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In-Vitro and In-Silico Analysis of the Anti-Proteinase Activity of *Cadaba Indica* Leaf Extract

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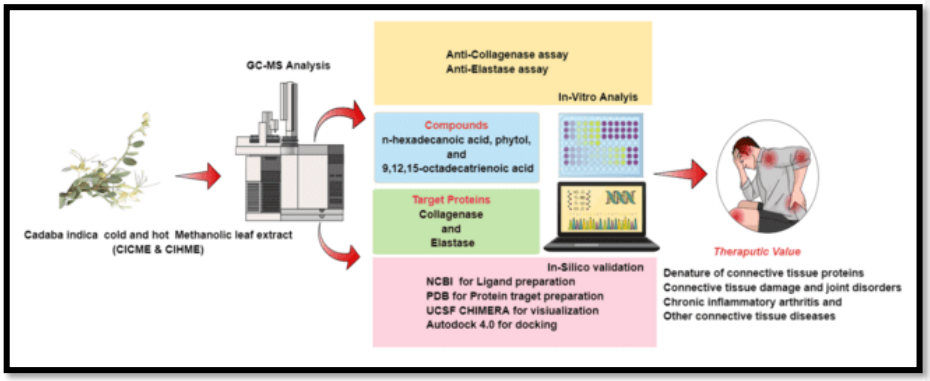
Abstract: Protecting collagen and elastin is essential for controlling connective tissue breakdown in joints and skin diseases. Therefore, this study aimed to evaluate the antiproteinase activity of *Cadaba indica* leaves using in-vitro andin-silico analyses. *Cadaba indica* is a traditional medicinal plant with anti-inflammatory, antiarthritic, and analgesic properties. The in vitro antiproteinase activity of *Cadaba indica* hot percolation methanolic extract (CIHME) and cold macerated methanolic extract (CICME) was assessed by collagenase and elastase inhibition assays using piroxicam as a standard. Three phytochemical compounds, n-hexadecanoic acid, phytol, and 9,12,15-octadecatrienoic acid from CICME, were subjected to molecular docking with the target proteins, collagenase and elastase, using AutoDock 4.0. The results showed that both CICME and CIHME effectively inhibited proteinase enzymes.In addition, the effects of both extracts were statistically significant when compared with piroxicam. Dunnett's t-test was used for the data analysis. The anti-collagenase activity assay showed that CICME (61.97%) resulted in a higher percentage of inhibition than CIHME (53.42%). In addition, CICME (61.38%) showed a higher percentage of inhibition than CIHME (50.41%). In silico results showed that all three phytochemical components were successfully bound to the target. In addition, 9,12,15, octadecatrienoic acid (-4.22 Kcal/mol) and phytol (-4.82 Kcal/mol) showed binding solid energy with the target. In-vitro and in-silicoantiproteinase activities showed that the phytochemicals of Cadaba indica can prevent connective tissue degradation in various diseases.

Keywords:*Cadaba indica*, cold maceration, antiproteinase, collagenase, elastase.

Graphical Abstract



1 Introduction:

Herbal plants are an incomparable wealth of our planet,which can prevent and treat various diseases. However, musculoskeletal diseases such as arthritis are a major threat in this era, causing severe functional impairment in patients. Cadaba indica is a traditional herbal plant with many therapeutic uses. In traditional medicine, this plant has been used as an analgesic and anti-inflammatory, totreat menstrual irregularities (Selvamani & Latha, 2005). Several studies have been conducted to evaluate their medicinal value scientifically. Acute toxicity and in vivo acute anti-inflammatory evaluations havebeen scientifically validated(Ramakrishnan et al., 2017).Thirumalai et al. (2020) evaluated the in vitro anti-arthritic activity through a protein denaturation inhibitory effect. Previousstudies have shown thatCadaba indica plant products may be used to treat and manage musculoskeletal disorders, such as arthritis.

Connective tissue damage and excess protein denaturation are the prominent pathological mechanisms of arthritis and other musculoskeletal diseases (John.J.Cush & Peter.E.Lipsky, 2013). Hence, connective tissue proteins are prevented from degrading to treat any joint disorders. Proteolytic enzymes primarily degrade connective tissue proteins. Inhibition of these enzymes may help prevent further connective tissue damage (Guo et al., 2021). Although several allopathic drugs are available to treat various joint disorders, cost and adverse effects are the main factors for the low economic public beingunable to use them. Hence, a new drug is required to overcome this problem (Alam et al., 2017). This study analyzed the inhibitory effect of Cadaba indica leaf extract on proteinase enzymes, such as collagenase and elastase, through in vitro and in silico methods.

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~~2~~1 Materials and Methods:

~~2.1~~1.1 Collection and processing of plant materials

Cadaba indica Lam leaves were collected during flowering in March 2017 from Melur, Madurai District, Tamil Nadu, India. The plant was authenticated by Dr.V. Chelladurai, formerly a research officer of botany, Central Council for Ayurveda and Siddha, Government of India. A voucher specimen (Dated 20/03/2017) was preserved in the laboratory for future reference.

~~2.2~~1.2 Preparation of extract:

~~2.2.1~~1.2.1 Cold Maceration Method:

100 g of coarse leaf powder was defatted with petroleum ether for 72 h at room temperature (Rajkumar & Sinha, 2010). The defatted plant material was soaked in 80% methanol at room temperature for 72 hours to prepare the methanolic extract(Okoduwa, et al., 2015)The extract solution was strained completely, filtered by What Man filter paper No-1 and labelledCadaba indica Cold macerated Methanolic Extract (CICME).

~~2.2.2~~1.2.2 Hot percolation Method:

100g The leaf-coarse powder was loaded into the Soxhlet apparatus and extracted by continuous hot percolation. Plant material was extracted with 80% methanol for 48h. The final extract was collected and labeled as Cadaba indicahot percolated Methanolic Extract(CIHME) (Okoduwaet al., 2015) (Adaramola & Onigbinde, 2017).

~~2.3~~1.3 AnticollagenaseActivity

Kim et al. (2004) method performed the collagenase inhibition assay with slight modifications. The reaction mixture contained 25µl of 50mM tricine buffer solution, 25µl of each concentration of CICME and CIHME solution (100, 200, 400, 800, and 1000 µg/ml), and 25µl of 0.1Unit of collagenase enzyme. 50 µl of 2mM FALGPA substrate was added to the reaction mixture, and the absorbance was measured immediately at 340nm in a 96-well microplate reader.The control solution contained all the reagents except the FALGPA substrate, whereas the mixture without the standard drug or text extract was considered a test-blank solution. The experiments were performed in triplicate. The percentage inhibition of collagenase activity was calculated as follows:

$$\text{Percentage of inhibition} = \{\frac{\text{The absorbance of control-Absorbance of sample}}{\text{Absorbance of control}} \text{x}100\}$$

~~2.4~~1.4 AntielastaseActivity

The anti-elastase assay was performed as described by Lee et al. (1999) with slight modifications. The reaction mixture contained 160µl of 0.2 M Tris-HCl buffer solution, 50µl of each concentration of CICME and CIHME solution (100, 200, 400, 800, and 1000 µg/ml), and 20µl of elastase enzyme. Each test extract and standard drug were incubated with elastase enzyme for 20 minutes at 25^0C before adding the substrate.20 µl of 0.8mM N-Succinyl-Ala-Ala-Ala-p-

Nitronilidesubstrate was added to the reaction mixture, and the absorbance was measured at 410 nm in the 96-wellmicroplate reader. The control solution contained all the reagents except the N-succinyl-Ala-Ala-Ala-Ala-p-Nitronilide substrate. In contrast, a mixture without a standard drug or text extract was considered a test blank solution. The experiment was performed in triplicate.

The percentage inhibition of elastase activity was calculated as follows:

$$\text{Percentage of inhibition} = \{\frac{\text{The absorbance of control-Absorbance of sample}}{\text{Absorbance of control}} \text{x}100\}$$

~~2.5~~1.5In silico antiproteinase and anti-inflammatory activities

Computational docking is broadly utilized to study target protein-ligand interactions for drug discovery and development(Yuriev & Ramsland, 2013). Typically,this strategy begins with a known protein structure target, such as the crystallographic structure of an enzyme(De Ruyck et al., 2016). Docking was then used to predict the conformation of the interaction and binding free energy of small molecules to the target enzyme. The phytochemical compounds detected in the cold-macerated methanolic leaf extract of Cadaba indica were selected for molecular analysis. Target proteins, such as collagenase, elastase, and cyclooxygenase-2, were docked with the selected phytochemicals using Auto Dock 4.0, and the binding energies were calculated. The binding energies and contact of each ligand were obtained and analyzed using UCSF CHIMERA.

~~3~~2 Preparation of ligands:

Phytochemical constituents such as n-hexadecanoic acid, 9,12,15-octadecatrienoic acid, and phytol were selected from the GC-MS chromatogram of the cold methanolic leaf extract of Cadaba indica (Thirumalai, et al., 2021) The two-dimensional (2D) chemical structures of selected phytochemical compounds were obtained from the PubChem database at NCBI (https://pubchem.ncbi.nlm.nih.gov). The ligands were prepared via energy minimization and hydrogen atom addition using the Chemsketch software building tool(Pettersen et al., 2004). The 3D structure was saved in structurest and developed fo,r docking analysis using Chemsketch software.

~~3.1.1~~2.1.1 Preparation of target proteins

The three-dimensional crystal structure of target enzymes such as human collagenase(Farrokhnia & Mahnam, 2017), neutrophil elastase (Narayanaswamy, et al., 2014)and cyclooxygenase-2 (Miladiyah, et al., 2017)were extracted from the Protein DataBank (PDB) with PDB IDs 1CGE, 1H1B, and 1CX2, respectively.

~~3.1.2~~2.1.2 Cavity prediction and binding site analysis

The target protein structures were then subjected to CASTP analysis. Computed Atlas of Surface Topography of Proteins (CASTP) is an online tool to locate, delineate, and measure protein structures' geometric and topological properties. CASTP is mainly used for detailed quantitative characterization of the

geometric and topological properties of proteins, including surface pockets, interior cavities, cross channels, and essential regions associated with target and ligand interactions. (Binkowski, et al., 2003) .

~~3.1.3~~2.1.3 Visualization of target proteins and ligands

The atomic charges of the amino acid residues were fixed and energy minimization was performed. The prepared target protein structures, H-bond, and non-bond interactions of ligands with the active site residues were analyzed using UCSF CHIMERA software to prepare high-resolution images.(Narayanaswamy, et al., 2014)

~~3.1.4~~2.1.4 Docking:

Geometrical optimization of the input compounds was performed usingArguslab software to obtaina stable structure of the prepared compounds. After preparing the ligand and target protein structures, molecular docking was performed using AutoDock 4.0. Molecular docking of the ligands with the target proteins (enzymes) followed a standard operational protocol.(Jemal, 2019)

~~4~~3 Results:

~~4.1~~3.1 Inhibition of Collagenaseenzymeactivity

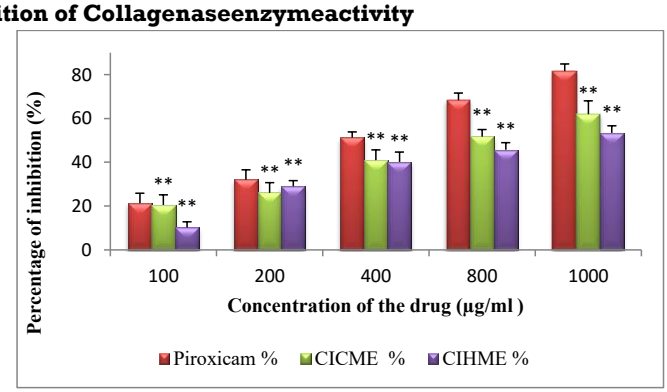

Figure 1. Effect of cold macerated (CICME) and hot percolated (CIHME) methanolic leaf extracts of Cadaba indica and piroxicam on inhibition of collagenase activity.Values are expressed in mean ± SD (n=3); statistical significance level *p< 0.01, **p<0.001

The percentage of collagenase inhibition by CICME and CIHME was statistically significant compared to the inhibitory activity of the standard piroxicam. The collagenase-inhibitory actions of CICME, CIHME, and Piroxicam are shown in Figure 1. The anti-collagenase activity of CICME (up to 61.97±6.1%) was significantly higher than that of CIHME (up to 53.42±3.2%).

3.2. Inhibition of elastase enzyme activity

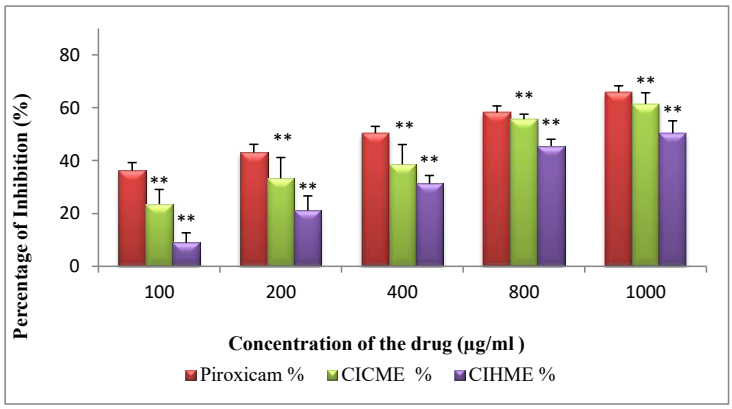

Figure.2. Effect of cold macerated (CICME) and hot percolated (CIHME) methanolic leaf extracts of Cadaba indica and piroxicam on elastase activity.

The percentage inhibition of elastase enzyme activity of CICME and CIHME was statistically significant compared to that of standard piroxicam. At 800µg/ml, the anti-elastase activity (55.69±1.9 %) was slightly similar to that of the standard piroxicam (58.13±2.5 %). The elastase inhibitory activity of CICME (up to 61.38±4.3%) was significantly higher than thatofCIHME (up to 50.41±4.6%).

3.3. In silico anti-proteinase and anti-inflammatory activity:

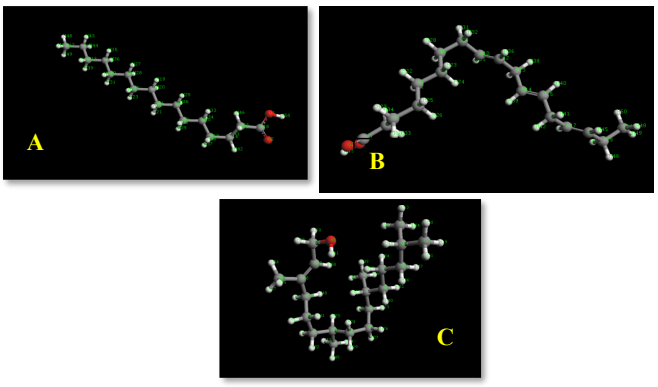

Figure 3: Molecular structures of bioactive compounds from the cold methanolic leaf extract of Cadaba indica Lam. A) n-Hexadecanoic acid, B) 9, 12, 15-Octadecatrienoic acid, C) Phytol

The three phytochemical constituents of cold methanolic leaf extract of Cadaba indica Lam was selected, and its molecular structure was obtained from

the PubChem database for chemical compounds in NCBI. The 3D structures were drawn using Chemsketch software, and the structures are shown in figure.3.

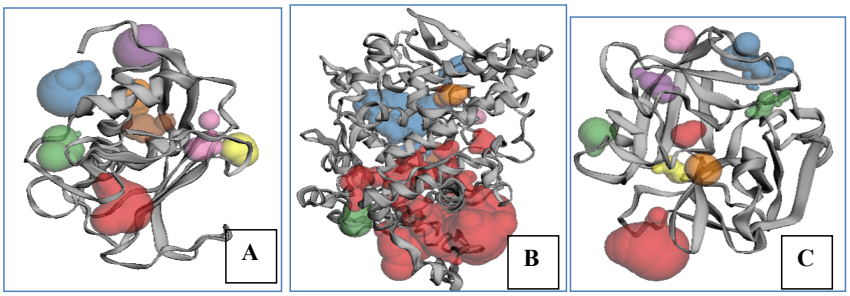

Figure 4: Prediction of active sites in 3D structures of target enzymes using the CASTp web server. The target enzymes were analyzed using the CASTp online tool A) human collagenase (PDB ID: 1CGL), B) Cyclooxygenase-2 (COX-2) (PDB ID: 1CX2), and C) neutrophil elastase (PDB ID: 1H1B).

The protein structures of target enzymes, such as collagenase, neutrophil elastase, and cyclooxygenase-2, were obtained from the Protein DataBank (PDB) and analyzed using the CASTp web server to characterize theactive sites. Figure.4. shows the active sites of the target enzymes in various colors.

Table.1: Docking results of phytochemical compounds from cold-macerated methanolic leaf extract of Cadaba indica Lam. with the human collagenase enzyme (PDB ID: 1CGL)

S.No	Target	Compounds	G (Kcal /mol)	Ki (μM)	Aminoacid interaction residues	No of H-bonds
1	Collagenase	n-Hexadecanoic acid	-3.10	5.36	1CGL:A:ARG214:HH12 - O :**LIG 1**,1CGL:A:ARG214:HH22 - O:**LIG 1**	2
2	Collagenase	9,12,15-octadecatrienoic acid	-4.22	810.61	1CGL:A:ARG214:HH12 - O :**LIG 1**,1CGL:A:ARG214:HH22 - O:**LIG 1**	2
3	Collagenase	Phytol	-4.82	291.32	1CGL:A:TYR237:O - H:**LIG 1**,1CGL:A:ARG214:HH12 - O:**LIG 1**	2

Note: LIG1- Ligand (phytochemical compound), Ki – Inhibition Constant, G - Binding energy (Kcal/mol)

Molecular docking analysis revealed a binding interaction between phytochemicals and human fibroblast collagenase. The n-Hexadeconoic acid and 9,12,15-octadecadienoic acid interacted with the ARG 214 residue of collagenase enzyme with the binding energy of -3.10 and -4.22 Kcal/mol, respectively. Phytol interacted with the ARG 214 and TYR 234 residues of collagenase with -4.82 a binding energy. All three compounds bound to the active sites of collagenase via two hydrogen bonds.

Table.2: Docking results of phytochemical compounds from cold-macerated methanolic leaf extract of Cadaba indica Lam. with the cyclooxygenase-2 (COX-2)enzyme (PDB ID: 1CX2)

S.No	Target	Compounds	G (Kcal/mol)	Ki (µM)	Aminoacid interaction residues	No of H-bonds
1	COX-2	n-Hexadecanoic acid	-4.58	438.35	1CX2:A:TYR385:HH - O:**LIG 1**	1
2	COX-2	9,12,15-octadecatrienoic acid	-6.21	28.21	1CX2:A:ALA378:NH - O:**LIG 1**	1
3	COX-2	Phytol	-3.71	1.90	1CX2:A:SER530 - H:**LIG 1**	1

Note: LIG1- Ligand (phytochemical compound), Ki – Inhibition Constant, G - Binding energy (Kcal/mol)

Docking results of cyclooxygenase-2 (COX-2) and phytochemical compounds showed a significant interaction between the ligand and target enzymes. n-Hexadecanoic acid interacted with the TYR 385 residue, and 9,12,15-octadecadienoic acid interacted with the ALA 378 residue of the COX-2 enzyme. Phytol interacts with the SER 530 residue of COX-2. All compounds were bound to the target enzyme (COX-2) with a single hydrogen bond, and the binding energies of n-hexadecanoic acid, 9,12,15-octadecadienoic acid, and phytol were -4.58, -6.21 and -3.71respectively.

Table.3: Docking results of phytochemical compounds from cold macerated methanolic leaf extract of Cadaba indica Lam. with the human neutrophil elastase enzyme (PDB ID: 1H1B)

S.No	Target	Compounds	G (Kcal/mol)	Ki (µM)	Aminoacid interaction residues	No of H-bonds
1	Neutrophil elastase	n-Hexadecanoic acid	-4.70	359.61	1H1B:A: SER195:OG -O**: LIG1**	1
2	Neutrophil elastase	9,12,15-octadecatrienoic acid	-3.23	4.26	1H1B:A:SER195:OG-O:**LIG 1**	1
3	Neutrophil elastase	Phytol	-6.06	37.35	1H1B:A:SER195:OG-H:**LIG 1**	1

Note: LIG1- Ligand (phytochemical compound), Ki – Inhibition Constant, G - Binding energy (Kcal/mol)

The neutrophil elastase enzyme and phytochemical compound docking results showed that all three compounds interacted with the target enzyme's SER 195 amino acid residue with a single hydrogen bond. The free binding energies of n-hexadecanoic acid, 9,12,15-octadecadienoic acid, and phytol are – -4.70, -3.23, and -6.06, respectively.

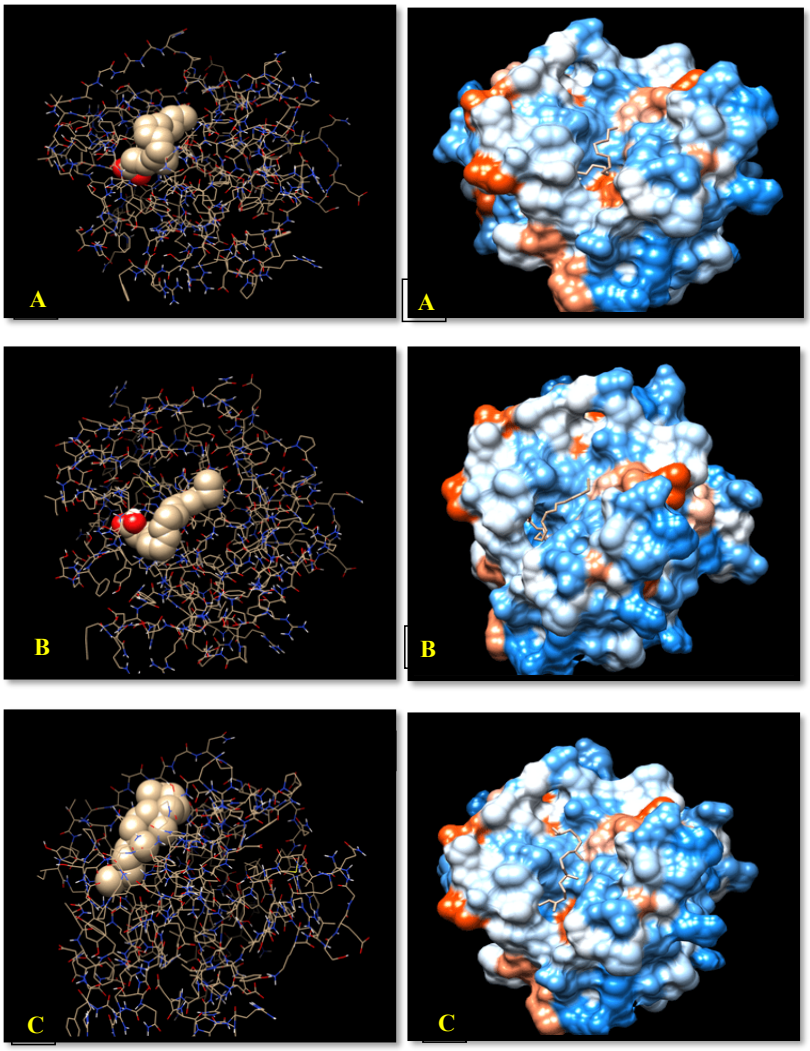

Figure5 : Molecular docking of bioactive compounds from cold-macerated methanolic leaf extract of Cadaba indica with collagenase. A) n-hexadecanoic acid with collagenase enzyme, B) 9,12,15-Octadecatrienoic acid with collagenase enzyme, C) phytol with collagenase enzyme.

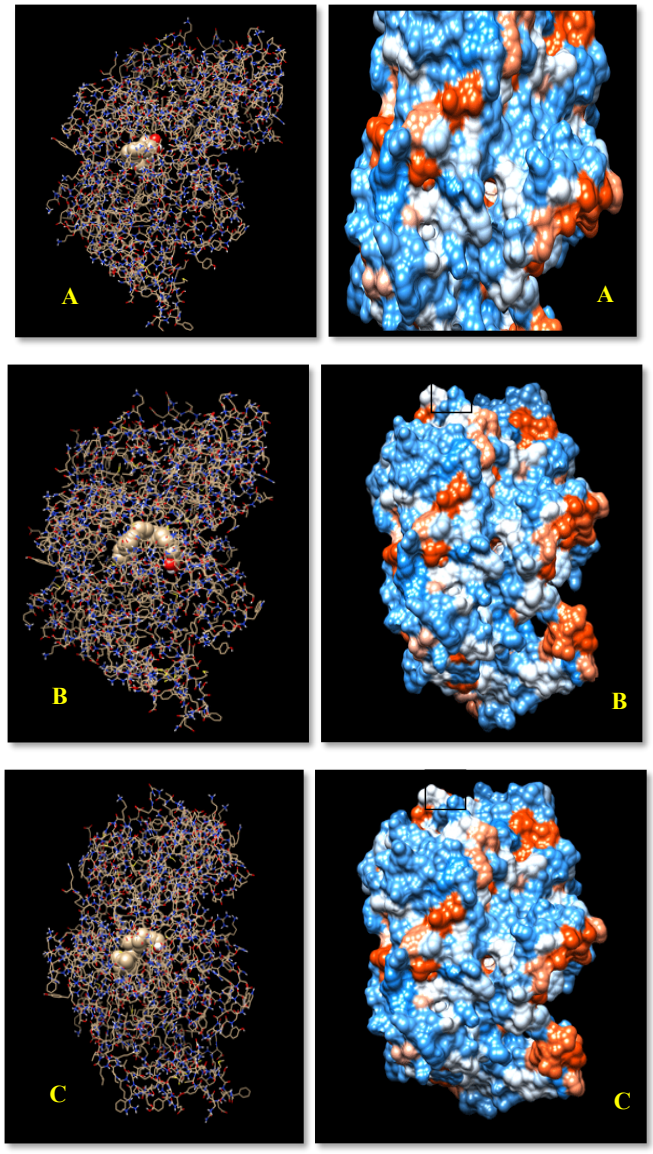

Figure 6: Molecular docking of bioactive compounds from cold macerated methanolic leaf extract of Cadaba indica with cyclooxygenase-2 (COX-2). A) n-Hexadecanoic acid with COX-2 enzyme, B) 9,12,15-Octadecatrienoic acid with COX-2 enzyme, and C) Phytol with COX-2 enzyme.

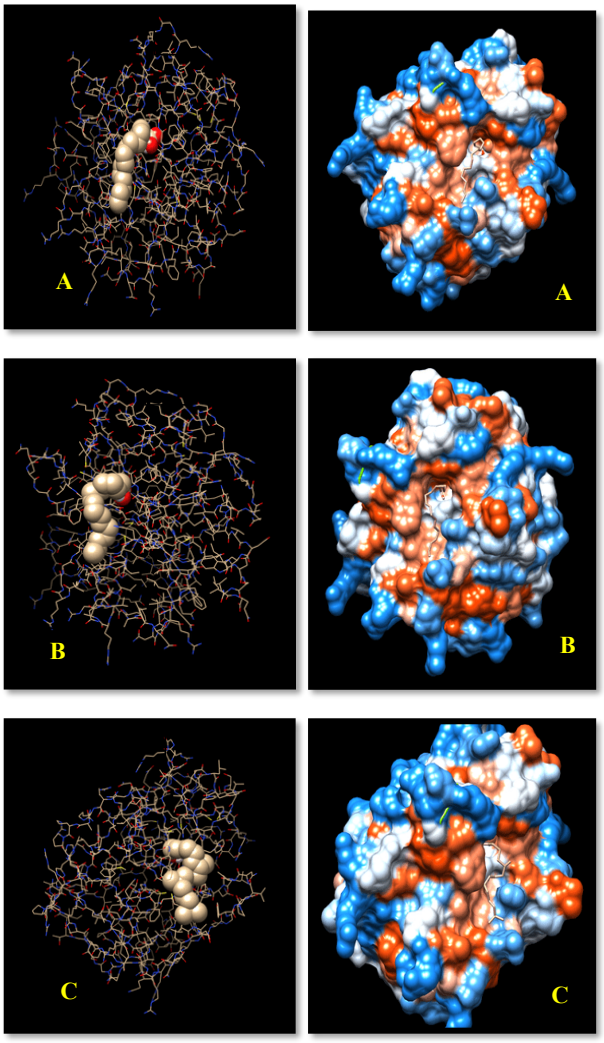

Figure 7: Molecular docking of bioactive compounds from cold-macerated methanolic leaf extract of Cadaba indica with neutrophil elastase enzyme. A) n-hexadecanoic acid with elastase enzyme, B) 9,12,15-Octadecatrienoic acid with elastase enzyme, C) phytol with elastase enzyme.

Discussion :

Cadaba indica is a traditional herbal plant that is extensively discussed in traditional tamil siddha texts. Mohan et al.(2015) reported the anti-inflammatory and antimicrobial properties of the Cadaba indica plant might be due to its rich flavonoids and phenolic acids. Similarly, Thirumalai et al.(2018) reported that the cold macerated methanolic leaf extract of Cadaba indica leaves is rich in rutin, quercetin, and gallic acid,which are responsible for its anti-inflammatory and antitumor activities.The pharmacological effects of plantsare related to their bioactive compounds. Hence,astandard phytochemical analysis is needed to confirm the medicinal values of such herbals. In our previous study, the advanced phytochemical analysis of the methanolic leaf extract was performed using the GC-MS method, and they listed the significant phytochemicals, especially free fatty acids, which are essential for the medicinal properties of the plant (Thirumalai, et al., 2021).

Computer-aided drug design is a new and more advanced technique used widely in biological research to study the molecular interaction between compounds and target proteins (Chaudhary & Mishra, 2016); three phytochemical constituents such as 9,12,15-Octadectatrionic acid (α-linolenic acid), n-hexadecanoic acid (palmitic acid), and phytol from the cold methanolic extract of Cadaba indica leaves were selected from the GC-MS chromatogram of our previous study. These three compounds had the highest peak area percentage among the several compounds identified in the methanolic leaf extract of Cadaba indica. These phytochemical compounds were subjected to molecular docking analysis with target enzymes such as collagenase, neutrophil elastase, and cyclooxygenase-2(COX-2).

The phytochemical constituent, phytol, strongly interacted with the collagenase and elastase enzymeswith -4.82 Kcal/ mol and -6.06 Kcal/mol, respectively. The n-hexadecanoic acid (palmitic acid) interaction with collagenase and elastase enzymes was -3.10 Kcal/mol and -4.70Kcal/mol, respectively. Likewise, the binding energy for the interaction of 9,12,15-Octadectatrionic acid (α-linolenic acid) with collagenase and elastase enzymes were -4.22 Kcal/mol and -3.23 Kcal/mol sequentially. The docking results of the three ligands with collagenase and neutrophil elastase showed that phytol strongly interacted with both enzymes.

At the same time, the docking analysis of three compounds with COX-2 enzyme revealed that the 9,12,15-Octadectatrionic acid (α-linolenic acid) had strong binding interaction with the binding energy of -6.21 Kcal/mol and then-Hexadecanoic acid (palmitic acid) and phytol binding energy was -4.58 Kcal/mol and -3.71 Kcal/mol respectively.

All three phytochemical constituents were found to bind successfully with these three target enzymes, which proved their anti-inflammatory and

antiproteinase enzyme activities. These effects contribute to understanding the anti-arthritic activity of cold-macerated methanolic leaf extract of Cadaba indica (CICME).

The anti-arthritic activity of the methanolic leaf extract of Cadaba indicamay be due to its phytochemical constituents.Alpha-linolenic acid suppresses the synthesis of Pro-inflammatory mediators, such as TNF-α, interleukin-1β, thromboxane B2, and prostaglandin E2, which are crucial in systemic inflammation. In addition, they induce the synthesis of leukotriene B_5 (LT B_5) and prostaglandin E_3 (PG E $_3$), which have anti-inflammatory effects and decrease arachidonic acid synthesis. (Erdinest, et al., 2012) .These actions may explain the systemic anti-inflammatory action of ALA. Flaxseed oil, which is rich in ALA, has been reported to have significant bone–protective and anti-osteoporotic effects. (Kim & Ilich, 2011) . ALA also inhibits the expression of cell adhesion molecules and the chemotactic response of leukocytes in joint tissues.(Das, 2008).

Dietary supplementation with ALA plays a significant role in preventing cardiovascular risk, lowering cholesterol levels, and hypertension, and reducing the risk of pneumonia. Previous studies reported that the ALA also has beneficial effects in treating asthma, other airway infections,and rheumatoid arthritis (Ganguly, et al., 2018; El-Fatah, et al., 2016)n-hexadecanoic acid is a saturated fatty acid that was detected as a significant phytochemical constituent in the cold methanolic extract of Cadaba indica leaves with the highest peak area percentage GC-MS chromatogram. n-Hexadecanoic acid, also known as palmitic acid, is commonly found in plants, animals, and microorganisms. Previous studies have reported that n-hexadecanoic acid has anti-inflammatory, antioxidant, anticancer, and hypocholesterolemic activities. (Krishnamoorthy & Subramaniam, 2014). The anti-inflammatory action of n-hexadecanoic acid is mainly due to the inhibitory effect of phospholipase A_2 (PLA_2), which plays a critical role in the conversion of membrane phospholipids into arachidonic acid during the synthesis of inflammatory mediators. (Aparna, et al., 2012). In addition, n-hexadecanoic acid has cytotoxic and antitumor activities, as previously reported using in vitro and in vivo methods.(Ravi & Krishnan, 2017)

Palmitic acid also inhibits macrophage invasion at the site of inflammation, which may reduce the accumulation of macrophages in the synovial fluid of joint tissues. (Widmer, et al., 2012). In addition, combining palmitic acid with oleic acid and stearic acid significantly affected cartilage destruction by inhibiting leukotriene synthesis, mast cell degradation, and matrix metalloproteinases. (Baugé et al., 2015).

The methyl ester of palmitic acid can inhibit liver Kupffer cells, the resident macrophages, to regulate the inflammatory process by synthesis. TNF-α and nitric acid.(Ning, et al., 2018)Phytol is one of the significant phytoconstituents present in the cold-macerated methanolic leaf extract of Cadaba indica. It is a diterpene

alcohol and a metabolic byproduct of chlorophyll in plants. Previous research has shown that diterpenes, such as phytol, have notable anti-inflammatory, anti-cancer, diuretic, and antimicrobial activities. (Islam, et al., 2018)

The mechanism of the anti-inflammatory action of phytol was reported to involve a significant interaction with the nuclear factor-κB (NF-κB) pathway and inhibition of neutrophil migration into the inflammatory sites, which further induces the production of pro-inflammatory mediators such as TNF-α and interleukin-6.Proteolytic enzymes are endogenous enzymes that denature proteins. They denature proteins by acting on specific peptide bonds in their sequence. According to the site of action in the sequence, proteolytic enzymes are classified into Proteases or exopeptidases and Proteinases or endopeptidases (Rawlings, 2020)Some proteinases act intracellularly,ly, while others act on extracellular regions. Aspartate, cysteine, and threonine proteinases are intracellular endopeptidases, whereas serine proteinases (elastases) and metalloproteinases are extracellular proteinases. Extracellular proteinases play a significant role in degrading connective tissue proteins. Endogenous substances called proteinase enzyme inhibitors mainly regulate the overactivity of proteinases and prevent excess protein denaturation in the ECM. However, proteinase inhibitors are either reduced or suppressed during pathological conditions such as arthritis, trauma, or inflammation (Close, 2001).This activity leads to unusual and excessive degradation of connective tissue proteins, leading to the destruction of tissues.(Okada, 2017)

Among the various ECM proteinase enzymes, collagenase (matrix metalloproteinase) and neutrophil elastase play vital roles in the degradation of primary connective tissue proteins such as collagen elastin. In this study, the antiproteinase activity of the plant extract was estimated using the collagenase and elastase inhibitory assay spectrophotometry method. The cold methanolic extract (CICME) and hot percolated methanolic extract (CIHME) were subjected to proteinase inhibitory action estimation using synthetic connective tissue proteins and their proteolytic enzymes.

The collagenase inhibitory effect was estimated using collagenase from Clostridium histolyticum and the synthetic substance,N-[3-(2-Furyl)acryloyl]-Leu-Gly-Pro-Ala, which resembles the collagen structure. Piroxicam, an oxicam derivative of NSAID, is used as a standard drug thatpotently inhibits these proteinases in synovial tissues. The dose-dependent inhibitory effects of both extracts (CICME and CIHME) showed a significant reduction in enzyme activity. Atan initial concentration of 100µg/ml, the collagenase inhibitory effect of the cold methanolic extract was similar to that of the standard drug piroxicam. The maximum proteinase inhibitory percentages of cold- and hot-percolated methanolic extract at 1000µg/ml were 61.97% and 53.42%, respectively. Piroxicam showed the highest percentage of collagenase inhibition (81.62% at the same dose. Among the two different methanolic extracts of Cadaba

indicaleaves, cold macerated methanolic extract (CICME) was more potent and efficacious than the hot percolated extract (CIHME).

Neutrophil elastases are the serine proteinases, the other most crucial endopeptidases that destroy the connective tissues. The anti-elastase assay demonstrated the ability of the Cadaba indica plant extractto protect connective tissue proteins. Synthetic elastin protein N-succinyl-Ala-Ala-Ala-Ala-p-Nitronilide (SANA) was used as a substrate in the anti-elastase assay using porcine pancreatic elastase enzyme. The maximum inhibitory effects of the standard drug piroxicam and the cold macerated methanolic leaf extract of Cadaba indica at a concentration of 1000 µg/ml were 65.85% and 61.38%, respectively. In contrast, for the hot-percolated methanolic extract, it was 50.41% at the same concentration. Compared to these results, the cold-macerated methanolic extract showed a better inhibitory percentage thanthestandard drug piroxicam.Neutrophil elastases are synthesized inthebone marrow as promyelocytes, which are their precursor forms. Active enzymes are stored in the azurophilic granules of polymorphonuclear leukocytes. (AL-Haik, et al., 1984)The in-vitro anti-proteinase results showed that the inhibitory effects were statistically significant. Compared to the standard drug, the cold methanolic extract (CICME) responded better than the hot percolated methanolic extract (CIHME).

The molecular docking results showed that the phytochemical constituent, phytol, strongly interacted with the collagenase and elastase enzymes with -4.82 Kcal/ mol and -6.06 Kcal/mol, respectively. The n-hexadecanoic acid (palmitic acid) interaction with collagenase and elastase enzymes was -3.10 Kcal/mol and -4.70 Kcal/mol, respectively. Likewise, the binding energy for interacting 9,12,15-Octadectatrionic acid (α-linolenic acid) with collagenase and elastase enzymes was -4.22 Kcal/mol -3.23 Kcal/mol sequentially. The docking results of three ligands with the collagenase and neutrophil elastase enzymes showed that the phytol strongly interacted with both enzymes. At the same time, the docking analysis of three compounds with COX-2 enzyme revealed that the 9,12,15-Octadectatrionic acid (α-linolenic acid) had strong binding interaction with the binding energy of -6.21 Kcal/mol and the n-Hexadecanoic acid (palmitic acid) and phytol binding energy was -4.48 Kcal/mol and -3.71 Kcal/mol respectively.

All three phytochemical constituents were found to bind successfully with these three target enzymes, which proved the anti-inflammatory and anti-proteinase enzyme activities. These effects contribute to understanding the anti-arthritic activity of cold-macerated methanolic leaf extract of Cadaba indica (CICME).The anti-arthritic activity of the methanolic leaf extract of Cadaba indicamay be due to its phytochemical constituents.

65 Conclusion:

The results obtained from the proteinase inhibitory assays indicated that the methanolic leaf extract of Cadaba indica obtained using cold maceration

(CICME) exhibited notable inhibitory activity against collagenase and elastase.The chosen extract for analysis and investigation was the cold-macerated methanolic extract of Cadaba indica (CICME), submitted to both GC-MS analysis and molecular docking study.The chosen chemical had a notable binding affinity towards the target enzymes collagenase, elastase, and cyclooxygenase, and the findings obtained from in vitro and in-silico assays provided clear evidence of the anti-proteinase effect exhibited by the leaves of Cadaba indica.

Conflict of interest: The authors declare that they have no competing interests.

References:

1. Adaramola, B. & Onigbinde, A., 2017. Influence of extraction technique on the mineral content and antioxidant capacity of edible oil extracted from ginger rhizome. Chemistry International , 3(1), pp.1-7.
2. Alam, J., Jantan, I. & Bukhari, S., 2017. Rheumatoid arthritis: recent advances on its etiology, role of cytokines and pharmacotherapy. Biomedicine & Pharmacotherapy, Volume 92, pp. 615-633.
3. AL-Haik, N., Lewis, D. A. & Struthers, G., 1984. Neutral protease, collagenase and elastase activities in synovial fluids from arthritic patients.. Agents and actions, 15(3), pp. 436-442.
4. Aparna, V. et al., 2012. Anti-Inflammatory Property of n-Hexadecanoic Acid: Structural Evidence and Kinetic Assessment. Chemical Biology and Drug Design, Volume 80, pp. 434-439.
5. Baugé, C. et al., 2015. Anti-inflammatory effects of an injectable copolymer of fatty acids (Ara 3000 beta®) in joint diseases. Journal of Inflammation , 12(17), pp. 1-9.
6. Binkowski, T. A., Naghibzadeh, S. & Liang, J., 2003. CASTp: Computed Atlas of Surface Topography of proteins.. Nucleic acids research, 31(13), p. 3352–3355.
7. Chaudhary, K. K. & Mishra, N., 2016. A Review on Molecular Docking:Novel Tool for Drug Discovery. JSM chemistry , 4(3), p. 1029.
8. Close, D. R., 2001. Matrix metalloproteinase inhibitors in rheumatic diseases. Annals of the rheumatic diseases, 60(3), pp. 62-67.
9. Das, U. N., 2008. Essential fatty acids and their metabolites could function as endogenous HMG-CoA reductase and ACE enzyme inhibitors, anti-arrhythmic, anti-hypertensive, anti-atherosclerotic, anti-inflammatory, cytoprotective, and cardioprotective molecules. Lipids in health and diseases, 7(37).
10. De Ruyck, J., Brysbaert, G., Blossey, R. & Lensink, M., 2016. Molecular docking as a popular tool in drug design, an in silico travel. Advances and applications in bioinformatics and chemistry: AABC, Volume 9, pp. 1-11.
11. El-Fatah, N. K. A., Kheder, S. M. M., El-Sayed, N. A. E.-R. & Helal, A.-. E. M. H., 2016. Effect of Omega 3 Polyunsaturated Fatty Acids Supplementation

on Osteoarthritic Knees among Females. International Journal of Health Sciences and Research, 6(4), pp. 362-369.
12. Erdinest, N. et al., 2012. Anti-Inflammatory Effects of Alpha Linolenic Acid on Human Corneal Epithelial Cells. Investigative Ophthalmology and Visual Science, 53(8), pp. 4396-4406.
13. Farrokhnia, M. & Mahnam, K., 2017. Molecular Dynamics and Docking Investigations of Several Zoanthamine-Type Marine Alkaloids as Matrix Metaloproteinase-1 Inhibitors. Iranian Journal of Pharmaceutical Research, 16(1), pp. 173-186.
14. Ganguly, R. et al., 2018. Alpha linolenic acid decreases apoptosis and oxidized phospholipids in cardiomyocytes during ischemia/reperfusion. Molecular and Cellular Biochemistry , Volume 437, pp. 163-175.
15. Guo, Y., Wei, T., Hu, N. & Zhou, X., 2021. Disrupted homeostasis of synovial hyaluronic acid and its associations with synovial mast cell proteases of rheumatoid arthritis patients and collagen-induced arthritis rats.. Immunologic Research, Volume 69, pp. 584-59.
16. Islam, M. et al., 2018. Phytol: A review of biomedical activities.. Food and chemical toxicology, Volume 121, pp. 82-94..
17. Jemal, K., 2019. Molecular Docking Studies of Phytochemicals of Allophylus serratus Against Cyclooxygenase-2 Enzyme. bioRxiv , Volume 866152.
18. John.J.Cush & Peter.E.Lipsky, 2013. Approach To Articular And Musculoskeletal Disorders. In: A. S. Fauci, ed. HARRISON'S RHEUMATOLOGY. New York: Mcgraw-Hill education, pp. 218-231.
19. Kim, Y. & Ilich, J. Z., 2011. Implications of dietary α-linolenic acid in bone health. Nutrition, 27(11-12), pp. 1101-1107.
20. Kim, Y.-J., Uyama, H. & Kobayashi, S., 2004. Inhibition effects of (+)-catechin–aldehyde polycondensates on proteinases causing proteolytic degradation of extracellular matrix. Biochemical and Biophysical Research Communications , Volume 320 , p. 256–261.
21. Krishnamoorthy, K. & Subramaniam, P., 2014. Phytochemical Profiling of Leaf, Stem, and Tuber Parts of Solena amplexicaulis (Lam.) Gandhi Using GC-MS. International Scholarly Research Notices, Volume 2014, pp. 1-13.
22. Lee, K., Kim, J., Cho, J. & Choi, J., 1999. Inhibitory Effects of 150 Plant Extracts on Elastase Activity, and Their Anti-inflammatory Effects. International Journal of Cosmetic Science, Volume 21, p. 71–82 .
23. Miladiyah, I., Jumina, J., Haryana, S. M. & Must, M., 2017. In silico molecular docking of xanthone derivatives as cyclooxygenase-2 inhibitor agents. International Journal of Pharmacy and Pharmaceutical Sciences, 9(3), pp. 98-104.
24. Mohan, V. R., Packiya Lincy, M. & Sakthi devi, G., 2015. Evaluation of phenolic and flavonoid contents and antioxidant activity of various solvent extracts of Cadaba indica lam. An International Journal of Advances in Pharmaceutical Sciences, 6(3), pp. 2849-2853.

25. Narayanaswamy, R., Wai, L. K., Abas, F. & Ismail, I. S., 2014. Molecular docking analysis of curcumin analogues as human neutrophil elastase inhibitors. Bangladesh Journal of Pharmacology , Volume 9, pp. 77-82.
26. Ning, W., Artur, K., Galyna, D. & Maik, G., 2018. Palmitic Acid Methyl Ester and Its Relation to Control of Tone of Human Visceral Arteries and Rat Aortas by Perivascular Adipose Tissue. Frontiers in Physiology , Volume 9, p. 583 .
27. Okada, Y., 2017. Proteinases and Matrix Degradation. In: G. S. Firestein, S. E. Gabriel, I. B. McInnes & J. R. O'Dell, eds. Kelley and Firestein's textbook of rheumatology. 10 ed. Philadelphia, PA: Elsevier, pp. 106-122.
28. Okoduwa, S. I. R. et al., 2015. Evaluation of extraction protocols for anti-diabetic phytochemical substances from medicinal plants. World journal of diabetes, 7(20), pp. 605-614.
29. Pettersen, E., Goddard, T., Huang, C. & Couch, G., 2004. UCSF Chimera--a visualization system for exploratory research and analysis. J Comput Chem, 25(13), pp. 1605-1612.
30. Rajkumar, T. & Sinha, B., 2010. Chromatographic fingerprint analysis of budmunchiamines in Albizia amara by HPTLC technique. Int.J.Res.Pharm.sci, 1(3), pp. 313-316.
31. Ramakrishnan, N., Vijayaraghavan, S., Ramarajan, K. & Tamizhazhagan, V., 2017. Preliminary in vivo evaluation of anti-inflammatory activities of various solvent extracts of Cadaba indica Lam on carrageenan-induced paw edema in swiss albino rats.. Innovare journal of agricultural science, 5(2), pp. 1-3.
32. Ravi, L. & Krishnan, K., 2017. Cytotoxic potential of N-hexadecanoic acid extracted from Kigelia pinnata leaves. Asian J. Cell Biol, Volume 12, pp. 20-27.
33. Rawlings, N., 2020. Twenty-five years of nomenclature and classification of proteolytic enzymes. Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics, 1868(2), p. 140345.
34. Selvamani, R. & Latha, S., 2005. Studies On The Antimicrobial Activity Of Cadaba Indica Lam. Indian journal of pharmaceutical sciences, 67(5), pp. 637-638.
35. Thirumalai, V., Nirmala, P. & Venkatanarayanan, R., 2018. Detection and Estimation of Phenolic Acid and Flavonods in Leaves of Cadaba indica Lam by High Performance Thin Layer Chromatography. Scholars Academic Journal of Pharmacy (SAJP) , 7(9), pp. 417-424.
36. Thirumalai, V., Nirmala, P. & Venkatanarayanan, R., 2020. In vitro Anti-arthritic activity of Methanolic leaf extract of Cadaba indica Lam. Research J. Pharm. and Tech, 13(3), pp. 1219-1223.
37. Thirumalai, V., Nirmala, P. & Venkatanarayanan, R., 2021. Phytochemical characterization of cold macerated methanolic leaf extract of Cadaba indica Lam. using GC-MS. Int J Pharm Sci & Res , 12(6), pp. 3185-92.

38. Thirumalai, V., Nirmala, P. & Venkatanarayanan, R., 2021. Phytochemical characterization of cold macerated methanolic leaf extract of Cadaba indica Lam. using GC-MS.. Int J Pharm Sci & Res, 12(6), pp. 3185-92.
39. Widmer, K. W., Jesudhasan, P. & Pillai, S. D., 2012. Fatty Acid Modulation of Autoinducer (AI-2) Influenced Growth and Macrophage Invasion by Salmonella Typhimurium.. Foodborne pathogens and disease, Volume 9, pp. 211-7.
40. Yuriev, E. & Ramsland, P., 2013. Latest developments in molecular docking: 2010–2011 in review. Journal of Molecular Recognition, 26(5), pp. 215-239.