, causing redness, swelling, heat, pain, and a loss of tissue function (Takeuchi and Akira, 2010). Vascular permeability modifications, leukocyte build-up, and the release of inflammatory mediators are all significant microcirculatory events that take place throughout the inflammatory phase (Jabbour et al., 2009). Common inflammatory mediators and regulatory mechanisms are involved in inflammatory pathways, have an impact on the pathogenesis of several chronic diseases. The synthesis of inflammatory mediators is activated by

inflammatory stimuli, which activates intracellular signalling pathways. Cytokine interactions between the IL-1R, IL-6, and TNF-α mediates inflammation can be triggered by initial primary inflammatory stimulus such as microbial products andinterleukin-1 (IL-1), interleukin-6 (IL-6), and tumour necrosis factor-alpha (TNF- α). Important intracellular signalling pathways, such as the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) and nuclear factor kappa-B (NF-κB) pathways activation leads to chronic inflammatory diseases (Hendrayani et al., 2016).

In clinical applications, biomarkers like cytokines are used to distinguish between healthy and inflammatory biological responses and the decreased levels of these biomarkers are evidenced for the successful therapeutic interventions (Miller et al., 2009). Inflammatory biomarkers have been linked to inflammation mediated disorders likehepatic ells, kidney cells, cardiovascular diseases, endothelial dysfunction, and other infections. Inflammatory cytokines, like IL-1, IL-6, and TNF-α, and inflammatory proteins, enzymes are produced from inflammatory cells, macrophages and adipocytes, when activated by noxious stimuli. These are important biomarkers to aid in the diagnosis, prognosis, and selection of appropriate treatments for certain diseases (Carrero et al., 2008).Inflammation is facilitated and inhibited by pro- and anti-inflammatory cytokines respectively (Turner et al., 2014). ILs, Colony Stimulating Factors (CSF), IFNs, TNFs, TGFs, chemokines and all types of inflammatory cytokines produced by cells attracts the migration of leukocytes to the site of infection or injury. Through a complex web of interactions, cytokines govern inflammation and the immune response to infection or inflammation. However, excessive synthesis of inflammatory cytokines can cause organ failure, tissue damage, hemodynamic abnormalities, and finally death. This study is mainly focussed to identify new chemical entities to develop in to new leads and drug candidates for the possible anti-inflammatory properties and other therapeutic benefits.

Results

Characterization of NBV

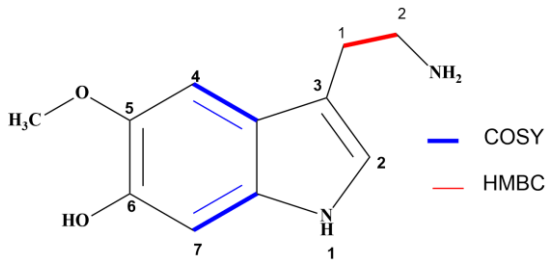

Figure.1. Novel Bufotenin Variant (NBV).

3-(2-aminoethyl)-5-methoxy-1H-indol-6-ol

^{1}H NMR: δ 8.24 (s, 1H, aromatic NH), 7.01(S-CH) 3.02,3.05(t, CH_2, aliphatic), 3.04,3.01 (t, 2H, CH_2, aliphatic),1.74(s-NH_2 aliphatic),7.07 (s-CH, aromatic) 3.83(s3H-CH_3), 7.01(s,1H aromatic),

IR (KBr): IR(KBr,cm^{-1}): 3434 (-NH), 3089.89 (C-H, aromatic), 2852.25 (C-H, aliphatic), 3451 (-OH), 1384 (C-N),2922.53cm-1(-NH2).

^{13}C NMR: 29.9,49.8,59.8,97.3,110.2,119.3,120.9,123.6,135.7 ,139.6,159.2

Mass (ESI-MS): (m/z) 207.1496 [M+H] +Elemental analysis for $C_{11}H_{14}N_2O_2$Calculated: C, 64.06; H, 6.84; N,13.58; O,15.52.

Spectral studies:

Isolated compound was obtained as a yellow colour crystal and the percentage yield found to be 39%, TLC was done to get single spot with R_f =0.61 and melting point found to be 167±5^{0}C.The functional groups were characterised with IR spectra (Fig.S1) and the absorption peaks were observed at 3089.89, 2852.25 for aromatic and aliphatic groups respectively. Indole amine broad stretching peak was observed at 3434cm$^{-1,}$ and the hydroxyl group attached to aromatic ring, was observed at 3451cm^{-1} and the stretching of amine showed at 2992.10cm^{-1}.The spectroscopic identification ^{1}H NMR of NBV (Table.1) and the spectra confirmed two methylene triplets and four singlets at different positions (Fig.S2). ^{13}C NMR spectra confirmed 11 carbons (Fig.S3). Detailed COSY (Fig.S4) and HMBC (Fig.S5) spectra confirmed that *δ*C110.2is also quaternary group, and *δ*C 139.6 is oxygenated methyl at δH 3.83 with singlet along with carbon and three hydrogens. HSQC (Fig.S6) and HMBC correlated and supported by the presence of methoxy-5, OH-6, and 3-amino ethyl at position 3. HMBC correlated with amine at *δ*H 7.07 and attached at *δ*C 123.6 indicating the presence of indole.

PXRD pattern of NBV shows a sharp peak is at 2.6 (Fig.S7) and conformed the powder crystalline indole compound. Based on the spectral data and XRD crystals analysis the structure was explicated, and the molecule was deduced as $C_{11}H_{14}N_20_2$. LC HRMS was used to identify $(M+H)^+$ and the presence of molecular ion at m/z 207.1496 was confirmed (Fig.S8). Based on the spectral analysis the IUPAC name was given as **3-(2-aminoethyl)-5-methoxy-1H-indol-6-ol,** and the compound structure was accessed. Docking study was performed with the NBV and demonstrated better docking interactions with highest binding score of -7.3 Kcal/mol against 2L3Y for IL-6 and -6.2 Kcal/mol against 2AZ5 for

TNF-α (Fig.2) and both were 2D super imposed (Table.S2)

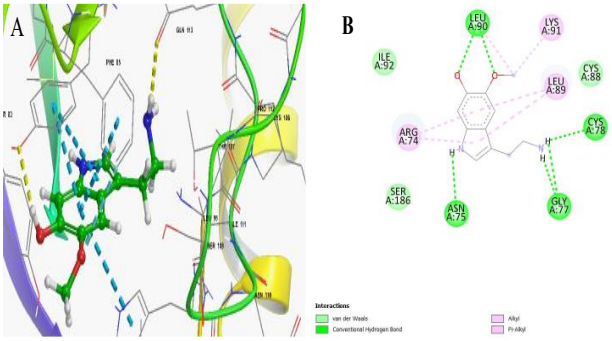

Figure.2 Binding poses and interactions of compound NBV with the binding site of 2L3Y and 2AZ5

Protein	Protein interacting atoms	Ligand interacting atoms	No. of H bonds	Distance (Å)	Binding Energy Kcal/mol
2L3Y	LEU-90	OH	6	2.23	-7.3
	LEU-90	CO		2.50	-6.9
	Gly-77	NH		2.30	-6.1
	Gly-77	NH		2.50	-5.9
	CYS-78	NH		2.16	-5.8
	ASN-75	NH		2.58	-5.6
2AZ5	TYR-83	OH	6	2.23	-6.2
	TRP-109	NH		2.51	-4.7
	PHE-85	CO		2.30	-4.7
	GLN-113	NH		2.49	-3.9
	ASN-88	NH		2.16	-4.3
	GLY-149	OH		2.58	-3.9

***In Silico* Studies of NBV with IL-6 and TNF-α.**

PDB IL-6=2L3Y; **PDB** TNF-α=2AZ5.

Acute toxicity studies

NBV mortality was observed at 2000mg/kgand the MTD (Maximum Tolerated Dose) was confirmed according to OECD 423 guidelines, as 500mg/kg. At the dose of 500mg/kg,animals don't show any significant toxic symptoms (Table.S1).

Anti-inflammatory activity of NBV

λ-carrageenan-induced Paw Edema levels of IL-6, TNF-α

This study was used the standard protocols for the evaluation of anti-inflammatory activity (Wu et al., 2002); λ-carrageenan was used for induction. It was observed that (TableS2), the increase of paw volume % inhibition is reduced from 74.83±3.18 to 54.00±2.36 with NBV 10 mg/kg, from 74.83±3.18 to 36.58±6.87 with NBV 20 mg/kg and this was comparable with standard indomethacin value from 74.83±3.18 to 65.16±1.47 as anti-inflammatory activity (Fig. 2A).

λ-carrageenan-induced Air Pouch Tissue levels of IL-6, TNF-α

The standard protocols were used to estimate IL-6, TNF-α in air pouch tissue by using ELISA kits and the results were compared (Table S3) with λ-carrageenan induced group. IL-6 levels reduced from Carr group 52.15±3.06 to 30.17±3.84, 26.50±1.60 and 20.62±1.71 with NBV 10 mg/kg, NBV 20 mg/kg and IND 1mg/kg respectively (Fig.2B). And TNF-α levels reduced from Carr group 49.17±1.72 to 28.22±1.43, 25.32±1.23 and 18.27±1.33 with NBV 10 mg/kg, NBV 20 mg/kg and IND 1mg/kg respectively (Fig. 2C).

λ-carrageenan-induced Serum levels of IL-6, TNF-α

The standard protocols were used to estimate IL-6, TNF-α in mice serum by using ELISA kits and the results were compared with Carr group (Table S4). IL-6 levels reduced from Carr group 374.85±17.98 to 213.79±9.42, 204.60±5.10 and 167.77±7.46 with NBV 10 mg/kg, NBV 20 mg/kg and IND 1mg/kg respectively (Fig. 2D). And TNF-α levels reduced from Carr group 674.40±16.9 to 319.25±5.68, 289.82±15.82 and

250.55.27±14.10 with NBV 10 mg/kg, NBV 20 mg/kg and IND 1mg/kg respectively (Fig. 2E).

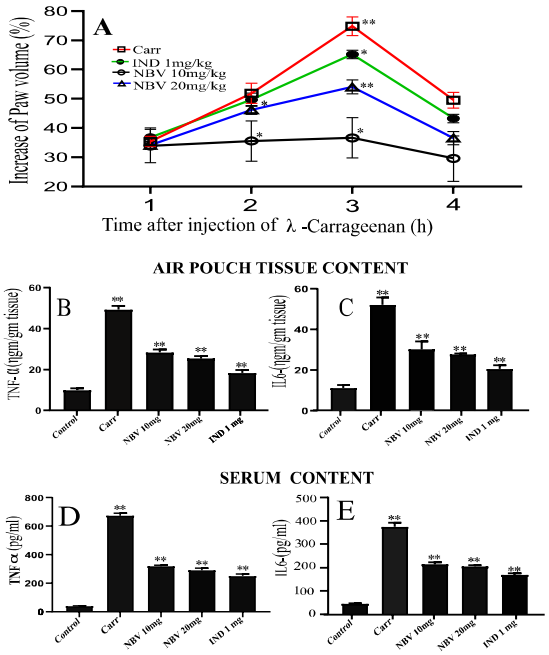

Fig 2. A. The percentage of increase volume of the effects of λ-carrageenin on paw edema at various time points. B. Air pouch Tissue contents of IL-6 and TNF-α. C. Air pouch Tissue contents of IL-6 and TNF-α.

Pre-treatment with NBV 10 and 20 mg/kg significantly reduced the percentage increase in paw volume considerably following the injection of λ-carrageenan, indicates that NBV suppressed λ-carrageenan -induced paw edema and the results were confirmed with H/E staining of air pouch tissue histological studies (Fig 3).Histopathology showed considerable alterations, includes the invasion of neutrophils and lymphocytes. Clearly visible inflammatory cells were found in Carr group. However, indomethacin and NBV (10, 20 mg/kg) significantly decreased inflammatory cellular infiltration, demonstrating that they had a protective effect on the histological alterations in the air pouch tissue.

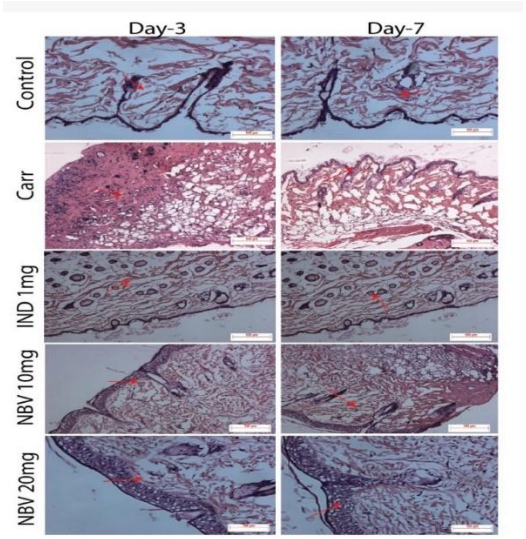

Figure.3. Effect of NBV on mice λ-carrageenan induced air pouch tissues histology. (H&E, 100 x magnifications)

Infiltration of epidermal cells and cellular damage was observed with in induction groups. The epidermal layer repair after the treatment with NBV10, 20mg/kg, was healed with considerable improvement as compared to the control group.This evidence showing with a definite influence on epidermal cells.The histopathological section of the air pouch tissue of mice showed marked atheromatous thickening and damage in the intima. The inflammatory changes were absent in both the doses of NBV-treated groups.

Bisphenol-A induced Biochemical parameters

BPA and NBV treatment for 14 days appears to have caused hepato toxicity as revealed by the assessment of LFT enzymes. Activities of LFT marker enzymes in the serum of control,BPA and treated groups are shown in (TableS5). BPA caused increase in activities of ALT, AST and ALP in mice serumwhen compared with the control (group I). NBV treated groups have shown a notable decrease of levels in different dosages preferably NBV with high doses (20mg/kg) shown highest decrease was noticed when compare to the low dose (10mg/kg) of NBV.

Effect of pro inflammatory cytokines induced by Bisphenol-A in liver and kidney

Effect of NBV on IL-6 and TNF-α" levels: Both IL-6 and TNF-α levels were significantly greater in the BPA group compare with the control group. Compared with the BPA group, NBV(10, 20mg/kg) caused a notable decrease in the levels of IL-6 and TNF-α. NBV at 20mg/kg exhibited more decrease to comparable they caused by BPA in liver and kidney (Table S6, S7).

HistoPathalogy findings:

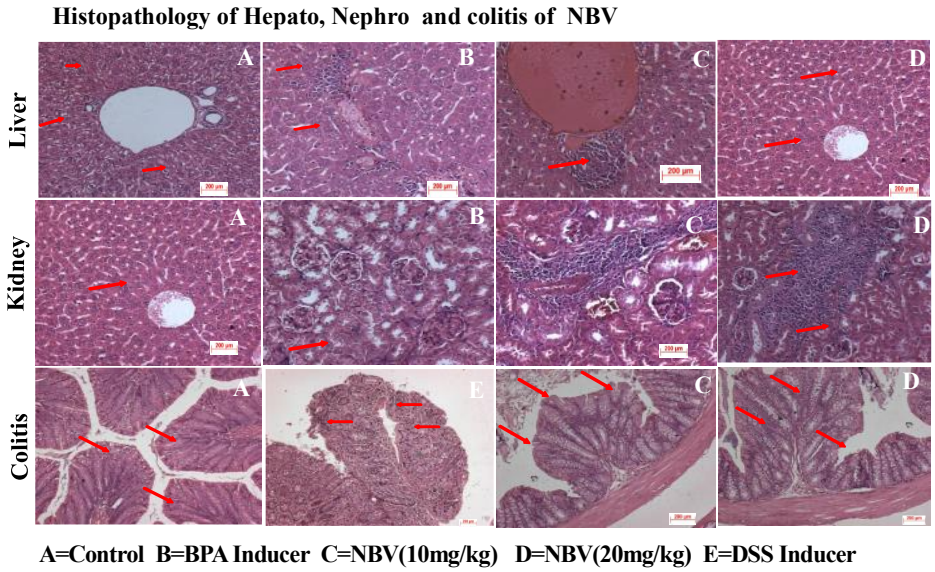

Figure.4 ProtectiveEffect of NBV on mice liver, kidney and colon. (H&E, 100 x magnifications)

The effect of NBV on histopathological changes was investigated as shown in Fig.4 . The results demonstrated that the Liverand kidneytissue from the control group presented a healthy tissue structure. Conversely, significant liverand kidney histopathological changes including the infiltration of cell were observed in the BPA group (Fig.4 B). However, Doses of NBV (10, 20mg/kg) significantly reduced cell infiltration indicating that they induced a protective effect on Bisphenol-A histopathological changes (Fig.4B). Ulcer colitis also observed in colon of mice colitis was compared with the control group (A) and DSS induced group(E) Themucosal layer repair after the treatment with NBV10, 20mg/kg, was recovered with considerable improvement as compared to the DSS group.This evidence showing with a definite influence on mucosal cells on colon tissue.

Immunohistochemistry observations:

Kidney liver and colon tissue samples were collected in 10% buffered formalin for histopathological examination. The tissues were processed by routine paraffin embedding technique, and 5 µm sections were stained with immunohistochemical staining by standard methods as described by protocol, using streptavidin-biotin complex (SB). For macrophages immunoreaction, we used the CD68 monoclonal mouse antibody at dilution 1:100 in PBS

Immunohistochemical labelling for each antibody was graded on scale

Score 1 - Less than 25 % of expression in inflammatory cells / inflammatory tissues

Score 2 - 25 to 50 % of expression of expression in inflammatory cells / inflammatorytissues

Score 3 - 50 to 75% of expression of expression in inflammatory cells /inflammatory tissues

Score 4 – Greater than 75% of expression of expression in inflammatory cells /inflammatory tissues

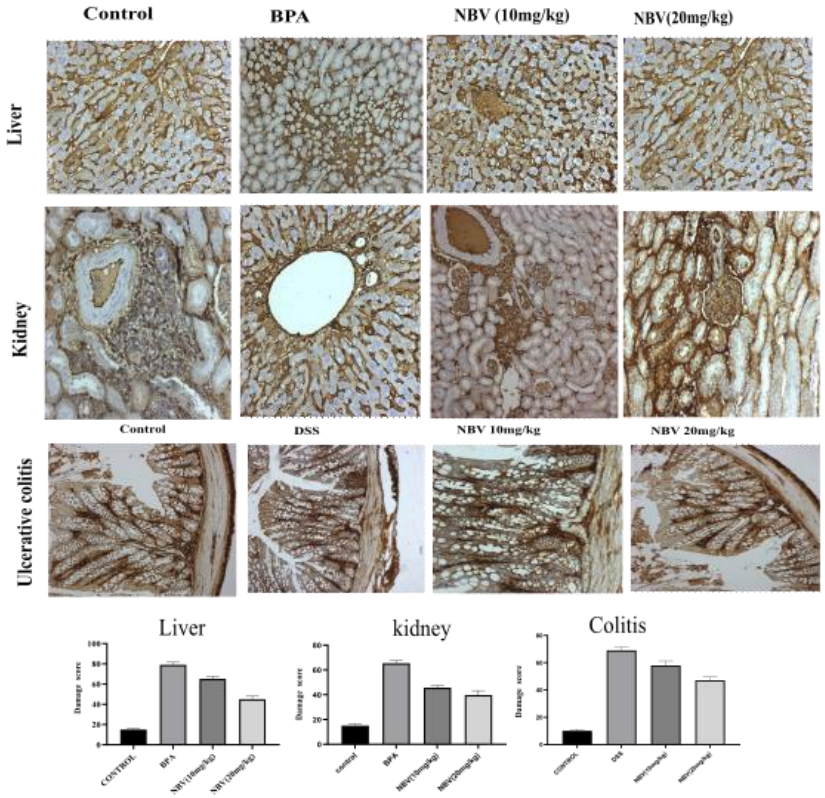

Figure 5. NBV Effect on CD68 Expression in Mice with BPA-Induced Hepatic and Nephrotoxicity and DSS-Induced Colitis Immunohistochemistry of macrophage markers (CD68) in inflammatory bio markers samples. Representative image macrophage markers determined by single labelling immune histochemistry in mucosal samples of healthy colon, induced group and treatment groups. Treatment group tissue with high or low doses of the different macrophage markers is shown. Magnification 200 (bar 200 mm)

Immunohistochemistry reveals that the cytoplasmic Bax immune reactivity in the different experimental groups. Control group showing -ve cytoplasmic reaction in glomerular and tubular cells, and BPA, DSStreated groups showing strong reaction in inflammatory cells, glomerular, tubular and colon cells. NBV treated group showing a decrease in the reaction in glomerular and tubular cells and colon.NBV treated groups showing reaction in both glomerular and tubular cells and colon tissue appeared more or less similar to control group is shown in Fig.5.

Effect of IL-6 and TNF-α Colitis activity induced by DSS:

After experimentally colitis induction by DSS, deregulated cytokines in pathological conditions promoting a pro-inflammatory effect, such as IL-6, TNF-α. mice with DSS induced colitis revealed a significant increase in IL-6, TNF-α, compared to the control indicates a systemic diseased condition in serum as well as in tissue. However, NBVtreated Groups showed a significant decrease in pro-inflammatory cytokines compared with the DSS colitis group (Table S8) ($p < 0.01$ & $p < 0.001$). These results indicate that NBV(10&20 mg/kg) prevent inflammatory reaction through down regulating cytokines expression and data are presented in tissue showed in Fig.6.

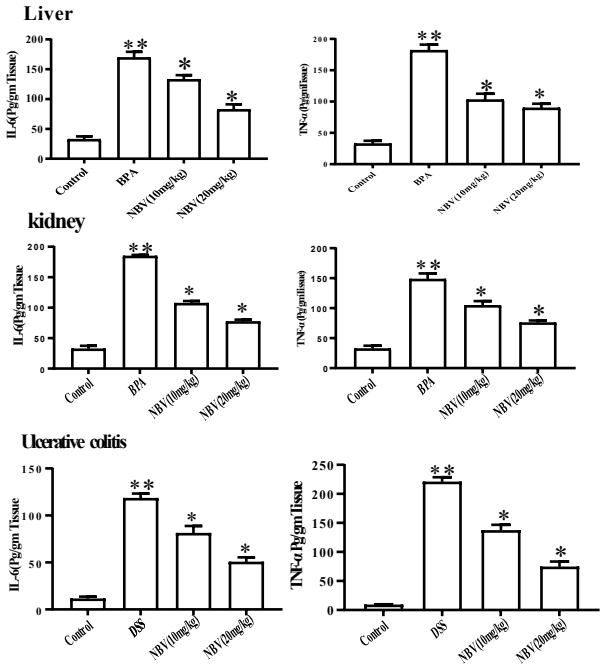

Figure 6 EffectNBV on IL-6 and TNF-α on tissue of liver, kidney and colon supernatant layer by ELISA Method.

DISCUSSION

In the process of drug discovery, the previous studies revealed that there are many molecules developed in to medicines from various animal poisons and venoms sources (Bordon et al., 2020). Toxins developed by venomous animals interfere with certain components of their victims with their essential physiological functions. Many animal species have independently evolved venomous systems, demonstrating the effectiveness of this approach. Toxins with great specificity and potency for their intended as molecular targets having predominating pharmacological activities and using in human therapeutics, and also as bioinsecticides(Herzig et al., 2020). Most venoms are mostly composed of peptides and proteins, which are frequently protease-resistant due to their disulphide-rich structures and there are limited drugs derived from venom that have received approval (King, 2011).From Toad skin and venom derived drugs are of Chinese medicine from Bufogargarizans Cantor and Bufo melanostictus Schneider. The hydrophilic components may be the key differentiating factors between them, yet they exhibit different characteristics in conventional applications. In toad skin and venom, 36 and 22 hydrophilic components, respectively, were found using UHPLC-HR-MS/MS and the quantitative findings demonstrated that the primary IAAs in toad venom were significantly greater than in skin and found to have potent anti-inflammatory properties (Zhang et al., 2018).

Previous research studies revealed that NF-κB signalling pathways are directly related to the regulation of inflammation. According to the literature pro-inflammatory cytokines like IL-6, IL-1, and TNF-α are transcriptionally upregulated by activation of NF-κB (Guolan et al., 2018). If it is controlled the signalling pathway activation it minimizes the severity of inflammatory damage. According to the present study findings, NBV at 10 and 20 mg/kg dramatically reduced IL-6 and TNF-α levels, it might be due to reduced NF-κB expression in both the cytoplasm and the nucleus. The previous research findings also proved that bufotenin prevented the activation of NF-κBto elicit anti-inflammatory activities. Therefore, as NBV is a derivative of bufotenin and proved to have anti-inflammatory activity by reducing IL-6 and TNF-α level and exact and mechanism of action of NBV needs to be explored further with additional inflammatory experimentalmodels.

In the liver and kidney tissues of mice, this study aimed to investigate the protective impact of NBV against damage caused by BPA. According to the available data, BPA significantly increased the serum levels of AST, ALP, and ALT, which indicated liver injury (Ahmed et al., 2015). The most reliable indicators of hepatocellular injury

are these enzymes. The increased serum liver marker activity indicates cellular leakage and the breakdown of the structural integrity of the membrane. The considerable reductions in the hepatic activity of both AST and ALT as shown in suggested that the BPA-induced hepatotoxicity was alleviated after the injection of NBV.

Bufotenin is an indole alkylamine hallucinogen and serotonin-2A receptor agonist. It can be found in the skin of several types of toads, as well as in higher plants, animals, and mushrooms. In our ongoing studies of the therapeutic potential of these extracts, bufotenine was found in many aqueous-based extracts of Australian cane toad (Bufo marinus) skins. Bufotenine was also identified from a traditional Chinese medicine made from the skin of the Chinese toad Bufo bufogargarizans cantor (Bufonidae). Bufotenine, a toad venom natural component, has showed significant potential anti-inflammation drug (Wang et al., 2021).

IL-6, TNF-α is regarded as one of the important cytokines generated in many inflammatory processes that stimulates signalling pathways linked to an inflammatory response. The immune system is the main producer of IL-6, TNF-α in response to infection and inflammation. Our research revealed that after BPA treatment, IL-6, TNF-α levels significantly increased in both liver and kidney tissues (Fig. 6). When mice were treated of BPA, the level of TNF- α and IL-6 in the liver tissues considerably raise. Our findings, however, showed that when NBV treated groups that, the levels of hepatic and renal TNF- α and IL-6 were much lower than in the BPA-treated group. NBV show that the levels of TNF-α, IL-6 in the liver and kidneys have significantly decreased.

The complex, heterogeneous factors behind intestinal development during inflammation include host genetics, environmental triggers, microbial community composition, and immunological dysfunction, and the degree of intestinal inflammation is connected to increased production of pro inflammatory cytokines (Ananthakrishnan et al., 2014). Chronic inflammation causes cellular proliferation, apoptosis evasion, and genetic instability, all of which eventually result in cancer. As a result, it is hypothesised that targeting aberrant, hyperactive inflammatory signalling pathways is an important chemo preventive technique.

The findings of this study showed that NBV treatment significantly improved colon lengths and histological studies and immune histochmemical analysis in DSS-induced acute colitis mice. The colons of mice given DSS showed deformation, mucosal erosion, significant chronic and acute inflammatory cell infiltration, and crypt loss. NBV was effective in minimising these inflammatory alterations, hence lowering the inflammatory state. The levels of proinflammatory chemokines and cytokines also decreased with the help of NBV treatment. Increased levels of several cytokines and chemokines support the development of IBD. To control the immune response during intestinal inflammation, a complex network of cytokines and chemokines is necessary. Immunocompetent cells in colitis release proinflammatory cytokines like IL-6 and TNF- α after activating NF-κB. According to the current investigation, NBV significantly decreased IL-6 and TNF-α overexpression. The findings above show that DSS-mediated suppression of the activation of inflammatory factors is associated with the protective role of NBV.

In this regard, earlier studies have shown that bufotenine can have a good anti-inflammatory effect. The studies determined that 5 methoxy DMT was the main anti-inflammatory to reduce the levels of pro-inflammatory substances like IL-6 and TNF-α from Sonoran desert toad venom (Sherwood et al., 2020), whereas our NBV also has a similar structure but is additionally hydroxy group was attached at 6^{th} position, which increases the potential activity over 5 methoxy DMT. The hydroxy group binds more strongly to cytokines and prostaglandins, causing them to be reduced faster than 5-methoxy DMT. The pharmacological characteristics of the compounds, such as their molecular weight, c Log P, solubility, TPSA, molecular PSA, drug score, and drug likeness, were further evaluated utilising the Osiris property explorer for drug bioavailability of NBV.

Experimental Section

Materials and methods

Analytical grade λ-carrageenan, Bisphenol-A, DSS (Dextran Sodium Sulphate) was purchased from Sigma Aldrich; Merck Life Sciences Pvt. Ltd., Mumbai. The Elisa Cytokines assay kits were purchased from DNA Bio, Hyderabad, Telangana, India. Indomethacin-Vasudha Pharmaceuticals Ltd., -Hyd, Silica gel-Vasudha Pharmaceuticals Ltd- Hyd. All other chemicals used in HPLC grade in the present study were purchased from Hi Media Laboratories Pvt Ltd and Sisco Research Laboratories Pvt Ltd (Mumbai, India) and Sigma-Aldrich (Merck Life Pvt ltd, Mumbai, India) unless specified

Collection of venom from Indian toad

Adult live toads (45 to 50 g) were collected for isolation and extraction of samples from the nearby places of Warangal, Kakatiya University surroundings during the period from April to September (Das et al., 2000). The samples were collected with standard procedure by gentle squeezing of toad paratoid glands. Initially the glands were cleaned thoroughly with the help of a dry brush and stimulated the parotoid gland by gentle rubbing, then the glandular secretion was collected by mechanical compression of both glands with the help of sterile forceps from living toads to gettheyellowish exudates and the animals were freed for living.

Isolation and Extraction

These yellowish and doughy secretions collected were placed on the smooth surface of a watch glass for complete air drying. About 5g of dried parotoid secretion was soaked in methanol for seven days in an amber-coloured bottle, and the supernatant was collected and evaporated to dryness using a Rota evaporator, dark brown solid mass methanolic extract (2g) was obtained and the percentage yield found to be 40%. This methanolic extract was labelled as toad Parotoid gland Extract of Methanol (TPGS-M) and is a crude extract with different biological activities, and this is further processed to get new chemical entities.

The dried sample (5g) was pulverized and extracted with ethyl acetate for seven days in an amber-colored bottle, the solvent was evaporated under vacuum to give an extract (2.5g). This extract was separated over column eluting with hexane: ethyl acetate (8:2 to 2:8 v/v) to yield seven fractions (Fr.1 to Fr.6). Fr-4 (250mg)(Meyers, 2001). was separated by column chromatography on silica gel with (ethyl acetate: water 9:1 to 1:9 v/v) and the fractions obtained are (Fr. A, B, C, D and E). Fr. D (39mg) was selected as it got single spot separated by TLC (Figure S9.) and then purified by preparative HPLC (CH_3CN: H_2O-42/58, v/v, 1.0ml/min) (1.7 mg, t_r 2.89 min) to give compound recorded at 300nm was subjected for the characterization (Figure S10.) This structural elucidation was done with sophisticated spectral analytical technique and deduced as NBV.

PXRD Studies

PXRD used to determine thecompound crystallographic structure and examined by using X-ray Diffraction Unit (Pan Analytical, X-pert pro, Netherland) with a Cu-Kα radiation source in the scattering range of 20–80 on an instrument running at 45 kV and 40 mA. X-ray diffraction spectroscopy was used to determine the compound existence, crystalline nature, phase variety, and grain size (Chhattise et al., 2016).

Molecular Docking

NBV was tested for its ability to bind to Drug-receptor interactions are frequently studied by using molecular docking. NBV was tested for its ability to bind to IL-6 and TNF-α with the PDB targets of 2L3Y and 2AZ5 respectively. IL-6 (2L3Y) docking study was done with Autodocking 4.2.1.5.6 software and TNF-α (2AZ5) Schrodinger Software version 2019-1. NBV structure was created using Chem Bio Draw Ultra 12.0. The structure was translated to PDB format using the Open Babel 2.4.0 tool. The UCSF Chimera 1.10.1 wizard program was used to generate targeted IL-6 protein structures from the RCSB PDB (www.rscb.org, PDB ID: 2L3Y, 2AZ5) (Thilakasiri et al., 2019) protein database for docking.

Acute Toxicity Studies

Animals

Swiss albinomale and female were acquired from Vyas Enterprises, Hyderabad and sufficient quarantine time was allowed with *ad libitum*. They were acclimatized for ten days before being utilized in the study animals were fated for 12-hours. For acute toxicity studies female mice were used, all the experiments were initiated with prior approval from Institutional Animal Ethics Committee and followed the standard protocols of Kakatiya University, the research protocol number is IAEC/48/UCPSC/KU/2018.According to the OECD-423 guidelines, the maximum tolerated dose (MTD) was determined using 15 female Swissalbinomice weighing 15 to 20g. mice were divided into four groups (n=3), the control group treated with normal saline, the second group given orally Novel BufoteninVariant (NBV, 2000mg /kg.p.o), third group received NBV (1000 mg/kg.p.o), and fourth group with NBV(500 mg/kg.p.o).Observations were drawn and toxic effects recorded during 14 days period, checked for mortality, physiological parameters like body weight changes, and behavioral changes in each animal noted according to standard protocols (OECD, 2002).

Anti-inflammatory activity of NBV

λ-carrageenan-induced Paw Edema levels of IL-6, TNF-α

The animals were divided into five groups (n=6), and treatment was given as mentioned below for 7 days.Control group was treated with normal saline, second group was treated with saline for seven days and on eighth day challenged with λ-carrageenan 0.1 ml/mouse in to the right paw sub plantar region (1% W/V), Groups 3 and 4 were pre-treated with NBV 10 and 20 mg/kg/po respectively for seven days and on eighth day also treated with NBV just before one hour of λ-carrageenan induction. Group 5 received IND 1 mg/kg/po for seven days and on eighth day also treated with IND just before one hour of λ-carrageenan induction. On eighth day after the λ-carrageenan induction, at predetermined time intervals like 0, 1, 2, 3 and 4hours mice right paw volume was determined with Plethysmography. Paw volume (edema) was measured, and the percentage reduction in paw volumewas calculated by the following equation.

Percentage reduction

% of inhibition = $V_{control}$-$V_{treated}$ /$V_{control}$x100.

Further, on the eighth day at the end of the study, 1.5 ml by retro orbital puncture blood samples were collected to estimate Serum cytokines like IL-6 and TNF-α by ELISA Kit method.

λ-carrageenan-induced Air Pouch Tissue levels of IL-6, TNF-α

Subcutaneous air oval pouches were prepared as described by the previous studies(Edwards et al., 1981), briefly on first day 5 ml subsequently on third and fifth days 2 ml of air was injected subcutaneously into the dorsal surface of mice. Then, after λ-carrageenan (1ml of 1% solution) was injected carefully in to each pouch for inflammation induction in the air pouch. The animals were divided into five groups (n=8), in which the control group air pouches were injected with 1ml normal saline, remaining all groups air pouches were injected with 1ml 1% λ-carrageenan were injected. On subsequent seven days, first and second group groups were treated with normal saline for seven days. Third and fourth groups were treated with NBV 10 and 20 mg/kg/po respectively for seven daysandgroup 5 received IND 1 mg/kg/po for seven days. After seven days treatment all the groups were subjected for collecting air pouch contents and tissues.

For cytokines like IL-6 and TNF-α levels estimationthe walls of the air pouch were put into saline, homogenized with a homogenizer, and centrifuged at 5000rpm to extract the supernatant layer cells, and pro-inflammatory mediators were estimated using the Elisa kit method (Cui et al., 2019) follows kit procedure as a standard. Further, on third and seventh day the pouch walls were removed and fixed with 10% neutral formalin solution sent to histopathological studies with H/E staining(100X). The histopathological studies were performed to identify cytokine proinflammatory infiltration.

Bisphenol-A induced Hepatic and renal toxicity Experimental Procedure

Mice were separated into four groups (n=6) to examine the effect of NBV on BPA-induced Hepatic and Nephrotoxicity (Zaulet et al., 2017). Control, BPA, NBV (10 mg/kg) and NBV (20mg/kg).1% BPA was dissolved in 5% ethanol and 100 ml of water. Mice in the control group received regular water by gavage every day for ten days. On the eleventh day of the experiment, all the mice were sacrificed. We collect the serum for Elisa pro-inflammatory agent estimation and biochemical parameter analysis. The cervical dislocation method was used to kill the mice. For histology and immune histochemistry purposes, liver and kidney samples were obtained and maintained in buffered formalin solutions, whereas electron microscopy investigation required glutaraldehyde solutions.

Bio-chemical Parameters

In order to separate the serum, the collected blood was centrifuged for 10 minutes at 3000 rpm after being allowed to coagulate at room temperature. The collected serum was utilised to quantify biochemical parameters such urea, uric acid, aspartate aminotransferase, alanine aminotransferase (Sangai et al.), alkaline phosphatise (Chhattise et al.), and creatinine using commercially available standard test kits with autoanalyzer (Aditi et al., 2020).

DSS Induced colitis

All animal experiments were conducted in accordance with the established procedure(Fang et al., 2018). Mice (n = 6) were divided into four groups according to their bodyweight: control, DSS and NBV 10mg/kg, and 20mg/kg (Fig.6). For two weeks, mice were given varying doses of an NBV solution that had been dissolved in methanol, while the control group was given saline via oral gavage only once per day. To induce colitis in mice, 1.2% DSS was diluted in drinking water for two weeks. At the end of the experiment, mice were sacrificed, and the colons were removed for histological and

immunohistochemical analysis. The serum was gathered to determine the cytokines levels that promote inflammation.

CONCLUSION

This study showed that Novel Bufotenin Variant, which was not previously reported from Indian Toad, has good protective properties by preventing the release of pro-inflammatory cytokines like IL-6 and TNF-α in tissue serum of air pouch, liver, kidney and colon. NBV has the potential to be an effective molecule for further research in therapies that provide protection from inflammation.

Statistical Analysis

The study's findings were presented as mean and SD (n=6), and one-way ANOVA was utilized to analyse significance using a p-value 0.01 cut off. Graph Pad Prism version 8.4.2 was used. See additional files for the remainder of the experimental part.

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SUPPORTING INFORMATION

Novel3-(2-aminoethyl)-5-methoxy-1H-indol-6-ol from Indian Toad Paratoid Glandular Secretion as protective effect by inhibiting IL-6 and TNF-α

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Table S1. Toxicity observational studies

Acute toxicity: NBV mortality was observed at 2000mg/kg. the MTD (Maximum Tolerated Dose) was confirmed as 500mg/kg. At the dose of 500mg/kg animals doesn't show any significant symptoms

Group	Treatment	Toxicity (TS/NS)	Mortality(D/S)
control	Normal saline	0/3	0/3
NBV	2000mg/kg	3/3	3/3
NBV	1000mg/kg	3/3	3//3
NBV	500mg/kg	0/3	0/3

TS: Toxicity sign, NS: No sign, D: Death, S: Survival

Table S2. Percentage of inhibition of Paw edema by λ-carrageenan induced

Time (hr.)	Treatment			
	Control	**NBV (10mg/kg)**	**NBV (20mg/kg)**	**Indomethacin(1mg/kg)**
1	35.33±1.75	26.83±1.71	27.12±1.23*	28.83±3.18*
2	43.83±1.43	31.50±2.87*	35.16±1.47*	43.66±2.25*
3	74.83±1.18	39.58±5.87*	46.00±2.36*	65.16±1.47*
4	54.54±2.66	29.56±1.16*	36.55±2.25*	31.23±1.41*

Results were expressed as Mean ±SD, n=6 animals in each group, analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. Comparison: *p<0.01

TableS3. Effect on IL-6 and TNF-α by ELISA method

SERUM (pg/ml)	**CONTROL**	**CARR**	**NBV (10mg/Kg)**	**NBV (20mg/Kg)**	**Indomethacin (1mg/Kg)**
IL-6	44.02±2.604	374.85±17.915**	213.79±9.42**	204.123±5.158 **	167.77±7.460**
TNF-α	38.50±1.532	674.46±16.912**	319.25±5.68* *	289.825±15.820* *	250.55±14.101**

Results were expressed as Mean ±SD, n=6 animals in each group, analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. Comparison: *p<0.01.

Table S4. Effect on IL-6 and TNF-α by ELISA method in supernatant layer of Air pouch tissue

Air pouch tissue	**CONTROL**	**CARR**	**NBV (10mg/Kg)**	**NBV (20mg/Kg)**	**Indomethacin (1mg/Kg)**
IL-6	11.17±1.43	52.15±3.656**	30.175±3.844**	26.512±1.623**	20.625±1.717**
TNF-α	9085±0.82	49.17±1.727**	28.225±1.438**	25.325±1.234**	18.275±1.338**

Results were expressed as Mean ±SD, n=6 animals in each group, analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. Comparison: *p<0.01

Table S5. Bio Chemical Kidney, Liver Parameter of Serum

Parameter(U/L)	Control	Bisphenol	10mg/kg	20mg/kg
		kidney		
Urea	18.75±2.87	45.225±8.19**	35.2±5.39*	29.375±5.69**
Uric acid	6.2±0.74	16.915±0.71**	14.23±1.47*	10.65±1.43**
creatinine	0.32±0.12	6.925±1.12**	3.325±0.66*	2.10±1.20**
		liver		
AST	11.76±0.78	81.8±12.19**	64.2±2.91*	51.4±5.27**
ALP	11.02±4.31	67.2±6.58**	40.4±2.99	28.6±6.35**
ALT	14.32±2.27	77.77±2.84**	62.36±3.39*	36.68±1.52**

AST: Aspartate aminotransferase ALT: Alanine amino transferase ALP: Alkaline phasahatase

Results were expressed as Mean ±Sem, n=6 animals in each group, analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. Comparison: *p<0.01,**P,0.05

Table S6. ESTIMATION OF IL-6 AND TNF-α IN Liver

Serum(pg/ml)	control	Bisphenol-A	10mg/kg	20mg/kg
IL-6	22.3 ±4.979	182±9.403	114.8±9.523*	77.2±3.701*
TNF-α	32.4±4.9796	148.4±9.502	120.3±6.010*	65.8±3.563*

Results were expressed as Mean ±SD, n=6 animals in each group, analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. Comparison: *p<0.01

Table S7.ESTIMATION OF IL-6 AND TNF-α IN kidney

Serum(pg/ml)	control	Bisphenol-A	10mg/kg	20mg/kg
IL-6	12.3 ±4.979	169.4±9.76	132.8±7.19*	82.4±8.712**
TNF-α	18.4±4.979	181.6±9.502	102.6±9.88*	89.4±7.127**

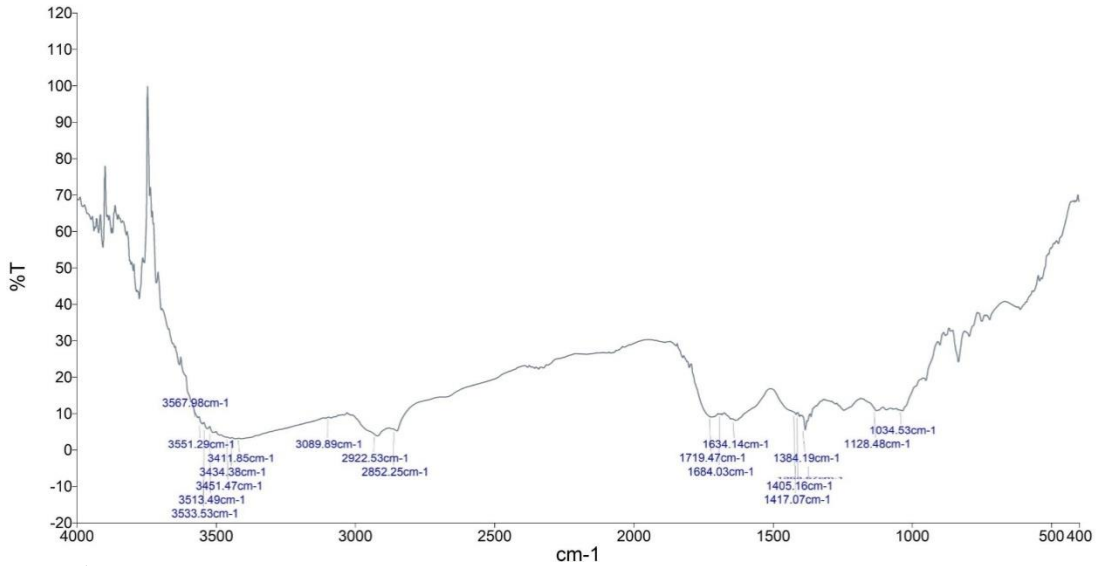
Results were expressed as Mean ±SD, n=6 animals in each group, analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. Comparison: *p<0.01,**P,0.05

Table S8.ESTIMATION OF IL-6 AND TNF-α IN Colon

Serum(pg/ml)	control	DSS	10mg/kg	20mg/kg
IL-6	11.4 ±2.30	118.4±4.87	81.2±7.66*	50.6±4.27**
TNF-α	9.1±0.741	220.8±7.85	137.2±9.59*	74.4±7.12**

Results were expressed as Mean ±SD, n=6 animals in each group, analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. Comparison: *p<0.01

Figure S1. IR Spectra of the isolated compound(NBV)



IR(KBr,cm^{-1}): 3434 (-NH), 3089.89 (C-H, aromatic), 2852.25 (C-H, aliphatic), 3451 (-OH), 1384 (C-N),2922.53cm-1(-NH2).

FigureS2. ^{1}H-NMR(400MHz) DMSO-d Spectra of the isolated compound(NBV)

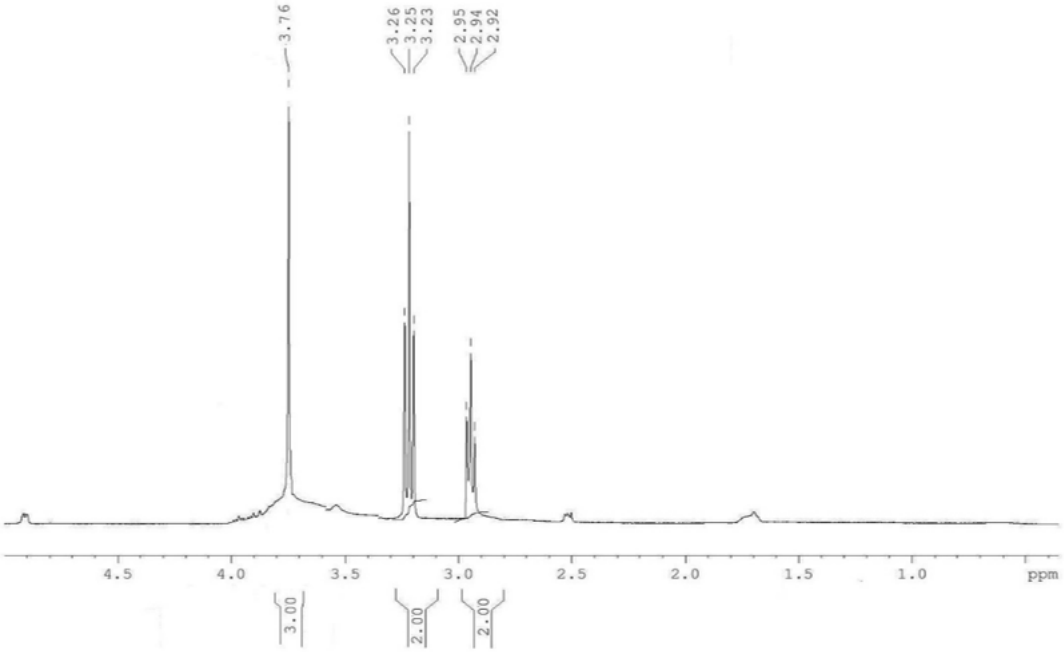


Figure S3.^{13}C-NMR(300MHz, DMSO) δ (ppm)Spectra of the isolated compound (NBV)

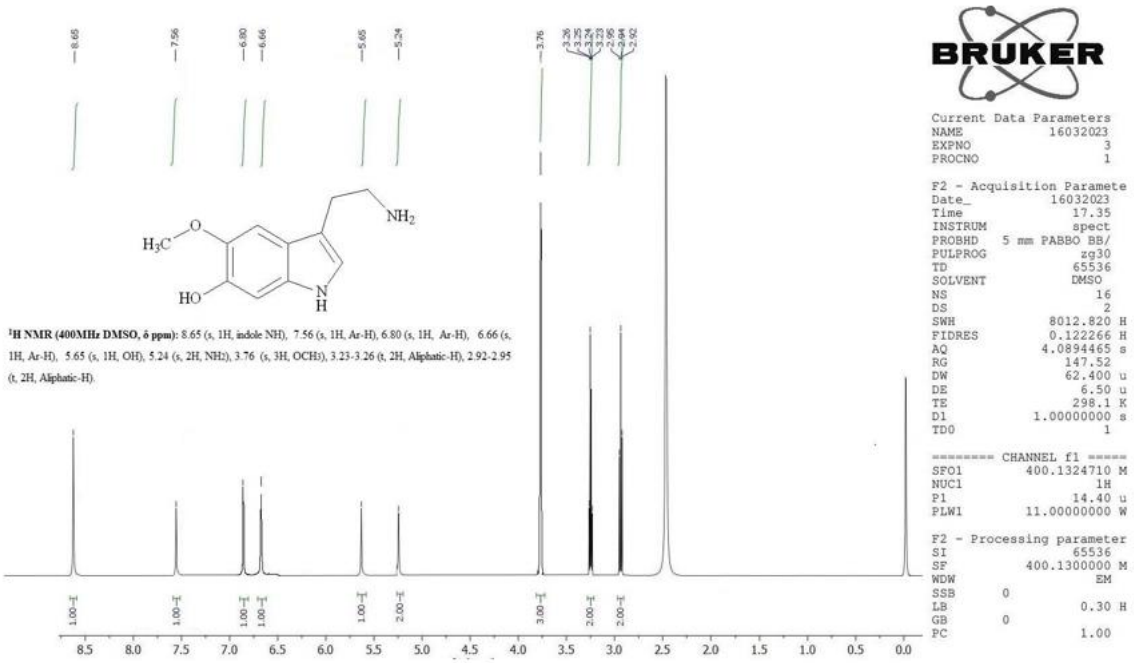


Figure S4. COSY spectra of the isolated compound(NBV)(400MHz, DMSO)

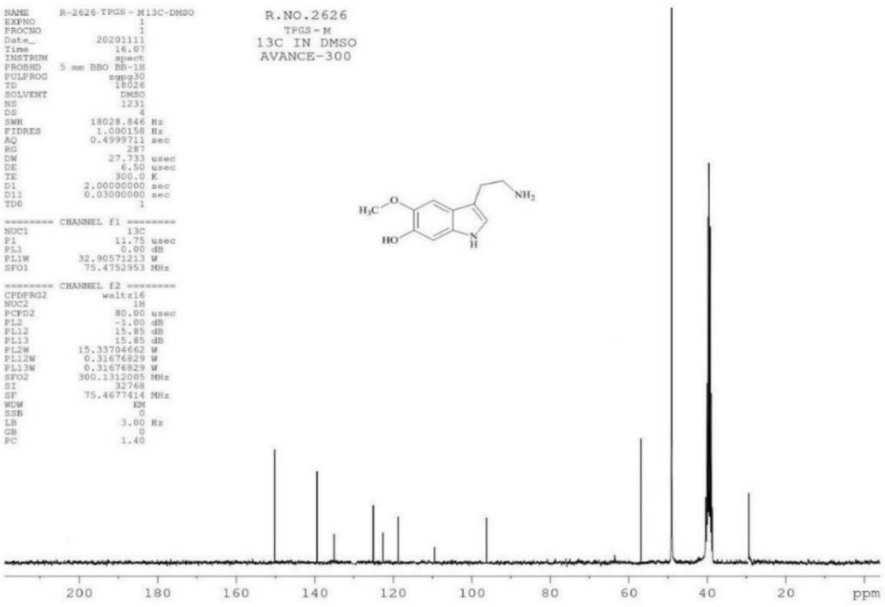


Figure S5. HMBC spectra of the isolated(NBV) compound(NBV) (400MHz,DMSO)

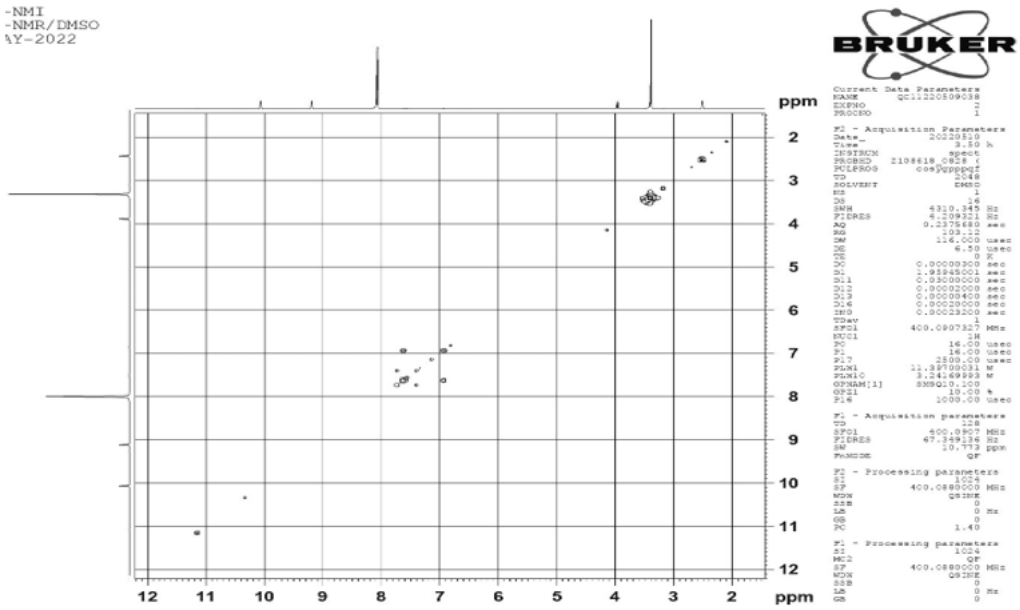


Figure S6. HSQC spectra of the isolated compound (NBV)(400MHz,DMSO)

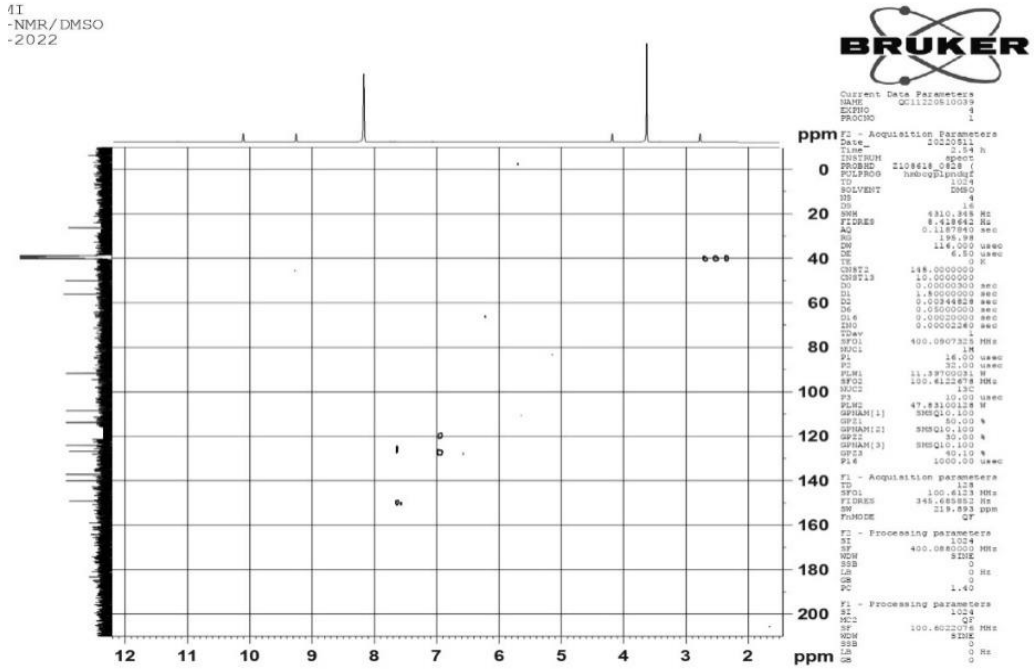


Figure S7. XRD Observations of the isolated compound(NBV)

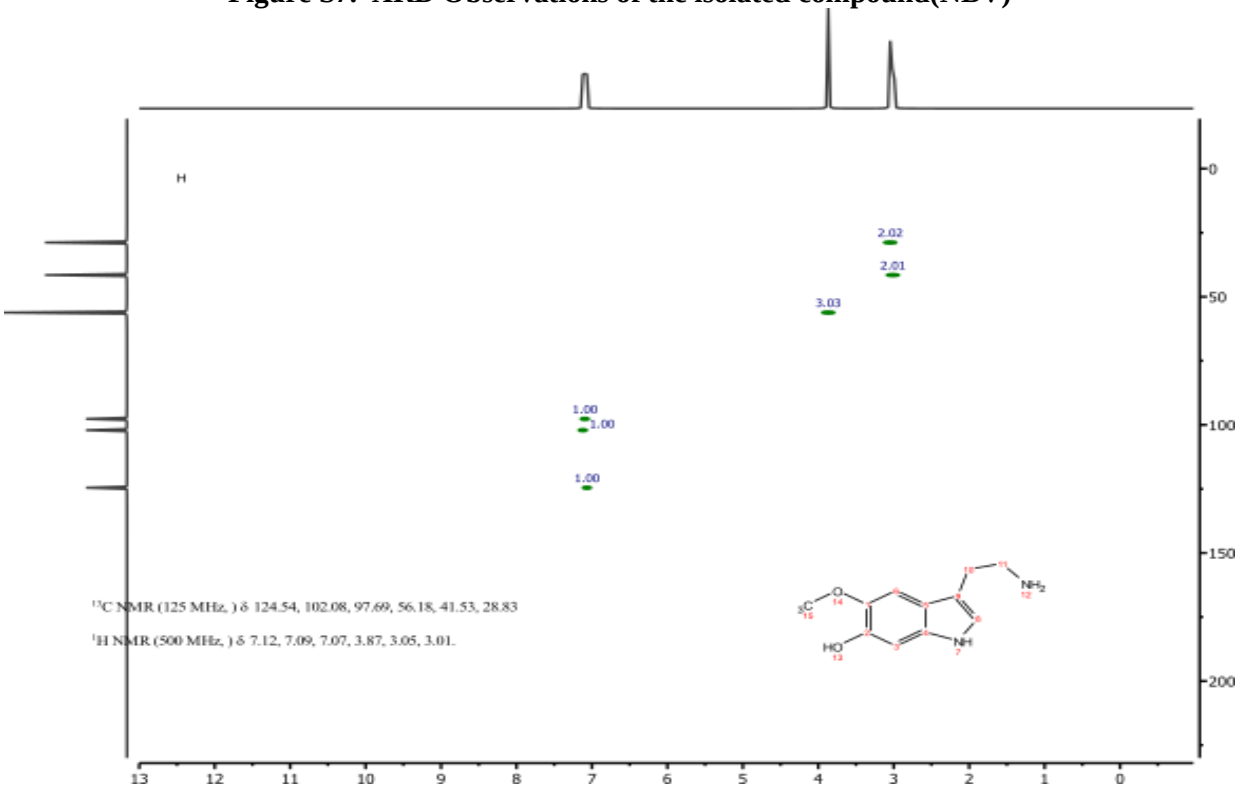


Figure S8. LC-HRMS spectra of the isolated compound(NBV)

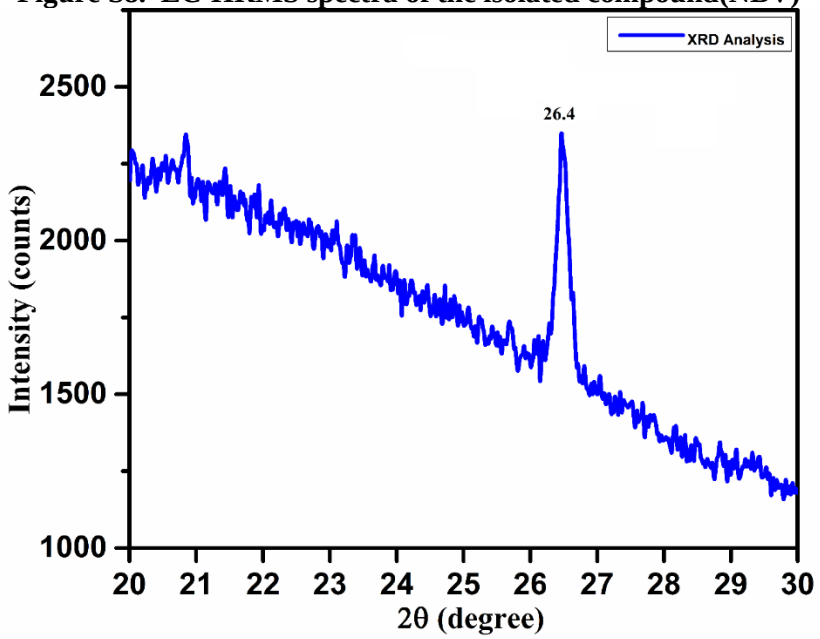
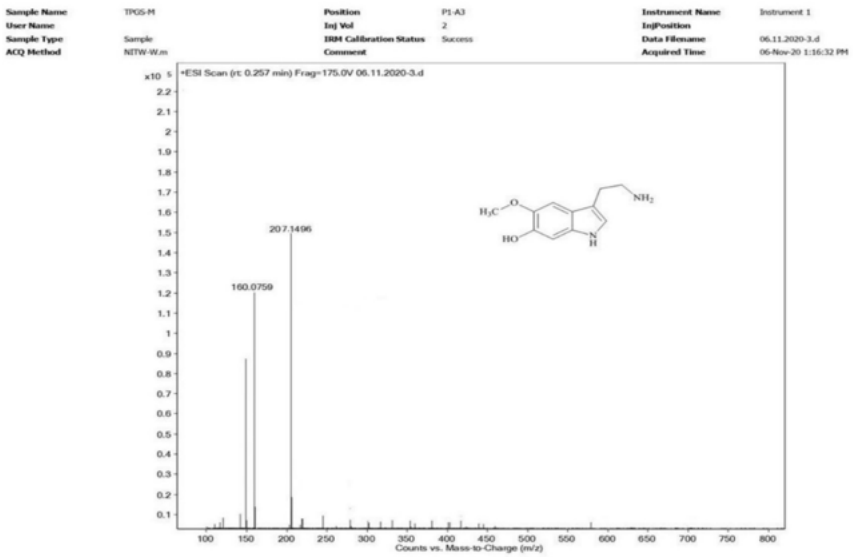


Figure S9. Isolation Process by Column Chromatogrphy



Mass (m/z) (ESI-MS): 207.1496 [M+H] +

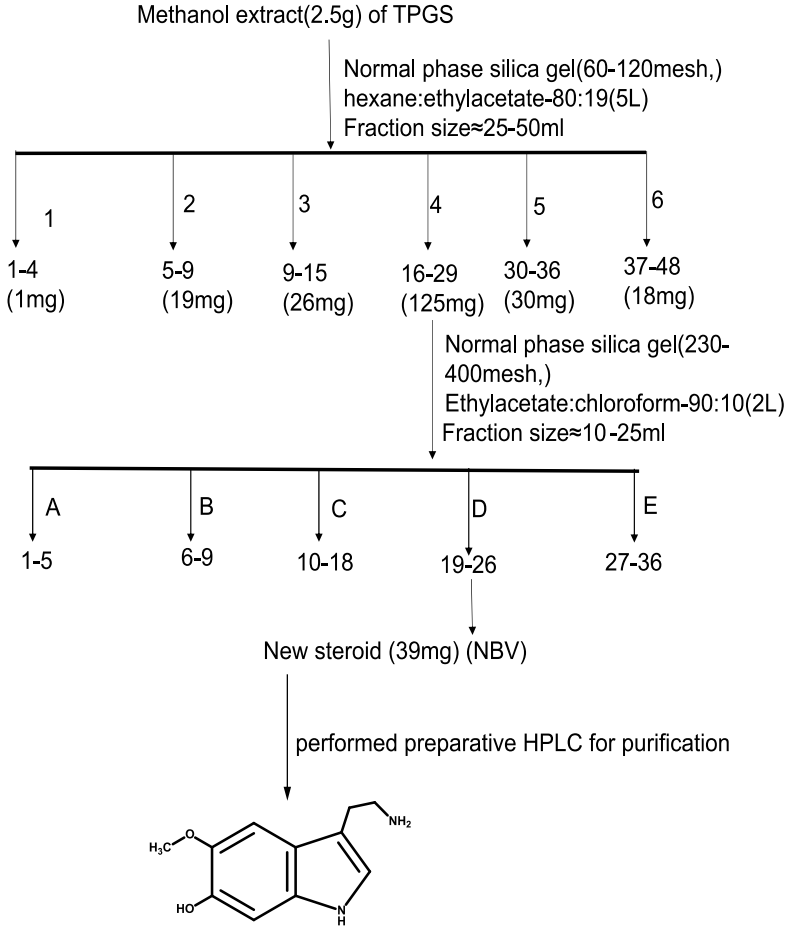


Figure S10. Purification of isolated NBV by preparative HPLC