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Anacardic acid inhibits the catalytic activity of matrix metalloproteinase-2 and matrix metalloproteinase-9.

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Running Title:

Inhibition of Gelatinases by Anacardic Acid

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Nonstandard Abbreviations

MMP, matrix metalloproteinase; CNSE, Cashew nut shell extract; PE, Petroleum Ether; 3T3-L1, Mouse embryonic fibroblast - adipose like cell line; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; SA, salicylic acid; NP40, Nonidet P-40; Mca,(7-methoxycoumarinyl) acetyl; Dpa, [3(2-dinitrophenyl 2,3-diaminopropionyl].

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Abstract

Cashew Nut Shell Liquid (CNSL) has been used in traditional medicine for the treatment of a wide variety of pathophysiological conditions. To further define the mechanism of CNSL action, we investigated extracts of CNSL (CNSE) on two matrix metalloproteinases, MMP-2/Gelatinase-A and MMP-9/Gelatinase-B that are known to have critical roles in several disease states. We observed that the major constituent of CNSE, anacardic acid, markedly inhibited the gelatinase activity of 3T3-L1 cells. Our gelatin zymography studies on these two secreted gelatinases, present in the conditioned media from 3T3-L1 cells, established that anacardic acid directly inhibited the catalytic activities of both MMP-2/9. Our docking studies suggested that anacardic acid binds into the MMP-2/9 active site, with the carboxylate group of anacardic acid chelating the catalytic zinc ion, and forming a hydrogen bond to a key catalytic glutamate side chain, and the C15 aliphatic group being accommodated within the relatively large S1′ pocket of these gelatinases. In agreement with the docking results, our fluorescence-based studies on recombinant MMP-2 catalytic core domain demonstrated that anacardic acid directly inhibits substrate peptide cleavage in a dose-dependent manner, with an IC_{50} of 11.11 μM. Additionally, our gelatinase zymography and fluorescence data confirmed that cardol-cardanol mixture, salicylic acid and aspirin, all of which lack key functional groups present in anacardic acid, are much weaker MMP-2/-9 inhibitors. Our results provide the first evidence for inhibition of gelatinase catalytic activity by anacardic acid, providing a novel template for drug discovery and a molecular mechanism potentially involved in CNSL therapeutic action.

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Introduction

Cashew nut shell liquid (CNSL), a by-product of processing the cashew (*Anacardium occidentale*), is a rich source of long chain non-isoprenoid phenolics that have been used in traditional medicine which includes being an anaesthetic in leprosy and psoriasis, in promoting wound healing and in treating conditions such as ulcers and tooth abscesses (Himejima M. et al., 1991). The major constituent of CNSL is anacardic acid (alkenyl salicylic acid), present in a few forms all containing a C15 alkenyl side chain but differing in the number of double bonds from zero to three (Paramashivappa et al., 2001), in addition to cardanols (3-alkenyl phenols) and cardols (5-alkenyl resorcinols). Anacardic acid is a phytochemical of interest due to its wide-ranging bioactivities that comprises of microbicidal, insecticidal and mulloscicidal properties (Kubo at al., 2003; Gellerman et al., 1969; Begum et al., 2002; Mendes et al., 1990).

The bactericidal properties of anacardic acid are more effective against gram-positive bacteria which include the medically relevant *Streptococcus mutans*, a causative agent in tooth decay, the acne causing *Propionibacterium acnes*, the stomach ulcer forming *Helicobacter pylori*, and the infectious methicillin-resistant *Staphylococcus aureus* (MRSA) (Muroi et al., 1993; Kubo et al., 1994; Kubo et al., 1999; Muroi et al., 1996). Anacardic acid also has a potent antioxidant effect (Trevisan et al., 2006), capable of protecting human cells from oxidative stress and providing a gastroprotective effect from ethanol induced damage (Morais et al., 2010). Due to these antioxidant functions, anacardic acid has been proposed to be a useful chemoprotectant (Trevisan et al., 2006) and for having roles in skincare (Kubo et al., 2006).

Due to the interesting chemical properties of anacardic acid, studies are beginning to define its effects on distinct classes of enzymes. This includes enzymatic inhibition by anacardic acid, at varying degrees, on xanthine oxidase, tyrosinase and lipoxygenase (Masuoka et al., 2004; Kubo et al., 1994; Ha et al., 2005; Grazzini et al., 1991). Effects on the post-translational cellular machinery have also been observed, where anacardic acid mediates the activation of aurora kinase (Kishore et al., 2008), while it inhibits Small Ubiquitin-like MOdifier E1 (SUMO) ligase activity and thus perturbs protein SUMOylation

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(Fukuda et al., 2009). Notably, potential anti-cancer related functions have been attributed to anacardic acid, including the inhibition of prostaglandin synthesis by cyclooxygenases (COX) (Grazzini et al., 1991) that are known to have roles in carcinogenesis (Langenbach et al., 1999; Arun et al., 2004). Other potential anti-cancer functions occur through the inhibition of estrogen receptor alpha DNA binding, diminishing both gene transcription and breast cancer cell proliferation (Schultz et al., 2010). Anacardic acid also inhibits the histone acetyltransferase (HAT) activity of transcription co-activators (Balasubramanyam et al., 2003). Furthermore, anacardic acid has been reported to suppress expression of nuclear factor-kB-regulated gene products that are involved in proliferation and in invasion, leading to potentiation of apoptosis (Sung et al., 2008).

However, in spite of these important recent advances, the key molecular mechanisms behind the traditional use of CNSL in wound healing and in treating several pathophysiological conditions, which are likely mediated by anacardic acid, have not been clearly defined. In this regard, possible protein targets for anacardic acid include the matrix metalloproteinases (MMPs), as this family of proteins is known to have critical roles in both extracellular matrix remodeling (Nagase et al., 1999; Vu et al., 2000) and inflammatory responses, in addition to pathological conditions that include cancer metastasis (Stamenkovic et al., 2003). Hence, we focused our efforts on two MMPs, MMP-2 and MMP-9, the gelatinases secreted by cells that display highly impaired regulation and elevated protein levels in both chronic wounds and in certain tumors (Jezierska et al., 2009). Our studies with 3T3-L1 mouse embryonic fibroblast cells clearly demonstrate that anacardic acid directly inhibits gelatinase enzymatic activity. Computational-based docking results indicate that anacardic acid readily binds to the MMP-2 or the MMP-9 active site. Our fluorescence studies reveal that anacardic acid inhibits peptide substrate cleavage by the MMP-2 catalytic core domain in a dose-dependent manner. Moreover, our combined fluorescence and gelatinase zymography studies agree with the docking-predicted binding mode of anacardic acid to MMP-2 or -9, since similar compounds lacking key functional groups compared to anacardic acid like aspirin, salicylic acid, and cardol-cardanol mixture from CNSE all inhibited gelatinase activity to a lesser

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extent. Thus, our results provide a novel molecular mechanism of action of anacardic acid, providing a new template for MMP-2/-9 drug discovery and a potential link to the therapeutic functions of CNSL.

Materials and Methods

Materials. 3T3-L1 mouse fibroblast cells were obtained from ATCC through the National Center for Cell Sciences, Pune, Maharashtra, India. Saturated anacardic acid was obtained commercially from Calbiochem. Cell culture media and supplements and the other chemicals were obtained from Sigma, USA.

Extraction of Anacardic Acid from cashew nut shell liquid. Locally available cashew shells from Kollam, Kerala, India were crushed and the cashew nut shell was subjected to extraction by shaking with Petroleum Ether (PE) in a rotary shaker for 24 hours at ambient temperature. The solvent was removed by rotary evaporation below 40 ˚C, obtaining a brown colored oily residue henceforth referred to as Cashew Nut Shell Extract (CNSE). CNSE contains a mixture of at least three analogous compounds, anacardic acid **(Figure 1A)**, cardol **(Figure 1B)** and cardanol **(Figure 1C)**, having C15 side chains that are either fully saturated or containing one, two or three double bonds **(Figure 1D)**. Thin layer chromatography (TLC) analysis of this extract was conducted using a solvent system containing PE (70 %), ethyl acetate (28 %) and formic acid (2 %) and visualized by spraying a mixture of (i) equal volumes of aqueous solution of ferric chloride (1 %) and potassium ferricyanide (1 %), and (ii) methanolic ferric chloride (1 %). Usually separation of individual components of CNSE is carried out by precipitation of anacardic acid as calcium salt (Paramashivappa et al., 2001). This procedure is useful for large-scale separation. However we found that anacardic acid could be conveniently separated from the other constituents by column chromatography on SiO_2 and eluting with PE containing increasing proportions of chloroform, different from previously described methods (Paramashivappa et al., 2001). Anacardic acid (450 mg) was obtained from 2.0 g of PE extract from CNSE. The identity of anacardic acid was established by: (1) HPLC, with a Shimadzu LC-20, a Phenomenex C-18 reverse phase Luna column with prominence diode

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array detector, with mobile phase of acetonitrile (72 %), H_2O (18 %), and acetic acid (10 %) and the absorbance was monitored at 245 nm), revealing that our anacardic acid extract contained 56.2 % triene 18.3 % diene, 24.2 % monoene forms and 1.3 % of the fully saturated C15 aliphatic chain **(Supplementary Figure 1)**. (2) The HPLC/MS data were generated by an Agilent 1290 series uHPLC coupled to an Agilent ion trap mass spectrometer (6340 series), with electrospray interface. The masses (m/e) of the three major molecular peaks (MH^+) corresponded to 343 for the triene, 345 for the diene and 347 for the monoene forms of anacardic acid **(Supplementary Figure 2).** (3) ^{1}H NMR spectra (Bruker AV II 500 spectrometer) were in agreement with those reported in the literature (Philip et al., 2008; Silva et al., 2008) **(Supplementary Figure 3)**. A 20 mg/mL (60 mM) solution of isolated anacardic acid mixture and cardol-cardanol extract, was prepared in 100 % dimethyl sulfoxide (DMSO), stored at -20 ˚C, and then diluted as needed in cell culture medium. Reconstitution of all the stocks were completed in such a way that the working concentration of the DMSO was kept to 0.5 % or below.

Cell culture. 3T3-L1 mouse fibroblast cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal bovine serum (v/v), 1 % penicillin, 1 % streptomycin and 0.1 % Amphotericin B.

Cytotoxicity Assay. Using MTT (3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide) cytotoxicity assays were performed for cells treated with and without anacardic acid in serum free DMEM. 3T3-L1 cells were seeded at a density of 7500 cells/well in a 96 well microtiter plate and incubated overnight. Cells were treated with and without anacardic acid (at a concentration range of 0.5-12.5 µM) in both serum free and serum containing DMEM for 24 hours. 20 µl of 5 mg/mL MTT was added to each well and incubated for 3 hours at 37 °C. The media was removed after incubation and 150 µl of MTT solvent (4 mM HCl, 0.1 % NP-40 in isopropanol) was added for solubilization. After shaking briefly for 5 mins, the absorbance was read at 590 nm with a reference filter of 620 nm using Synergy HT Multi-Mode Microplate Reader (BioTek).

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Gelatin Zymography. The zymography assay (Boris et al., 2002) used gelatin as a substrate for MMP-2 and MMP-9. Gelatin at a concentration of 0.1 % was incorporated into 10 % polyacrylamide gel containing 0.4 % SDS. Electrophoresis under non-reducing conditions was performed using Bio-Rad mini-gel system at 125 V for 90-120 mins. After electrophoresis the gels were washed twice for 30 mins in 2.5 % Triton X-100 (v/v) to remove the SDS and then incubated overnight in the developing buffer [50 mM Tris–HCl (pH 7.6), 200 mM NaCl, 5 mM $CaCl_2$, 0.2 % (v/v) Brij-35] at 37 ˚C. Digestion bands were quantitated by Quantity One (Bio-Rad, USA).

Cellular studies. The 3T3-L1 cells seeded in a 12 well plate, on reaching confluency, were washed twice with PBS and then treated with or without quercetin (50 µg/mL), commercial anacardic acid (1 µg/mL), a mixture of cardol-cardanol (1 µg/mL), CNSE (1 µg/mL) and anacardic acid isolated form CNSE (1 µg/mL). After 24 hours the conditioned media was collected, centrifuged to avoid cellular debris, then mixed with 4X sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 10 % glycerol, 1 % sodium dodecyl sulfate (SDS) and 0.00625 % (w/v) bromophenol blue and then loaded for electrophoresis on a 10 % SDS-PAGE gel for zymography studies. All the experiments were performed in triplicates.

Conditioned Media Studies. 3T3-L1 cells were trypsinized and seeded on a 10 cm plate. After reaching confluency, cells were washed twice with PBS and treated with serum free DMEM and incubated at 37˚C for 24 hours. The media was collected after 24 hours, centrifuged to avoid cell debris and aliquots were stored in -20˚C before subsequent experimentation. This was referred to as conditioned media. To study the dose dependent inhibition of anacardic acid, the conditioned media aliquots were incubated with, or without anacardic acid, at a concentration range of 10-100 µM for 1 hour at 37 ˚C. To ensure that the long chain of anacardic acid plays an important role in MMP inhibition, conditioned media was treated with different concentrations of (10-100 µM) salicylic acid (SA) and aspirin (50 µM). To study the effect of anacardic acid on MMP-9 inhibition, conditioned media was treated with anacardic acid isolated from CNSE and SA (10-100 µM). The samples were mixed with 4X sample buffer containing 62.5 mM Tris–

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HCl (pH 6.8), 10 % glycerol, 1 % SDS, and 0.00625 % (w/v) bromophenol blue, and loaded on a 10 % SDS-PAGE gel for zymography analysis. Control experiments confirming the presence of MMP-2 included the addition of 20 mM EDTA and 5 mM DTT to the incubation buffer and the sample buffer respectively.

Purification of MMP-2 catalytic domain. MMP-2 catalytic domain, amino acid sequence as in PDB code: 1QIB, residues 88-250, was cloned into a plasmid (DNA 2.0). Protein expression was induced by 0.4 mM IPTG being added to the media of *E. coli* BL21 (DE3) cells grown at 37˚C at O.D. 600 nm of 0.5, and cells were grown for additional 5 hours. The expressed MMP-2 protein was then purified as previously described (Dhanaraj et al., 1999).

Fluorescence Assay. A fluorogenic substrate Mca-Pro-Leu-Ala-Nva-Dap (Dnp)-Ala-Arg-NH_2 (Cat. No.ALX-260-123-M001, Enzo life sciences) was used for fluorescence studies (Knight et al., 1992). 30 µM substrate and 20 ng of purified MMP-2 catalytic domain were used for the assay. The working concentration of substrate, enzyme, anacardic acid, cardol-cardanol and CNSE extract was prepared in assay buffer (50 mM HEPES, 10 mM $CaCl_2$, 0.05 % Brij-35, pH 7.5). The MMP-2 catalytic domain was incubated with anacardic acid for 30 mins before the fluorescent substrate was added. The experiment was performed in a 96-well black plate, and the plate was read at Ex/Em=360/460 (using Synergy HT Multi-Mode Microplate Reader (BioTek)) 10 mins after the substrate was added. Percent inhibition was calculated using the formula

$$Inhibition\ \% = 1-(F-F_{min})/(F_{max}-F_{min})\times 100$$

Where F_{min} is the negative control which contains only the fluorescent labeled ligand or fluorescent labeled ligand and anacardic acid as required, F_{max} is positive control which is a mixture of fluorescent labeled ligand and the enzyme.

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Computational Docking. The AutoDockTools (http://mgltools.scripps.edu) package was used to generate input files for the docking runs and ligand site characterization, using our previously defined approach (Perry et al., 2009). Briefly, the size and characterization of the optimal ligand binding site of the catalytic MMP-2 domain, pdb code 1QIB, and the MMP-9 catalytic domain, pdb code 2OVX.pdb, were performed using AutoLigand (Harris et al., 2008). A grid box of 40 by 44 by 44 points on a 1 Å grid was used to enclose the entire MMP-2 catalytic domain structure to generate affinity maps for use by AutoLigand. For MMP-9 catalytic domain, a grid box of 44 by 44 by 44 points on a 1 Å grid was used to generate affinity maps for use by AutoLigand. For docking studies a grid box of 44 by 46 by 60 points on a 0.375 Å grid spacing, centered on the ligand binding sites for MMP-2 or MMP-9 identified by AutoLigand, were used to generate affinity maps for use by AutoDock4 (Morris et al., 1998). Anacardic acid was docked to either MMP-2 or MMP-9 catalytic domains with a Lamarckian genetic algorithm starting with an initial population of 500 randomly positioned inputs due to the large number of active torsions. The maximum number of energy evaluation was set to 2.5×10^7 and used a mutation rate of 0.02 with a crossover rate of 0.8. Results were clustered at 2.0 Å root-mean-square deviation.

Statistical Analysis Statistical analysis was conducted using Prism (GraphPad Software). Statistical comparisons were performed using student's t-test, one-way analysis of variance followed by Dunnett's or Tukey's test. A value of $p<0.05$ was considered significant. All the values are expressed as the mean ± S.E.M. of triplicate determinations from three independent experiments.

Results

Regulation of MMP-2 activity by components of CNSE

To study the regulation of MMP-2 by the major constituents of CNSE, we incubated 3T3-L1 cells in serum free DMEM with CNSE extract, the extracted mixture of cardol-cardanol, extracted anacardic acid as well as commercially available anacardic acid containing a saturated C15 chain as a control. Quercetin

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that is known to inhibit the activity of MMP-2 and MMP-9 in cells (Vijayababu et al., 2006) was also used as additional control. The gelatin zymography studies **(Figure 2A, B)** determined that the CNSE extract inhibits cellular gelatinase activity and that the anacardic acid component of CNSE is the most active compound in this regard. Both anacardic acid isolated from CNSE and the commercially available compound significantly inhibited secreted MMP-2 gelatinase activity at a concentration of 1 µg/mL (3 µM). These values are significantly greater than the inhibition observed on incubation with 1 µg/mL of CNSE extract containing a mixture of anacardic acid, cardol-cardanol, or the inhibition by 1 µg/mL of cardol-cardanol mixture.

Determining Anacardic Acid cytotoxicity in 3T3-L1 cells

To confirm that the observation of reduced MMP-2 gelatinase activity was not due to cytotoxicity we conducted studies to determine the concentration at which anacardic acid is toxic to the cells, by both analyzing morphological changes upon incubating cells with anacardic acid, and by using the classical MTT cell viability assay (Mosmann et al., 1983). Importantly, we observed that there is no visible morphological alteration of 3T3-L1 cells when treated with 1.5, 3 or 6 µM concentration of anacardic acid for 24 hours (**Figure 3A**) when compared to control 3T3-L1 cells that were not exposed to anacardic acid. Results of our MTT analysis (**Figure 3B)** determined that no significant cytotoxic effects occur in cells that are incubated with anacardic acid in serum free DMEM at the 3 µM concentration used in the previous assay. An increase in cytotoxicity is observed in concentrations of anacardic acid that are notably higher than that used in our assay, and the observed cytotoxicity at these concentrations is mitigated when cells are incubated in serum containing media (**Supplementary Figure 4).**

Fluorescence-based studies on MMP-2 with CNSE components

The relative contribution of MMP-2 inhibitory activities of CNSE, cardol-cardanol mixture and anacardic acid were compared by conducting fluorescence-based studies with a direct assay of the MMP-2 activity

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using the fluorogenic substrate Mca-Pro-Leu-Ala-Nva-Dap (Dnp)-Ala-Arg-NH2. Controls for these studies (**Supplementary Figure 5**) show a distinct 6 fold increase in MMP-2 activity (**Supplementary Figure 5-B**) over basal (**Supplementary Figure 5-A**) upon incubating the fluorogenic substrate with the purified recombinant catalytic core domain of MMP-2. We observed that anacardic acid significantly inhibits MMP-2 activity over the entire concentration range tested (1–50 μg/ml). Additionally, as a control, the commercially available saturated anacardic acid (Calbiochem) demonstrated a concentration dependent increase in the percent inhibition of MMP-2 activity, which is very similar to CNSE purified anacardic acid **(Supplementary Figure 6).** The cardol-cardanol mixture was inhibitory to a lesser extent, even at the highest concentration (50 μg/ml) **(Figure 4).** Upon comparing the structures of cardol (**Figure 1B)**, cardanol (**Figure 1C)** and anacardic acid (**Figure 1A),** the major difference in these structures is the presence of the COOH group in anacardic acid which is absent in both cardol and cardanol, implicating this group to plays a crucial role in the inhibition of MMP-2 activity.

In silico Docking Studies

The observed cellular inhibition of gelatinase activity by anacardic acid could be mediated through either perturbation of catalytic activity, inhibition of secretion, or through more indirect effects, such as inhibition of transcription or stabilization of the inactive pro-form of each enzyme. Hence, we used *in silico* docking methods to help define whether anacardic acid was capable of directly inhibiting catalytic activity, through binding the active site of MMP-2 and of MMP-9. AutoDock has been previously defined as the most reliable method for studying MMP-inhibitor complexes, in a comparative study of fully automated docking programs (Hanessian et al., 2001). Therefore, we used AutoDock-based molecular docking to characterize the binding site and to predict the binding mode of anacardic acid, to previously determined MMP-2 and MMP-9 catalytic domain structures (Dhanaraj et al., 1999; Tochowicz et al., 2007). First, the AutoLigand code (Harris et al., 2008) was used to characterize the ligand-binding sites. AutoLigand works by finding a set of contiguous points that make up the best total affinity for a given

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volume. By searching the space encompassing the entire target protein, the code was used to find the optimal binding site based on total energy per volume. The AutoLigand results **(Figure 5)** indicate that the optimal binding site in both MMP-2 and in MMP-9 contains their zinc catalytic centers and their S1′ pockets, which in both gelatinases constitutes an aliphatic tunnel with a few hydrogen bond acceptor sites. In MMP-2 the optimal result was a fill of 100 points with a total volume of 295 $Å^3$ and a total energy per volume of -0.195 kcal/mol $Å^3$. In MMP-9 the optimal result was a fill of 100 points with a total volume of 284 $Å^3$ and a total energy per volume of -0.219 kcal/mol $Å^3$. Next, anacardic acid was docked to the MMP-2 and the MMP-9 Autoligand target sites, using AutoDock4 (Morris et al., 1998). Due to the large number of free rotations in the C15 aliphatic segment of anacardic acid, AutoDock produced a large number of binding poses. Strikingly however, in MMP-2, 94 out of the 100 dockings placed the head group in the aliphatic pocket, with the carboxylate group functioning as zinc binding group and forming a hydrogen bond to the active site Glu404 side chain that functions in hydrolyzing peptide substrate **(Figure 5A, B)**. The hydroxyl group of anacardic acid also forms a hydrogen bond to backbone oxygen of A192. Notably, the extensive, lipophilic C15 chain binds in the aliphatic S1′ tunnel. The reasonable docking energies ranged from -7.5 to -9.6 kcal/mol with 37/100 in the –9.6 cluster (**Supplementary video)**. Similarly, for MMP-9, anacardic acid bound in 71 out of the 100 dockings with the head group in the aliphatic pocket and the C15 chain in the aliphatic S1′ tunnel **(Figure 5C, D)**, with the largest cluster of 28/100 having a docking energy of -10.6 kcal/mol. The anacardic acid carboxylate group also functions as a zinc-binding group in MMP-9, and forms a hydrogen bond to the Glu402 side chain. The hydroxyl group of anacardic acid forms a hydrogen bond to backbone oxygen of Ala189 in MMP-9. Notably, the docked anacardic acid and the AutoLigand fill volume for MMP-2 and MMP-9 are consistent with previously structurally defined inhibitors bound to MMPs, such as batimastat ligand in the MMP-2 crystal structure (Dhanaraj et al., 1999).

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Anacardic Acid inhibits the catalytic activity of MMP-2 and MMP-9.

To confirm and further characterize the *in silico* results suggesting a direct inhibition of gelatinase activity by anacardic acid, conditioned media containing the secreted MMP-2 enzyme was obtained from 3T3-L1 cells that were not previously treated with anacardic acid. The presence of gelatinolytic activity in our zymography studies corresponding to MMP-2 (**Supplementary Figure 7A**), loss of this activity due to a reduction of the disulfide bond in the presence of 5 mM DTT (**Supplementary Figure 7B**), as well as a loss of activity in the presence of 20 mM EDTA (**Supplementary Figure 7C**), most likely resulting from the chelation of the essential catalytic Zn^{+2} ion, confirms the presence of MMP-2 in the conditioned media. Anacardic acid exhibited a clear dose dependent inhibition of secreted MMP-2 gelatinolytic activity in the conditioned media **(Figure 6A).** Salicylic acid contains the same head group of anacardic acid but lacks the C15 aliphatic chain, indicated to be important in MMP-2/-9 binding as inferred from our *in silico* studies. When compared with anacardic acid, treatment with salicylic acid results in significantly lower levels of inhibition throughout the concentration range tested **(Figure 6B).** We observed that while anacardic acid at a concentration of 100 μM inhibited MMP-2 activity at 72 %, salicylic acid at the same concentration exhibited merely 26 % inhibition (**Figure 6C**). Acetylation of the hydroxyl group of salicylic acid generates aspirin (acetyl-salicyclic acid). Our docking studies predict the involvement of this hydroxyl group in anacardic acid to have a role in binding to MMP-2 active site, and that acetylation of this group is likely to produce a steric hindrance in the binding. Additionally, aspirin also lacks the long C15 side chain that is predicted to bind within the S1′ tunnel. In concurrence with these observations, our studies demonstrated that aspirin at 50 μM had no significant inhibitory effect on MMP-2 (**Supplementary Figure 8**). It is noteworthy that the active site of the related MMP-9 is structurally highly conserved with MMP-2, and in keeping with this conservation we also clearly observed by gelatin zymography that anacardic acid significantly inhibits MMP-9 activity from the conditioned media **(Figure 6D).** As observed in the case of MMP-2, salicylic acid was not found to inhibit MMP-9 as effectively as anacardic acid **(Figure 6E, F).**

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These results were further confirmed using the fluorescence-based assay employing the catalytic core domain of MMP-2 and comparing the inhibitory activities of anacardic acid isolated from CNSE and salicylic acid. While Anacardic acid significantly inhibited (98 %) MMP-2 activity, salicylic acid exhibited only 17 % inhibition even at the highest concentration tested (200 μM) **(Figure 7)** underscoring the importance of the aliphatic side chain of anacardic acid in binding and inhibition of the gelatinolytic activity. Interestingly, the extent of inhibition demonstrated by commercially available saturated anacardic acid (Calbiochem) was similar to that from CNSE, indicating that the partial unsaturation of the C15 chain is not critical to the inhibitory activity **(Supplementary Figure 6).** Additionally, to further establish the role of the carboxylic group in the inhibition of MMP-2 and MMP-9 by anacardic acid, we isolated, purified and characterized cardanol from CNSE (**Supplementary Figure 9A-C**). Cardanol is also identical to anacardic acid, but with the one important difference being that it lacks the carboxylate group. Cardanol also shows minimal inhibition of MMP-2 catalytic activity, as compared to anacardic acid under the same conditions, thus supporting our *in silico* docking studies which emphasizes the importance of the carboxylate group of anacardic acid in binding the zinc present in the catalytic site of MMP-2 (**Supplementary Figure 10**). Finally, we further characterized this direct inhibition of the MMP-2 catalytic core domain by CNSE isolated anacardic acid, using the fluorogenic substrate described earlier. We observed that anacardic acid exhibits a distinct dose-dependent inhibition of the MMP-2 activity with an IC_{50} of 11.11 μM **(Figure 8A, 8B)**.

Discussion

Selective inhibition of MMPs could have substantial benefits in treating a number of disease states, and in promoting wound healing. However, despite major efforts towards developing MMP inhibitors, only Periostat (doxycycline), a tetracycline used for treating periodontal disease, and glucosamine sulfate for treating osteoarthritis, are commercially available. Natural products form one source of potential MMP inhibitors and, interestingly, CNSL has been noted in traditional medicine for its use in promoting wound

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healing. Additionally, the major constituent of CNSL is anacardic acid, and some indirect links for anacardic acid modulating MMP function have been previously observed. Anacardic acid can down regulate the expression of matrix metalloproteinase-1 (MMP-1) through inhibition of p300 HAT activity (Kim et al., 2009) and can also reduce expression of MMP-9 through the effects on nuclear factor-κ B-regulated gene products (Sung et al., 2008). Therefore, we analyzed whether anacardic acid and the other major extracted components of CNSE, could have any direct effects on two secreted gelatinases, MMP-2 and MMP-9, which are known to play key roles in several pathological conditions. Indeed, CNSE components inhibit gelatinase activity of 3T3-L1 cells, with the anacardic acid component having the greatest effect.

Regulation of MMP activity can occur at different levels including transcriptional control, altered processing of the inactive zymogenic form or catalytic inhibition by a group of tissue inhibitors of matrix metalloproteinases (TIMPs) that interact with the MMP active site. Our computational docking analyses suggested that MMP-2 inhibition occurs through anacardic acid directly binding to the MMP-2 active site involving interactions similar to those observed during structure-based inhibitor design studies on the MMPs. As carboxylate groups are the second most common zinc binding group in MMP inhibitors developed so far (reviewed in Lia et al. 2009), it is noteworthy that the interaction of anacardic acid with MMP-2 also likely involves its carboxylate group functioning as a zinc binding moiety; where cardanol that lacks the carboxylate group shows minimal inhibition when compared to anacardic acid. Different members of the MMP family all contain a similarly folded enzymatic domain that uses a zinc ion for catalysis. Thus, in case of both natural substrates and newly developed MMP inhibitors, it is the nearby sites, and particularly the S1′ pocket, which are observed to form their basis for substrate selectivity (reviewed in Maskos et al., 2003). Our docking studies suggest that the large C15 aliphatic chain of anacardic acid readily binds into the relatively deep S1′ pocket of MMP-2 and MMP-9 (Lovejoy et al., 1999) used in recognition of gelatin substrate. It is conceivable that anacardic acid may preferentially inhibit MMPs that have a deeper S1′ pocket as compared to MMPs with a shallow pocket. Therefore, the

potential use of anacardic acid as a natural 'bio-drug', suggests that it now joins a small list of previously defined natural product compounds that have interesting activities against MMPs (reviewed in Lia et al., 2009; Mannello et al., 2006). This list includes the long chain fatty acid molecules, such as oleic acid and elaidic acid, which are micromolar inhibitors of MMP-2 (Berton et al., 2001). Interestingly, our docking results indicate a molecular mechanism for these fatty acid compounds, where their carboxylate groups bind to the active site zinc ion and their fatty acid chains are incorporated into the large S1′ site pocket of MMP-2 and MMP-9.

These docking results agreed with our gelatin zymography studies carried out on these two secreted gelatinases present in conditioned media that was isolated from 3T3-L1 cells, which had not previously been exposed to any CNSE components. Inhibition of both MMP-2 and MMP-9 occurred in a dose-dependent manner, suggesting a direct interaction of anacardic acid with the catalytic activity of the enzyme. We also analyzed the effects of salicylic acid and aspirin, two compounds sharing similarities to anacardic acid in the ring structure but lacking a C15 chain, and, in the case of aspirin, an additional acetylation of the ring hydroxyl group. Salicylic acid shows significantly lower inhibition than anacardic acid suggesting that the long C15 chain of anacardic acid plays an important role in gelatinase binding and inhibition. Further, aspirin had no significant effect in inhibiting the activity of MMP-2 from conditioned media, which is most likely due to steric hindrance resulting from acetylation of the hydroxyl group that appears to play a key role in the binding of anacardic acid to MMP-2/-9.

Anacardic acid is already being used as a template for initial drug discovery research against a number of interesting targets. This includes the use of derivatives of anacardic acid as inhibitors of glyceraldehyde-3-phosphate dehydrogenase from the *Trypanosoma cruzi* pathogen that causes Chagas disease (Pereira et al., 2008). Several studies have focused on analogues of anacardic acid being used as anti-bacterial agents against MRSA (Green et al., 2007), against *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* (Swamy et al., 2007) and *Streptococcus mutans* (Green et al., 2008). Another

approach has used anacardic acid analogues for targeting bacterial histidine protein kinase (HPK)-mediated two-component regulatory systems (Kanojia et al., 1999). Additionally, analogues of anacardic acid are being developed for HAT inhibition (Eliseeva et al., 2007), which could provide a new avenue for the treatment of cancer. Notably, our analyses provide a novel molecular mechanism for anacardic acid that involves inhibition of MMP-2 and MMP-9 function. Our fluorescence-based study on recombinant MMP-2 catalytic core domain clearly demonstrated that anacardic acid directly inhibits the catalytic activity in a dose dependent manner with an IC_{50} of 11.11 μM. This suggests that anacardic acid could be used as a novel template for design and synthesis of analogues, which have drug-like properties and improved binding characteristics to selectively inhibit the gelatinases MMP-2 and MMP-9. This is significant as MMP-2 and -9 are most strongly correlated to metastatic potential, with metastatic tumor cell lines expressing higher levels of these MMPs than non-metastatic varieties (Liotta et al., 1980). Also, the *MMP-2* gene was observed to be one of the key genes mediating aggressive metastasis of breast cancer to the lungs (Minn et al., 2005). In addition, MMP-2 and MMP-9 are produced by the non-malignant cells present in a tumor (Wilhelm et al., 1989) and have key roles in angiogenesis (Brooks et al., 1996). Thus, the development of new gelatinase inhibitors are likely to be of importance in combating a wide range of diseases such as arthritis, cancer and inflammatory disease states including chronic wounds, the latter being of particular relevance to individuals with diabetes. In conclusion, these studies provide a molecular basis for the regulation of MMP-2 and MMP-9 by anacardic acid, and give a strong impetus for the natural products drug discovery paradigm. Further, these studies also provide the basis for exploring cost-effective, novel therapeutic applications for CNSL components and future synthetic derivatives.

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Authorship Contributions

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Contributed new reagents or analytic tools: Bose, Pandurangan, Banerji, and Perry

Performed data analysis: Omanakuttan, Harris, Nambiar, Kumar, Banerji, Perry and Nair

Wrote or contributed to the writing of the manuscript: Omanakuttan, Nambiar, Kumar, Perry and Nair

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Figure Legends

Figure 1. Structure of anacardic acid and related compounds. The main components of CNSE are anacardic acid **(A)** cardol **(B)** and cardanol **(C)**. Anacardic acid consists of a salicylic acid group, with a substituted alkyl chain of 15 carbon atoms, which occurs as saturated, monoene, diene and triene ('n'- indicates the number of H atoms removed) **(D)**. Acetylation of the hydroxyl group of salicylic acid **(E)** generates aspirin **(F).**

Figure 2. Regulation of MMP-2 activity by components of CNSE. (A) Zymogram showing MMP-2 activity of conditioned media from 3T3-L1 fibroblast cells treated with 0.5 % DMSO (lane 1), quercetin at 50 µg/mL (lane 2), commercial anacardic acid (Calbiochem, Cat. No. 172050) (lane 3), cardol-cardanol mixture (lane 4), CNSE (lane 5) and anacardic acid isolated form CNSE (lane 6) each at 1 µg/mL **(B)** Representative plot of % inhibition observed in the zymogram. Each bar represents the mean ± SE of triplicate determinations from three independent experiments. ***$P < 0.001$ (one-way ANOVA with Dunnett's multiple-comparison post-test).

Figure 3. Effect of anacardic acid isolated from CNSE on 3T3-L1 cells. (A) Morphological examination of confluent cells treated with and without anacardic acid isolated from CNSE (at concentration range of 1.5-6 µM) in serum free DMEM after 24 hours. **(B)** Cell viability using MTT cytotoxicity assay was performed for the cells treated with anacardic acid isolated from CNSE (at a concentration range of 0.5-12 µM) in serum free DMEM. Each bar represents the mean ± SE of triplicate determinations from three independent experiments. ***$P < 0.001$ (one-way ANOVA with Dunnett's multiple-comparison post-test).

Figure 4. Fluorescence- based studies on MMP-2 inhibition by CNSE components. Plot showing % inhibition of MMP-2 catalytic core domain in the presence of different concentrations of CNSE, cardol-cardanol mixture and anacardic acid isolated from CNSE (at a concentration range of 1-50 µg/ml). ***$P < 0.001$ (one-way ANOVA).

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Figure 5. Predicted binding mode of anacardic acid to gelatinase. (A) Surface diagram of docked structure of anacardic acid into the MMP-2 active site, with the AutoLigand fill points on a 1 Å grid spacing. The gray points represent optimal locations for carbon atoms and the red points optimal locations for hydrogen bond acceptor atoms. **(B)** Ribbon structure of MMP-2 with the docked structure of anacardic acid shown in sticks. The large green sphere is the co-ordinated zinc atom and the transparent surface surrounding anacardic acid represents the AutoLigand optimized binding pocket. Hydrogen bonding depicted as black dashed lines. **(C)** Surface diagram of docked structure of anacardic acid into the MMP-9 active site. Surface Diagram Figures generated using PMV (56), ribbon diagrams generated by pymol (http://www.pymol.org/).

Figure 6. Effect of different modulators on MMP-2 and MMP-9 enzymatic activity. Zymogram showing MMP-2 activity of conditioned media from 3T3-L1 cells (not previously exposed to either anacardic acid or salicylic acid (SA)) **(A)** Treated with 0.5 % DMSO (lane 1), 10 µM, 20 µM, 30 µM, 40 µM, 50 µM, 75 µM and 100 µM anacardic acid isolated from CNSE (lanes 2-8). **(B)** Treated with 0.5 % DMSO (lane 1), 10 µM, 20 µM, 30 µM, 40 µM, 50 µM, 75 µM and 100 µM salicylic acid (lanes 2-8). **(C)** Representative plot of the zymogram showing % MMP-2 inhibition at different concentrations of anacardic acid and salicylic acid. Zymogram showing MMP-9 activity of conditioned media from 3T3-L1 cells (not previously exposed to anacardic acid and salicylic acid). Each bar represents the mean ± SE of triplicate determinations from three independent experiments. ***$P < 0.001$ (Student's *t*-test). **(D)** Treated with 0.5 % DMSO (lane 1), 20 10 µM, 20 µM, 30 µM, 40 µM, 50 µM, 75 µM and 100 µM anacardic acid isolated from CNSE (lanes 2-8). **(E)** Treated with 0.5 % DMSO (lane 1), 10 µM, 20 µM, 30 µM, 40 µM, 50 µM, 75 µM and 100 µM salicylic acid (lanes 2-8). **(F)** Representative plot of the zymogram showing % MMP-9 inhibition by anacardic acid and salicylic acid. Each bar represents the mean ± SE of triplicate determinations from three independent experiments. ***$P < 0.001$ (Student's *t*-test).

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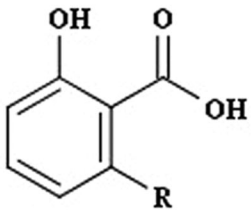
Figure 7. Comparison of MMP-2 inhibition by anacardic acid or salicylic acid using fluorescence-based studies. Plot showing % inhibition of MMP-2 catalytic core domain in the presence of different concentrations of anacardic acid isolated from CNSE or salicylic acid (10-200 μM). ***$P < 0.001$ (Student's *t*-test).

Figure 8. Dose response of MMP-2 inhibition by anacardic acid. (A) Plot showing % inhibition of MMP-2 catalytic core domain in the presence of different concentrations of anacardic acid isolated from CNSE (1-100 μM). ***$P < 0.001$ (one-way ANOVA with Dunnett's multiple-comparison post-test). **(B)** Dose response curve and determination of IC_{50} of anacardic acid mediated inhibition of MMP-2 activity. Each bar represents the mean ± SE of triplicate determinations from three independent experiments.

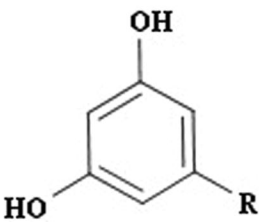
Figure 1

(A)

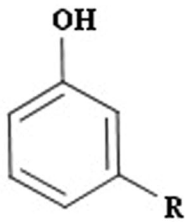


$R=C_{15}H_{31-n}$

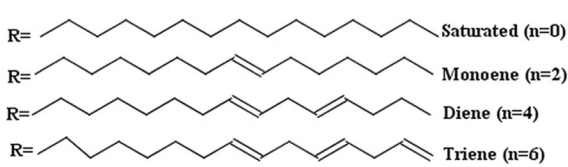
(B)



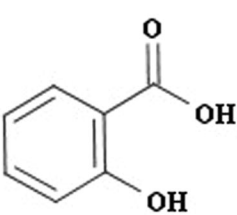
(C)



(D)



(E)



(F)

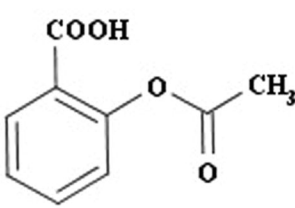
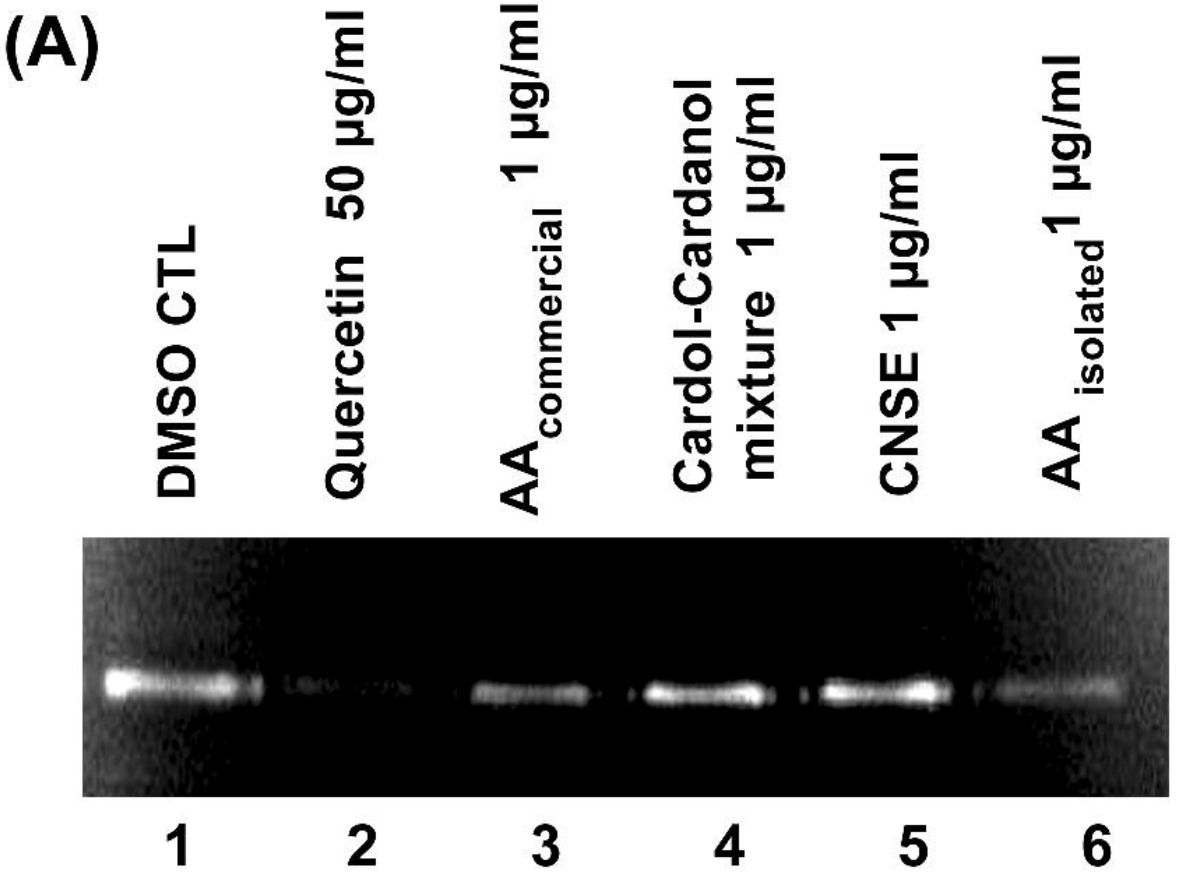


Figure 2


(A)
DMSO CTL
Quercetin 50 µg/ml
AA commercial 1 µg/ml
Cardol-Cardanol mixture 1 µg/ml
CNSE 1 µg/ml
AA isolated 1 µg/ml
1
2
3
4
5
6

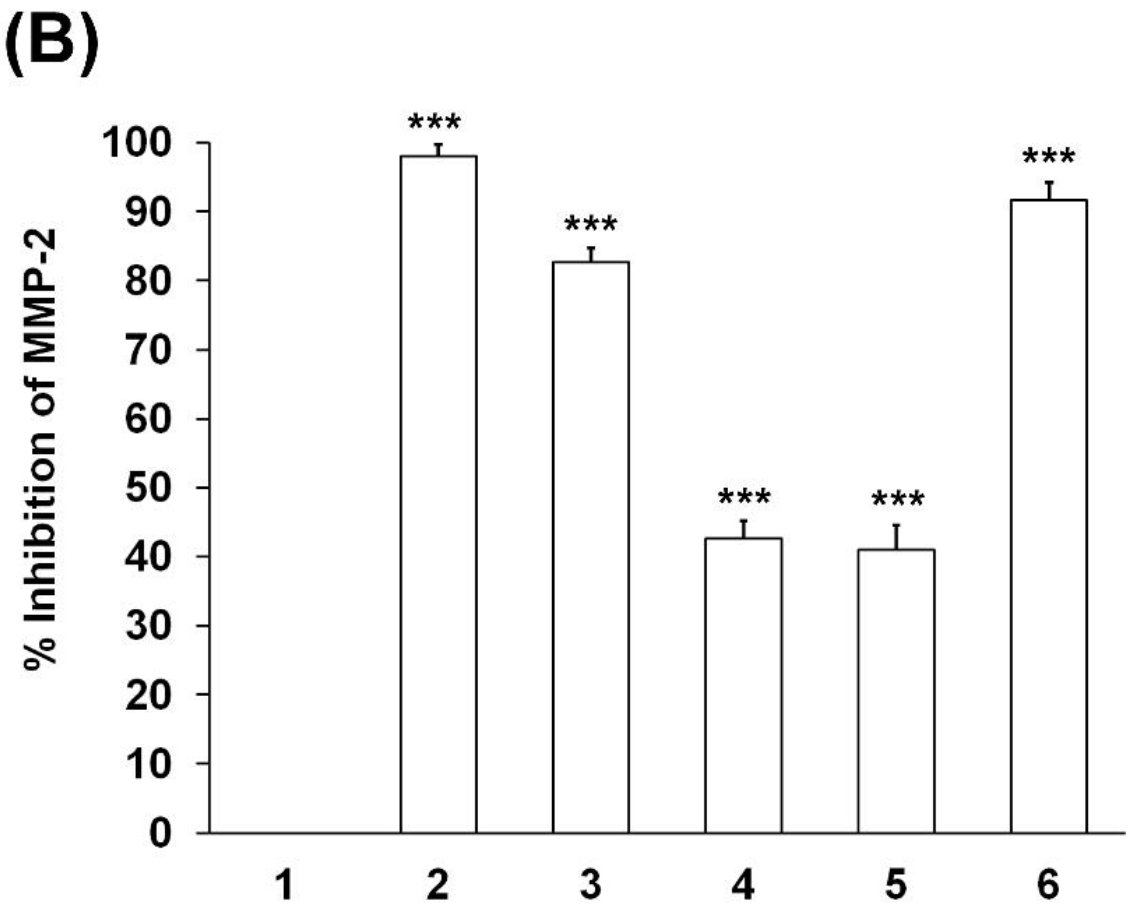
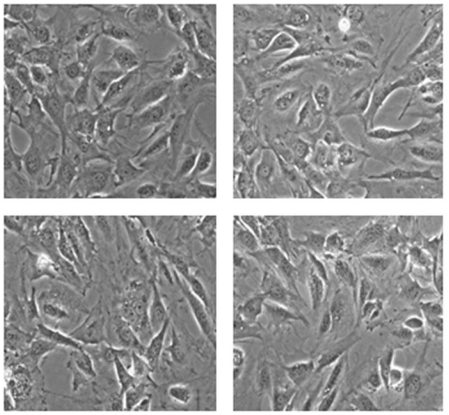

(B)
% Inhibition of MMP-2
100
90
80
70
60
50
40
30
20
10
0
***
***
***
***
***
1
2
3
4
5
6

Figure 3

A


CTL
AA 1.5μM
AA 3μM
AA 6μM

B

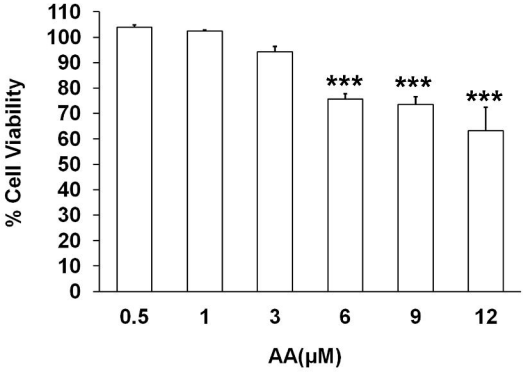

% Cell Viability
110
100
90
80
70
60
50
40
30
20
10
0
***
***
***
0.5
1
3
6
9
12
AA(μM)

Figure 4

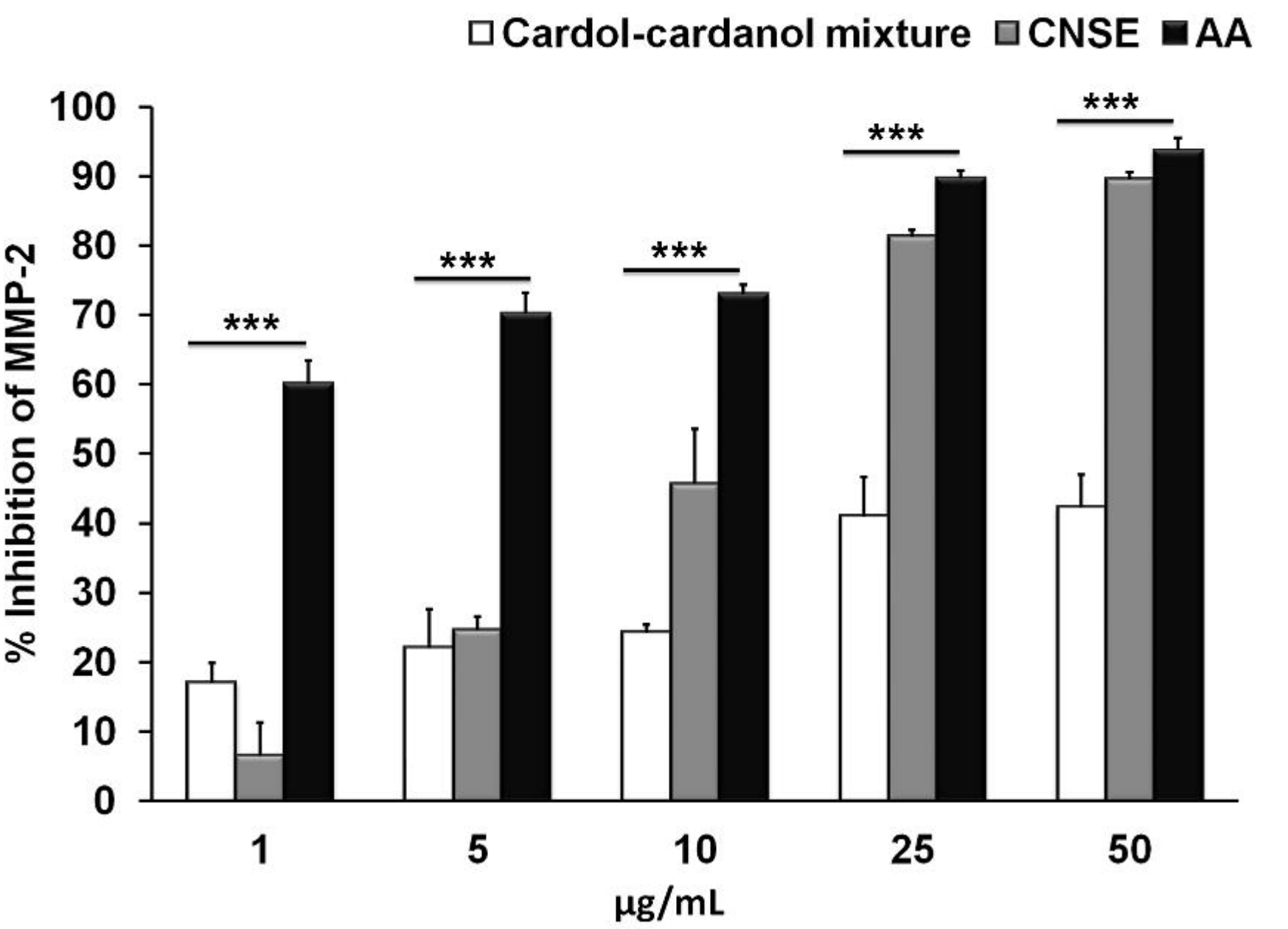

Cardol-cardanol mixture
CNSE
AA
% Inhibition of MMP-2
100
90
80
70
60
50
40
30
20
10
0
***
***
***
***
***
1
5
10
25
50
µg/mL

Figure 5

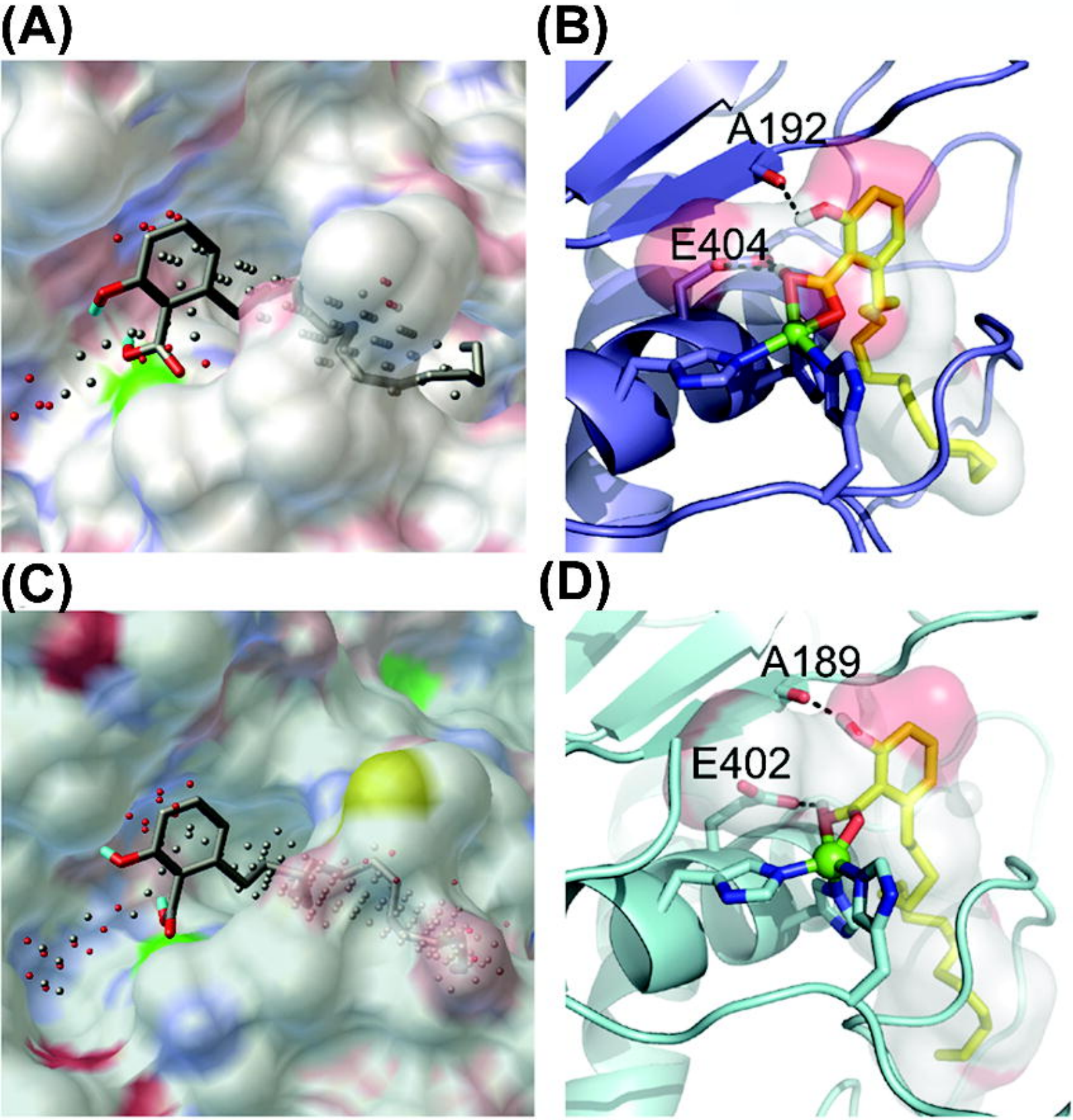


Figure 6

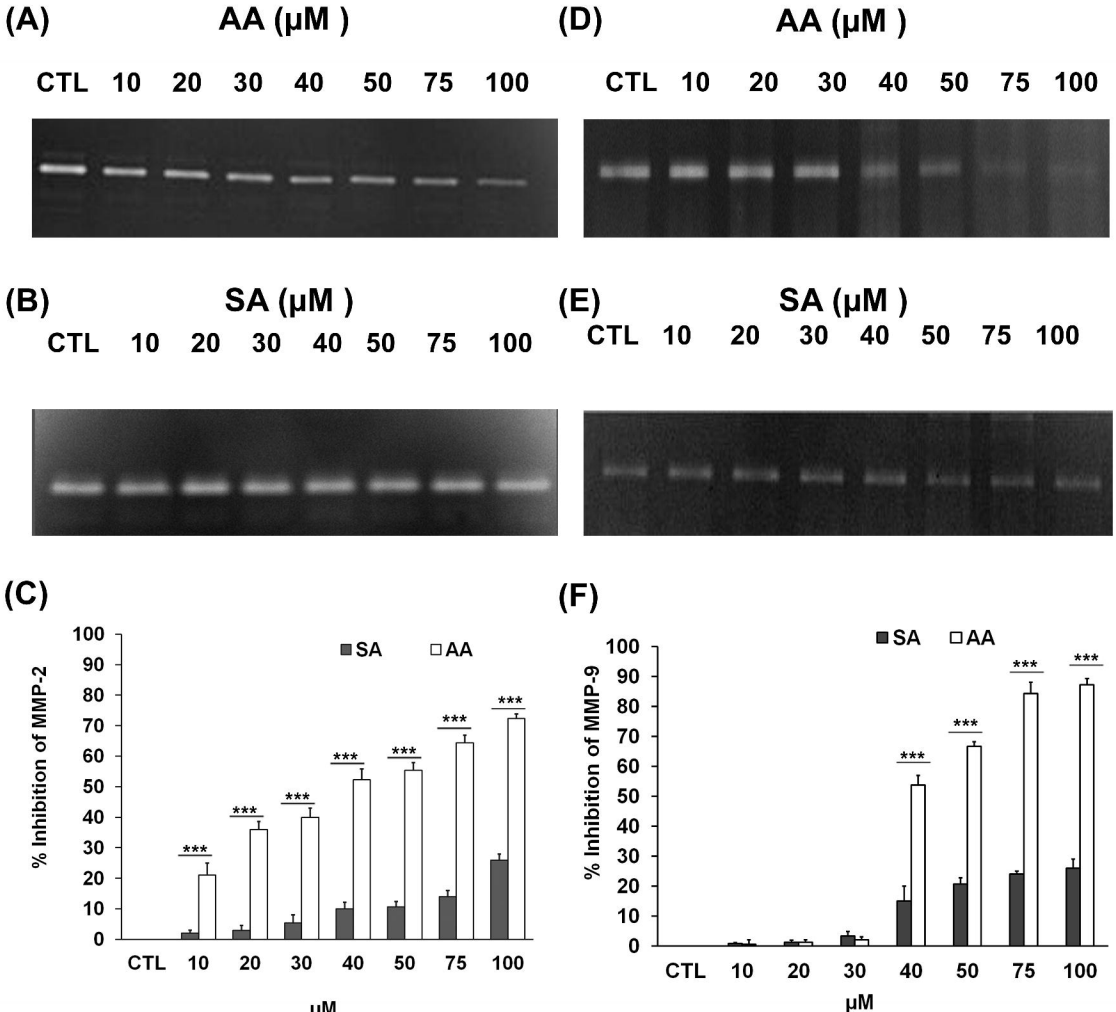

(A)
AA (μM)
CTL 10 20 30 40 50 75 100
(D)
AA (μM)
CTL 10 20 30 40 50 75 100
(B)
SA (μM)
CTL 10 20 30 40 50 75 100
(E)
SA (μM)
CTL 10 20 30 40 50 75 100
(C)
% Inhibition of MMP-2
SA
AA
***
CTL 10 20 30 40 50 75 100
μM
(F)
% Inhibition of MMP-9
SA
AA
***
CTL 10 20 30 40 50 75 100
μM

Figure 7

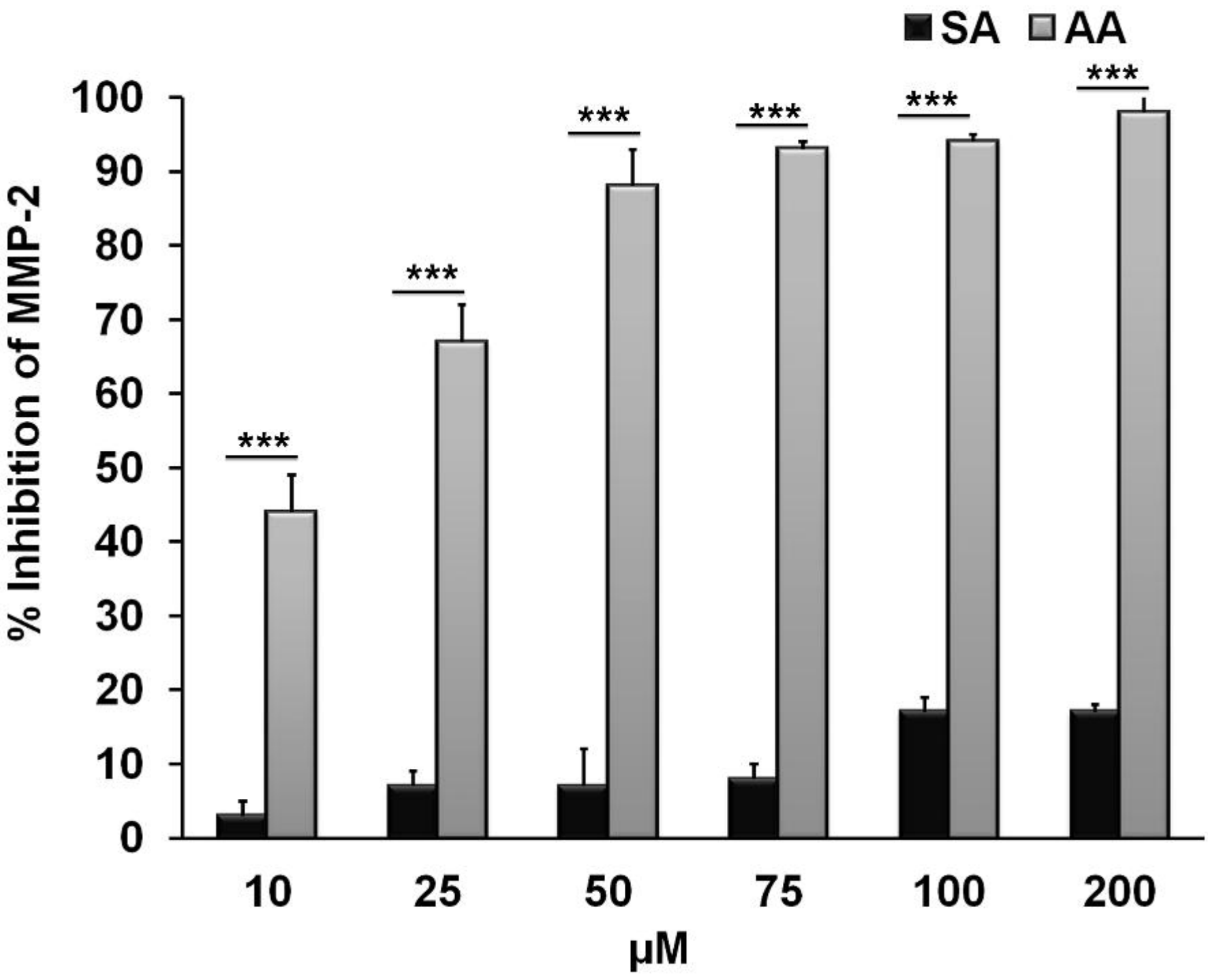
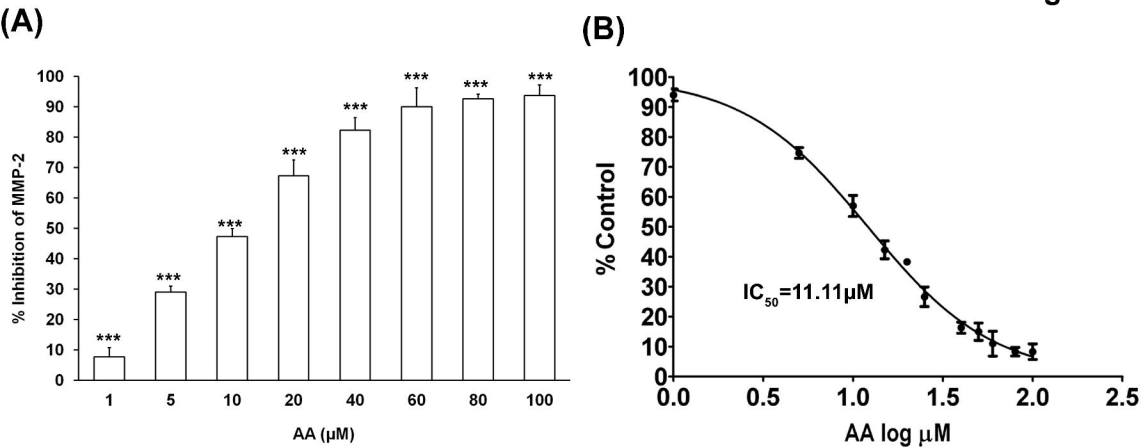

SA
AA
% Inhibition of MMP-2
100
90
80
70
60
50
40
30
20
10
0
***
***
***
***
***
***
10
25
50
75
100
200
μM

Figure 8


(A)
% Inhibition of MMP-2
100
90
80
70
60
50
40
30
20
10
0
***
1
5
10
20
40
60
80
100
AA (μM)
(B)
% Control
IC50=11.11μM
0.0
0.5
1.0
1.5
2.0
2.5
AA log μM