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Anti-Gout arthritic activity of ethanolic and methanolic seed extracts of *Pedalium Murex*: An in vitro and in silico studies

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***Abstract*---**The present investigation is undertaken to evaluate the comparative anti gout activity of the ethanolic (EEPM) and methanolic (MEPM) seed extracts of Pedalium murex. The Invitro anti gout arthritic activity of the plant extracts was evaluated using xanthine oxidase and protease enzyme inhibition as well as membrane stabilization using well established protocols. In silico anti arthritic activity was studied using molecular docking analysis from 10 GCMS derived compounds with that of the immunuo stimulatory proteins TLR2, TLR4, MMP2 and MMP7 to find out the suitable antagonistic ligand for these proteins. In the present investigation the EEPM extract showed better anti gout activity comparable to the MEPM extract in terms of xanthine oxidase and protease enzyme inhibition as well as membrane stabilization. The anti out arthritic activity of EEPM was comparable to the positive control used in the present study. In the In silico studies among the 10 compounds docked for the proteins TLR2, TLR4, MMP2 and MMP7, three compounds namely 11 -Eicosenoic Acid Methyl ester, Docosanoic Acid, Methyl ester and 1,2Benzendicarboxylic Acid, mono (2-ethylhexyl) ester could inhibit the above mentioned immuno stimulatory proteins and there by suppress the reactions observed in the gouty arthritis. It can be

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concluded that the ethanolic seed extract of Pedalium murex (EEPM) can be used as a potential drug in the treatment of gouty arthritis as it has the ability to suppress the immunological reactions involved in the disease.

***Keywords*---**Pedalium murex, anti - gout arthritic, MSU, xanthine oxidase.

1. Introduction

Gouty arthritis is a form of arthritis characterized by severe pain,redness and tendency in joints due to deposits of uric acid crystals (MSU) in the synovial fluid and synovial lining. The inflammation process occurs by vascular tissue Intense joint inflammation as white blood cells engulf the uric acid crystals, causing pain, heat, and redness of the joint tissues followed by release of cytokines (IL-1, TNF-), chemokines, prostaglandins, free radicals and matrix metalloproteinase which ultimately destroys the tissues and bones by triggering the inflammatory response [1].Thus inflammation is a complex response . However, if prolonged and untreated, it may cause chronic inflammatory condition due to inflammation as seen in several auto immune disorders such as arthritis and asthma [2]. Gout is caused initially by an excess intake of purine rich foods , beverages that leads to hyperuicemia and also high level expression of xanthine oxidase [3]

Pedalium murex L. (Pedaliaceae) is commonly known as Large Caltrops or Gokhru is gaining its importance due to its multifaceted medicinal value [2] .The entire plant is used in preparation of several drugs for ailments like cold and cough.It is highly reported as a traditional cure in reproductive disorders like impotency in men [4] ,gonorrhea, as well as diseases like leucorrhoea in women.and its participation also exyends to cure of ulcers, fevers, wounds [5] general debility. Several papers have been published supporting the importance of this plant in the medicinal area [6]. The leaves of *Pedalium murex* were found to have plenty of flavonoids [7] . Seeds of *Pedalium murex* are reported to have rich flavonoids and vitamin C, where both are potent antioxidants, that act as an effective anti-cancer agent by preventing oxidative cell damage and protection against all stages of carcinogenesis. The seeds are rich in stigmasterol, flavonoids, alkaloids, glycosides, triterpenoid, carbohydrates, amino acids and phenols. On comparing with methanolic seed extract, the ethanolic extract of seed is reported to be an excellent hypolipidemic agent [8]. As a result the different parts of *Pedalium murex* confirmed the presence of various bioactive phytochemical compounds which showed significantly higher anti-inflammatory activity . Therefore, focusing with above scenario an attempt was performed to evaluate the Anti-gout arthritic activities of Ethanolic and Methanolic extracts of *Pedalium murex* as a promising drug with prolonged anti-inflammatory activity and minimum side effects from natural resources.

2.1 Materials and methods
2.1.1 Chemicals

The chemicals such as ethanol, methanol, chloroform and dimethyl sulfoxide(DMSO) and other analytical chemicals were obtained from SD Fine Chemicals Ltd. Bovine serum albumin and enzymes such as Xanthine oxidase, and trypsin were purchased from Hi media chemicals.

2.2 Preparation of Ethanolic (EEPM) and Methanolic (MEPM) seed extract of *Pedalium Murex*

Pedalium murex seeds were collected and dried. After which the seeds were washed 2-3times using distilled water and then dried in shade for 7–14 days.The ethanolic extract EEPM was prepared by submerging the seeds of *Pedalium murex* in 90% (v/v) ethanol and methanol separately for three days in room temperature with occasional shaking by cold maceration process. The extracts were then filtered and the excess solvent present in the extracts were removed by vacuum evaporation method and the dry yield of the EEPM and MEPM was calculated which were found to be 1.34% (w/w) and 0.94% (w/w) respectively.

2.3 In Vitro studies –Anti Gout Arthritic activity

To analyze the anti-gout arthritic activity of EEPM and MEPM extracts standard protocols which were routinely followed were employed. The In-vitro xanthine oxidase enzyme inhibitory activity was analyzed based on the Owens and Johns (1999) method since this enzyme is the triggering force for the gouty arthritis by elevating the uric acid level in the blood. The hyperuricemia further induces the protein denaturation and membrane destabilization leading to gouty arthritis in joints. In the present investigation we analyzed the inhibitory activity of our EEPM and MEPM extracts on protein denaturation and membrane destabilization. The Mizushima and Kobayashi method was applied for the estimation protein denaturation inhibitory activity. The membrane stabilization of RBC was carried out according to the studies by *Sadique et.al.*[9]

2.4 In silico Anti- Gout arthritic activity
2.4.1. GC-MS Analysis

GC-MS analysis was performed for the Ethanolic extract of *Pedalium murex* (EEPM) to find out the phytochemicals present in it which can be further investigated through the molecular docking analysis to evaluate the In silico anti gout arthritic activity.

2.4.2. Molecular docking analysis

In silico molecular docking studies were carried out for 10 compounds obtained from GCMS analysis by Auto Dock Vina software to identify the appropriate ligands for the inhibition of TLR2, TLR4, MMP2 and MMP7 proteins from the *Pedalium murex* seed Phyto compounds. The three-dimensional structures of toll-like receptor 4 (TLR-4) (PDB ID: 5NAO) ,toll-like receptor 2 (TLR-2) (PDB ID:2Z80) Matrix metallopeptidase-2(MMP-2)(PDB ID: 1GXD) and Matrix metallopeptidase-7

(MMP-7) (PDB ID: 2Y6C) proteins were downloaded from protein data bank (http://www.ncbi.nim.nih.gov). Molecular docking was performed and the ligands exhibiting appropriate docking score, protein interaction in terms of amino acid residues with the ligand and the active site were determined. The docking results were stored as pdf file and their binding affinity, molecular interaction between the compounds and protein receptors were analyzed using PyMol Molecular Graphic System (Ver. 1.0) and Discovery Studio (Ver. 3.1) software, respectively. The standard drug colchicine is used as the reference drug.

2.4.3. Statistical Analysis

The values reported are Mean±SE. The statistical analysis was carried out using analysis of variance (ANOVA) followed by Dunnett's 't' test. The < 0.05 p values were considered as significant.

3. Results

3.1. Xanthine oxidase inhibitory assay

The Figure-1 shows the inhibitory activity of xanthine oxidase by EEPM and MEPM. In the present study the ethanolic extract of *Pedalium murex* showed the higher inhibitory percentage when comparable to methanolic extract. At the concentration of 800 and 1000µg/ML the inhibitory activity was quite similar to the positive drug Allopurinol.

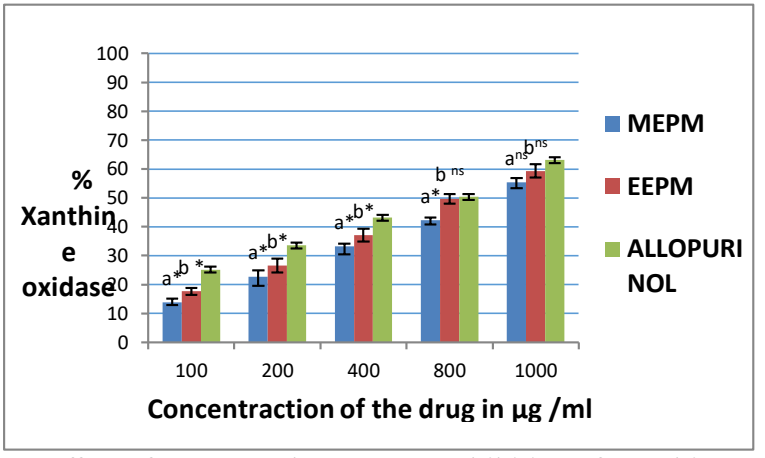


Figure –1 Effect of EEPM and MEPM on Inhibition of Xanthine Oxidase

Values are expressed in mean ± SE (n=3), statistically significant test for comparison was done by ANOVA followed by Dunnett's 't' test. Comparison between a –Allopurinol vsMEPM and b – Allopurinol vs EEPM *p<0.05, **p<0.1 and ns – Non-Significant.

3.2. Inhibition of Protein denaturation

The inhibitory potential of protein denaturation was depicted in Table -1. Though a dose dependent inhibitory action of Xanthine oxidase enzyme was observed for

both the extracts, the EEPM exhibited better inhibitory potential of protein denaturation than the methanolic extract of *Pedalium murex* seeds. At the concentration 800µg and 1000µg of EEPM maximum inhibition potential was noted which was comparable to the inhibition percentage of Acetylsalicylic acid the positive control used.

Table – 1 Effect of EEPM and MEPM on Inhibition of Protein Denaturation

Conc of Extract (in µg)	% Inhibition of MEPM	% Inhibition of EEPM	% Inhibition of Acetylsalicylic acid
100	15.26± 3.0 a**	20.3±1.53 b **	29.32±3.17
200	29.3±1.69a**	33.6±2.59 b**	40.49±2.20
400	38.2±2.18 a**	45.5±3.05 b*	52.85±3.18
800	42.5± 2.46 a **	60.5±1.60 b ns	62.50 ±3.15
1000	54.9 ±2.20 a**	65.56±1.39 b ns	68.66 ±2.09

Values are expressed in mean±SD (n=3), statistically significant test for comparison was done by ANOVA followed by Dunnett's 't' test. Comparison between a –Acetylsalicylic acid vs MEPM b –Acetylsalicylic acid vs EEPM *p<0.05, **p<0.1 and ns – Non-Significant.

3.3. Membrane stabilization study

The protective effect of the MEPM and EEPM extracts on RBC membrane against the heat and hypotonic saline induced damage was shown in Figure -3.Here both the extracts protected the RBC membrane and at higher concentration of 800µg/ml and 1000 µg/ml a similar type of protection was observed for both the extract and the positive control acetylsalicylic acid exhibited higher activity than these extracts.

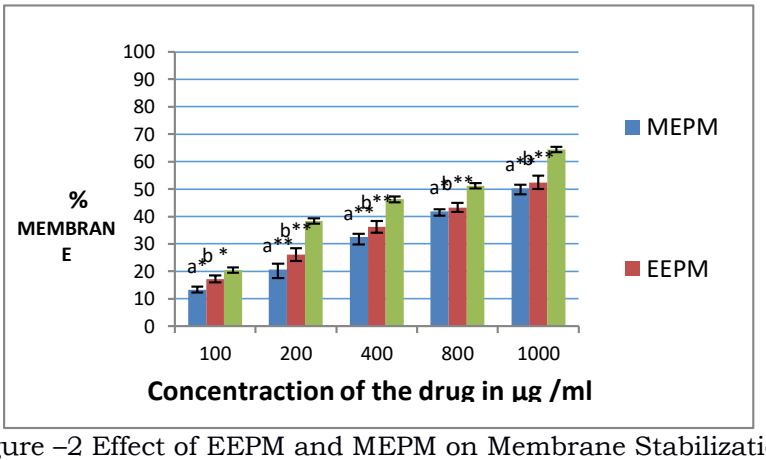


Figure –2 Effect of EEPM and MEPM on Membrane Stabilization

Values are expressed in mean±SE (n=3), statistically significant test for comparison was done by ANOVA followed by Dunnet's 't' test.Comparison

between a –Acetylsalicylic acid vs MEPM, b –Acetylsalicylic acid vs EEPM *p<0.05, **p<0.1 and ns – Non-Significant.

3.4. GC-MS Analysis

GCMS analysis was performed to determine the presence of active ingredients in ethanolic extract of *Pedalium murex* seeds. A total of 10 compounds were identified. Table 2 and Figure 3 explicit the list of phyto chemical compounds identified in the chromotogram along with the peak, Retention time,molecular weight, molecular formula and % of peak area.

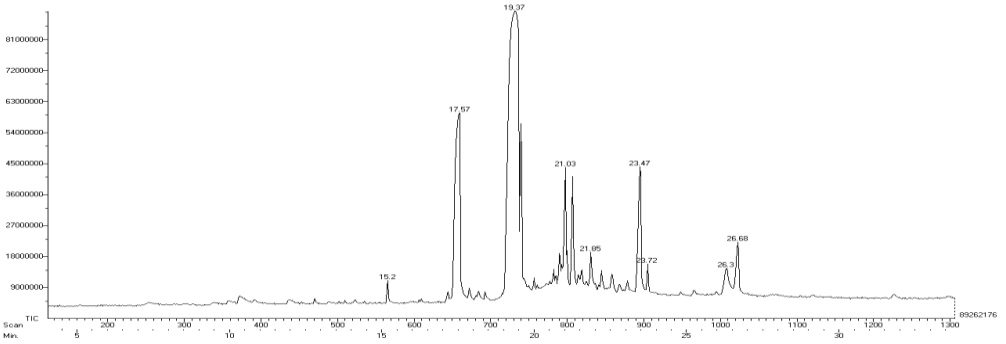


Figure –3 GC-Ms chromatogram of Ethanolic seed extract of *Pedalium murex*

Table 2: List of compounds identified in GC-MS analysis

Peak	R.Time	Name of the compound	Molecular Formula	Molecular Weight	Peak Area%
1	15.200	Methyl Tetracadecanoate	C15H30O2	242.40	5.138
2	17.370	Pentadecanoic Acid ,14 -methyl-methyl ester	C17H34O2	270.50	10.381
3	21.03	11 -Eicosenoic Acid , Methyl ester	C21H40O2	324.5	20.332
4	21.28	Eicosanoic Acid , Methyl ester	C21H42O2	326.6	11.556
5	21.85	12-Methyl_E,E-2,13-Octadecadien 1_Ol	C19H36O	280.5.	8.902
6	23.48	Docosanoic Acid ,Methyl ester	C23H46O2	354.6	19.305
7	23.72	1, 2 Benzendicarboxylic Acid , mono (2-ethylhexyl) ester	C16H22O4	278.34	7.345
8	26.3	9-Octadecenoic Acid (Z)-2-hydroxy-1-((hydroxymethyl) ethyl ester	C21H38O4	354.52	6.738
9	26.68	Tetracosanoic Acid , Methyl ester	C25H50O2	382.7	10.302
10	19.05	9,12-Octadecadienoic acid (Z,Z),Methyl Ester	C19H34O2	294.5	1.01

3.5. Insilico Anti gout arthritic activity

The Insilico anti gout arthritic activity was evaluated in terms of the inhibitory action of proteins TLR2 and TLR4,where these are important proteins that are responsible for triggering the inflammatory reaction against the monosodium urate crystals leading to gouty arthritis. In addition the inhibitory action of the MMP2 and MMP7 the important proteases involved in the degeneration reactions responsible for arthritis were also evaluated. The ligands Eicosanoic Acid , Methyl ester, Docosanoic Acid Methyl ester, 1,2Benzendicarboxylic Acid , mono (2-ethylhexyl) ester, were selected as the best antagonistic ligand based on the docking score among the 10 ligands identified in the GCMs analysis. The Table 3 explains the docking score values of the compounds interaction with MMP2, MMP7, TLR2 and TLR4 proteins.

Table 3: Docking score values of phytochemical compound with MMP2, MMP7, TLR2 and TLR4 Receptors

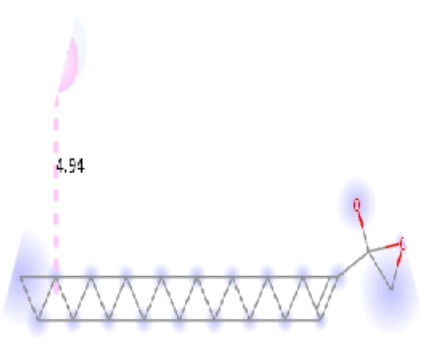
S.NO	Compound Name	Binding energy expressed in (kJ mol-1)			
		MMP2	MMP7	TLR2	TLR4
1	Methyl Tetradecanoate	-3.6	-2.9	-3.5	-3.9
2	Pentadecanoic Acid ,14 -methyl-methyl ester	-3.4	-3.7	-3	-3.2
3	9,12-octadecadienoic acid (z z)- methyl ester	-3.6	-2.1	-3	-2.4
4	11-Eicosenoic Acid , Methyl ester	-7.1	6.6	-5.6	-5.6
5	Eicosanoic Acid , Methyl ester	4.7	-4.3	-5.2	-6
6	12-Methyl_E,E-2,13-Octadecadien 1_Ol	-3.9	2.7	-3.3	-3.6
7	Docosanoic Acid ,Methyl ester	-6.4	-5.6	-5.7	-5.9
8	1,2Benzendicarboxylic Acid , mono (2-ethylhexyl) ester	-5.7	-5.4	- 6.0	-5.3
9	9-Octadecenoic Acid (Z)-2-hydroxy-1-((hydroxymethyl) ethyl ester	-3.6	5.3	-2.9	-3.7
10	TetracosanoicAcid, Methyl ester	-4.6	-3.1	-4.5	-5.8
11	Colchicine	-7.0	-6.8	-6.7	-6.2

Upon the analysis of docking scores in the present study, the three compounds namely 11 -Eicosenoic Acid,Methyl ester, Eicoanoic Acid , Methyl ester and 1,2Benzendicarboxylic Acid , mono (2-ethylhexyl) ester were chosen for the ligand protein interaction study using LIPGLOT analysis.

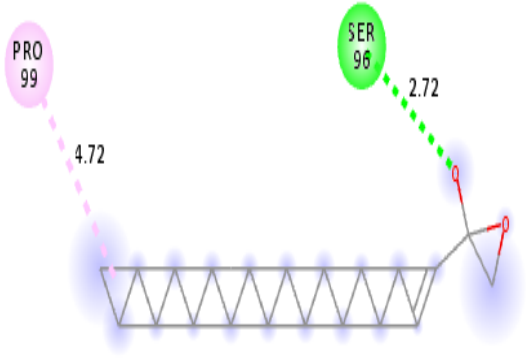
The Table 4,5 and Figure 4,5,6and 7 explains the antagonistic activity of the ligand withTLR4 MMP2, MMP7and TLR2 proteins in terms of amino acid interactions(hydrophilic and hydrophobic).

Table 4- Interaction of MMP2, TLR2 receptors with the phytochemicals of *Pedalium Murex*

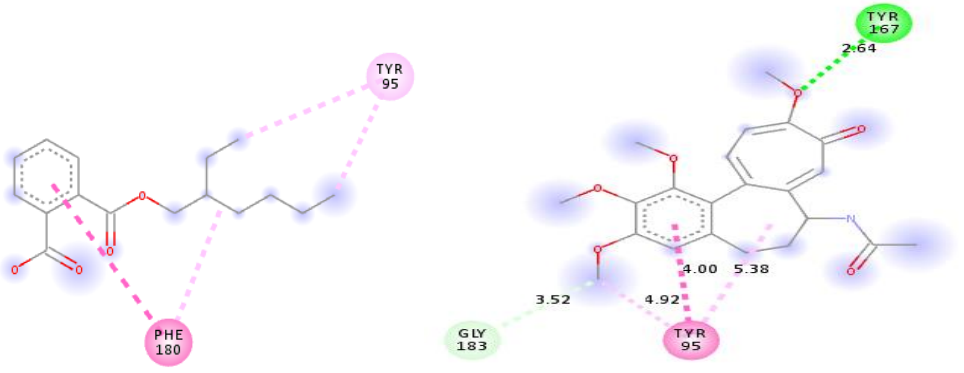
S.No	Compound	Amino acid binding site - MMP2		Amino acid binding site – TLR2	
		H bonding sites	Hydrophobic contact sites	H bonding sites	Hydrophobic contact sites
1	11 - Eicosenoic Acid, Methyl ester	0	2	0	1
		-	PRO290, PHE256	-	LEU717
2	Docosanoic Acid, Methyl ester	1	3	1	1
		PRO459	PHE398,VAL150,PHE431	GLU529	LYS480, PRO456, TYR440
3	1,2Benzendicarboxylic Acid, mono(2-ethylhexyl) ester	4	2	3	6
		SER246,ASN245, CYS349, SER338	CYS363, PRO348	LEU443, THR446, SER427	LYS457, LEU459, LEU476, HIS426, PHE472, ILE448
4	Colchicine	2	1	1	4
		TYR395,TYR425	PRO546	LYS 208	ILE261,LEU234,MET270 ,HIS248



(A) 11 -Eicosenoic Acid , Methyl ester

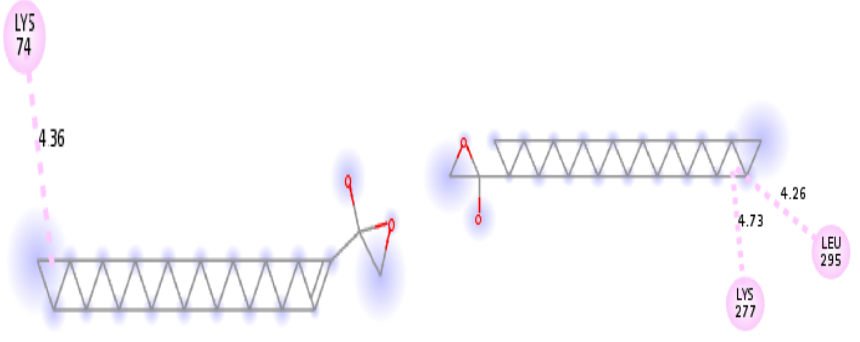


(B) Docosanoic Acid ,Methyl ester

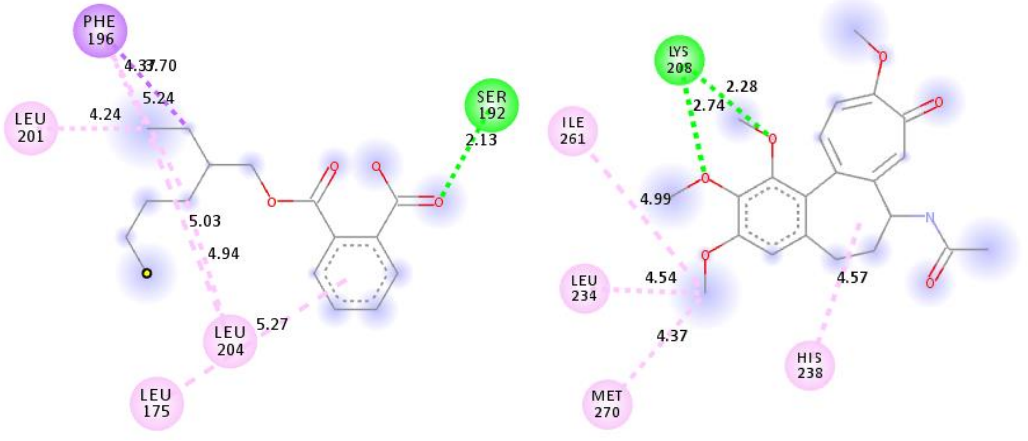


(C) 1,2Benzendicarboxylic Acid , mono (2-ethylhexyl) ester (D) Colchicine

Figure:6 Amino acid Interaction of Matrix metalloproteases VII enzyme with specific ligands



(A) 11 -Eicosenoic Acid , Methyl ester (B) Docosanoic Acid ,Methyl ester



(C) 1,2Benzendicarboxylic Acid, mono (2-ethylhexyl) ester (D) Colchicine

Figure: 7 Amino acid Interaction ofTLR2 with specific ligands

In the present investigation the selected ligands has the ability to interact with the hydroxy amino acids serine and threonine residues present in the TLR2, TLR4, MMP2 ,MMP7 proteinsthrough both hydrophobic and hydrophilic interactions confirms the antagonistic nature. The standard drug colchicine also shows the same type of interaction in the present study indicating that the selected phyto constituents mimics the standard drug in reducing the immunological reactions triggered by the MSU crystals through these proteins.

3.6 Discussion

Gout is common disease that is characterised due to the deposition of MSU crystals in joints or in the subcutaneous tissues, which further causes acute inflammatory flares or chronic arthritis in the body [10]. The MSU crystals result in acute gout attacks characterised by interleukin 1 beta driven acute inflammation, fever and intense pain caused by neutrophil accumulation as well as in the activation in joints [11]. Further, few studies stated that the deposition of MSU crystals in the articular tissue will trigger the response in the neutrophils, leading to molecular events causing pain and inflammation. The treatment for gouty arthritis is primarily initiated to reduce the hyperuricemia by the use of xanthine oxidase inhibitors. Secondarily, immune response has to be suppressed by reducing the inflammation and pain so as to protect the bones as well as tissues from destruction. In the present investigation, comparable to methanolic seed extract, ethanolic seed extract of Pedalium murex exhibited dose dependent inhibitory activity of xanthine oxidase enzyme. The researchers Arokiyaraj et al, have reported the presence of phytochemical compounds like terpenoids, phenols, gums, flavonoids, saponins, sugars, alkaloids and steroids in the ethanolic extract which is found to be more responsible for the xanthine oxidase enzyme inhibitory activity observed in the present study.

Upon the recognition of MSU crystals the neutrophils infiltration occurs leading to the release of lysosomal proteolytic enzymes causing inflammation and injury to the cell membrane. As the lysosomal membrane mimics the RBC membrane, any kind of substances that protects the RBC membrane, thus inhibiting the hemolysis will also protect the lysosomal membrane and prevent the release of inflammatory mediators especially protein degrading enzymes. In the present study the investigations made with the ESEPM exhibited not only the membrane stabilization effect but also the inhibitory effect against the protease enzyme and protein denaturation as they are the common cause of inflammation, joint tissue destruction and synovial proliferation occurring in gouty arthiritis [12].

Complex inflammatory reactions play a predominant role in any type of arthiritis, which will induce the immune system to produce several mediators and cause destruction of cells and severe pain leading to disability. A potential drug should act in multifaceted manner to treat this pain and disability. So in the present investigations we made an attempt using Insilico methods, whether the phyto constituents present in the seed extract could acts as an antagonist ligand to the inflammatory proteins and thereby act as a potential drug for gouty arthritis. Recognition of the naked MSU crystal by Toll-like receptor TLR2 and TLR4, which are normally involved in triggering innate host defense responses to infectious pathogens, was recently discovered to be a primary trigger of the inflammatory

and degenerative tissue reactions associated with gouty arthritis [13,14]. The binding of MSU ligands as an antagonist against TLR2,TLR4,MMP2 and MMP7 receptors in neutrophils acts as a initial trigger to the immune response and inflammatory stimuli which mobilize the multiple signalling pathways by activating the inflammasome which results in processing and secretion of IL-1β, increased NF-kB activity, triggering the cytokines, such as interleukin IL-6, IL-8,and tumor necrosis factor, matrix metalloproteinase (MMPs) leading to the pathogenesis of gouty arthritis. [15,16].

Moreover MSU crystals deposited in synovial and cartilaginous tophi can directly activate chondrocytes and synovial lining cells, thereby promoting cartilage degradation via induction of IL-1, TNF-α, matrix metalloproteinase (MMPs), and certain other mediators [15,16]. With the above scenario we analysed the suppressive activity of the immune stimulatory proteins TLR2, TLR4 as well enzymes MMP2, MMP7 by means of the plant phytocontituents present in the EEPM through docking analysis. Based on the docking score and ligand amino acid interaction three ligands namely 11 -Eicosenoic Acid , Methyl ester, Docosanoic Acid ,Methyl ester and of 1,2Benzendicarboxylic Acid , mono (2-ethylhexyl) ester were found to be potential ligand as they show antagonistic nature towards the inflammatory proteins TLR2, TLR4 ,MMP2, MMP7.The positive drug colchine used in the present study also exhibited similar kind of interactions confirms that phytochemicals shall be used as a promising drug in the treatment of gouty arthritis.

3.7 Conclusion

The management of gouty arthritis requires a dual approach of reducing the pain and join destruction which is triggered due to the multiple immunological reactions due to hyperureciemia. The present study suggests the insight for the discovery of the new phyto drug in the Pedalium murex for the treatment of gouty arthritis as the ethanolic extract shows better anti arthritic activity by inhibiting the enzymes xanthine oxidase, and other proteases as well as protect the RBC membrance. The Insilico analysis shows three ligands -11 -Eicosenoic Acid , Methyl ester, Docosanoic Acid ,Methyl ester and of 1,2Benzendicarboxylic Acid , mono (2-ethylhexyl) ester could act as a best antagonistic ligand for the TLR2, TLR4 ,MMP2, MMP7 proteins thereby halt the immunological reactions observed in the gouty arthritis.

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