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L-Proline-based-cyclic dipeptides from *Pseudomonas sp.* (ABS-36) inhibit pro-inflammatory cytokines and alleviate crystal-induced renal injury in mice

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ABSTRACT

Study on the constituents of bioactive culture broth extract (CBE) of *Pseudomonas sp.* (ABS-36) explored the secretion of an array of cyclic dipeptides (CDPs) and twenty of them had been isolated and reported in the present paper. Six major CDPs [(cyclo(Leu-Pro) (**1**), cyclo(Val-Pro) (**2**), cyclo(Leu-hydroxy-Pro) (**9**), cyclo(Pro-Tyr) (**10**), cyclo(Pro-Ala) (**11**) and cyclo(Gly-Pro) (**12**)] exhibited pan cytokine inhibition effect by inhibiting key pro-inflammatory cytokines IL-1β, TNF-α and IL-6 tested under various cell based assays. With this background, the effect of these six CDPs in treating renal inflammation was screened using crystal-induced nephropathy model in mice at 50 mg/kg body weight through oral administration. *cis*-Cyclo(Val-Pro) (**2**) exhibited 57% inhibition of plasma IL-1β protein expression and 35.2% inhibition of elevated blood urea nitrogen. Further, *cis*-cyclo(Val-Pro) (**2**) attenuated renal injury as demonstrated by significant reduction of mRNA expressions of IL-1β ($P < 0.01$) and kidney injury marker-1 ($P < 0.001$). Furthermore, evaluation of tubular-necrosis, -dilation and -cast in the histological sections exhibited moderate protection of renal tissues by *cis*-cyclo(Val-Pro) (**2**). All the tested CDPs reduced the nitrite production and were interestingly non-cytotoxic.

1. Introduction

Pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6 are important in determining early phases of the inflammation, which then progress to systemic inflammatory response syndrome [1]. Role of pro-inflammatory cytokine IL-1β has been implicated in various crystallopathies involving inflammation and tissue remodeling such as atherosclerosis caused by cholesterol crystals [2], gout caused by mono sodium urate crystals, pseudo gout due to calcium pyrophosphate crystals [3], asbestosis developed by asbestos particles [4], silicosis with silica crystals [5], renal nephropathy caused by calcium oxalate crystals [6] and peri-implantitis caused by titanium ions present in the implant-supporting bones [7]. Hence, discovering pan-cytokine inhibitors will result in developing drugs to treat various chronic inflammatory diseases.

Pseudomonas of Gamma proteobacteria genus is widely present in the environment such as soil, water, plant surface and animals. *Pseudomonas* species have enormous ability to produce proteins and secondary metabolites that exhibit diverse biological activity. In view of this, *Pseudomonas* species (ABS-36) isolated from the rhizospheric soil was exploited for its ability to produce biologically active secondary metabolites to develop drug lead molecules for treating inflammation. The study was directed towards developing cytokine inhibitors from the culture broth extract of *Pseudomonas* sp. ABS-36, which was identified to inhibit TNF-α secretion in our earlier study [8].

The chemical investigation on the culture broth extract (CBE), which lead to the discovery of secretion of twenty cyclic dipeptides (CDPs) is described in this paper along-with the pro-inflammatory cytokine inhibition property and effect of the isolated major proline-based-CDPs in mouse model of oxalate nephropathy. Also, the paper discusses the pan-cytokine inhibition effect of *cis*-cyclo(Val-Pro) (**2**) and its future perspectives. This is the first report of isolation of seventeen CDPs (**1–3**, **5–8**, **10**, **11**, **13–20**) from this source.

2. Materials and methods

2.1. General

Multi detection reader (Spectramax M4, California, USA), IR (Jasco FT/IR-4200, Maryland, US), MS (LCMS-2020, Shimadzu, Japan), HPLC

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(LC-8A, Shimadzu, Japan) equipped with an ODS column (Inertsil ODS-3, 25 cm × 4.6 mm i.d., GL Sciences, Japan), and NMR (JNM-AL300, JEOL, Japan) instruments were used as per the requirements. mRNA was converted to cDNA using Thermo scientific verso cDNA synthesis kit. The real-time PCR (RT-PCR) was performed in an iCycleriQ apparatus associated with the iCyclerOptical System software (version 3.1) using SYBR Green PCR Master Mix (KAPA SYBR® FAST qPCR Master Mix Kit). Protein was isolated from cultured cell and tissue samples using RIPA buffer (Sigma-Aldrich, MO, USA), transferred using Immunoblot PVDF membrane (BIO-RAD, laboratories, USA) and detected using Enhanced Chemiluminescence Detection System (BIO-RAD, laboratories, USA). Histological sections were taken using Yidi microtome (Jinhua YIDI Medical Appliance CO. Ltd). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Himedia Laboratories Pvt. Ltd., Mumbai, India. Mouse macrophages cell line RAW 264.7 was obtained from the Cell Bank of National Center for Cell Sciences, Pune (Maharashtra, India). Silica gel (100–210 or 40–50 μm, Kanto Chemical, Japan) and silica gel 60 F_{254} glass plate (1.05715.0001, Merck) were used for column chromatography and for TLC/p-TLC, respectively. Dimethyl sulfoxide (DMSO) was procured from Merck Specialties Private Limited, India. Prednisolone (Sigma-Aldrich, MO, USA) and lipopolysaccharide (LPS) (*E. coli* serotype 0111:B4, Sigma-Aldrich, MO, USA), TRIzol Reagent® (Thermo Fisher Scientific) were used. ELISA kits for TNF-α, IL-6 and IL-1β were purchased from R and D systems (Biotechne Brand, USA and Canada) and Blood Urea Nitrogen (BUN) kits (AUTOSPAN Liquid Gold Urea) were procured from Arkray Healthcare Pvt. Ltd. (India). All reagents used were of analytical grade. Isolated compounds and prednisolone were dissolved in DMSO and added directly to the culture media before the addition of LPS. The final concentration of DMSO was never allowed to exceed 0.1%.

2.2. Preparation of culture broth extract (CBE)

Culture broth of *Pseudomonas* sp. ABS-36 strain was prepared by cultivating under King's B media (20 L; 30 °C). After 7 days of incubation, the broth was centrifuged at 10,000 rpm for 10 min cautiously to collect the supernatant and precipitate, separately. The supernatant was then extracted with ethyl acetate (EtOAc) thrice and the combined EtOAc layer was evaporated under reduced pressure to yield a dry residue (CBE) (13.0 g).

2.3. Isolation and characterization of secondary metabolites

CBE was chromatographed on silica gel (100–210 μm) eluting with EtOAc–MeOH gradient to give fractions 1–7. Fraction 6 (eluted with EtOAc–MeOH (50:1), which exclusively contained various CDPs, was rechromatographed over silica gel (40–50 μm) eluting with $CHCl_3$ _ acetone followed by acetone–MeOH gradient. The fraction eluted with $CHCl_3$–acetone (6:1) afforded cyclo(Leu-Pro) **1** (15 mg) and cyclo(Val-Pro) **2** (21 mg) as pure amorphous solid. The fraction eluted with $CHCl_3$-acetone (1:3) was separated by HPLC on an ODS column (solvent, CH_3CN–H_2O (1:4) containing 0.03% TFA, flow 0.5 ml/min, detection 215 nm) to give cyclo(Val-Leu) **3** (5.1 mg), cyclo(Phe-Pro) **4** (7.2 mg), cyclo(Val-Phe) **5** (5.5 mg), cyclo(Ile-Phe) **6** (2.2 mg), cyclo (Leu-Ile) **7** (2.4 mg) and cyclo(Leu-Leu) **8** (3.2 mg). The fraction eluted with acetone was separated by preparative TLC (developing solvent, $CHCl_3$–MeOH (95:5)) to afford pure cyclo(Leu-hydroxy-Pro) **9** (11 mg), cyclo(Pro-Tyr) **10** (19 mg), cyclo(Ala-Pro) **11** (20 mg). The fraction eluted with acetone–MeOH (8:2) was rechromatographed over silica gel (40–50 μm) eluting with $CHCl_3$–acetone gradient gave sub-fractions A (eluted with $CHCl_3$–acetone (20:1)), B (eluted with $CHCl_3$–acetone (20:1.5)), C (eluted with $CHCl_3$–acetone (20:2)), and D (eluted with $CHCl_3$–acetone (20:3)). Sub-fraction A was suspended in MeOH and an insoluble part was subjected to HPLC (solvent, MeOH–H_2O (2:3) containing 0.03% TFA) to afford cyclo(Gly-Pro) **12** (9.4 mg) and cyclo(Gly-Phe) **13** (1.0 mg). The MeOH-soluble part was also separated by HPLC (solvent, MeOH–H_2O (2:3) containing 0.03% TFA) to afford cyclo(Gly-Pro) **12** (10 mg), cyclo(Gly-Phe) 13 (8.5 mg), cyclo(Ala-Phe) **14** (6.3 mg) and cyclo(Ile-Phe) **6** (2.2 mg) and cyclo(Ala-Ile) **15** (0.9 mg). HPLC separation (solvent, MeOH–H_2O (1:2) containing 0.03% TFA) of the sub-fraction B yielded cyclo(Gly-Tyr) **16** (2.4 mg), cyclo(Ala-Tyr) **17** (2.0 mg) and cyclo(Val-Tyr) **18** (6.1 mg) and cyclo(Leu-Tyr) **19** (1.9 mg). HPLC separation (solvent, MeOH–H_2O (1:2) containing 0.03% TFA) of the sub-fraction C yielded cyclo(Ala-Ala) **20** (2.1 mg). HPLC purification (solvent, MeOH–H_2O (1:2) containing 0.03% TFA) of the sub-fraction D yielded cyclo(Gly-Tyr) **16** (2.2 mg), cyclo(Ala-Tyr) **17** (7.0 mg) and cyclo(Gly-Phe) **13** (7.0 mg). Fraction 7 (eluted with EtOAc-MeOH (30:1–10:1)) of the initial silica gel column yielded additional amount (15 mg) of cyclo(Gly-Pro) **12**.

2.4. In vitro studies

2.4.1. Cell culture

RAW 264.7 macrophage cell lines were cultured in DMEM media (Dulbecco's Modified Eagle's Medium) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Cells were seeded in 96-well plate for ELISA estimations and in T-25 culture flask for RNA/protein estimation using western blot.

2.4.2. Cytokine protein estimations using ELISA

RAW 264.7 cells were treated with varying concentrations of the CBE (12.5, 25, 50, 100, 250 and 500 μg/mL) and CDPs (3 μM, 10 μM, 30 μM and 100 μM). After one hour of incubation, cells were primed either with 1 μg/mL LPS for TNF-α and IL-6 estimations or with LPS and 250 μM calcium oxalate crystals for IL-1β estimations. Cell free supernatant was collected after 24 h of incubation and was further subjected to ELISA estimations using commercially available ELISA kits as per the manufacturer's instruction [1].

2.4.3. Cell viability assay

Cell viability was analyzed using rapid colorimetric MTT assay. 50 μL of MTT reagent dissolved in phosphate buffer saline (5 mg/mL) was added to each well containing cells pretreated with CBE and CDPs and incubated at 37 °C for 3 h. 100 μL of DMSO was added after removing the whole media to dissolve the formed crystals of formazan. Absorbance was measured at 570 nm in multiplate reader [9].

2.4.4. Griess assay

Nitrite (NO) levels were determined using Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid). RAW cells were pretreated for 6 h with different concentrations of CBE / isolated compounds as per the experimental design, followed by incubation with LPS for 24 h. Cell free supernatant was collected, then mixed with equal volume of Griess reagent and incubated at room temperature for 10 min. Thereafter absorbance was measured at 540 nm using multiplate reader [10,11].

2.4.5. Real time (RT)-quantitative PCR study of compound (2): In vitro

Total RNA was isolated from cultured cells (treated with compound (**2**) 100 μM) using Trizol reagent followed by cDNA synthesis using cDNA synthesis kit. TNF-α, IL-6 and IL-1β expression was evaluated using following primers: TNF-α forward, CCGCTCGTTGCCAATAGTG ATG; TNF-α reverse, CATGCCGTTGGCCAGGAGGG; IL-6 forward, GCACTACAGGCTCCGAGATGAA; IL-6 reverse, GCCTCCGACTTGTGAA GTGGTA; IL-1β forward, GCACTACAGGCTCCGAGATGAA; IL-1β reverse, GTCGTTGCTTGGTTCTCCTTGT. SYBR Green PCR Master Mix was used. All gene expressions were normalized using GAPDH as housekeeping gene [12].

2.5. In vivo study

2.5.1. Oxalate crystal induced renal nephropathy model

Anti-inflammatory efficacy of the isolated compounds was tested using oxalate induced mouse model of renal nephropathy. Approval for the study protocol was obtained from Institutional Animal Ethics Committee (BITS-Hyd/IAEC/2017/10). Briefly, study was carried out in eight different groups of C57/BL6 mice. Mice were fasted overnight before the administration of test compounds at a dose of 50 mg/kg. Compounds (**1**, **2** and **9–12**) were prepared as suspension using methylcellulose and Tween 20 mixture (9:1) and administered using oral gavage at dose volume of 10 mL/kg. 75 mg/kg sodium oxalate solution was then injected by intraperitoneal route after one hour of drug administration and immediately after oxalate administration the mice were fed with normal diet and 3% *w/v* sodium oxalate water. Mice were sacrificed after 24 h of sodium oxalate administration to harvest the blood samples and kidney tissue samples [13].

2.5.2. ELISA estimations and BUN analysis

ELISA studies were carried out on isolated plasma to estimate the levels of IL-1β using IL-1β kit as per the manufacturer's instruction [1]. Isolated plasma was also subjected to BUN level estimations using commercially available kits for BUN measurements [14,15].

2.5.3. RT–PCR analysis of inflammatory markers in renal tissue

The renal damage caused by the oxalate nephropathy was evaluated by determining the expression of KIM-1 (kidney injury molecule-1, a renal injury marker) and expression of IL-1β, IL-6 and TNF-α (inflammatory markers) in the isolated renal tissue. Total RNA was isolated from kidneys using Trizol reagent. After quantification and purity analysis of isolated RNA, cDNA was prepared using cDNA synthesis kit. RT-PCR was carried out using primers for IL-1β, IL-6 and TNF-α. All gene expressions were normalized using GAPDH as housekeeping gene [12].

2.5.4. Assessment of renal injury by histology

After the processing of renal tissue in tissue processor unit, 5 μm sections were taken using microtome followed by staining of the kidney sections using hematoxylin and eosin. Histological scoring was done by quantifying tubular injury by semiquantitative scoring method and scores were given on a scale of 0–5 (0 for being nil damage and 5 for being severe damage) based on renal damage such as tubular dilation, tubular necrosis and casts [16].

2.6. Statistics

All values were presented as mean ± SEM. Groups were compared using Graph Pad Prism 6.0, one way ANOVA followed by Dunnett's multiple comparisons. P value < 0.05 was considered significant.

3. Results

3.1. CBE as potential source for pro-inflammatory inhibitors

The rhizospheric soil of peanut plant was used as a source for isolating the bacteria. The isolated fluorescing bacterial strain was identified as *Pseudomonas species* by comparison of 16S rDNA sequence and registered as ABS-36 strain (GenBank Accession No. KT625586). This bacterium was cultivated in King's B broth and the culture broth was extracted with EtOAc. The lyophilized extract (CBE) was tested for its effect on the expression of cytokines using inflammatory markers such as IL-1β, TNF-α and IL-6 in the culture supernatant of RAW 264.7 through LPS-induced ELISA assay. CBE inhibited the production of TNF-α, IL-6 and IL-1β with IC_{50} values of 73.66, 95.49 and 132.09 μg/mL, respectively (Fig. 1). Thus, the bioactive CBE was subjected to chemical investigation to isolate the individual constituents.

3.2. Cyclic dipeptides as constituents of CBE

CBE was subjected to a combination of column chromatography on silica gel, p-TLC and HPLC. Twenty compounds were isolated and they were characterized as diketopiperazines, commonly known as cyclic dipeptides (CDPs), based on ^{1}H and ^{13}C NMR spectroscopy (Fig. 2). The identity of the compounds was established based on the comparison of measured NMR data (see supplementary data) with that of reported data. Compounds were identified as cyclo(Leu-Pro) (**1**) [17], cyclo(Val-Pro) (**2**) [17,18], cyclo(Val-Leu) (**3**) [19], cyclo(Phe-Pro) (**4**) [20], cyclo (Val-Phe) (**5**) [21], cyclo(Ile-Phe) (**6**) [22], cyclo(Leu-Ile) (**7**) [22], cyclo(Leu-Leu) (**8**) [23], cyclo(Leu-hydroxy-Pro) (**9**) [18,24], cyclo(Pro-Tyr) (**10**) [17], cyclo(Ala-Pro) (**11**) [17,20], cyclo(Gly-Pro) (**12**) [17,25], cyclo(Gly-Phe) (**13**) [24,26], cyclo(Ala-Phe) (**14**) [20,24], cyclo(Ala-Ile) (**15**) [20,24], cyclo(Gly-Tyr)(**16**) [27], cyclo(Ala-Tyr) (**17**) [20], cyclo(Val-Tyr) (**18**) [20], cyclo(Leu-Tyr) (**19**) [21] and cyclo (Ala-Ala) (**20**) [28]. Relative and absolute stereochemistry of compound **2** was established as cyclo(L-Val-L-Pro) by comparing the NMR data with those of the *cis*- and *trans*-isomers and based on the specific rotation, $[\alpha]_D^{25}$–133 (*c*, 0.11, ethanol) (lit. -149 (c, 1.09, ethanol) [17]. The NMR data of compound **4** was not consistent with that of *cis*-cyclo (Phe-Pro), but in good agreement with that of *trans*-cyclo(Phe-Pro). Compound **4** was determined to be cyclo(L-Phe-D-Pro) based on the specific rotation, $[\alpha]_D^{25}$ 69.3 (*c*, 0.75, ethanol) (lit. 67 (methanol) for cyclo(L-Phe-D-Pro) [29] and (lit. -79 (c, 0.94, ethanol) for cyclo(D-Phe-L-Pro)) [17]. Compounds **1–3**, **5–11**, **14–15** and **17–20** were assigned as *cis*-cyclic dipeptides by comparing their NMR data with the reported values and assumed to be common natural L,L-forms.

3.3. Effect of CBE and CDPs on pro-inflammatory (IL-1β, TNF-α and IL-6) protein expression, nitrite production and cell viability

RAW cells were treated with different concentrations of isolated L-proline-based-cyclic dipeptides **1**, **2** and **9–12** as per the experimental design and the results were found to be significant. The suppressive effect was found to be more against IL-1β secretions compared to TNF-α and IL-6 with IC_{50} values ranging between 44 and 61 μM (Fig. 3). Cyclo (Pro-Tyr) (**10**) and cyclo(Pro-Ala) (**11**) exhibited IC_{50} values of 44.70 and 44.82 μM to inhibit IL-1β expressions. Cyclo(Val-Pro) (**2**) showed highest inhibition potential against IL-6 and TNF-α with IC_{50} values of 40.2 and 115.38 μM, respectively.

Overexpression of pro-inflammatory cytokines up-regulates genes that produce inducible nitric oxide synthase (iNOS), increasing oxygen consumption and thereby, producing many reactive species like NO. Hence the effect of CDPs (**1**, **2 and 9–12**) on NO production was tested. Cell-free supernatant collected after the treatment of macrophages with varying concentrations of compounds were analyzed for nitrite levels using Griess reagent. Results revealed inhibitory potential of isolated compounds to be significant (P < 0.001 at 12.5 μg/mL for CBE, P < 0.05 at 100 μM for **1**, **2 and 9–12**) as shown in Figs. 2–3.

Cytotoxic effect of CDPs (**1**, **2** and **9–12**) was tested on RAW 264.7 cells using rapid colorimetric MTT assay. Cells treated with concentrations ranging from 3 μM to 100 μM were evaluated for viability. Data revealed CDPs to be non-cytotoxic with IC_{50} values ranging from 250 to 450 μM (Fig. 3). CBE exhibited no cytotoxicity with IC_{50} value of 1867.3 μg/mL.

3.4. Effect of CDPs in crystal-induced renal nephropathy animal model

As the CDPs (**1**, **2** and **9–12**) effectively controlled crystal-induced IL-1β secretions under in vitro model, their anti-inflammatory potential was tested under mouse model of renal nephropathy. Renal nephropathy developed using sodium oxalate crystals can be well identified by the formation of calcium oxalate crystals in renal tubular and interstitial tissues that lead to tubular injury, renal inflammation that ultimately progresses to renal failure [6]. All the tested CDPs [**1** (48.1%), **2**

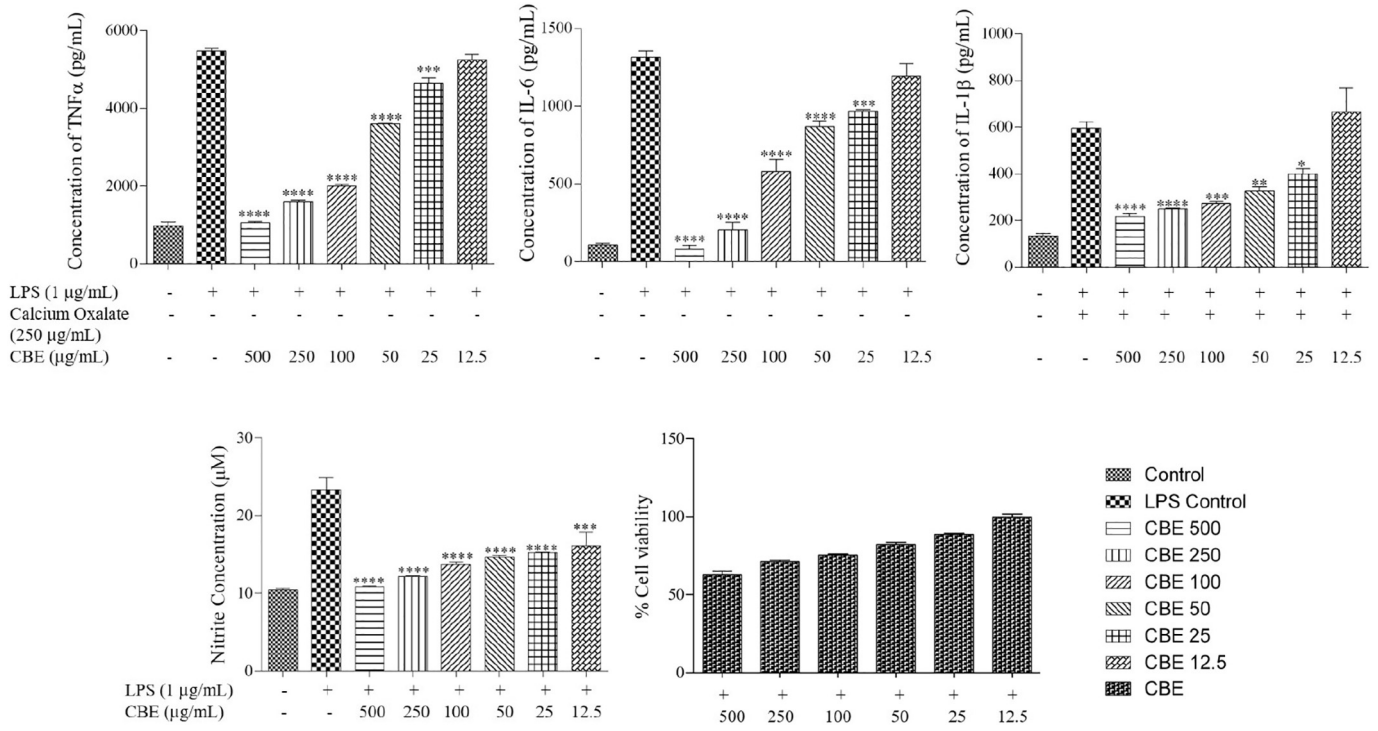

Fig. 1. Effect of CBE on production of pro-inflammatory cytokines TNF-α, IL-6 and IL-1β as well as on nitrite production and viability of RAW 264.7 cells at diverse concentrations ranging from 12.5 μg/mL to 500 μg/mL. All values were presented as mean ± SEM ($n = 3$).(*$P < 0.05$ vs LPS control, **$P < 0.01$ vs LPS control, ***$P < 0.001$ vs LPS control, ****$P < 0.0001$ vs LPS control).

(57.1%), **9** (37.0%), **10** (29.3%), **11** (31.5%) and **12** (31.5%)] showed significant control on release of IL-1β levels (Fig. 4).

Kidney sections (5 μm) of significantly active compound (**2**) were stained using hematoxylin and eosin. Tubular injury index was scored by assessing tubular necrosis, tubular dilation and tubular cast. Tubular necrosis was characterized by morphological changes such as loss of brush border, flattening of cells, disruption and detachment of tubular cells from basement membrane. Tubular cast involved acellular casts (e.g. granular, hyaline, waxy, or fatty casts) and cellular casts (e.g. red or white blood cell casts) formed as a result of protein precipitation. Tubular dilation referred to widening of luminal tubular tissue [30]. Cyclo(Val-Pro) (**2**) exhibited marginal protection of renal tissue as demonstrated by histological analysis data (Fig. 5).

3.4.1. Attenuation of plasma IL-1β levels and blood urea nitrogen (BUN) levels

Effects of CDPs on plasma IL-1β levels in oxalate crystals treated mice are shown in Fig. 4. All six proline-based-CDPs reduced plasma IL-1β levels with significant levels of $P < .0001$ (**1**, **2** and **9**) and $P < 0.001$ (**10**−**12**). Among them cyclo(Val-Pro) (**2**) was found to be most effective with 57% inhibition at 50 mg/kg dose ($P < 0.0001$). Plasma BUN reached peak level in 24 h as a result of damage to renal structure [16]. Among the tested CDPs, cyclo(Val-Pro) (**2**) exhibited significant reduction of the elevated level of BUN (Fig. 6), indicating the protection of renal function against the damage induced by oxalate nephropathy ($P < 0.05$). It showed 35.2% inhibition of elevated plasma BUN compared to oxalate nephropathy control group at a dose of 50 mg/kg.

3.4.2. Effect of Cyclo(Val-Pro) (2) on mRNA expression levels of pro-inflammatory and renal injury marker

In order to reconnoiter the active targets of highly active cyclo(Val-Pro) (**2**), mRNA expression levels of IL-1β, IL-6, TNF-α (pro-inflammatory markers) and KIM-1 (renal injury marker) were measured and the results are delineated in Fig. 7. Cyclo(Val-Pro) (**2**) attenuated renal injury, as displayed by the significant reduction in the renal RNA expression of IL-1β ($P < 0.01$). Also, the expression of renal injury marker, KIM-1 was very significantly reduced ($P < 0.001$). Further, a reduction ($P < 0.05$) in the pro-inflammatory markers (TNF-α and IL-6) was demonstrated by Cyclo(Val-Pro) (**2**). The primary cytokine IL-1β released during oxalate nephropathy was significantly attenuated by Cyclo(Val-Pro) (**2**), compared to the relative expression of other cytokines TNF-α and IL-6.

3.5. Anti-inflammatory efficacy of cyclo(Val-Pro) (2): In vitro

Corroboration of the predominant suppression effect of cyclo(Val-Pro) (**2**) over IL-1β protein was made by determining mRNA expression of three pro-inflammatory cytokines TNF-α, IL-6 and IL-1β in LPS-stimulated RAW macrophages using real time qPCR. No basal change was observed in the cytokines expression following incubation with only tested samples without LPS. Results demonstrated significant diminution of the mRNA expression level of all cytokines. A highly significant attenuation of mRNA expression level of 78% of IL-1β was found as shown in Fig. 8.

4. Discussion

Small molecule inhibitors of pro-inflammatory cytokines that cause various inflammatory disorders have become the area of major interest in anti-inflammatory drug discovery. CDPs of natural origin particularly from microbial sources have demonstrated vast potential to be used as antioxidants, immunomodulators and anticancer, antimicrobial or antifungal agents. CDPs are found to be more beneficial than their non-CDP counterparts as they exhibit inherent property of chemical and enzymatic stability, and structural and conformational specificity [31].

Several reports describing the isolation of CDPs especially from microbial origin are found in the literature. Cyclo(Val-Pro), cyclo(Gly-Phe), cyclo(Phe -Tyr), cyclo(Leu-Tyr) and cyclo(Val-Leu) derived from *Streptomyces kunmingensis*, had exhibited cytotoxic action on MCF-7 cell

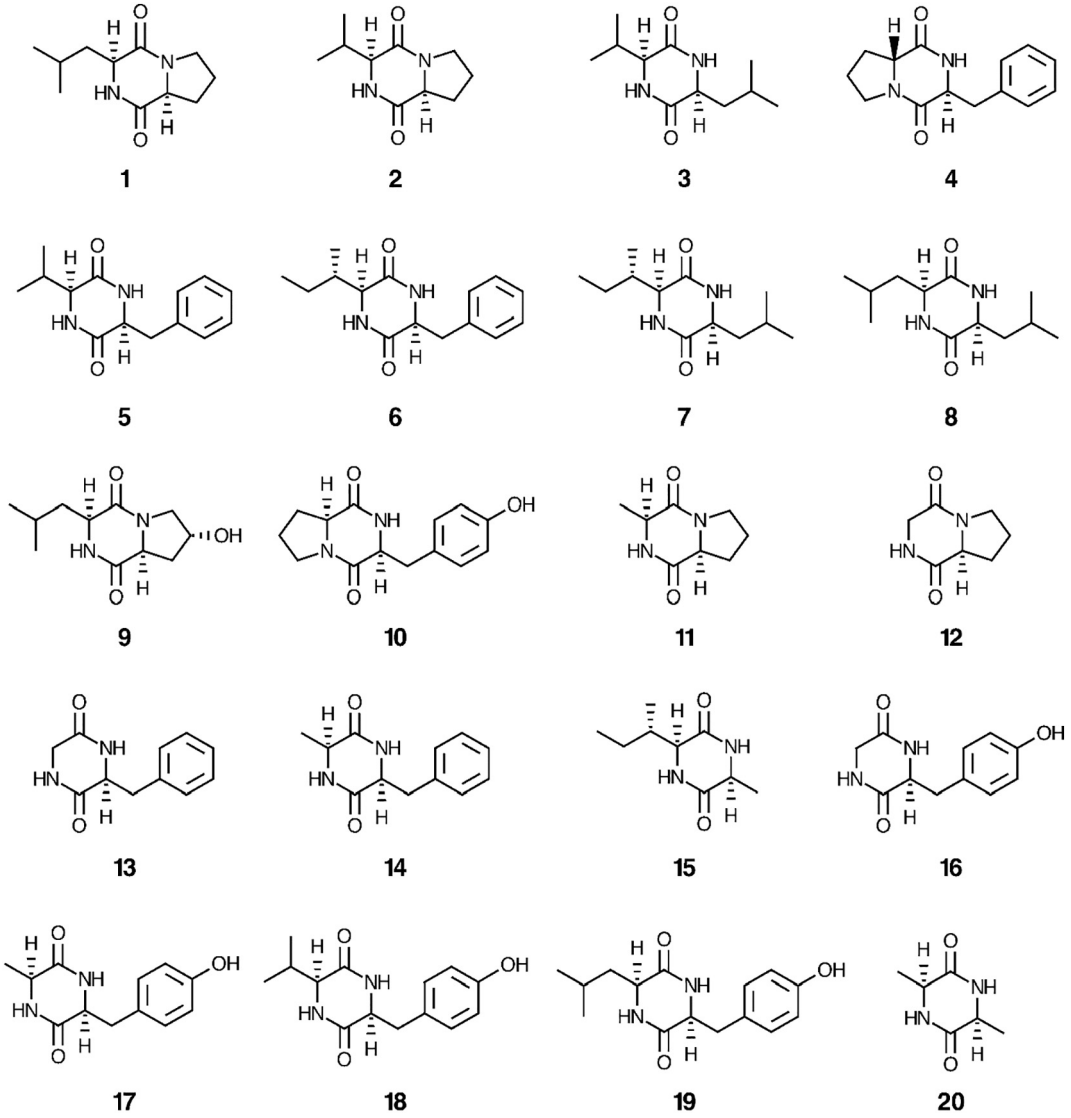

Fig. 2. Structures of isolated CDPs (**1 − 20**) from CBE. *cis*-Cyclo(Leu-Pro) (**1**), *cis*-cyclo(Val-Pro) (**2**), *cis*-cyclo(Val-Leu) (**3**), *trans*-cyclo(Phe-Pro) (**4**), *cis*-cyclo(Val-Phe) (**5**), *cis*-cyclo(Ile-Phe) (**6**), *cis*-cyclo(Leu-Ile) (**7**), *cis*-cyclo(Leu-Leu) (**8**), *cis*-cyclo(Leu-hydroxy-Pro) (**9**), *cis*-cyclo(Pro-Tyr) (**10**), *cis*-cyclo(Ala-Pro) (**11**), *cis*-cyclo(Gly-Pro) (**12**), *cis*-cyclo(Gly-Phe) (**13**), *cis*-cyclo(Ala-Phe) (**14**), *cis*-cyclo(Ala-Ile) (**15**), *cis*-cyclo(Gly-Tyr) (**16**), *cis*-cyclo(Ala-Tyr) (**17**), *cis*-cyclo(Val-Tyr) (**18**), *cis*-cyclo (Leu-Tyr) (**19**), *cis*-cyclo(Ala-Ala) (**20**).

line [32]. *Actinomycetes* and *Pencillium oxalicum* HSY-P-17 species had been reported as good sources for CDPs [33]. Cyclo(Pro-Tyr), cyclo(Val-Pro) and cyclo(Pro-Met) derived from *Pseudomonas aurantiaca* had exhibited antifungal properties [34]. Cyclo(Val-Pro) extracted from *Asperigillus oryzae* [35] and *Nigrospora* species had been found to possess cytotoxic action on HCT-116 cell lines [36]. Further, CDPs with antifungal effect had been obtained from *Bacillus cereus* [37]. *Actinomycetes* 11,014-derived CDPs were found to possess antitumor properties [38] and cyclo(Val-Pro), cyclo (Leu-Pro) and cyclo(Phe-Pro) originated from *Streptomyces* had been demonstrated for antineoplastic activity [39]. Further, reports of secretions of cyclo(Val-Pro) and cyclo(Pro-Tyr) from *Pseudomonas fluorescens* [40], *Halobacillus litoralis* [41] and sponge *Tedania anhelans* [42] corroborate the emerging interest on these compounds to develop as lead molecules. Through the present study, *Pseudomonas sp.* (ABS-36) have been identified as a new source for CDPs **1–3**, **5–8**, **10**, **11**, **13–20**.

4.1. CDPs as anti-inflammatory drug lead molecules

CDPs have shown tremendous potential to be used as anti-inflammatory agents in various studies such as protective effect in neurological disorders [43,44], septic responses [45], contact hypersensitivity [46], etc. Reported evidences for the anti-inflammatory property of various CDPs were examined in the literature. Cyclo(His-Pro) had shown protection against neuroinflammation by modulating NFκB and nrf2 pathways [43,44]. CDPs from *Aspergillus* species SCSIOW2 had demonstrated beneficial effect in the treatment of Alzheimer's disease by inhibiting NO pathway [47]. Also, several CDPs containing formulation have exhibited their protective role in neurological disorders such as dementia, schizophrenia, Alzheimer's disease, Parkinson's syndrome [48]. A composition containing CDPs had demonstrated prevention of intestinal inflammation by affecting GLP2 mediated secretions [49]. *Streptomyces*-derived CDPs had down-regulated pro-inflammatory cytokines TNF-α and IL-6 when tested in LPS-induced in vitro and in vivo models [50]. Cyclo(D-Val-L-Pro), cyclo (L-Pro-L-Tyr) and cyclo (L-Pro-D-Leu) derived from marine based bacterium have shown suppressive effects on septic responses [45,51] as well as inhibited LPS induced endothelial inflammatory responses [52]. Cyclo(Gly-Pro) attenuated nociceptive behavior and inflammatory responses in paw edema model [53]. Fungus-derived CDPs demonstrated

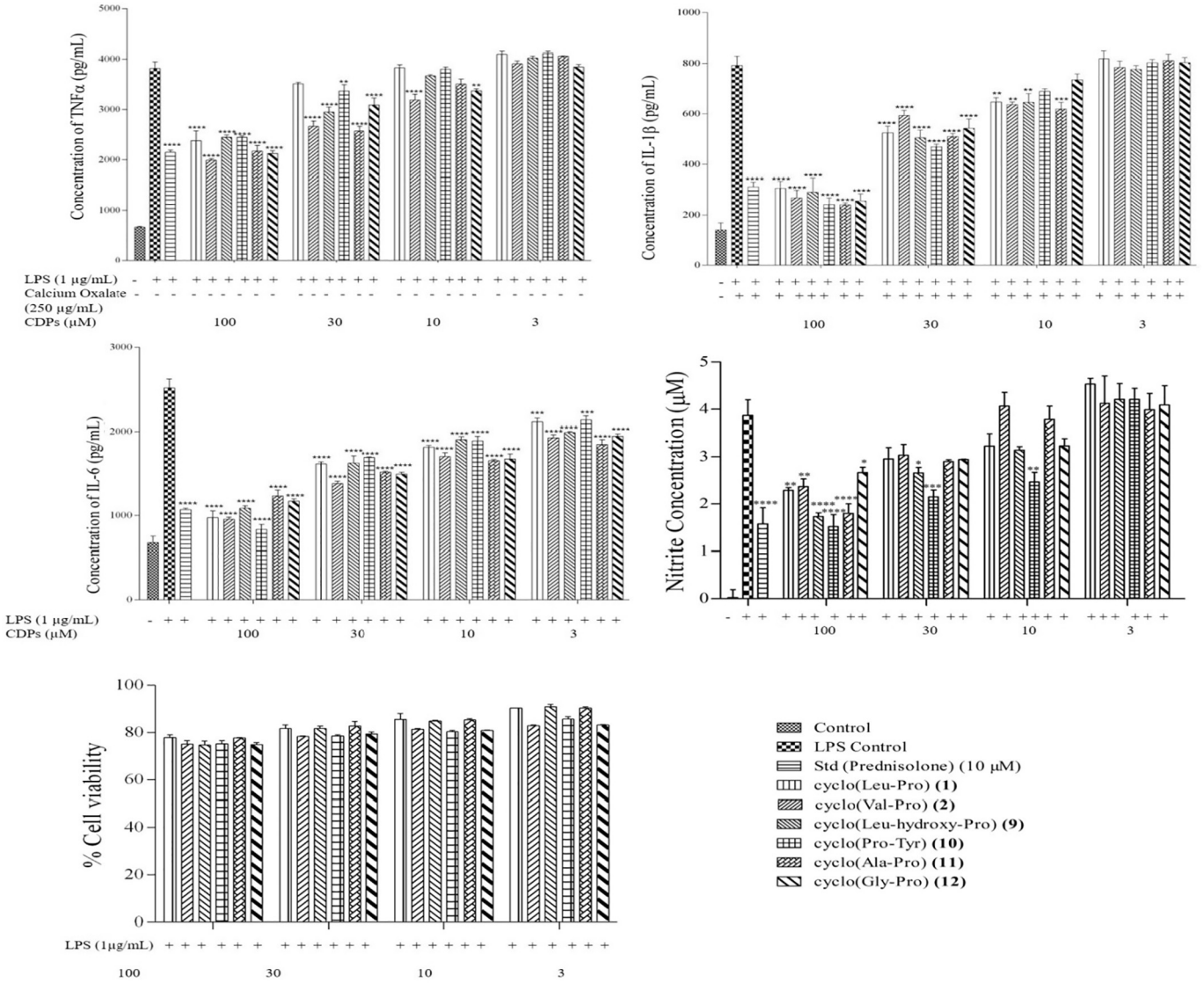

Fig. 3. Effect of CDPs (**1**, **2**, **9–12**) on TNF-α, IL-6, IL-1β and NO levels at different concentrations (3 μM, 10 μM, 30 μM and 100 μM). Figure also shows the effect of CDPs on viability of RAW 264.7 cells. All values were presented as mean ± SEM ($n = 3$). (*$P < 0.05$ vs LPS control,**$P < 0.01$ vs LPS control, ***$P < 0.001$ vs LPS control, ****$P < 0.0001$ vs LPS control).

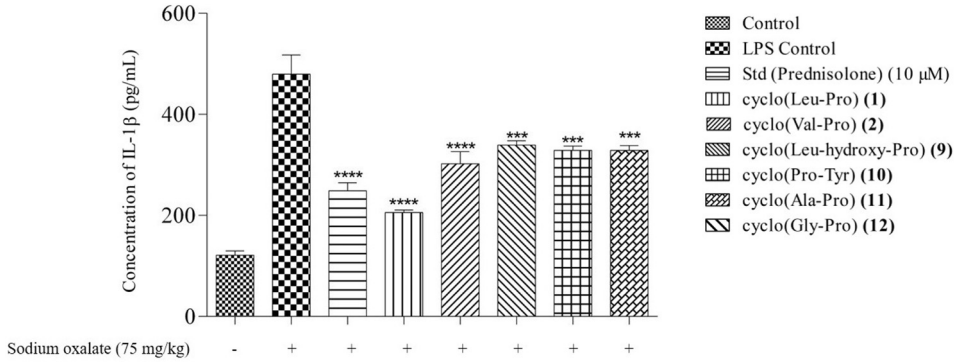

Fig. 4. Effect of CDPs (**1**, **2**, **9–12**) in the reduction of plasma IL-1β levels at 50 mg/kg dose in oxalate crystals-treated animals. All values were presented as mean ± SEM ($n = 6$). (***$P < 0.001$ vs oxalate control, ****$P < 0.0001$ vs oxalate control).

hepatoprotective action in WB-F344 cell lines [54]. Cyclo(L-4-hydroxy-Pro-L-Ser) derived from placental extract had been found to be beneficial in contact hypersensitivity [46]. Cyclo(dehydrohistidyl-tryptophyl) showed anti-inflammatory activity through inhibition of inducible nitric oxide synthase (iNOS) [55]. CDPs from *Achromobacter* species had demonstrated anti-inflammatory potential by stimulating the production of IL-10 and IL-4 anti-inflammatory cytokines [56].

Crystallopathies and inflammatory conditions concerning tissue

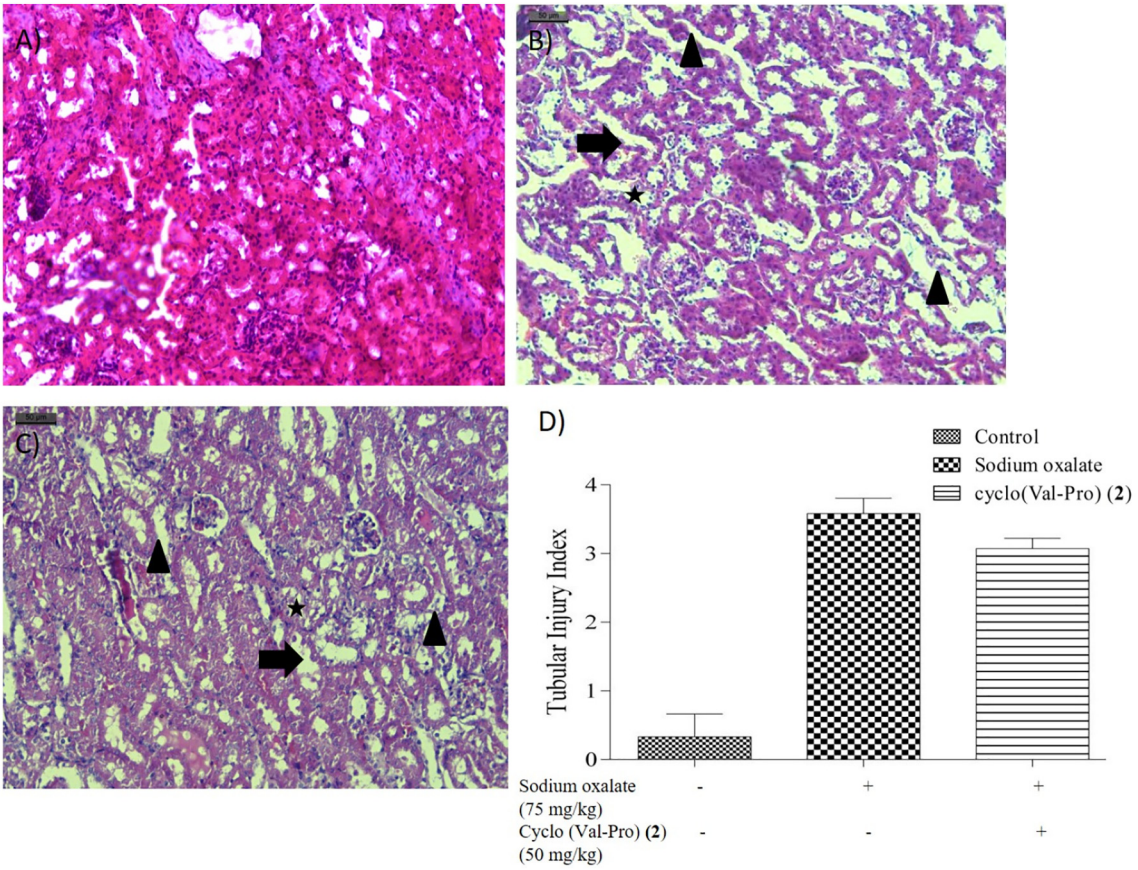

Fig. 5. The protective effect of cyclo(Val-Pro) (**2**) at the dose of 50 mg/kg on histology of renal tissue in calcium oxalate-induced renal nephropathy model. Representative photomicrograph of renal histological sections (H & E) from (A) control group (B) oxalate crystal control group (C) cyclo(Val-Pro) (**2**) treated group. (D) indicates Tubular Injury Index which was quantified by semi-quantitative scoring (Tubular dilation indicated by triangle, tubular necrosis indicated by arrows and tubular casts indicated by star). All values were presented as mean ± SEM (n = 6).

remodeling such as rheumatoid arthritis, gout, and oxalate induced renal injury, pseudogout, atherosclerosis encompass the role of IL-1β [57]. In the present study, based on their in vitro efficacy against inflammatory cytokines and NO, the proline-based-CDPs were evaluated under calcium oxalate crystal-induced renal nephropathy model. Up-regulation of BUN and IL-1β levels indicated the induction of the renal injury. All the tested CDPs showed significant reduction of IL-1β expression. Cyclo(Val-Pro) (**2**) at 50 mg/kg showed significant ($P < 0.0001$) reduction in the levels of IL-1β (57%) and BUN (35.2%) as compared to other isolated CDPs. Therefore, cyclo(Val-Pro) (**2**) was further subjected to other mRNA expression studies and histopathological evaluation. Significant decrease in mRNA expression levels of inflammatory cytokines and kidney injury marker indicated the protective role of **2** in renal injury model. However, a moderate effect was observed in histopathological studies. Literature search was done to identify the reasons for the striking inhibition of inflammatory cytokine transcription with no significant impact on histology. As reported in a study by Mulay et al., [16], treatment with "Anakinra – a recombinant IL-1R antagonist" (25 mg/kg) under the crystal-induced model had shown a marginal protection in tubular injury score with significant improvement in the renal function parameter (plasma creatinine). While such effect was observed with a low dose (25 mg/kg) of anakinra, a more protection in injury scores as well as plasma creatinine was observed with the higher dosage (75 mg/kg). Thus, it is assumed that cyclo(Val-Pro) (**2**) which has shown a marginal protection in histology with significant protection in plasma IL-1β and BUN could

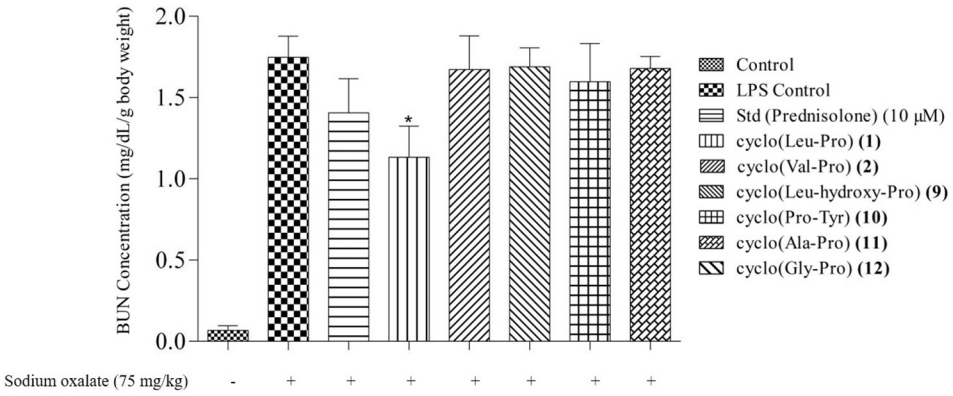

Fig. 6. Effect of CDPs on BUN (Blood Urea Nitrogen) levels at 50 mg/kg dose in oxalate crystal induced renal nephropathy model. As a result of renal tissue damage BUN levels peaked after 24 h of disease induction. Cyclo(Val-Pro) (**2**) significantly reduced the BUN level indicating protection against the renal damage. All values were presented as mean ± SEM (n = 6). (*$P < 0.05$ vs oxalate control group).

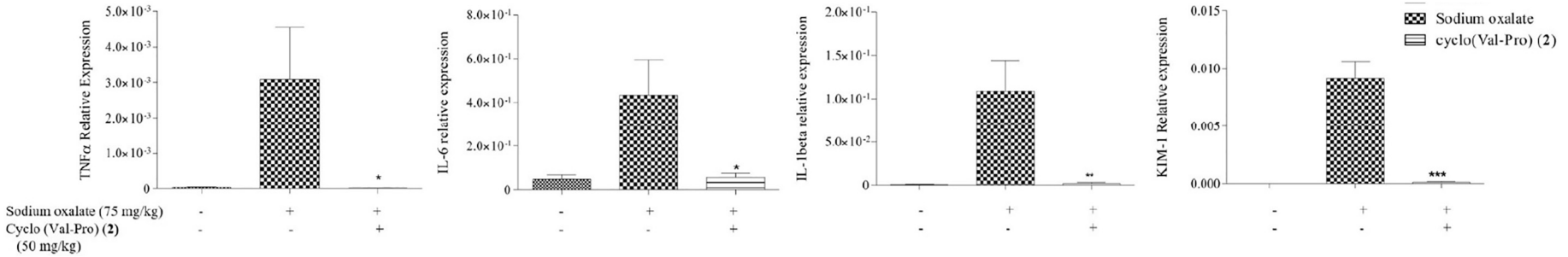

Fig. 7. mRNA expression level of pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) and Kidney Injury Marker (KIM-1) in renal tissues were significantly decreased by administration of cyclo(Val-Pro) (**2**) at 50 mg/kg dose in oxalate-induced renal nephropathy model. All values were presented as mean ± SEM (n = 6). (*$P < 0.05$ vs oxalate control, **$P < 0.01$vs oxalate control, ***$P < 0.001$ vs oxalate control).

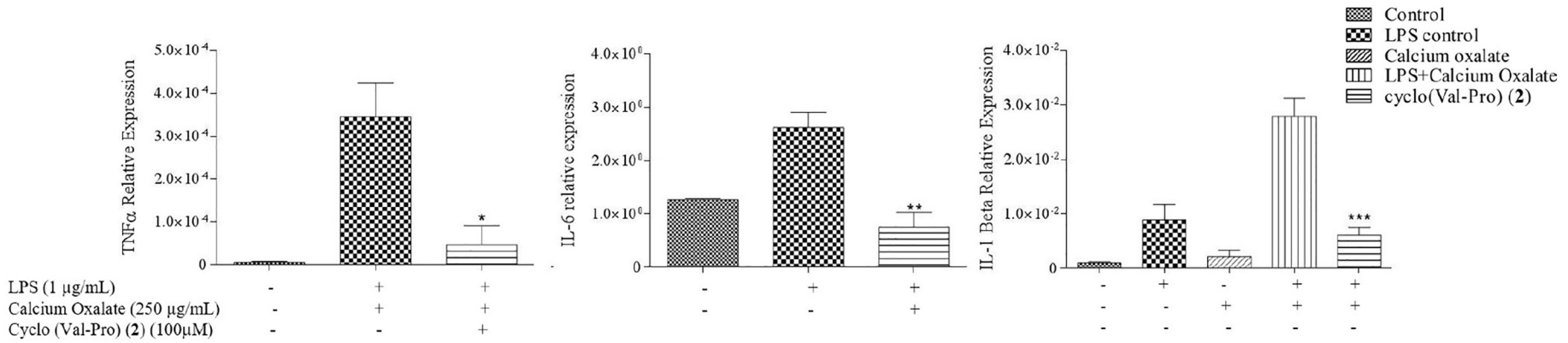

Fig. 8. Effect of cyclo(Val-Pro) (**2**) (100 μM) on mRNA expression of pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) in RAW 264.7 cells. All values were presented as mean ± SEM (n = 3) (*$P < 0.05$ vs LPS control, **$P < 0.01$ vs LPS control, ***$P < 0.001$ vs LPS + crystal control).

exhibit a better histological protection at higher doses. However, this warrants elaborative studies.

Following the protective effect of cyclo(Val-Pro) (**2**) in renal injury model, furthermore investigation using RT-PCR mRNA expression studies on RAW cells were performed. The mRNA expression levels of pro-inflammatory cytokines were significantly reduced, further confirming the pan-inhibition effect and anti-inflammatory efficacy of **2**.

5. Conclusion

The study concludes with the identification of *Pseudomonas sp.* (ABS-36) as a new source for CDPs **1–3**, **5–8**, **10**, **11** and **13–20,** which secretes non-cytotoxic pan-cytokine inhibitory L-proline-based cyclic dipeptides (**1**, **2** and **9–12**) possessing protective effect in oxalate-induced renal nephropathy. Cyclo(Val-Pro) (**2**), a more active CDP is reported as a newer anti-inflammatory lead. Further, elaborative mechanistic studies need to be carried out to get in-depth knowledge regarding the underlying pathway. Also pharmacokinetic studies are warranted to corroborate the variation in efficacy between the in vitro and in vivo studies.

Ethics statement

This study was carried out in accordance with the recommendations of approved guidelines of Animal Research Ethics Committee, BITS-Pilani Hyderabad Campus. The protocol was approved by the Institutional Animal Ethics Committee (BITS-Hyd/IAEC/2017/10).

Author contributions

SBA, YF, HA, KH, ABS and PPP performed the experiments. SBA, YF and OPK designed the experiments and wrote the manuscript. All authors agree to the submission of the manuscript.

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Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.05.044.

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