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In vitro and *in silico* Approach to Evaluate the Urease and Collagenase Inhibitory Activity of *Embilica officinalis* Gaertn Fruit

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Authors' contributions

This work was carried out in collaboration between all authors. Author SB designed the study, performed the experiments, wrote the protocol and wrote the first and final draft of the manuscript. Authors AM, LS and PB performed the statistical analysis. Author SD managed the analysis of the study and literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The key virulent factors of bacteria are enzymes. Urease and collagenase enzyme play a vital role in pathogenesis of wide array of bacterial strains and cause numerous diseases. So the aim of present study was to find out the potent drug candidate from

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Emblica officinalis Gaertn. fruit for these pathogenically important enzymes.
Study Design: A study was done to screen out the bacteria producing urease and collagenase from a stack of 19 bacterial strains and the positive strains were checked for their susceptibility to methanol and ethyl acetate extracts of *Emblica officinalis* Gaertn. fruit. Further extracts were investigated for their potential to antagonize these enzymes.
Place and duration of study: Department of Biotechnology KUK, Jwahar Lal University, Delhi between February 2012 and December 2013.
Methodology: Screening of bacteria and their susceptibility to methanol and ethyl acetate extracts of *E. officinalis* was done by using agar diffusion assay. Further investigation of extracts to antagonize urease and collagenase enzymes was checked by using phenol hypochlorite and gelatin diffusion assay respectively. GC-MS analysis, docking and ADME studies were conducted to screen for plant-based urease and collagenase inhibitors.
Results: Methanol extract inhibited Jack bean urease enzyme (IC_{50}:0.74 mg/ml) more potently than collagenase Type 1 (IC_{50}:1.13 mg/ml), while ethyl acetate extract inhibited collagenase completely (IC_{50}:4.19 mg/ml) and was observed to be more effective than methanol extract (IC_{50}:5.51 mg/ml). GC-MS analysis revealed an array of 28 and 30 compounds in methanol and ethyl acetate extract respectively. *In silico* study identified xylenol and erucylamide as active compounds of *E. officinalis* having good binding score with better ADME properties compared to standard compounds.
Conclusion: So our observations find application for the consideration of *E. officinalis* compounds for further validation towards development of effective drugs against these significant bacterial enzymes.

Keywords: E. officinalis; urease; collagenase; GC-MS; xylenol; virence factors; in silico study; GLIDE; Gelatin digestion assay.

1. INTRODUCTION

Pathogenic bacteria present an astonishing stash of virulence factors that enable them to conquer many different niches during the course of infection and enhance their potential to cause disease. Four general classes of virulence factors include bacterial toxins, adhesins, extracellular enzymes and antiphagocytic factors. Particularly fascinating virulence factor are microbial enzymes which play a major role at various stages of infection. Several enzymes like leukocidins, hemolysins, lecithinase, proteases, phospholipases, neuraminidases, collagenases and hyaluronidase have been implicated in microbial virulence by destroying white blood cells, red blood cells, plasma membrane of cells and damaging tissues, making the host permissive for microbial infection[1,2].Other enzymes, such as urease, contribute to virulence by facilitating survival inside phagocytic cells [3]. Innovative pharmacological approaches to control the expression of these bacterial enzymes are thought to be beneficial to combat the bacterial infections.

Because of their great pathogenic significance and virulence nature, our investigation deals with two bacterial enzyme- urease and collagenase. Urease is a known virulence factor for number of bacterial species such as *Helicobacterpylori*, *Clostridium perfringens, Klebsiella pneumoniae, Proteus* sp., *Salmonella* sp., *Staphylococcus aureus, Staphylococcus saprophyticus, Pseudomonas aeruginosa,* and *Ureaplasma urealyticum* which are responsible for various diseases like gastritis, urolithiasis, pyelonephrities, ammonia and hepatic encephalopathy, hepatic coma, urinary catheter encrustation [4,5,6].Bacterial species such as *Clostridia* produce collagenase [1] that breaks down protein collagen and

helps in spreading of bacteria in infected body tissues and organs. It is responsible for many pathological conditions including skin ulceration, metastasis, arthritis, chronic inflammation, osteoporosis, tumor invasion, cardiovascular disease, nephritis, neurological disease, breakdown of blood brain barrier, gastric ulcer, corneal ulceration, liver fibrosis, emphysema, fibrotic lung disease, etc. [7]. Collagenase is also a potential virulence factor expressed by *Porphyromonas gingivalis* associated with periodontal disease [8].

Though antibiotic strategies are highly effective in the management of bacterial infections, they have been responsible for emergence of drug and multi-drug resistant strains of bacteria. Targeting the virulence factors is one of the alternative approaches to find new molecules to treat infections due to resistant bacteria [9]. Natural products have been used as sources for the discovery of new drugs on the basis of their widespread application in traditional medicine to cure several human diseases. Recently, Amin et al. examined the anti-*H. pylori* and urease inhibiton activity of some traditional plants used in folk medicine [10] *Emblica officinalis* Gaertn. is a plant from *Phyllanthaceae* family and its fruit is highly valued in traditional Indian medicine. Fruits are globose, fleshy, pale yellow with six obscure vertical furrows enclosing six trigonous seeds. Amla fruit is sour, sweet and astringent in taste and colling in action. It strengthen the lungs, helping to fight chronic lung problems as well as upper respiratory infections. The dried fruits of amla in Unani and Ayurvedic medicine has been reported for the treatment of haemorrhage, diarrhoea and dysentery [11]. Preclinical studies carried out in the past three decades have validated many traditional uses of amla having gastroprotective and antibacterial properties [11]. *In vitro* Anti- *H. pylori* activity of *E. officinalis* fruit has also been claimed recently [12]. In addition, the amla fruit pulp is reported to have properties like diuretic [13], adaptogenic [14], hepatoprotective [15], antitumorous and hypocholestrolemic [16]. In several folk medicines it is used in treatment of dyspepsia, gastritis, hyperacidity, constipation, inflammation, colitis, hemorrhoids, hematuria, menorrhagia, anemia, diabetes, cough, asthma and osteoporosis [17] and ulcer [18].Since the *E. officinalis* is being used in treatment for ulcers and as anti-inflammatory agent in various folk medicines, we have assumed that there might be few compounds which can curtail the enzymes responsible for these conditions. Following this perception, we tested jointly the *E. officinalis* extracts as urease and collagenase inhibitor and the potential of the major principles present in extracts for antagonizing the tested enzymes by virtual screening through docking analysis of interactions between active compounds and enzymes. *In silico* ADME study was further done to evaluate the drug likeliness of docked compounds.

2. MATERIALS AND METHODS

2.1 Chemicals

Urease Type IX (Specific activity: 50,000 to 100,000 units/g) from *Canavalia ensiformis* (L.) DC. (Fabaceae) commonly known as Jack Bean and Collagenase Type 1 (Specific activity: 50,000 to 100,000 units/g) from *Clostridium histolyticum* were purchased from Sigma Aldrich. Other important chemicals used in the study were methanol, ethyl acetate, peptone, dextrose, sodium chloride, calcium chloride, potassium phosphate, urea phenol red, thiourea, EDTA, gelatin, agarose, Coomassie Blue dye.

2.2 Bacterial Strains

A total of 19 bacterial strains (*Bacillus cereus* (MTCC 430), *Bacillus polymyxa* (NCDC 68), *Bacillus pumilus* (MTCC 7411), *Bacillus stearothermophilus* (MTCC 8505), *Bacillus subtilis*

(MTCC 8509 and MTCC 121), *Lactobacillus brevis* (NCDC 371), *Lactobacillus plantarum* (NCDC 20), *Staphylococcus aureus* (MTCC 3160 and MTCC 109), *Staphylococcus epidermidis* (MTCC 3086 and MTCC 435), *Staphylococcus hominis* (MTCC 4435), *Escherichia coli* (MTCC 1885), *Klebsiella pneumoniae* (MTCC 4030), *Pediococcus acidilactici* (NCDC 252), *Proteus vulgaris*(MTCC 426), *Pseudomonas aeruginosa* (MTCC 424 and MTCC 7453))were procured from Microbial Type Culture Collection (MTCC), Chandigarh and National Collection of Dairy Cultures (NCDC), Karnal, cultured in nutrient broth at 37℃ by picking-off technique [19] for 24 h before use and stored in nutrient agar slants at 4℃.

2.3 Extraction of Plant Material

E. officinalis fruits were gathered from Rewari region of Haryana. Dr. Narendera Singh, Department of Botany, Kurukshetra University, identified and authenticated the plant material. Voucher plantspecimen is deposited at the Wild Life Institute of India, Dehradun, under specimen number GS 426.The extraction was done in methanol and ethyl acetate solvents by hot continuous Soxhlet extraction for 48 hrs. Resulting extracts in were evaporated and concentrated to dryness using the rotatory evaporator and were stored at -4℃ till further uses.

2.4 Urease Inhibition Study

2.4.1 Screening of urease producing bacteria and inhibition assay for bacterial urease

Urease producing strains of bacteria were screened by using Christensen's urea agar. Urea base was prepared by dissolving peptone, dextrose, sodium chloride, potassium phosphate monobasic, urea and phenol red in 100 ml of distilled water, added aseptically to the autoclaved cooled agar solution. Inoculum from 18 to 24 h pure culture was used to streak the urea base slant surface and observed for a color change after incubating at 37℃. Urease production by bacterial strain was indicated by a bright pink color on the slant. Further antiurease activity of extracts against bacterial urease was determined by Lin et al. [20] method with slight modifications which is based on the verity that if urease is secreted by bacteria then bromocresol purple will be converted to purple due to the pH increase. Briefly, Urea and Bromocresol purple were added to the standard plating medium of the agar diffusion assay to a final concentration of 10 mM and 0.01 g per liter respectively (pH 6.0). Urease positive bacterial strains were cultured on nutrient agar and bored wells were loaded with plant extract. The plates were incubated at 37℃ for 24 h and the observed yellow zone due to low pH surrounding each well indicates the urease inhibition by tested plant extract.

2.4.2 Jack bean urease inhibition assay

Jack bean urease inhibition was measured through catalytic effects of urease on urea by measuring change in absorbance in the absence and in the presence of extracts. Thiourea was used as standard inhibitor. Reaction mixture comprising 1.2 ml of phosphate buffer solution pH 8.2, 0.2 ml of urease enzyme solution, and 0.1 ml of extract was subjected to incubation for 5 min. After pre-incubation, 0.5 ml (66 mM) of urea was added to the reaction mixture, and incubated for 20 min. Urease activity was determined by measuring the ammonia released during the reaction by modified spectrophotometric method described by Weatherburn [21]. The increase in absorbance was measured after 30 min at 640nm using spectrophotometer.

2.5 Collagenase Inhibition Study

2.5.1 Screening of collagenase producing bacterial strain

Nineteen bacterial strains were screened for collagenase production potential by using Balan et al. [22] method with slight modifications. Briefly, bacteria were cultured on gelatin agar plates and incubated for 24 h at 37℃. After incubation period the plates were observed for gelatin digesting bacteria by using 15% mercuric chloride in 20% concentrated HCl solution. Mercuric chloride solution precipitated proteins present on plate and gave it an opaque appearance. A clear zone around colonies, indicating the hydrolysis of gelatin substrate was checked for collagenase producing bacteria.

2.5.2 Gelatin digestion assay for collagenase type 1 inhibition

Collagenase inhibition was performed by gelatin digestion assay [23] with slight modifications. Agarose solution (1%) was prepared in collagenase buffer (50 mM TrisHCl, 10 mM $CaCl_2$, 0.15 M NaCl, 7.8 pH) with 0.15% gelatin and allowed to solidify in 6-well plate for 45 min at room temperature. Different concentrations of *E. officinalis* extract (30µl) were incubated with 50µl of bacterial collagenase-1 in 50 µl of collagenase buffer for 30 min. The reaction products (50 µl) were loaded into the wells made in gelatin–agarose gel and incubated for 18 h at 37℃. The degree of gelatin digestion was visualized by Coomassie Blue staining. Following destaining, the area of light translucent zone over blue background was determined to estimate collagenase activity. EDTA was used as positive control.

2.6 Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

The GC-MS was performed as previously described [24]. Briefly, Shimadzu Mass Spectrometer-2010 model was used for all measurements. 1 µl of sample dissolved into appropriate solvents was injected in GC-MS equipped with a split injector and a PE Auto system XL gas chromatograph interfaced with a Turbo-mass spectrometric mass selective detector system. The analytical column connected to the system was an Rtx-5 capillary column (length-60 m × 0.25 mm i.d., 0.25 µm film thickness).

2.7 Computational Study

2.7.1 Ligand and protein preparation

The structures of ligands which were selected from the GC-MS chromatogram were downloaded from PubChem and were geometrically minimized using OPLS_2005 force field by Ligprep module of Maestro 9.1 (Schrödinger suite, LLC). Ligprep produces a single, low energy, 3D structure for each input structure with various ring conformations, ionization states and tautomers using various criteria including molecular weight or specified numbers and types of functional groups present. Protein preparation and refinement studies were performed on urease (PDB ID: 3LA4) and collagenase (PDB ID: 1NQD) using protein preparation module (Schrödinger suite, LLC), which included removal of water molecules, charges stabilization, addition of hydrogen atoms, bond orders assignment and orientation of hydroxyl groups. Finally, energy minimization was carried out using default constraint of 0.3 Å RMSD and OPLS 2005 force field.

2.7.2 Molecular docking using GLIDE

GLIDE uses a hierarchical series of filters to search for possible locations of the ligand in the active site region of the receptor. The receptor grid was generated at the receptor site bound by a ligand. The ligands were then docked to the target proteins using Glide module of Schrodinger. The docking was done in Extra Precision mode (XP). Glide XP scoring protocols were used for the docking. The docked protein and the ligands were viewed with Glide Pose Viewer. Non-bonded interactions like hydrophobic was observed using LigPlot program and these interactions can increase the binding affinity between target drug interfaces.

2.7.3 ADME (Absorption, Distribution, Metabolism and Excretion) studies

The drug likeliness of docked compounds was done by ADME studies using QikProp 3 module of Schrodinger to get an idea whether the compounds are able to enter the higher phases of drug development process or not.

2.8 Statistical Analysis

All values are expressed as means ± standard deviation for separate groups for determinations in triplicates. The results were analyzed using analysis of variance (ANOVA). A value of $p < 0.05$ was considered statistically significant.

3. RESULTS AND DISSCUSSION

3.1 Urease Inhibition Study

Medically bacterial ureases are important virulence factors, which are implicated in the pathogenesis of many clinical conditions, detrimental for human and animal health [5], discovery of its inhibitors has, therefore, gained growing interest with time. In pharmaceutical applications, only acetohydoxamic acid and its derivative have gained the attention to be used as a potent drug element till date, which are even further associated with a number of side effects. So still there is a need and scope to explore extensively the potential of other inhibitors in pharmaceutical scenario, particularly from natural sources [25]. *E. officinalis* fruit and leaves extracts have been utilized for thousand years in the traditional medicine of India, China, Indonesia and Bangladesh for cure of several diseases.

3.1.1 Bacterial urease inhibition

In present investigation, to determine the potency of *E. officinalis* fruit extracts against bacterial ureases, firstly screening of 19 bacterial strains was done to select out the urease positive bacteria. The culture medium remains yellowish for urease negative bacterial strains. Out of the nineteen tested bacteria, five strains (*Staphylococcus aureus* 109, *Staphylococcus aureus* 3160, *Proteus vulgaris* 426*, Klebsiella pneumonia* 4030*,* and *Pseudomonas aeruginosa* 7453) were found to be urease positive. Further the bacterial urease inhibition assay was performed for these five urease positive bacterial strains. The transition range of bromocresol purple is between pH 5.2 to 6.8. The pH of the plates was 6.0 and the color was yellowish before inoculation with the tested bacterial strains. After inoculation and 24 h of incubation at 37℃, the are a with bacterial growth turned into purple, indicating that the bacteria were able to counter the pH of the medium by producing

ammonia through urease activity. When the plant extracts were loaded into the wells, the area around the wells did not change to purple, indicating that the bacteria did not counter the pH of medium and urease was likely inhibited. The results for the assessment of inhibitory activity of extracts on bacterial urease are listed in Table 1. *E. officinalis* methanol extract was found to be the more potent inhibitor of bacterial ureases as its Minimum Inhibitory Concentration (MIC) was very much lower than ethyl acetate fraction for all the bacterial strains.

3.1.2 Jack bean urease inhibition

In vitro study was also done on Jack bean urease enzyme which is reported to shares more than 50% similarity with bacterial urease and also it has been found that the inhibitors mechanism of action and the kinetics of inhibition for bacterial urease and Jack bean urease are similar [26]. Again the methanol extract (70.13%) showed more inhibitory potency for the enzyme than ethyl acetate (43.22%) fraction. Concentration dependent activities against Jack-bean urease were observed for the extracts (Fig. 1) and inhibitory effect increased together with increasing the concentration in the range of (100-1000 µg/mL).

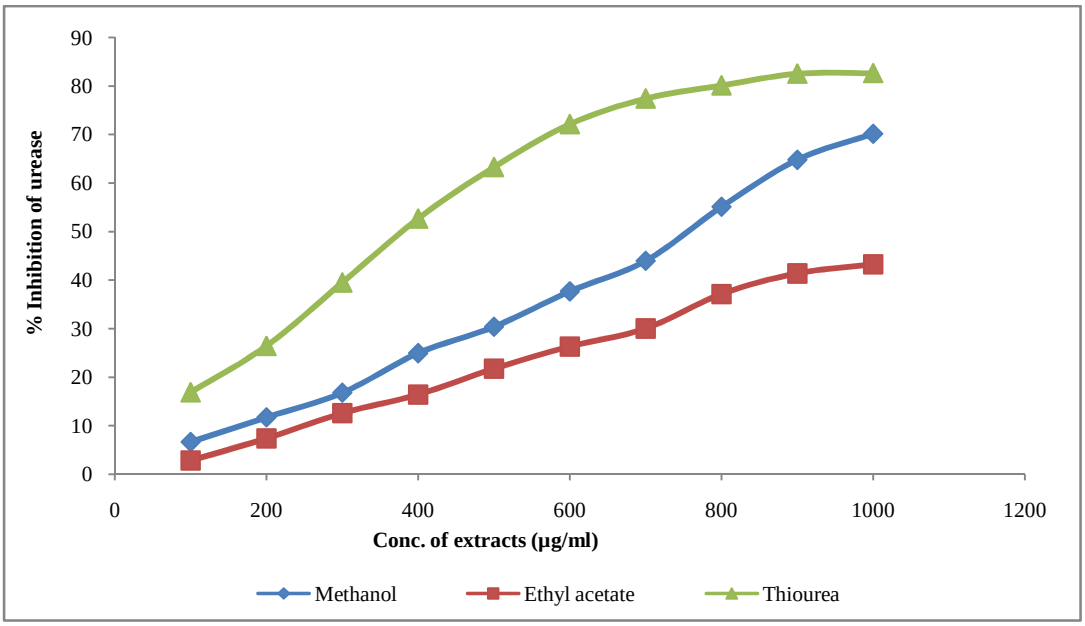


Fig. 1. Inhibition profile of *E. officinalis* against Jack bean urease

Further IC_{50}determination was done from dose response curve and the relevant data is presented in Table 1. Thiourea inhibited urease with an IC_{50}value of 0.49 mg/ml. Other studies are contradictoryto our results for the IC_{50} value of thiourea [27,28]. However, it is noteworthy that the value may vary with different assay conditions like temperature, enzyme concentration, cofactor required, substrate concentration etc.As long as we could explore, this study has not yet been conducted on this plant, so it can be considered a very first report on the simultaneous study of urease inhibition by *E. officinalis* extracts on purified enzyme as well as on urease producing bacterial strain.

3.2 Collagenase Type 1 Inhibition Study

Collagenase enzyme secreted by bacteria results in breakdown of natural defensive tissue planes in order to assist bacterial spread or to provide an environment suitable for the growth and burgeoning of bacteria. Enzyme inhibitors, particularly from natural sources are now an important part of the modern drug discovery research. A quinazolinedione alkaloid isolated from the fruits of *Evodia officinalis* have been reported to have Collagenase-1 inhibitory activity [29]. Aucubin isolated from *Eucommia ulmoides* has been found to inhibit MMP-1 [30]. Following this track, we studied extracts from *E. officinalis* for their potential inhibitory activity on bacterial collagenase 1.

3.2.1 Screening of bacteria for collagenase production

In present investigation, an attempt was made to screen out the collagenase producing bacterial strains, but in our 19 tested strains we didn't observe any collagenase activity. An earlier study also reported no collagenase production by different strains of *P. aeruginosa*, *S. epidermidis* and *S. aureus* collagenase, except a single strain of *S. aureus*, which showed collagenase activity only under anaerobic conditions [31]. Our results do not support the studies [32], who found collagenase activity in many strains of *Pseudomonas.*

3.2.2 Inhibitory effect of *E. officinalis* fruit on bacterial collagenase 1

After incubation of bacterial collagenase-1 with different concentrations of *E. officinalis* extracts (from 10 to 0.62 mg/ml in wells 2-6), the remaining gelatinolytic activity was compared with initial enzyme activity represented by the control in well 1 (Fig. 2.2).The initial group treated with reaction products of bacterial collagenase-1 and buffer exhibited the highest gelatinolytic activity in the discrete zone, represented no enzyme inhibition. However, gelatin digestion was clearly decreased following addition of *E. officinalis* extracts, and showed the dose dependent manner of collagenase inhibition (Fig. 2.1, 2.2).

An earlier report suggested that aqueous extract of *E. officinalis* fruit significantly inhibited the activity of collagenase type 2 [33]. Amla (*E. officinalis*) extract promotes also procollagen production and inhibits matrix metalloproteinase-1 in human skin fibroblasts [34]. Chanvorachote et al. demonstrated maximum of 78.67% inhibition of collagenase by *E. officinalis* extract in mouse fibroblasts [35]. We observed complete inhibition of collagenase type 1 by *E. officinalis* ethyl acetate extract (Fig. 2.2), while methanol extract inhibited 75.00%of the enzyme. A significant reduction was observed with 0.62 mg/ml or higher concentrations of extracts.IC_{50} value of the ethyl acetate and methanol extract of *E. officinalis* was 4.19 and 5.51 mg/ml respectively, so ethyl acetate extract was found to be more potent for inhibiting bacterial collagenase, almost equal to standard inhibitor EDTA.

3.3 GC-MS Analysis

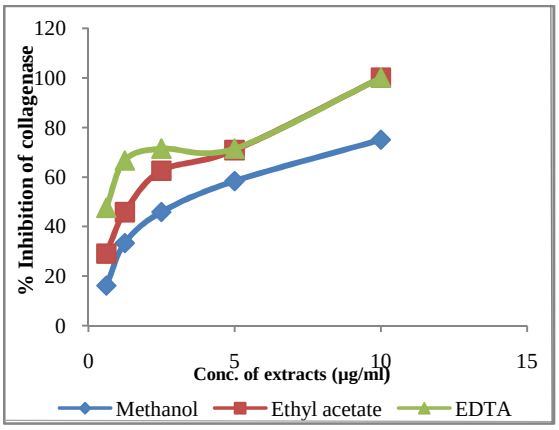
There is growing awareness in correlating the phytochemical constituents of a medicinal plant with its pharmacological activities. The demand for medicinal plant products has increased considerably because phytocompounds target the biochemical pathway which makes them safer than synthetic medicines. Hence keeping this in context, both the extracts of *E. officinalis* were studied thoroughly by GC-MS and their secondary metabolites were identified by doing NIST library search of the acquired mass spectral data**.** The results pertaining to GC-MS analysis led to the identification 28 and 30 compounds from methanol and ethyl acetate extracts of *E. officinalis* fruit respectively (Table 2 and 3).

Table 1. Inhibitory activity of *E. officinalis* extracts against baterial and Jack bean urease

***E. officinalis* extracts**	**Bacterial urease study (Minimum Inhibitory Concentration µg/ml)**					**Jack bean urease study**	
	***Staphylococcus aureus* 109**	***Staphylococcus aureus* 3160**	***Proteus vulgaris* 426**	***Klebsiella pneumonia* 4030**	***Pseudomonas aeruginosa* 7453**	**% Inhibition**	**IC_{50} value (mg/ml)**
Methanol	125	7.3	31.2	31.2	15.6	70.13±0.02	0.74
Ethyl acetate	125	31.2	500	62.2	62.2	43.22 ± 0.04	1.13

Table 2. GC-MS spectral analysis of methanol extract of *E. officinalis*

	Compound name	**Retention Time**	**Peak Area (%)**
1	Glucatonic anhydride	6.283	10.39
2	4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	9.655	3.09
3	5-hydroxymethylfurfural	12.300	14.91
4	1-Tridecene	14.932	0.52
5	Pentadecane	15.091	0.24
6	DL-Proline, 5-oxo-, methyl ester	15.400	0.62
7	2,6-Xylenol, 4,4'-dimethylene	15.524	16.85
8	Pyrogallol	16.093	23.96
9	Levoglucosan	17.707	2.36
10	1-Pentadecene	18.996	0.74
11	Tetradecane	19.131	0.26
12	Caprylone	20.576	0.18
13	1-Nonadecene	22.667	0.66
14	Palmitic acid, methyl ester	24.958	0.92
15	Palmitic acid	25.670	5.83
16	1-Nonadecane	25.997	0.36
17	Linoleic acid, methyl ester	27.629	0.59
18	9-Ooctadecenoic acid, mehyl ester	27.714	0.90
19	Heptadecane-(8)-carbonic acid-(1)	28.399	0.84
20	Oleic Acid	28.476	1.07
21	Stearic acid	28.744	1.53
22	1,2-Benzenedicarboxylic acid, 3-nitro-	33.974	0.18
23	D:A-Friedooleanan-7-one, 3-hydroxy-	34.488	0.39
24	Stigmast-5-en-3-ol, (3.beta.)-	41.505	0.39
25	Stigmast-5-en-3-ol, oleat	41.755	2.28
26	Stigmast-5-en-3-ol, (3.beta.)-	43.499	0.43
27	4,4,6a,6b,8a,11,11,14b-Octamethyl-1,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a,14,14a,14b-octadecahydro-2H-picen-3-one	43.594	9.04
28	Lup-20(29)-en-3-yl acetate	44.012	0.47



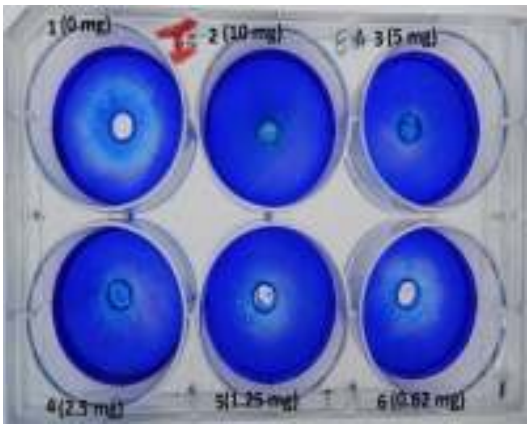


Fig. 2.1. Inhibition profile of *E. officinalis* extracts on bacterial collagenase-1.
Fig. 2.2. Collagenase inhibition demonstration on six well plate by *E. officinalis* ethyl acetate extract

3.4 Computational study

3.4.1 Docking studies

Molecular basis of interactions between target enzymes and phytocompounds can be implicit by docking analysis. We found an array of compounds in *E. officinalis* methanol and ethyl acetate extracts by GC-MS analysis. For docking analysis, we selected 10 compounds on the basis of their considerable percentage in respective extract and these were docked with urease and collagenase to screen out the potential compound(s) that could best interact with the target enzymes. Selected compounds with their PubChem ID, docking score and number of bonds are summarized in Table 4.

Glide predictions revealed that with urease enzyme, except docosane, all compounds showed negative dock score and good score was obtained in case of palmitic acid, xylenol and oleic acid. We noticed that Lys716, Asp730, Lys709, Glu718, Val744, Glu742 amino acids were playing vital roles in bond formation and they are the crucial amino acids for efficient binding of the docked compounds. Xylenol made maximum number of bonds, 2 hydrogen bonds by cl¯of xylenol and Lys709 and Glu718 amino acids of urease and a salt bridge by its oxygen atom and Lys716 residue of urease were observed (Fig. 3.1, 3.2). Besides these bonds, xylenol made a number of hydrophobic interactions with Val444, Leu839, Phe838, Met746, Phe712, Tyr837 and Pro717 residues of urease (Fig. 3.2). These interaction patterns help to anchor compounds in the binding pocket of enzyme.

When the compounds were docked with collagenase enzyme, almostall of them showed good dock score (-4.08688 to-9.34014) in comparison to reference EDTA(-2.7882). Sitostenone, erucylamide, vitamin E, and xylenol interacted strongly with collagenase having dock score in range of -8.00 to-9.00. Asp947, Gly921. Tyr932, Tyr957, Asp930, Asp947, Glu921 and His959 were common amino acids which played role in bond formation with all the docked compounds. We depicted the binding pattern of erucylamide in Fig. 4.1, which have dock score comparable to sitostenone and formed 2 hydrogen bonds, one between NH_2 of erucylamide and Asp947 and other between its oxygen atom and Gly921 amino acid residue of enzyme. Besides hydrogen bonds, several hydrophobic interactions (Phe952,

Tyr931, Leu958, Phe915, Phe934, Val942, Trp956, Tyr932, Val954, Leu946, Leu923, Met919, Ile944, Ile912) with active site residues of collagenase were also found and are clearly revealed in Fig. 4.2.

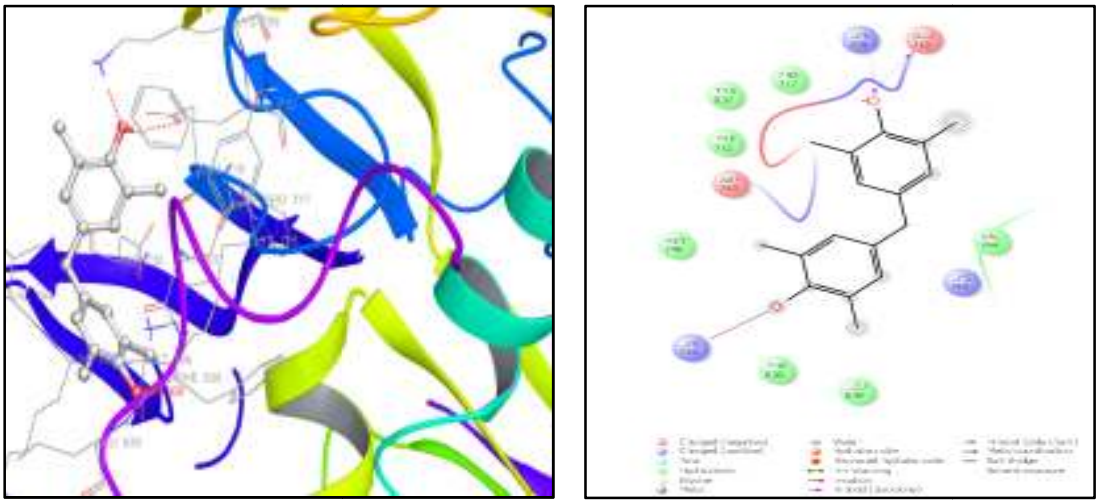


Fig. 3.1. Docking structure between xylenol and urease
Fig. 3.2. Interaction of xylenol with amino acid residues of urease

Our *in vitro* study clearly revealed methanol and ethyl acetate extracts as more potent inhibitor of urease and collagenase respectively. These *in silico* findings are well supporting our *in vitro* studies. Since dock score in urease was best among compounds of methanol extract, while in collagenase study, ethyl acetate extract constituents were more acceptable.

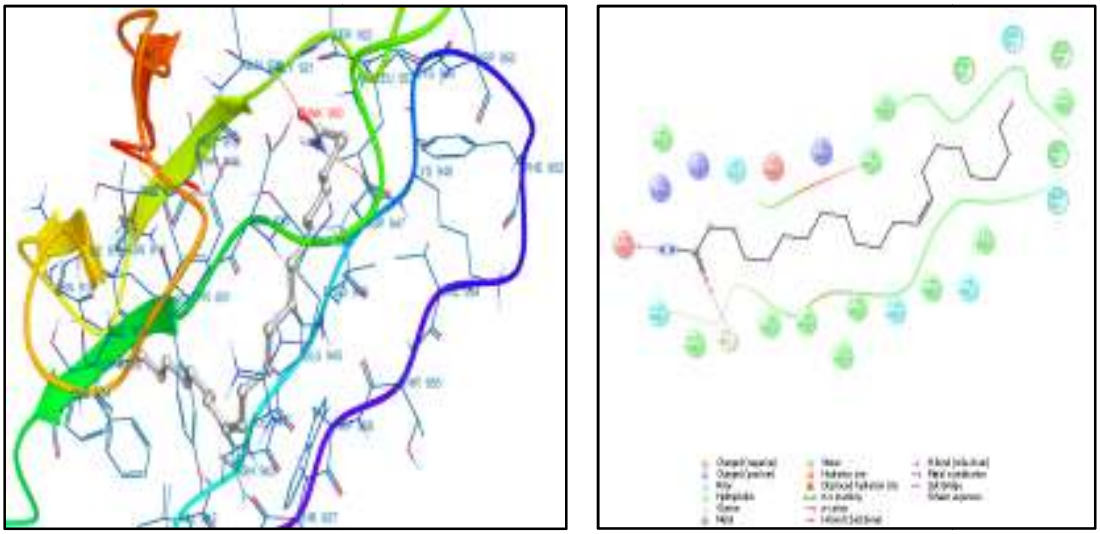


Fig. 4.1. Docking structure between erucylamide and Collagenase
Fig. 4.2. Interaction of erucylamide with and acid residues of collagenase

3.4.2 Principal descriptors and ADME analysis

Poor drug-likeness and pharmacokinetic properties of lead compounds are considered to be major reasons for drug failures. Therefore, the preclinical pharmacokinetic evaluation should be comprehensive enough to ensure that compounds do not fail in the clinic. Computer aided method is an easy tool to search such kinds of biologically active compounds with drug-likeness properties. Ten principal descriptors and nineteen predicted ADME properties were calculated by QikProp software. Principal descriptors, for compounds and their range in 95% of drugs are revealed in Table 5.

A large number of ADME properties of the docked compounds were calculated and are given in Table 6 with their range in 95% of drugs.

ADME properties predicted were not found to be in favor of glucatonic anhydride, docosane, vitamin E, sitostenone, and also the standard inhibitors EDTA and thiourea, so these may not be a potent drug candidate. It is observed that xylenol and erucylamide are very closely similar to the drug-like compounds and these two didn't even show a single violation for principal descriptors and ADME properties.

Table 3. GC-MS spectral analysis of ethyl acetate extract of *E. officinalis*

Peak no.	Compound name	Retention time	Peak area (%)
1	Oxalic acid, isobutyl pentyl ester	6.235	0.77
2	Undecane, 3,7-dimethyl-	7.427	3.30
3	2,3,6,7-Tetramethyloctane	8.249	0.91
4	Decane, 3,7-dimethyl-	8.406	4.62
5	3-Tetradecene, (Z)-	10.443	0.88
6	Tridecane	10.625	0.93
7	5-isobutyl nonane	12.433	2.85
8	1-Tridecanol	13.028	0.75
9	Hexadecane	13.327	0.64
10	Docosane	13.457	9.25
11	5-isobutyl nonane	13.671	1.55
12	Heptadecane	15.075	1.35
13	1-Hexadecanol	18.989	1.12
14	Oxalic acid, ethyl neopentyl ester	19.124	4.78
15	Phthalic acid, diisobutyl ester	24.102	0.97
16	Palmitic acid, methyl ester	24.865	1.59
17	Pentadecanoic acid	25.563	3.75
18	Dichloroacetic acid, heptadecyl ester	25.998	1.05
19	9-Tetradecen-1-ol	27.112	0.84
20	9-Octadecenoic acid, methyl ester	27.633	1.51
21	Oleic acid, methyl ester	27.732	1.30
22	Oleic Acid	28.369	26.09
23	Octadecanoic acid	28.629	3.29
24	Erucylamide	28.958	5.00
25	3-Bromocholest-5-ene	40.986	3.20
26	Gamma-tocopherol	41.437	4.67
27	Acetic acid, (1,2,3,4,5,6,7,8-octahydro-3,8,8-trimethylnaphth-2-yl)methyl ester	41.852	1.03
28	Vitamin E	41.994	4.14
29	1,3-Cyclopentanedione, 2-bromo-	42.333	2.74
30	Sitostenone	44.696	5.12

Table 4. Docking score of compounds and standard inhibitors with target enzymes

Compounds	CID	Uresae		Collagenase	
		Docking Score	Bonding	Docking Score	Bonding
Docosane	12405	0.1553	0	-6.14	0
Oleic acid	445639	-2.01716	2 H-bond	-5.17353	1 H-bond
Erucylamide	5365371	-1.50113	2 H-bod	-8.92922	2 H-bond
Vitamin E	2116	-1.48625	1 H-bond, 1n-cation	-8.83529	1 H-bond
Sitostenone	5484202	-1.19849	1 H-bond	-9.34014	1 H-bond
Glucotonic anhydride	574367	-1.54638	1 H-bond	-4.08688	1 H-bond
5-Hydroxymethylfurfural	237332	-1.86329	1 H-bond	-5.82994	1 H-bond
Xylenol	79345	-2.09333	2 H-bond,1 salt bridge	-8.17329	1 H-bond
Pyrogallol	1057	-1.03549	3 H-bond	-5.86316	2 H-bond
Palmitic acid	985	-2.35846	1 H-bond	-4.40745	1 H-bond
Thiourea	2723790	-2.18737	3H-bond	-	-
EDTA	6049	-	-	-2.7882	3 H-bond

Table 5. Principal descriptors for compounds and their range in 95% of drugs

Principal descriptors	Docosane	Oleic acid	Erucyla-mide	Vitamin E	Sitostenone	Glucotonic anhydride	5-Hydroxy methyl furfural	Xylenol	Pyrogallol	Palmitic acid	EDTA	Thiourea	Range of 95% drugs
MW	310.605	282.465	337.588	430.713	412.698	112.085	126.112	256.344	126.112	256.428	292.245	76.120	130-725
SASA	814.769	709.768	850.986	799.839	686.762	270.638	319.07	547.108	300.357	667.169	506.04	578.037	300-1000
FOSA	814.769	592.174	725.13	740.572	599.152	53.045	76.288	344.252	0	558.833	129.406	392.471	0-750
FISA	0	97.505	105.903	35.653	57.292	136.477	143.404	70.936	148.112	108.336	376.634	137.486	7-330
PISA	0	20.089	19.953	23.632	30.318	81.115	99.378	131.92	152.245	0	0	14.392	0-450
WPSA	0	0	0	0	0	0	0	0	0	0	0	0	0-175
MV	1410.5310	1207.178	1463.877	1533.448	1372.615	398.257	474.622	945.363	445.549	1116.717	874.274	723.322	500-2000
donorHB	0	1	2	1	0	0	1	2	3	1	4	3	0-6
accptHB	0	2	2.5	1.5	2	4.5	4.2	2.5	2.25	2	12	14	2-20
glob	0.746538	0.772496	0.732677	0.804036	0.869743	0.96728	0.92224	0.851448	0.939271	0.780233	0.873799	0.832937	0.75-0.95

**MW: Molecular Weight, SASA: Total solvent accessible surface area, FOSA: Hydrophobic SASA, FISA: Hydrophilic SASA, PISA: Carbon Pi SASA, WPSA: Weakly Polar SASA, MV: Molecular Volume, donorHB: Donor-Hydrogen Bonds, accptHB: Acceptor- Hydrogen Bonds and Glob: Globularity*

Table 6. Predicted ADME properties for compounds and their range in 95% of drugs

Proper -ties predict -tion	Docosa-ne	Oleic acid	Erucyla-mide	Vitamin-E	Sitoste-none	Glucoto-nic anhydride	5-Hydroxy methyl furfural	Xylenol	Pyrogal-lol	Palmitic acid	EDTA	Thiourea	Range of 95% drugs
Stars	15	1	3	5	4	9	3	1	3	3	5	7	0-5
CNS	2	-2	-2	0	0	-1	-1	0	-1	-2	-2	1	-2-2
Polrz	38.733	32.718	40.421	47.093	45.215	10.559	11.863	30.371	11.204	29.548	22.058	22.732	13-70
logPC16	11.849	10.582	13.655	13.092	11.511	3.814	4.545	8.863	5.272	9.59	10.063	13.557	4-18
logPoct	10.901	12.55	16.488	16.889	15.957	6.831	8.024	13.137	9.005	11.599	20.303	28.068	8-35
logPw	-5.665	2.353	6.414	2.122	2.614	6.283	7.078	5.94	8.433	2.408	18.766	20.605	4-45
logPo/w	12.433	5.886	5.877	8.133	6.915	-1.148	-0.192	3.99	0.104	5.206	-3.381	-1.573	-2-6.5
logS	-13.496	-6.058	-6.395	-8.258	-7.207	1.01	-0.707	-4.733	-0.335	-5.432	0.91	-4.575	-6.5-0.5
CIlogS	-13.496	-4.111	-4.286	-7.808	-7.201	0.339	-0.714	-4.331	-1.207	-3.576	1.192	-4.046	-6.5-0.5
logHERG	-5.676	-3.502	-4.578	-4.816	-3.784	-2.448	-3.264	-4.565	-3.241	-3.281	2.289	-5.032	Concern below -5
PCaco	9906.038	298.455	516.501	4549.65	2835.3	503.138	432.513	2104.825	390.259	235.587	0.003	48.639	≤25 poor, ≥500 great
logBB	1.842	-1.43	-1.897	-0.587	-0.303	-0.409	-0.741	-0.33	-0.737	-1.467	-2.572	2.174	-3-1.2
PMDCK	5899.293	170.285	484.502	2544.171	1526.005	235.456	199.944	1105.88	178.917	131.874	0	33.259	≤25 poor, ≥500 great
logKp	6.575	-1.806	-1.577	-0.845	-1.893	-3.749	-3.524	-1.978	-3.425	-2.172	-11.573	-5.559	-8- -1
Metab	0	3	4	5	2	1	2	7	3	1	6	3	1-8
logKhsa	1.952	0.727	0.881	2.013	1.813	-1.43	-0.869	0.59	-0.81	0.514	-1.825	-0.558	-1.5-1.5
% HOA	100	92.748	96.957	100	100	68.58	73.002	100	73.934	86.924	0	45.631	≥80% high, ≤25% poor
Rule of 5	1	1	1	1	1	0	0	0	0	1	0	1	Max is 4
Rule of 3	1	1	1	1	1	0	0	1	0	0	1	1	Max is 3

Stars: Number of property or descriptor values that fall outside the 95% range of similar values for known drugs, CNS: Predicted central nervous system activity, Polrz: Polarizability, logPC16: logP for hexadecane/gas, logPoct: logP for octanol/gas, logPw: log P for water/gas, logPo/w: log P for octanol/water, logS: aqueous solubility, CIlogS: logS-conformation independent, logHERG: HERG K+ channel blockage, PCaco: apparent Caco-2 permeability, logBB: brain/blood barrier, PMDCK: apparent MDCK permeability, logKp: skin permeability, Metab: No. of primary metabolites, logKhsa: serum protein binding, pHOA: percentage human oral absorption, Rule of 5: Lipinski rule of 5 violations, Rule of 3 : Jorgensen rule of 3 violations

4. CONCLUSION

In vitro and *in silico* experiments validate the activity of *E. officinalis* extracts by effectively inhibiting the virulence enzymes viz. urease and collagenase. *In vitro* study revealed that methanol extract (1 mg/ml) inhibited 70.13% urease activity and ethyl acetate extract (10 mg/ml) completely inhibited the collagenase enzyme. GC-MS chromatogram displayed compounds, xylenol and erucylamide further confirmed the urease and collagenase inhibitory activity by various parameters of *in silico* study including docking and ADME prediction. Therefore, these two compounds from *E. officinalis* fruit are putative target for designing drugs against pathogenic enzyme urease and collagenase.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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