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In vitro antioxidant, antidiabetic, and *in silico* docking studies on diabetic wound healing potential of identified compounds of methanolic extract of marine sponge, *Agelas clathrodes* (Schmidt, 1870) by GC-MS and LC-MS analysis

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Abstract

Marine sponge, *Agelas clathrodes* (Schmidt, 1870) is an excellent producer of secondary metabolites, including glycosphingolipid derivatives (GSLs) and bromopyrrole alkaloids. Different secondary metabolites from this species showed immunomodulatory, anti-inflammatory, cytotoxicity to cancer cells, antimicrobial, antifungal, and antihistaminic activity. An enervating complication of diabetes mellitus is diabetic wounds/ulcers, which lead to morbidity in patients. The majority view is that changes in vascular basement membrane in tissues, down-regulation of collagen expression, and disorders in immune response exacerbate pain and wound healing disorders. In this study, antioxidant and antidiabetic properties of methanolic extract of marine sponge were investigated using various *in vitro* assays. The methanolic extract showed good activity. The methanolic extract was subjected to GC-MS and LC-MS analysis to identify chemical constituents. This showed the presence of pyrrole alkaloids and terpenoids. *In silico* docking studies of identified compounds were carried out using Auto Dock Vina against 4 different proteins matrix metalloproteinases-9 (MMP-9), human interleukin-6 (IL-6), fibroblast growth factors (FGF), histone deacetylase 4 (HDAC4). The compounds asthaxanthine and oxysceptrin from the methanolic extract showed more affinity towards all the proteins and the compounds considered as good choice for diabetic wound healing.

1. Introduction

Globally, more than 500 million people suffer with diabetes. One in thirty of these DM patients will experience chronic wound healing, which might result in the amputation of a lower limb and negatively impact the patient's quality of life. Due to deregulated angiogenesis, high oxidative stress, a chronically sustained suboptimal inflammatory response, neuropathy, microvascular complications, and persistent bacterial colonization, as well as psychological issues with the patient's mental health, self-esteem, and family cohesion, the pathophysiology of diabetic wounds is exceedingly complex (Jozic *et al*., 2021; Brem and Tumic-Canic, 2007). Antioxidants preserve non-toxic reactive oxygen species (ROS) levels in the injured tissue, which may promote healing (Aggarwal *et al.,* 2022). Enzymatic or nonenzymatic processes that take place in the external environment, in organelles like the mitochondria, or intracellularly in the cytosol can be the mechanism by which antioxidants function (Devi and Signh, 2021). Humans with chronic wounds have a high MMPs and the endogenous regulatory process are not properly regulated, which causes the newly created extracellular matrix (ECM) to degrade (Mokthar, 2021).

IL-6 function dysregulation is frequently seen in a variety of diabetes problems. The inflammatory and tissue remodeling stages of wound healing are linked to the pleiotropic cytokine IL-6. A lack of IL-6 causes an early phase delay in the healing of wounds. Fibroblast growth factors are a class of cell signaling proteins that promote angiogenesis and granulation tissues that produce fibroblasts in multiplicity. It is an effective chemoattractant and mitogen for fibroblasts and endothelial cells. Tissue covers the wound's cavity and surrounding space during the early phases of the wound-healing process (Liu *et al.,* 2021). The marine sponge, *A. clathrodes* was a great source of alkaloids and glycosphingolipid derivatives (GSLs), among other secondary metabolites. Sipekema *et al.* (2005) reported that alkaloids had antibacterial activity against *Staphylococcus aureus*, *Klebsiella pneumonia*, and *Proteus vulgaris*. Secondary metabolites such as glycosphingolipid derivatives (GSLs) and bromopyrrole alkaloids were produced in large quantities by the marine sponge, *A. clathrodes*. Various secondary metabolites from this species have shown antibacterial, antifungal, antihistaminic, cytotoxic to cancer cells, and immunomodulatory properties (Lee *et al*., 2020).

Figure 1: Marine sponge, *A. clathrodes* (Schmidt, 1870).

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2. Materials and Methods

2.1 Preparation of sample

From Rameswaram, Tamil Nadu, India's subtidal and intertidal zones, marine sponges were collected. Depending on how many of each species there were, samples were gathered in large quantities and then cleaned with freshwater to get rid of any attached detritus and related biota. The laboratory received the collected samples and kept them in a chilled box. Further, the sponge samples are labeled properly and stored at -7°C.

Ground crude marine sponge powder is placed in a Soxhlet extractor using methanol as a solvent and is filtered through Whatman filter paper. The filtrate was concentrated on a rotary evaporator to remove the solvent completely.

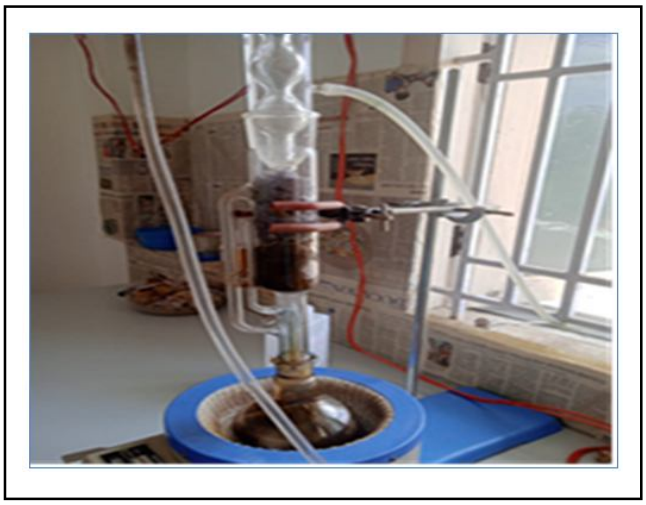

Figure 2: Extraction of marine sponge, *A. clathrodes*.

2.2 GC-MS examination of methanolic extract of marine sponge, *A. clathrodes*

Using Agilent, the chemical components of the extract were identified. A 1.0 ml/min flow rate of helium gas was employed as the carrier gas. The National Institute of Standard and Technology's NIST library's known chemicals were compared to the mass spectra and fragmentation patterns that were produced (Hajji *et al.,* 2010; Sawada and Hirai, 2013).

2.3 LC-MS examination of a methanolic extract of marine sponge, *A. clathrodes*

Agilent system was used for the LC-MS analysis of the methanolic extract. We used an X-bridge C-18 column for chromatographic separation. 5% solution B (100% acetonitrile) and 95% solvent A (0.1% formic acid in water) were maintained in the column. By comparing spectra with those of existing legitimate standards, substances can be identified using their UV, FTIR, and mass spectra and by comparing the spectra to accepted, trustworthy standards (Hajji *et al*., 2010; Sawada and Hirai, 2013; Turkan *et al*., 2019).

2.4 Antioxidant activity-DPPH assay

2.4.1 Preparation of the reagent

4 mg of DPPH were dissolved in 100 milliliters of methanol to create a 0.1 ml DPPH solution.

2.4.2 Operating process

Various loudness levels LC-MS study of methanolic extract from *A. clathrodes* marine sponges. After adding 2.96 ml of DPPH (0.1 mM) solution, sample extracts were prepared up to 40 µl using DMSO. The reaction mixture was incubated for 20 min at room temperature under dark circumstances. The UV-Vis spectrophotometer was used to measure the mixture's absorbance at 517 nm after 20 min. A control of 3 ml of DPPH was used (Turkan *et al*., 2019)

$$\% \text{ of antioxidant activity} = [(Ac - As) \div Ac] \times 100$$

where, Ac: Control reaction absorbance; As: Testing specimen absorbance.

2.5 Antidiabetic activity α-amylase inhibition assay

Following a 10 min preincubation period at 25°C, 100 µl of α-amylase solution (4.5 units/ml/min) and 0.02 M sodium phosphate buffer (pH 6.9) were added to varying concentrations of sample extract (Worthington *et al*., 1993). After that, 100 µl of 1% starch solution was added, and the reaction was stopped by adding 1.0 ml of dinitro salicylic acid reagent. This was then incubated at 25°C for 30 min. After five min of incubation in a boiling water bath, the test tubes were allowed to cool to room temperature. After diluting the reaction mixture ten times with distilled water, the absorbance at 540 nm was determined (Wickramaratne *et al*., 2019). The percentage of α-amylase enzyme and the control, which has buffer instead of extract, were the subjects of the readings.

2.6 Molecular docking studies

2.6.1 Protein preparation

The three-dimensional structure of selected proteins was retrieved from the RCSB database in the protein data bank in PDB format. The selected proteins with PDB code and active sites are listed in Table 1.

Download the co-crystallized structure of the protein from pdb (pdb file). Download the ligand from Pubchem (sdf file). Conversion of sdf to pdbqt using open babel gui. Prediction of active binding site using Discovery Studio (Arya *et al,* 2022). Using the current selection of the co-crystallized ligand, we can obtain the attributes of the binding site. sphere xyz coordinates which are required in the config file for docking. Prepare the protein by deleting water applying polar hydrogen adding Kollman charges and saving it as pdbqt file. Prepare config file which contains receptor, ligand, output, and XYZ coordinate information software, protein, ligand, and config files are added to a new folder. Open the terminal in the folder and give the commands of config and log to conduct molecular docking (AutoDock Vina.v.1.2.7). The binding affinity of different conformation is listed. The first conformation with the most binding affinity is considered the most suitable ligand (Mokthar, 2021).

3. Results

3.1 DPPH free radical-scavenging activity

Different concentrations of methanolic extract were taken for assay and IC_{50} values (63 µg/ ml) were determined. Results were compared with positive standard ascorbic acid (36.5 µg/ml). Results obtained suggest that methanolic extract significantly showed free radical scavenging activity. Results were tabulated in Table 2.

Table 1: Protein with protein data bank code and their active sites

S .No.	Name of protein	Protein data bank code	Active sites
1.	Matrix metalloproteinases -9 (MMP-9)	4XCT	SITE 1 AC1 16 LEU A 187 LEU A 188 ALA A 189 LEU A 222 SITE 2 AC1 16 HIS A 226 GLU A 227 HIS A 230 HIS A 236 SITE 3 AC1 16 LEU A 243 TYR A 245 PRO A 246 MET A 247 SITE 4 AC1 16 TYR A 248 ZN A 302 PGO A315 HOH A 501
2.	Human interleukin-6 (IL-6)	1IL6	SITE 1 AC1 9 TYR A 32 ARG A 100 GLU A 101 SER A 102 SITE 2 AC1 9 TYR A 103 SER A 108 HOH A2069 HOH A2351 SITE 3 AC1 9 HOH A2353
3.	Fibroblast growth factors (FGF)	1BAS	SITE 1 HPA 18 ASN A 18 LYS A 112 LYS A 113 ASN A 114 SITE 2 HPA 18 GLY A 115 SER A 116 CYS A 117 LYS A 118 SITE 3 HPA 18 ARG A 119 GLY A 120 PRO A 121 ARG A 122 SITE 4 HPA 18 THR A 123 HIS A 124 TYR A 125 GLY A 126 SITE 5 HPA 18 GLN A 127 LYS A 128
4.	Histone deacetylase -4	HDAC4	SITE 1AC1 16 LEU A 243 TYR A 245 PRO A 246 MET A 247 SITE 2HPA 18 ARG A 119 GLYA 120 PRO A 121 ARG A 122

Table 2: Antioxidant activity of methanolic extract of *A. clathrodes* by DPPH assay

S. No.	Sample	20 µg	40 µg	60 µg	80 µg	100 µg	IC_{50}
1.	Methanolic extract	12.8 %	30.7%	48.9%	66.0%	79.5%	63
2.	Ascorbic acid (standard)	25.7%	55.6%	84.3%	115%	147%	36.5

3.2 *In vitro* antidiabetic activity by α-amylase inhibition

In vitro the antidiabetic activity of methanolic extract showed an IC_{50} value of 48.92. The percentage α-amylase inhibition was carried out in 5 different concentrations (20, 40, 60, 80,100 µg/ml). The result is shown in Table 3. Methanolic extract showed good antidiabetic activity by inhibiting α-amylase enzyme.

Table 3: Percentage α-amylase inhibition (%) by methanolic extract of *A. clathrodes*

Concentration (µg/ml)		Percentage α-amylase inhibition (%)
Control	Methanol	Acarbose (standard)
20	10.27	22.41
40	24.23	29.06
60	40.5	38.74
80	52.5	43.27
100	83.7	48.45
IC_{50}	48.92	52.6

3.3 GC-MS analysis of the methanolic extract of *A. clathrodes*

GC-MS analysis was performed on the marine sponge's methanolic extract. There were twenty-five components found in the methanolic extract. Terpenoids, alkane hydrocarbons, steroids, and saturated and unsaturated fatty acids make up a large number of these. Oxime-, methoxy-phenyl-,2(3H)-furanone, dihydro-3-hydroxy-4,4-dimethyl-, methyl acetic acid, 2-tridecyl ester 1-bromodocosane alpha -ergost-8(14)-ene, cholest-5-ene, and β-sitosterol is among the major compounds. (Zang *et al.,* 2023). The list of components examined by GC-MS together with their retention duration is displayed in Table 4 (Thilak *et al.,* 2023).

Table 4: GC-MS analysis of methanolic extract of marine sponge, *A. clathrodes*

S. No	Retention time (min)	Compound identified in methanol extract
1.	4.463	Oxime-, methoxy-phenyl-
2.	5.58	Phenol
3.	5.94	1,4-Benzenediamine, 2-methyl-
4.	6.604	2(3H)-Furanone, dihydro-3-hydroxy-4,4-dimethyl-
5.	6.987	1-Pyrrolidinecarbonitrile
6.	7.975	Benzoic acid
7.	8.997	Propanedioic acid,
8.	7.975	Benzoic acid
9.	8.997	Propanedioic acid
10.	9.953	Phthalic anhydride
11.	10.786	1H-Imidazole, 1-methyl-4-nitro
12.	10.789	Pyrazole, 1-methyl-4-nitro-
13.	10.987	3,4-Difluorobenzoic acid, 4-dodecyl ester
14.	11.124	2 2-Propenoic acid,
15.	12.563	4-Amino-2,6-dihydroxy pyrimidine
16.	12.164	Benzoic acid, 4-ethoxy-, ethyl ester
17.	17.518	cis-Vaccenic acid
18.	17.418	9,12,15-Octadecatrienoic acid, methyl ester,
19.	17.185	9,12-Octadecadienoic acid (Z, Z)-, methyl ester
20.	16.037	3,5-Difluorobenzaldehyde carbamoyl hydrazone
21.	19.429	1-Docosene
22.	20.007	15-Hydroxypentadecanoic acid
23.	17.697	Octadecanoic acid
24.	19.429	1H-Imidazole, 1-methyl-4-nitro
25.	22.051	β-Sitosterol

3.4 LC-MS analysis of the methanolic extract of *A. clathrodes*

The methanolic extract of the marine sponge was subjected to LC-MS analysis. A total of 12 compounds were identified in the methanolic extract. The compounds include pyrrole alkaloids, antioxidant pigments, and anti-histaminic, anti-inflammatory marine sponge compounds. Table 5 showed 12 compounds including cyclic carbomoroidinylpyridone derivative, hydroxy indole 3- acetic acid, kaempferal, oroidin, bromo 2- pyrrole carboxamide, oxysceptrin,4,5-dibromopyrrole-2-carboxyllicacid, longamide B, dihydrosp-ongacidine, asthaxanthine, fucoxanthine, bromohymeniladisine.

Table 5: LC-MS analysis of methanolic extract of marine sponge, *A. clathrodes*

S. No	Detected compounds	Retention time (min)	Relative abundance
1.	Bromo 2- pyrollecarboxamide	1.7	171.0
2.	Oxysceptrin	2.0	151.1
3.	4,5-dibromo pyrrole -2-carboxylic acid	2.4	184.2
4.	Longamide B	2.87	225.2
5.	Cyclic carbomylpyridoneE derivative	5.416	272.6
6.	Hydroxy indole -3-acetic acid	7.106	181.1
7.	Kaempferol	7.74	331.2
8.	Oroidin	8.60	184.2
9.	Fucoxanthine	9.53	576
10.	Dihydrospongacidine	9.32	334.5
11.	Axaxanthine	11.4	386.5
12.	Bromohymenialdisine	11.9	141.1

3.5 Molecular docking studies of GC-MS and LC-MS identified compounds

Compounds identified by GC-MS and LC-MS were used in molecular docking investigations with AutoDockvina 1.2.7 software. The chemicals detected by GC-MS revealed that 1-methyl-4-nitro (-9.7) and 1H-imidazole had the highest affinity to the HDAC4 protein. Dihydrospongacidine (-9.7), the ligand, exhibited greatest interaction with protein HDAC4 according to the LC-MS investigations. Fucoxanthine and astaxanthine, two substances, had a higher binding affinity of -7.2 with fibroblast growth factor. Oxysceptrin demonstrated an affinity of -7.3 for human interleukin-6.

Table 6: Binding energies of ligand-receptor from Auto Dock Vina (LC-MS identified compounds)

S.No.	Name of compounds	PDBID -1BAS (kcal/mol)	PDB ID -1IL6 (kcal/mol)	PDB ID - 4XCT (kcal/mol)	PDB ID2VQM (kcal/mol)
1	Cyclic carbomoroidinylpyridone derivative	-6.9	-7.3	-9.8	-6.2
2	Hydroxy indole 3- acetic acid	-5.2	-5.4	-8.6	-9.5
3	Kaempferol	-7.9	-8.3	-11.8	-6.7
4	Oroidin	-6.1	-6	-9.2	-5
5	Bromo 2- pyrollecarboxamide	-3.4	-3.6	-5.3	-8.3
6	Oxysceptrin	-7	-7.3	-10.9	-6
7	4,5-dibromo pyrrole -2-carboxylic acid	-3.9	-4.1	-6.2	-5.8
8	Longamide B	-4.9	-5	-6.8	-3.4
9	Dihydrospongacidine	-4.1	-4.2	-6.7	-9.7
10	Asthaxanthine	-7.2	-6	-10.2	-9.1
11	Flucoxanthine	-7.2	-6.9	-10	-4.9
12	Bromohymeniladisine	-7.9	-8.3	-11.8	-5.2

Table 7: Binding energies of ligand-receptor from Auto Dock Vina (GC-MS identified compounds)

S. No	Name of compound	PDB ID -1BAS (kcal/mol)	PDB ID -1IL6 (kcal/mol)	PDB ID – 4XCT (kcal/mol)	PDB ID:2VQM (kcal/mol)
1	1-Docosene	-4.9	-5.3	-4.9	-5.4
2	1-dodecanol	-4.8	-4.6	-4.5	4.6
3	1-pyrrolidone carbonitrile	-4	-3.7	3.3	3.6
4	1,4 benzenediamine 2 methyl	-5	-4.7	-4.4	-4
5	Bromo 2- pyrrole carboxamide	-3.4	-3.6	-5.3	-8.3
6	1H -imidazole,1 methyl 4-nitro	-7.1	-6.9	-5.9	9.8

7	2,2 propionic acid	-5.9	-4.8	-5	-4.8
8	2(3H) furanone	-4.9	-4	-3.8	-3.4
9	3,4 difluoro benzoic acid	-6.1	-5.2	-6.7	-6
10	9,12, 15octadecatrionic acid	-5.2	-4	-4.2	-5.1
11	15- hydroxy pentadecanoic acid	-6.6	-5.5	-3.7	-6.1
12	Benzoic acid	-5.6	3.8	-4.8	-5.2
13	Cholest-5-ene -	-4.9	-4.7	-4.1	-4.6
14	Cis -vaccinic acid	-5.8	-5.1	-4.9	-5.9
15	Octadecanoic acid	-5.2	-5.1	-4	-3.9
16	Oxime methoxy phenyl	-6.1	-5.1	-4.7	-4.7
17	Phenol	-4.9	-3.7	-5.3	-4.2
18	Phthalic anhydride	-6	-5.9	-5.3	-5.3
19	Beta-sitosterol	-7.6	-6.9	-7	-6.4
20	Pyrazole 1 methyl 4 nitro	-5	-4.3	-4.8	-5
21	Propanedioic acid	-7.3	-5.8	-7.1	-4.3

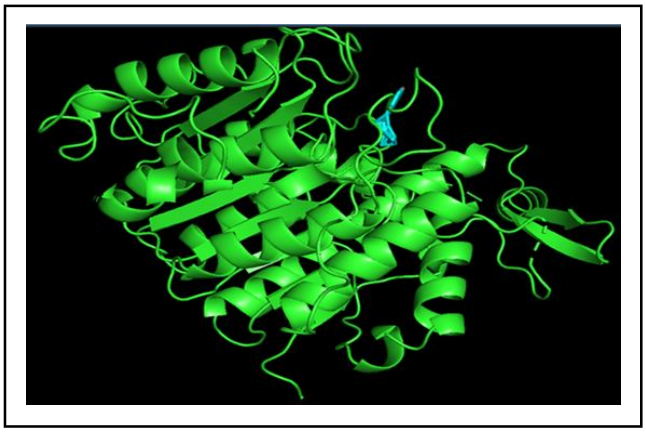

Figure 3: GC-MS identified compounds, 1H-imidazole, 1-methyl-4-nitro (-9.7) with maximum binding HDAC4 protein.

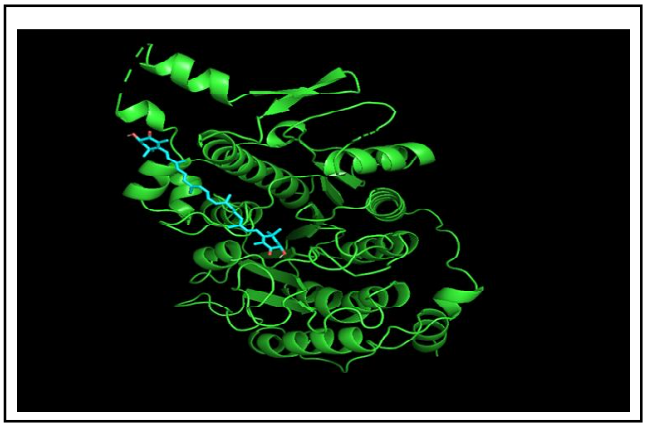

Figure 4: THE LC-MS identified the ligand dihydrospon-giacidine (-9.7) showed maximum binding with protein HDAC4 protein.

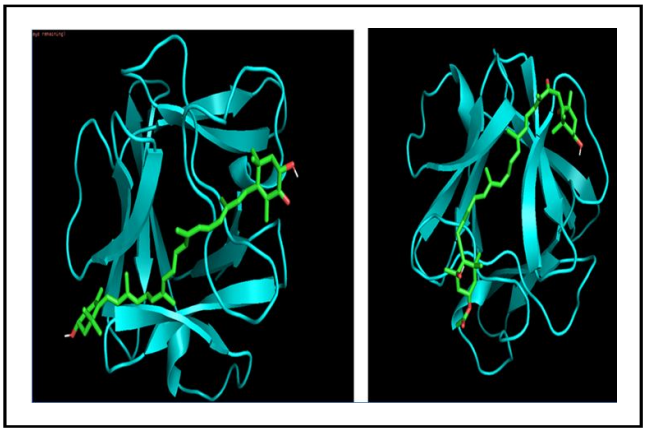

Figure 5: The two compounds astaxanthine, fucoxanthine with maximum binding affinity (-7.2) with fibroblast growth factor.

Figure 6: The compound oxysceptrin (-7.3) with maximum affinity towards IL-6.

Figure 7: Oxysceptrin showed maximum affinity (-10.9) with MMP -9 protein.

4. Discussion

A. clathrodes, the marine sponge, has been shown in numerous studies to be a rich source of various compounds with anti-cytotoxic, anti-inflammatory, antibacterial, and antioxidant properties. The potential of methanolic extract to cure diabetic wounds was examined in this work using *in silico* molecular docking analyses.

To determine the chemical components of the methanolic extract, GC-MS and LC-MS analyses were performed. To identify the volatile ingredients in the methanolic extract of the marine sponge, *A. clathrodes,* GC-MS analysis was performed. Using mass fragmentation patterns and standard data from the NIST library, the retention times of the 25 compounds were determined. In LC-MS analysis highly potential flavonoids and pyrrole alkaloid-like compounds were identified. Their presence was confirmed by using combined data of HPLC, UV spectra, FTIR, and mass spectra of available standards.

Using the AutoDockVina 1.2.7 program, *in silico* molecular docking analyses of the aforementioned compounds were carried out. Matrix metalloproteinases-9 (MMP-9), human interleukin-6 (IL-6), fibroblast growth factors (FGF), and histone deacetylase 4 (H DAC4) were the four proteins that were utilized. During the various phases of diabetic wound healing, these four proteins demonstrated a significant involvement. The majority of the marine sponge compounds that were discovered had strong affinity for the four proteins.

An extensively used technique to assess a crude extract's capacity to scavenge free radicals produced by DPPH reagents is the DPPH radical scavenging assay. DPPH is a free radical with a purple colour that reacts with antioxidants to produce a stable yellow colour. The absorbance of DPPH was found to fall by 517 nm, which indicated the compound's reduction capacity.The IC_{50} value of standard acarbose was compared with the extract's β-amylase inhibition assay.

The marine sponge, *A. clathrodes'* methanolic extract demonstrated both *in vitro* antidiabetic efficacy through α-amylase inhibition and *in vitro* antioxidant activity using the DPPH assay.

5. Conclusion

It can be concluded that the *in silco* docking study revealed the presence of two compounds asthaxanthine and oxysceptrin from the methanolic extract of marine sponge, *A. clathrodes* may be recommended as the best candidate for diabetic wound healing potential. The identified compounds are potent molecules that require further pharmacological research. These substances might be recommended as the greatest choice for potential diabetic wound healing. The antioxidant investigation using the DPPH assay method of methanolic extract and the α-amylase inhibition assay demonstrated good results in their respective *in vitro* antidiabetic activities. The antioxidant and antidiabetic properties were also beneficial to diabetes wound healing studied. A limitation of this study is that its results only in *in vitro a*nd *in silico* studies and the pharmacological activity and toxicity need to be studied in living organism.

Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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