Research article

Identification of three coagulins as MMP inhibitors from *Withania coagulans* Dunal fruits

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ABSTRACT

Introduction and Aim: *W. coagulans* Dunal has been reported to contain an array of bioactive compounds. The present investigation was carried out to investigate MMP inhibitory molecules from *W. coagulans* Dunal fruit.

Materials and Methods: Isolation of active principle compounds and anti-ChC activity from methanol extract of *W. coagulans* Dunal fruit were carried out using chromatographic techniques and dot-blot assay on X-ray film. Active principle compounds were identified by ESI MS, ^{1}H, and ^{13}C NMR spectroscopy. The Bioefficacy of compounds was assessed by *in vitro* solution assay and gelatin zymography.

Results: Dot blot assay of methanol extract of *W. coagulans* Dunal fruit exhibited inhibitory activity against ChC (*Clostridium histolyticum* collagenase). Bioactivity assay guided chromatographic fractionation revealed the presence of five compounds out of which three were tentatively identified as Coagulin-H, Coagulin-L, and Coagulin-O by ESI MS, ^{1}H, and ^{13}C NMR spectroscopy. *In vitro* solution assay revealed Coagulin-H exhibits the highest MMP-2 and MMP-9 inhibitory activity. Coagulin-L and Coagulin-O exhibit 80.57 ± 2.1 % and 70.96 ± 2.8 % inhibitor activities against MMP-2 and 78.94 ± 3.6% and 63.15 ± 3.8% inhibitor activities against MMP-9 at 150 µg/ml respectively. In gel inhibition assay by gelatin zymography revealed that crude extracted residue of fruit exhibits dose-dependent inhibitory potential against MMPs of NIH3T3 fibroblast and HeLa cervical cells.

Conclusion: Our results advocate the anti-MMP potential of purified Coagulin-H, Coagulin-L, and Coagulin-O of *W. coagulans* Dunal fruits. These bioactive principles could be further investigated in detail for anti-cancer properties.

Keywords: MMP inhibitors; *Withania coagulan* Dunal; Column chromatography; dot-blot assay; coagulins.

INTRODUCTION

Matrix metalloproteinases (MMPs) or matrixins are zinc endopeptidases that degrade extracellular matrix (ECM) proteins. The ECM structure is continually remodelled by MMPs under physiological events such as wound repair, and immune response modulations (1). Pathological complications such as osteoarthritis, cardiovascular, inflammatory diseases, metastasis, and tumour invasion are perceived attributes of dysregulation of MMPs (2). Cancer research literature surveys demonstrated MMPs as diagnostic and prognostic biomarkers that are involved in the progression of varied cancer types (3).

Although 28 MMPs have been identified with six families in humans, overexpression of MMP-2 (also known as gelatinase A, 72 KDa) and MMP-9 (also known as gelatinase B, 92 KDa) has been evidenced to promote bladder carcinoma, ovarian carcinoma, oral carcinoma, and lung adenocarcinoma (4-5). Conformationally both MMP-2 and MMP-9 are composed of three domains with an additional fibronectin domain introduced into the catalytic domain (6-7). Functionally they are able to degrade collagen, gelatin, elastin, laminin, and fibronectin exerting pro-inflammatory and anti-inflammatory action on various tissues. These MMPs are usually minimally expressed but when overexpressed, the regulation becomes crucial to control cancer progression. Therefore, these MMPs have been a target for pharmaceutical and biotechnological research for developing an anti-cancer therapy.

Earlier, several synthetic inhibitors have been shown to inhibit MMPs but none of them were successfully passed clinical trials. Two low molecular weight synthetic MMP inhibitors namely Batimastat (BB-94) and marimastat (BB-2516) were reported previously (8). Indeed, Batimastat was the first MMP inhibitor explored in humans with advanced malignancies. Clinical administrations of these synthetic inhibitors have caused 'musculoskeletal syndrome', and severe joint pain (9). In abnormal pathological conditions monitoring the uncontrolled activity of MMPs remains of great interest to the scientific community and the failure of synthetic MMP inhibitors in clinical trials steered to find out an alternative way. According to scientific literature, MMP inhibitors from natural sources have satisfactory results in clinical trials with potent efficacy and reduction of side effects (10). The

most general strategy to inhibit MMPs is regulating the active site zinc (11). The MMPs are produced as zymogens (pro-enzymes) in the secretions (12).

Several plants and their active principle compounds were found to be effective in the inhibition of MMPs (13). The *W. coagulans* Dunal belongs to the *Solanaceae* family and is commonly found throughout India, Pakistan, North-West India, and Afghanistan. The *W. coagulans* Dunal fruit possesses milk coagulating properties due to the presence of milk coagulating proteases hence, it is utilized for 'cheese' and 'paneer' preparation in North-West India and adjoining country (14). Various parts of *W. coagulans* Dunal have been reported to contain steroids, anthraquinone, flavonoids, tannins, phenolic compounds, glycosides, triterpenoids, and withanolides (15). Crude extract of this plant exhibit anti-microbial, anti-inflammatory, anti-tumor, anti-carcinogenic, cardiovascular, anti-hyperglycemic, free radical scavenging, immunosuppressive and central nervous system sedative activities (16).

Notwithstanding *W. coagulans* Dunal has been found to possess an array of biological (including milk cougulin) and therapeutic activities, there is insufficient data available on anti-cancer potency in the scientific literature prompted us to find and identify MMP inhibitors, which could be anti-cancer agents. Thus, the present study emphasizes on isolation, purification, and characterization of a novel lead molecule as MMP (MMP-2 and MMP-9) inhibitors from the methanol extract of *W. coagulans* Dunal fruit.

MATERIALS AND METHODS

Procurement of chemicals

ChC, Gelatin skin porcine, Brij-35, Azocasein, Human recombinant MMP-2, and MMP-9 were procured from Sigma Aldrich. Trypsin and trypsin inhibitors were obtained from Anaspec. X-ray films, Methanol (MeOH), Chloroform ($CHCL_3$), Ethyl Acetate (EtOAc), n-Hexane, Tris, and Trichloroacetic acid were brought from Spectrochem. TLC plates were brought from Merck. DMEM, FCS, gentamycin, DMEM, and Fetal bovine serum were purchased from Hi-media. The NMR spectrum was undertaken on a Bruker AMX 500 (^{1}H, 300 MHz; ^{13}C, 125 MHz) spectrometer. All chemicals used in this study were of analytical grade.

Purification of active principles from *W. coagulans*

Dried whole fruits of *W. coagulans* Dunal were purchased from the local market of Aurangabad (M.S.) India and pulverized into fine powder by using a mixer grinder. The completely dried powder (350 gm) was sequentially soaked in solvents namely in n-Hexane, $CHCl_3$, EtOAc, and MeOH (1:10 w/v) respectively. Extraction was carried out at room temperature for 10-12 hrs with intermittently shaking and filtered. The resulting extracts were tested for anti-*Clostridium histolyticum* collagenase (anti-ChC) activity.

MeOH extracts exhibited significant anti-ChC activity and were used for the purification of active principles. Isolation of active principles was carried out by bioassay-guided fractionation. Silica gel column and thin layer chromatography were used for fractionation and purification. Extract (20 gm, blackish and gummy) was loaded on the silica gel (mesh size 60-120) column chromatography and elution of fractions was carried out with increasing order of linear gradients of MeOH in $CHCl_3$. A total of 12 fractions each of 500 ml was collected. The fractions F1, F2, and F3 were concentrated at room temperature and anti-ChC activity was detected using dot blot assay. Fractions F2 and F3 exhibited inhibitory activities. Fraction F2 (1.49 g) was loaded on the silica gel (mesh size 60-120) column chromatography and the remaining fractions were stored at -20 ^{0}C. Fractionation was carried out by linear gradients of MeOH in $CHCl_3$. Seventeen fractions (each 35-40 ml) were collected, according to TLC pattern and anti-ChC activity the fractions 7th (F2G) and 8th (F2H) were pooled together and subjected to preparative TLC using a mobile phase MeOH: $CHCl_3$ (1:4 v/v) and two compounds were purified C1 (9.5 mg) and C2 (8.9 mg). Fractions 9th (F2I) and 10th (F2J) were pooled together and subjected to preparative TLC using a mobile phase MeOH: $CHCl_3$ (1:4 v/v) and one compound was purified C3 (20 mg). Similarly, fractions 11th (F2K), 12th (F2K), 13th (F2M), 14th (F2N), and 15th (F2O) were pooled together, subjected to preparative TLC using a mobile phase MeOH: $CHCl_3$ (1:4 v/v) and two compounds were purified C4 (3.9 mg) and C5 (14.2 mg). The purity and inhibitory activity of each compound was confirmed by a single spot on TLC and dot blot assay against ChC respectively. Compounds C1, C2, C3 C4, and C5 were analysed by ESI MS, ^{1}H, and ^{13}C NMR spectroscopy.

Dot-blot assay for anti-ChC activity

Anti-ChC activity from crude MeOH extract and purified compounds was carried out by using a dot-blot assay on the X-ray film (17). In a typical reaction mixture 5 µl ChC (200 µg/ml in 50 mM Tris-HCl buffer pH 7.5, 50 mM $CaCl_2$, 100 mM NaCl) was mixed with 5 µl buffer, 5 µl test solution and incubated at 37^{0}C for 10 min. Simultaneously an aliquot was kept without a test solution as a control. After incubation, all samples were placed on X-ray film and X-ray film was incubated at 37°C for 15 min. Then X-ray film was washed with tap water and dried at room temperature. Inhibitory activity was checked by comparing it with the control spot. Spots appeared as unhydrolyzed gelatin revealing the presence of inhibitory activity in the extract. Films were visually assessed and scanned on an EPSON scanner at 300 dpi.

Cell culture for expressions of MMP-2 and MMP-9

Cell lines of cervical cancer (HeLa) and fibroblast were obtained from National Centre For Cell Science, Pune (MS) India. The cell cultures were maintained at MGM College of biosciences, Aurangabad (MS) India. HeLa cells were cultivated in a 1:1 mixture of DMEM/Ham's F-12 added with 10% FCS and 10 ng/ml gentamycin at 37°C in the presence of 5 % CO_2 (18). Similarly, human fibroblast NIH3T3 cells were grown in Dulbecco's modified Eagle medium (DMEM) and added with 10% fetal bovine serum at 37ºC in the presence of 5 % CO_2 (19). The growth of cell cultures was observed under an inverted microscope for 24-48 hrs and cell-cultured media was used for the detection of MMPs.

MMP-2 inhibitor assay of active principle compounds

Inhibition of MMP-2 by each purified compound was carried out using azocasein as substrate (20). Pro-MMP-2 was activated by incubating with 10 µg/ml trypsin at 37 ^{0}C for 1 h and trypsin activity was inhibited by the addition of 100 µg/ml trypsin inhibitor. Activated MMP-2 was kept in cold condition until further use. Briefly, 20 µl (120 ng) activated MMP-2 was added in 1.5 ml centrifuge tubes along with 160 µl buffer (50 mM Tris-HCl pH 7.5, containing 1 mM $CaCl_2$, 100 mM NaCl, and 0.05 % Brij-35) and each purified compound in 20 µl methanol (150 µg/ml) in the total volume of 200 µl. After 10 min of incubation at 37^{0}C, each reaction was initiated by the addition of 50 µl azocasein (1 % w/v). After 5 hrs of incubation at 37 ^{0}C, the proteolytic activity was arrested by the addition of 60 µl 5 % TCA and all tubes were centrifuged at 6000 rpm for 15 min. Each supernatant (150 µl) was taken out in a microtiter plate and an equal volume of 1 N NaOH was added to each well and the optical density was documented at 405 nm on a microtiter plate reader. Percent inhibition of extracted residues was calculated by considering the absorbance of control as 100 % MMP-2 activity.

MMP-9 inhibitor assay of active principle compounds

The activation and inhibition assay of MMP-9 was carried out like MMP-2. Briefly, 20 µl (100 ng) activated MMP-9 was added to 1.5 ml centrifuge tubes along with 160 µl buffer (50mM Tris-HCl pH 7.5, containing 1 mM $CaCl_2$, 100 mM NaCl, and 0.05% Brij-35) and each purified compound in 20 µl methanol (150 µg/ml) in the total reaction mixture of 200 µl.

Gelatin zymography for MMP inhibitors

The inhibitory effect of crude methanol extracted residue and purified compounds against NIH3T3 and HeLa cell MMPs was studied by gelatin zymography (21). Media from cultured cells was collected and centrifuged immediately at 6000 rpm for 15 min and the supernatant was preserved at -20 ^{0}C. For electrophoresis 50 µl media of each cell was mixed with sample buffer (0.1 mM Tris-HCL pH 6.8, 20 % glycerol (w/v), and 0.01 % bromophenol blue) and loaded onto 10 % SDS-PAGE co-polymerized with 0.1 % gelatin (porcine skin, Sigma).

Electrophoresis was performed at room temperature with 150 volts and 25 mA current. After electrophoresis SDS from the gel was removed by washing with 2.5 % Triton X-100 for 1 h followed by distilled water. Then gel was cut into strips, one strip was incubated separately overnight in activation buffer (50 mM Tris-HCl pH 7.5, containing 150 mM NaCl, 10 mM $CaCl_2$, 1 µM $ZnCl_2$) at 37°C, other strips were incubated in activation buffer containing various concentrations of MeOH extracted residue such as 0.2, 0.4 0.6 and 0.8 mg/ml and all purified compounds (0.5 mg/ml). After incubation, gels were vigorously washed with distilled water, stained with Coomassie brilliant blue R-250 (0.5 % w/v in 50 % methanol and 10 % acetic acid), and de-stained by a solution of 30 % methanol and 10 % acetic acid. Gels were visually assessed and images were scanned using a gel-documentation system.

Statistical analysis

An inhibitory assay of the purified compound against human recombinant MMPs was performed in triplicate. Means and standard deviations of the assays were calculated using Microsoft excel 2010.

RESULTS

Identification of active principle compounds

Bioactivity assay guided fractionation was performed for the isolation of active compounds by using different polarity solvents and silica gel column chromatography (Fig. 1).

MeOH extract was found to exhibit prominent activity as compared to extracts in other solvents. Further fractionation of MeOH extract resulted in the isolation of anti-ChC activity exhibiting five compounds and the purity of compounds was checked as the appearance of a single spot on TLC. Each compound appeared as a single spot on TLC when observed under UV light (Fig. 2).

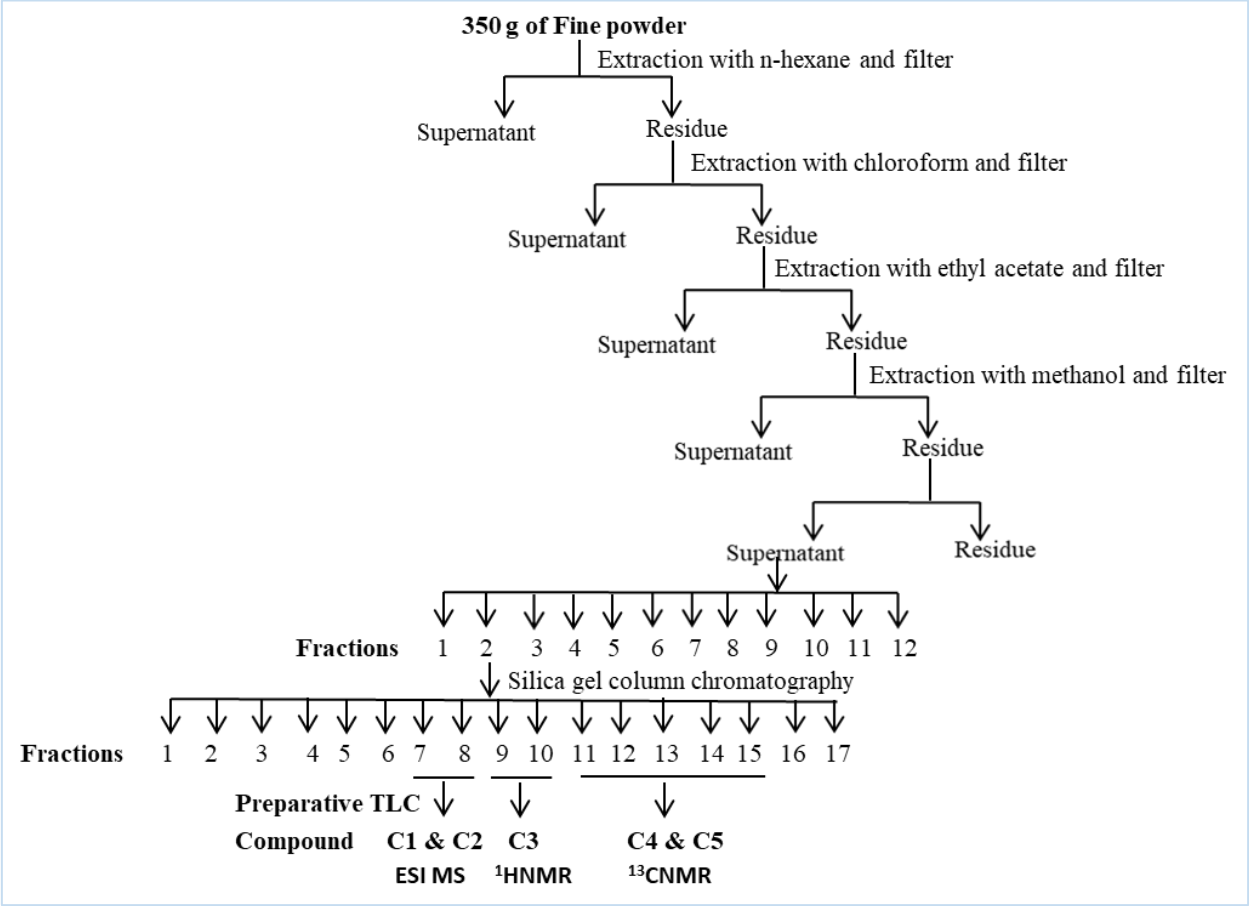


Fig.1: Schematic representation of bioactivity assay guided fractionation and isolation of active principle compounds from *W. coagulans* Dunal fruit. *W. coagulans* fruits were procured, pulverized into a fine powder and fine powdered was utilized for sequential extraction and fractionation which resulted in the isolation of five active compounds (Detailed procedure is given in the methodology section).

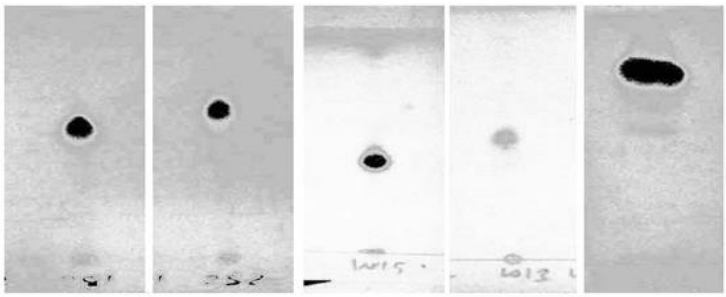
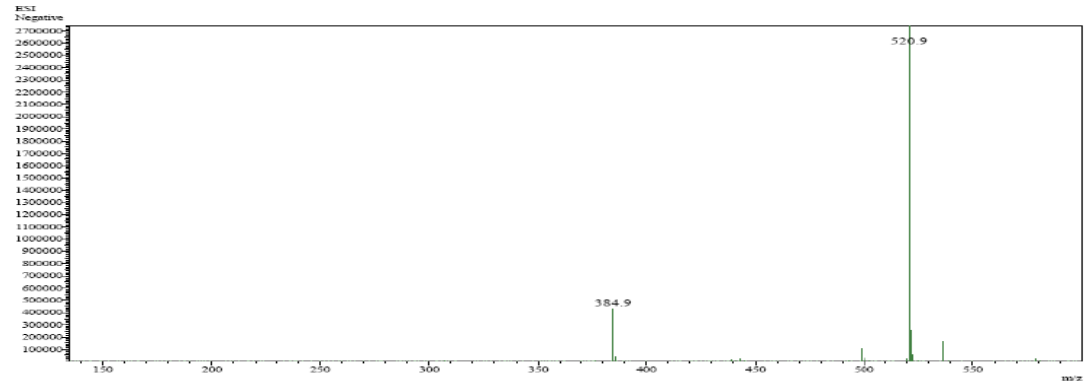


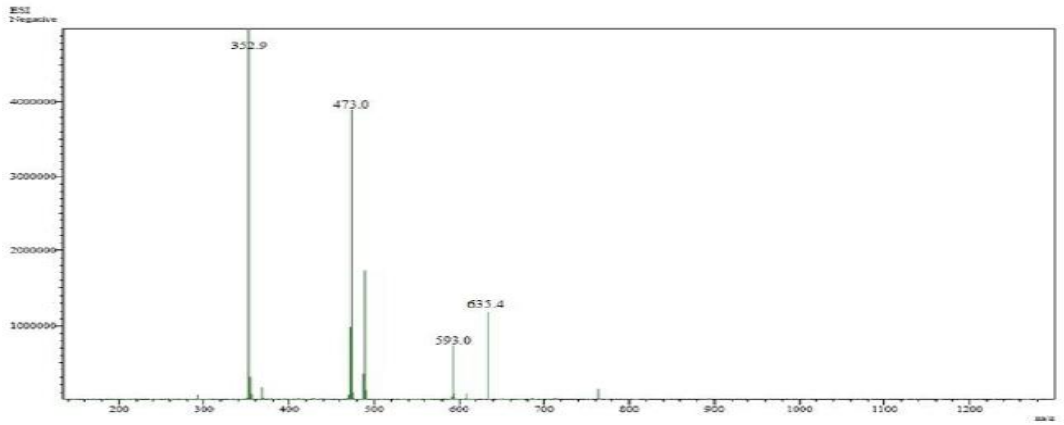
Fig. 2: Separation of isolated active compounds on thin layer chromatography (TLC). Isolated active principle compounds were dissolved in methanol and loaded on thin layer chromatography using a mobile phase of different amounts of methanol in chloroform (v/v).

The tentative structures of compounds were explicated based on ESI MS, ^{1}H, and ^{13}C NMR data by comparing available literature. Three compounds (C1, C3, and C5) were identified when compared their spectroscopic data with the data from reported literature. Structures of all identified compounds were similar to C28-steroidal lactone triterpenoids with four A, B, C, and D rings, and also their structures were similar to withaferin A. Coagulin-H (Compound C1) [5α, 6β, 14α, 15α, 17, 20- hexahydroxy-1-oxo- with a-2, 24-dienolide] was obtained as a whitish coloured compound. ESI MS spectra showed a peak at molecular ion m/z 520 [M+H]-. The molecular formula was deduced as $C_{28}H_{40}O_9$ and the tentative structure was confirmed by ^{1}H and ^{13}C NMR spectrum. ^{1}HNMR (DMSO) Bruker-300MHz, δ (ppm): 0.79 (s, 1H), 1.10 (d, J=6.3Hz, 3H), 1.13 (s, 3H), 1.22-1.26 (m, 2H), 1.76 (s, 4H), 1.92 (s, 4H), 2.27 (s, 1H), 2.73 (s, 1H), 3.56-3.84 (m, 6H), 4.29 (m, 2H), 4.43 (s, 3H), 4.69-4.72 (m, 5H), 5.21 (d, J=3.6Hz, 2H), 5.64 (s, 1H), 7.16 (dd, J=3.3 and 8.4Hz, 1H), 7.26 (d, J=8.4Hz, 1H). Coagulin-O (Compound C3) [(14R, 20S, 22R)-14, 20-

Dihydroxy-3β-(O-β-D-glucopyranosyl)-1-oxo-with a-5, 24-dienolide] was yielded as a whitish coloured compound. ESI MS spectra showed a peak at molecular ion m/z 635 $[M+H]^-$. The molecular formula was deduced as $C_{34}H_{50}O_{11}$ and the tentative structure was confirmed by matching the ^1H and ^{13}C NMR spectrum with reported data. ^{1}HNMR (DMSO) Bruker-300MHz, δ (ppm): 0.99 (s, 3H), 1.14 (s, 1H), 1.12 (s, 3H), 1.25 (s, 3H), 1.29-1.42 (m, 2H), 1.42 (s, 1H), 1.49-1.60 (m, 3H), 1.75 (s, 5H), 1.88 (s, 3H), 2.02 (s, 1H), 2.06 (d, J=3.9Hz, 1H), 2.18 (s, 1H), 2.33-2.39 (m, 2H), 2.44 (s, 2H), 2.58-2.60 (m, 1H), 2.66-2.73 (m, 1H), 2.92 (t, J=8.1Hz, 1H), 3.02 (d, J=9Hz, 1H), 3.12 (t, J=9Hz, 2H), 3.17 (s, 1H), 3.66 (d, J=11.1Hz, 1H), 3.75-3.77 (m, 1H), 4.12 (s, 1H), 4.25 (d, J=7.5Hz, 1H), 4.49 (s, 1H), 4.68 (s, 2H), 4.99 (s, 2H), 5.64 (s, 1H), 5.76-5.79 (m, 1H), 6.66-6.74 (s, 1H). ^{13}CNMR (DMSO) δ (ppm): 210.59 (C1), 165.95 (C26), 150.81 (C24), 134.57 (C5), 125.74 (C6), 120.08 (C25), 101.37 (C-1'), 87.33 (C22), 81.19 (C14), 80.89 (C20), 78.17 (C-3'), 76.81 (C-5'), 74.51 (C3), 73.36 (C-2'), 70.05 (C-4'), 61.05 (C-6'), 53.91 (C17), 52.25 (C10), 45.86 (C13), 37.60 (C2), 35.80 (C4), 35.24 (C16), 35.02 (C8), 34.27 (C9), 33.47 (C23), 31.87 (C12), 29.84 (C7), 25.40 (C21), 21.68 (C15), 20.27 (C11), 19.80 (C28), 19.12 (C19), 18.37 (C18), 12.12 (C27) Coagulin-L (Compound C5) [(14R, 17S, 20S, 22R)-14, 17, 20-trihydroxy-3b-(O-b-D-glucopyranosyl)-1-oxowitha-5, 24-dienolide] was obtained as whitish coloured compound. ESI MS spectra showed a peak at molecular ion m/z 650 [M+H]-. The molecular formula was deduced as $C_{34}H_{50}O_{12}$ by matching the ^1H and ^{13}CNMR spectrum by comparing reported literature data. ^{1}H NMR (DMSO) Bruker-300MHz, δ (ppm): 1.0 (s, 2H), 1.1-1.3 (m, 6H), 1.35-1.42 (m, 1H), 1.46-1.60 (m, 2H), 1.75 (s, 3H), 1.82 (s, 3H), 1.89 (s, 2H), 2.01-2.06 (m, 2H), 2.33-2.74 (m, 5H), 2.92 (t, J=8.1Hz, 1H), 3.03 (d, J=8.7Hz, 1H), 3.06-3.15 (m, 2H), 3.16 (s, 1H), 3.37-4.25 (m, 14H), 4.25 (d, J=7.8Hz, 1H), 4.64-4.69 (m, 2H), 5.63 (s, 1H), 6.68 (s, 1H). ^{13}C NMR (DMSO) δ (ppm): 210.62 (C1), 172.71 (C26), 165.97 (C24), 150.83 (C5), 134.59 (C6), 125.76 (C25), 120.10 (C-1'), 101.38 (C17), 87.34 (C14), 81.21(C22), 80.91(C20), 78.19 (C3), 76.83(C-3'), 76.75 (C-5'), 74.53 (C-2'), 73.38 (C-4'), 70.07 (C-6), 61.11 (C10), 53.93 (C13), 52.26 (C2), 48.59 (C4), 45.87 (C16), 37.62 (C8), 35.82 (C9), 35.41 (C23), 35.04 (C12), 34.29 (C15), 31.88 (C7), 29.86 (C11), 25.42 (C18), 22.21 (C21), 21.70 (C19), 20.28 (C28), 19.14 (C27) (Fig. 3 and 4).



a



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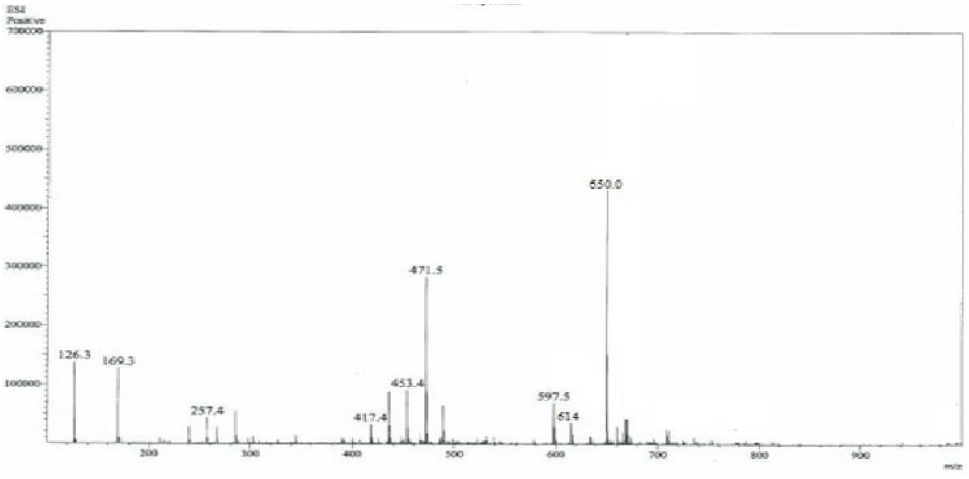

c

Fig. 3: ESI MS spectra of the active principle compounds identified (a) Coagulin-H (b) Coagulin-O and **(c)** Coagulin-L. After purification of active compounds, their molecular weight was identified by the analysis with ESI MS as m/z ratio and the obtained molecular weight as mentioned in the spectrum.

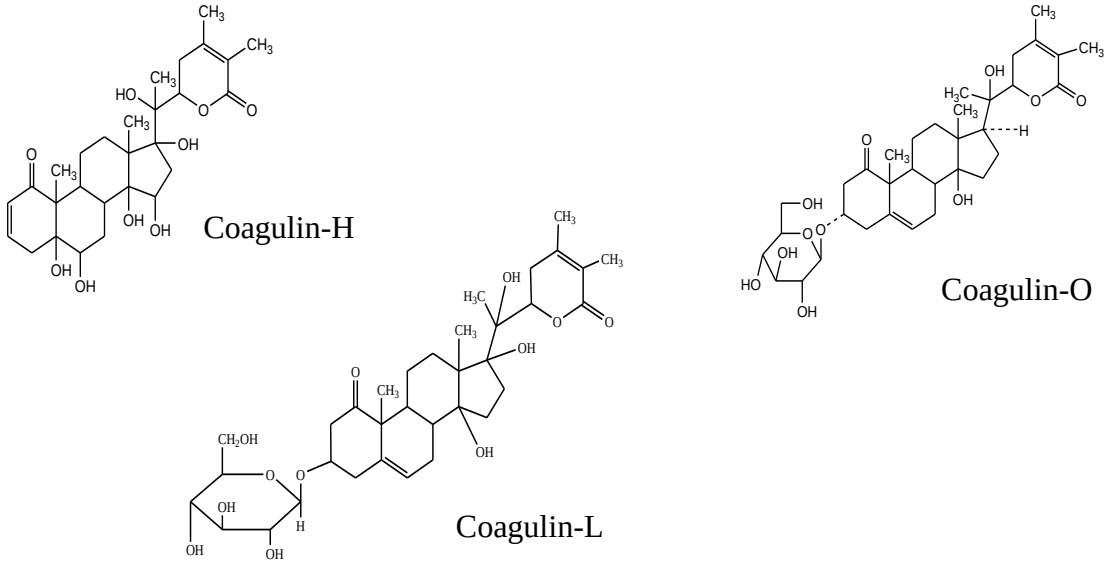

Fig. 4: Structure of identified active principle compounds. For the identification and confirmation of molecular structure, the isolated active compounds were also analysed with ^{1}H and ^{13}C NMR spectroscopy. The tentative identification and structure were confirmed by matching obtained data of spectrum with previously reported data.

MMP-2 and MMP-9 Inhibitory activity of active principle compounds

The crude methanolic extracted residue of *W. coagulans* Dunal fruits exhibited inhibitory activity against ChC on X-ray film. Therefore, active compounds were isolated by different fractionation processes, and their inhibitory activities against ChC were detected by dot blot assay. The inhibitory activities of all fractions during purification were assessed against ChC on X-ray film. Visual observation of X-ray film confirmed that all isolated compounds (C1, C2, C3, C4, and C5) exhibited inhibitory activity against ChC (Fig. 5). It was assumed that compounds that exhibited inhibitory activity against ChC may also possess inhibitory activity against MMPs. For the confirmation of this hypothesis, the inhibitory assay of purified compounds against human recombinant MMP-2 and MMP-9 was performed. Proteolytic activities of MMP-2 and MMP-9 were assessed by solution assay and the percent inhibitory activity of each compound was calculated.

Coagulin-H showed the highest inhibitory potential while compound C4 showed the lowest inhibitory potential against MMP-2. Inhibitory potentials of all compounds against MMP-2 were in order of Coagulin-H (89.03 ± 1.8 %) > Coagulin-L (80.57 ± 2.1%) > Coagulin-O (70.96 ± 2.8%) > Compound C2 (65 ± 2.3%) > Compound C4 (44.80 ± 3.8%) at 150 µg/ml. Similarly, Coagulin-H displayed the highest inhibitory potential while compound C2 showed the lowest inhibitory potential against MMP-9. Inhibitory potentials of all compounds against MMP-9 were observed in order of Coagulin-H (91.05 ± 0.9%) > Coagulin-L (78.94 ± 3.6%) > Coagulin-O (63.15 ± 3.8 %) > compound C4 (61.22 ± 2.8%) > compound C2 (55.78 ± 2.4%) at 150 µg/ml. (Fig. 6).

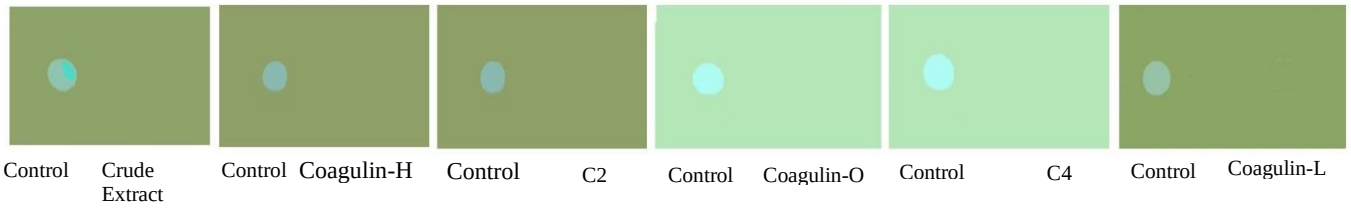

Fig. 5: Dot blot assay on X-ray film showing anti-ChC activity of active principle compounds. Crude methanolic extract of *W. coagulans* fruits and each isolated compound were treated with ChC in a buffer medium and incubated at 37 ^{0}C for a few minutes. (Rest of the procedure is given in the methodology section)

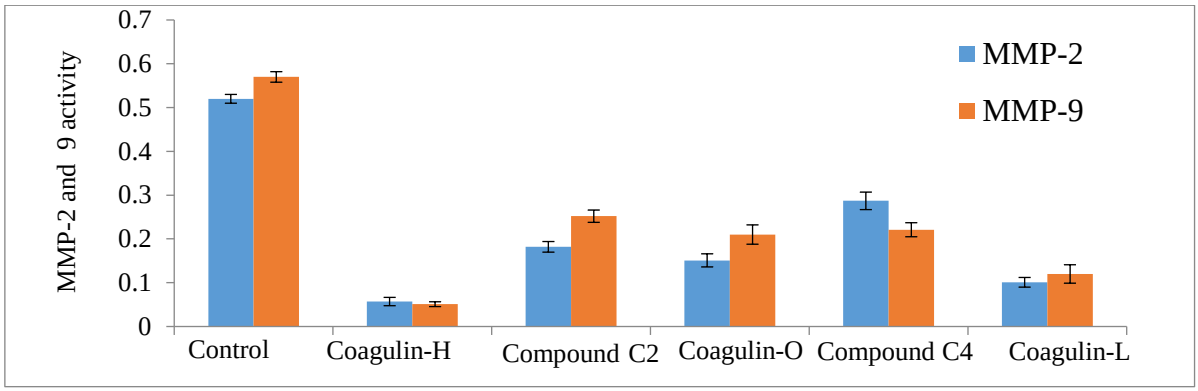

Fig. 6: Inhibitor efficacy of purified active principle compounds against human recombinant MMP-2 and 9. The proteolytic activity was carried out using azocasein as substrate and % inhibition was calculated with control having 100% enzyme activity (without inhibitor). Values given as mean ± standard deviation (SD), *n* = 3.

Effects of crude methanol extract and active principle compounds on NIH3T3 and HeLa cells MMPs

MMPs activities were detected from a cultured medium of NIH3T3 fibroblast and HeLa cervical cells by using gelatin zymography. Also, dose-dependent inhibitory effects of various concentrations of methanolic extracted residue on both cell MMPs have been studied by using gelatin zymography. It was observed that methanolic extracted residue is capable to inhibit MMPs of both cells (HeLa and NIH3T3) in a dose-dependent manner.

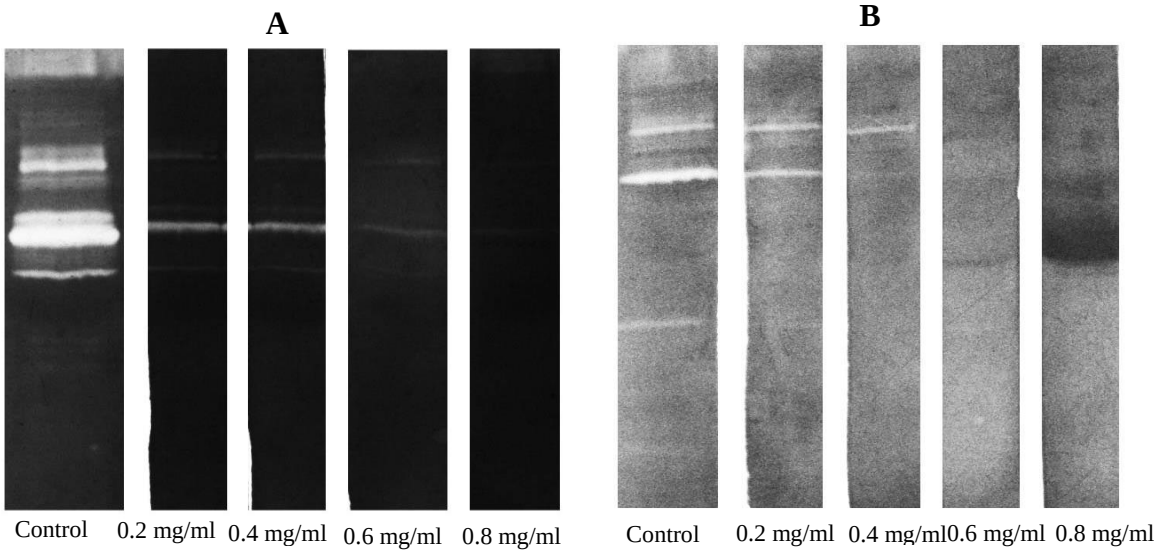

Fig. 7: Representative zymograms showing the dose-dependent inhibitory effect of methanolic extracted residue against MMPs of HeLa (A) and NIH3T3 cells (B). After electrophoresis, gels were cut into strips and incubated with different concentrations of methanolic extracted residue at 37˚C overnight. After incubation, all strips were rinsed with distilled water and stained with Coomassie brilliant blue R-250 (0.5 % w/v in 50 % methanol and 10 % acetic acid) and de-stained.

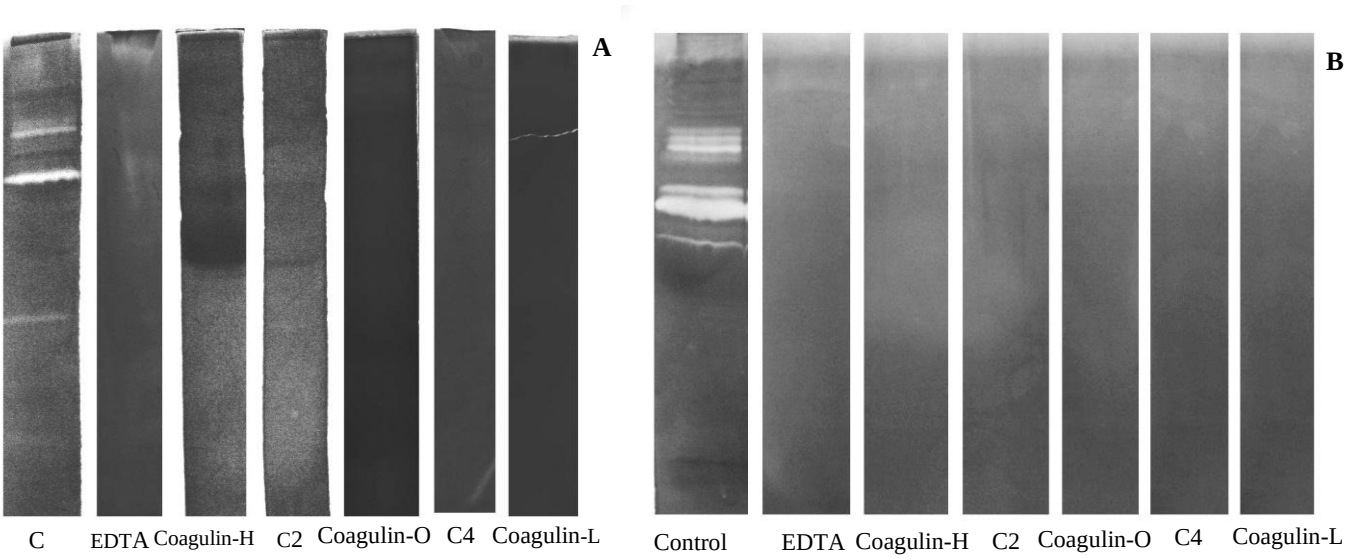

Fig. 8: Zymograms represent the inhibitory potential of active principle compounds against MMPs of HeLa (A) and NIH3T3 cells (B). After electrophoresis gel was cut into strips and incubated with purified compounds (0.5 mg/ml) at 37˚C overnight. After incubation, all strips were rinsed with distilled water and stained with Coomassie brilliant blue R-250 (0.5 % w/v in 50 % methanol and 10 % acetic acid) and de-stained.

The gelatinolytic activities of all HeLa cell MMPs were completely inhibited in presence of 0.6 mg/ml crude methanolic extracted residue (Fig. 7 A). Also, the gelatinolytic activities of all NIH3T3 cell MMPs were completely inhibited at 0.8 mg/ml of extracted residue (Fig. 7 B). It was observed that all purified compounds exhibited inhibitory activity against MMPs of NIH3T3 and HeLa cells when treated overnight at 0.5 mg/ml concentration of each compound (Fig 8. A and B).

DISCUSSION

Many studies have evidenced that imbalanced MMPs are involved in the progression of cancer and their inhibitors could be utilized to control the development of cancer (1). It was studied that natural MMP inhibitors have the potential to control cancer progression with fewer side effects (10). Hence, the main purpose of this study was to search for MMP inhibitors in natural sources for that, different 95 plant materials were procured and were verified to have inhibitor potential against ChC (Data not shown). Out of which, 32 plant materials were found to have inhibitor potential against ChC. The ChC was used for the assessment of inhibitor potential instead of MMP in the crude extract as well as in fractions and purified active principle compounds due to structural similarity with human MMPs (22). Crude extract of *W. coagulans* Dunal fruits plant and purified compounds exhibited inhibitor potential against ChC. Out of five purified compounds, three were tentatively identified as an analysis done by ESI MS, ^{1}H, and ^{13}C NMR and comparing their obtained spectroscopic data with reported literature (23).

The identified active principle compounds in the present study were Coagulin-H, Coagulin-O and Coagulin-L could be promising approaches as therapeutic agents for the treatment of various malignant and inflammatory disorders. In this study, MMP-2 and MMP-9 were selected to search for their inhibitors based on their involvement in the progression of various cancers and malignant disorders among other MMPs (24). Identified compounds exhibited inhibitory activity against human recombinant MMP-2 and MMP-9 and MMPs of NIH3T3 and HeLa cells that indicates these compounds could be utilized for the preparation of the anti-cancer drug. All identified compounds were structurally similar to withaferin-A and 3-azidowithaferin-A. Withaferin-A and 3-azidowithaferin-A have been reported to exhibit inhibitory activity against cell motility and gelatinases (MMP-2 and MMP-9) (25). It has been reported that Coagulin-H exhibit an inhibitory effect against lymphocyte proliferation, Th-1 cytokine production, and IL-2 production. The identified compounds are structurally similar to steroidal/terpenoid compounds and these types of compounds have been reported to exhibit different biological activities. Therefore, isolated Coagulin-H could be utilized as a powerful drug to control MMPs associated inflammatory diseases. Similarly, inhibitory activities of crude extract and purified compounds against MMPs of NIH3T3 and HeLa cells represent a promising approach to controlling the progression and growth of these cells.

CONCLUSION

W. coagulans is believed to be an herbal medicine used for the prevention of MMPs relevant inflammatory disorders. Our study first time reports active principle, anti-MMP compounds namely Coagulin-H, Coagulin-L, and Coagulin-O from methanol extract of *W. coagulans* Dunal fruit. These active principle

compounds exhibit inhibitor potential against ChC, MMPs of NIH3T3 and HeLa cells, and human recombinant MMP-2 and MMP-9 indicating their likely use in the formulation of anti-cancer drugs.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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