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Exploring Metabolites Of Green Algae *Caulerpa Spp*. To Discover Putative Inhibitors Of Dpp-4, A New Antidiabetic Target Protein

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Abstract

Diabetes mellitus (DM) remains a serious global health threat, claiming a million lives every year and affecting nearly 9% of the adult population who suffer from this impaired insulin sensitivity disease. The Dipeptidyl peptidase-4 (DPP-4) enzyme has recently attracted attention because of its crucial role in insulin signaling, making this enzyme an interesting and emerging target for antidiabetic drug discovery. This study aimed to explore reported metabolites from marine algae of the genus *Caulerpa* as DPP-4 inhibitors through computational studies using CB-dock 2, Protein-Ligand Interaction Profiler, SwissAdme, and pkCMS. Molecular docking allowed the identification of 7 hit compounds with strong binding affinities (□8.4 kcal to □9.3 kcal/mol) against DPP-4 target enzyme PDB ID: 3G4I. Four hits showed stronger binding affinity than two DPP-4 specific inhibitors and FDA-approved antidiabetic drugs, sitagliptin (□8.4 kcal/mol) and linagliptin (□9.0 kcal/mol). Following a molecular modification of the hit compounds using a bioisosterism-like approach and ADMET evaluation with pkCMS, three putative DPP-4 inhibitors were identified. They showed either stronger binding affinities or better ADMET profiles than sitagliptin and linagliptin, suggesting their promising potential as DPP-4 inhibitors. However, further optimized bioisoterism, *in silico* and *in vivo* studies, and clinical-based trials are required to confirm their antidiabetic activity.

Keywords: Antidiabetic, Caulerpa racemosa, caulerpin, DPP-4

INTRODUCTION

Diabetes mellitus (DM) remains a real therapeutic challenge to the world. Characterized by persistent hyperglycemia because of the inability of the body to use glucose (Shrivastava, 2013), DM represents one of the top 10 leading causes of death, claiming 463 million people worldwide in 2019 or 8.8 percent of the adult population with type 2 diabetes (T2D) responsible for around 90 percent of the cases (Williams et al., 2019). This figure is further exasperated by the fact that diabetes is a major risk factor for many other chronic diseases including coronary diseases, heart failure, stroke, and various cardiovascular conditions (Laakso & Lehto, 1998). Ironically, while the world is facing a global diabetic crisis, there is a substantial shortcoming of drugs available for treating type-2 DM (T2DM) even after the recent introduction of new drugs and targets (Kanwal et al., 2022). Also, the authors' further argument on the recent finding of a carcinogenic contaminant called N-nitroso dimethylamine (NMDA) in metformin, the last resort drug for antidiabetic, only exacerbated the issue. Collectively, these facts suggest that there is a sore need in discovering new and safer antidiabetic drugs to cure the debilitating insulin-resistant disease.

Because of its involvement in insulin signaling, dipeptidyl peptidase IV (DPP-4) has now gained much attention (Barchetta et al., 2022). Specifically, this enzyme degrades incretin hormones such as glucagonlike peptide-1 (GLP-1) and glycose-dependent insulinotropic polypeptide (GIP) by cleaving the N-terminal of the GLP-1 and GLP (Janardhan & Narahari Sastry, 2014), thus inactivating the hormones (Kieffer, 1995). Because 60% of insulin secreted after a meal is produced by GLP-1 and GLP (Salehi et al., 2010), the degradation of these hormones by DPP-4 means that the body cannot

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produce sufficient insulin which can lead to hyperglycemia. This condition is similar to T2DM patients who suffer from impaired insulin response to GL-1 and GIP (Gilbert & Pratley, 2020). Hence, molecules that can inhibit DPP-4 from degrading GLP-1 and GLP hormones or act as DPP-4 inhibitors to increase the levels of GLP-1 and GLP are promising drugs for treating diabetes (Janardhan & Narahari Sastry, 2014). This makes DPP-4 enzyme an attractive target for the discovery of new antidiabetic drugs.

Curiously, accumulating evidence is showing the green algae *Caulerpa racemosa* is a promising source of molecules with antidiabetic potential. Last year, Mandlik et al. (2022) reported that the extract of the green *C. racemosa* reduced glucose levels, restored impaired glycosylated hemoglobin levels and liver glycogen level in diabetic rats. Also, Dissanayake et al. (2022) found that the extract of the same species exhibited alpha-amylase inhibitory and anti-glycation activities. To date, however, there is no single report on the activity of the green algae metabolites against the emerging antidiabetic drug target DPP-4 even though over 35 molecules have been published from the green algae of the genus *Caulerpa* (Mert Ozupek et al., 2022). The antidiabetic potential, the metabolite diversity and the development in computational technology encouraged the author to perform an *in-silico* study on the reported metabolites of the green algae against the DPP-4 enzyme. This study aimed to find the putative DPP-4 inhibitors as candidates for treating DM from the known metabolites of the alga *Caulerpa* spp. through molecular docking, a simple molecular optimization and drugability of the genus Caulerpa's metabolites to find putative inhibitors of the DPP-4 enzyme.

METHOD

Molecular docking

Target protein preparation. Docking of all the ligands in the target protein was carried out using CB-dock 2 (Liu et al., 2020). The pdf file for the protein target crystal structure was retrieved from the protein data bank website (http://www.rcsb.org/pdb) PDB ID: 3D4I resolution 2.10 Å. Before docking, all water molecules and heteroatoms will be removed from the protein to be uploaded for which blind docking is executed by default on CB-dock 2. Only molecules with the binding affinity of <-7.5 kcal/mol were further optimized and evaluated.

Ligand preparation. Structures of all ligands shown below were obtained from marine natural products research articles and PubChem. The 3D structures were obtained by first extracting the corresponding canonical SMILES in Pubchem, copying the SMILES into Chem3D Pro, and minimizing the molecule before saving it into the file mol2 format. The ligands prepared in this study include 10,11-epxycaulerpenyne, caulerpenyne, fucosterol, taraxerol, b-sitosterol, sulfoquinovosyldi acylglycerol, trans phytol, furocaulerpin, flexilin, palmitic acid, trifarin, racemosin A, racemosin B, rasemosin C, D-alpha tocopherylquinone, cacospongionolide C, alpha tocospyrol, monomethyl caulerpinate, caulerchlorin, caulersin, caulerpin, caulerpicin, alpha-tocospiro A, racemobutenolide A, racemobutenolide B, alpha-tocospiro A, alpha-tocospiro B, a-tocoxylenoxy, a-tocopherol quinone.

Protein ligand interaction profiler. PLIP focuses on the one-click processing of protein structures for the detection of interaction patterns. There are other tools, web pages, and databases. The protein-ligand complex obtained from the docking through the CB-2 dock was uploaded to the PLIP web server and analyzed using the tool on PLIP. https://plip-tool.biotec.tu-dresden.de/plip-web/plip/index (Liu et al., 2020).

ADME/Tox

The drug-likeness of the compounds found in Caulerpa spp. was calculated using SwissADME (http://www.swissadme.ch/)(Daina, 2017). SMILES of all molecules were obtained by converting the 2D structure of each molecule using ChemiDraw Ultra 12. pkCMS server https://biosig.lab.uq.edu.au/pkcsm/predicti

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on were used to determine the toxicity estimation of selected compounds from Caulerpa in ADME analysis.

RESULTS AND DISCUSSION

Prior studies have documented the antidiabetic potential of the green algae *Caulerpa recemosa in vitro*. As mentioned introduction, two research on extracts of the green algae *Caulerpa racemosa* showed promising antidiabetic activity with Mandlik et al. (2022) reporting the effect of the extract of *C. racemosa* on reducing glucose levels and restoring impaired glycosylated hemoglobin levels and liver glycogen levels in diabetic rats and Dissanayake et al. (2022) on inhibiting the a-amylase and exerting antiglycation activity. However, those studies were conducted on crude extracts, which normally consist of a mixture of compounds and undesirable residues. They have to be purified if we are to discover particular bioactive compounds such as antidiabetic, antimalaria, antibiotic or any other bioactive compounds (Zhang, 2018). As such, the active compounds responsible for the observed antidiabetic activities in those studies remain unknown. In present study, the author tested 30 compounds as the representative of all molecules that have been reported over the years from *Caulerpa* spp. to identify compounds responsible for an antidiabetic activity specifically by molecular docking of those metabolites against DPP-4 target protein (PDB: ID, 3DFI) through an *in-silico* study. In addition, a bioisoterism like approach was performed to improve the activity of the natural products, their binding affinities and ADMET profiles. Therefore, this research sheds light on potential metabolites of *Caulerpa* spp. as a source of DPP-4 inhibitors and the possibility to improve their binding affinity and ADMET profiles of the inhibitors as potential antidiabetic drug leads.

Molecular docking

Molecular docking of 30 known molecules reported from the genus Caulerpa resulted in 7 hit molecules with strong binding affinity including caulerpin (**1**) (□9.2 kcal/mol), caulersin (**2**) (□8.7 Kcal/moll), caulerpisin (**3**) (□7.8 Kcal/mol) caulerchlorin (4) (□9.3 kcal/mol), monomethyl caulerpinate (**5**) (□9.2 Kcal/moll), teraxerole (**6**) (-7.9 kJ.mol) and b-sisterol (**7**) (□7.8 Kcal/mol) (Table 1). The remaining metabolites of the Caulerpa spp. showed binding affinities less than □7.5 kcal/mol) and were considered weak to moderate. Compared to the DPP-4 specific inhibitors and the antidiabetic drugs linagliptin (**8**) (□9.0 kcal/mol) and sitagliptin (**9**) (□8.4 kcal/mol), ligands **3**, **6** and **7** had slightly weaker binding affinities than sitagliptin but ligands **1,2,4** and **5** had stronger binding affinities than both sitagliptin and linagliptin.

The results also showed that binding sites and binding affinities varied among the ligands with the majority of the ligands bound to chain B than chain A of the 3D4I target proteins two ligands binding to pocket C3 chain B, four ligands to pocket C1 chain B and three ligands to pocket C2 chain A of the 3D4I target protein. Binding to pocket C3 chain B, caulerpin (**1**) and monomethyl caulerpinate (**5**) share virtually the same molecular structure (the caulerpin skeleton) except for the absence of a methyl group in 5. They both interacted with nearly identical amino acid residues of the target proteins including Tyr381, Thr401, Trp402, Asn420, Gly424, Me416, Pro426, Thr522, Lys523, Gly586 and Ile20 except for the missing of Gly403 from 5 and Trp525, Tyr585, Ile590, Glu521 from 1 (Table 1). Of the four ligands binding to pocket 1 chain B, teraxerole (**6**) and sitagliptin (**9**), only caulersin (**2**) and caulerchlorin (**4**) have the same structure (the bisindole caulerpin type) with the difference being the presence of oxygen atom in caulersin in place of chlorine in caulerchlorin while taraxerol is a pentacyclic triterpenoid and sitagliptin is a triazolopyrazine.

Of these four ligands, only the first three bound to identical amino acid residues namely Tyr48 Asp545 Val546 Tyr547 Lys554 Asn562 Trp563 Ala564 Trp627 Gly628 Trp629 His740 Gly741 Tyr752 with extra amino acid residue Ser630 in 2 and Asp545 in 6. In contrast, compound 9 interacted with entirely

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different amino acid residues from 2, 4 and 6 Pro75 Gly476 Leu477 Asp501 Leu504 Met509 Pro510 Ser511 Lys512 Ile529 Thr557 Val558 Phe559 Arg560 Leu561 Asn562 Thr565. While the different structures and different interacting amino acid may explain the different binding affinities between these four compounds binding to pocket C1 B, the presence of different functional groups in compounds 2 and 4 dictated with the replacement of oxygen atom in **2** by a fluorine atom in **4** caused □0.5 kcal/mol difference in binding affinities of the two ligands (Table 1). The last three ligands include caulerpisin (**3**), b-sisterol (**7**) and linagliptin (**8**) bound to pocket 2 chain A of the 3D4I. Of these ligands, only ligands **7** and **8** showed similar interaction as they revealed 60% similarity of interacting amino acids including Glu452, Pro475, Gly476 Leu477 Asp501, Met509 Pro510 Ser511 Lys512 Thr557 Phe559 (Table 1) despite their different molecular structures, being steroid for 7 and a xanthine aminopiperidine for **8**. As for caulerpisin (**3**), however, it interacted with entirely different amino acids compared not only to different structures such as b-sisterol (**7**) and linagliptin (**8**) but also to similar structures such as the caulerpin structure class (Table 1).

Whereas the different binding sites and affinities arise from molecules of different structure is commonly expected, the fact that molecules with the same structure such as caulerpins showed different binding sites and affinities is puzzling. It was reported by (Boström, 2006) that there was 83% different in binding sites of the pairs of proteins complexing structurally similar ligands with water molecules and side chain movement as determining factors. Nevertheless, the authors discovered that the majority of the ligand pairs occupied the same region in the binding sites. This was the case for most of the members of the caulerpin class molecules tested in this study except for caulerspisinin.

Of the five caulerpin typed molecules, four ligands (caulerpin (**1**), caulersin (**2**), monomethyl caulerpionate (**3**) and caulerchlorin (**4**) bound to chain B (blue ribbon) of the dimmer cartoon of 3D4I indicated by a solid red circle line for each molecule whereas caulerpisin bound to chain a (red ribbon). One reason is the replacement of one of the carboxylates in ligand **1** by relatively the same size of functional groups or atoms such as a ketone in caulersin (**2**), a carboxylic acid in monomethyl caulerpinate (**5**) and chlorine in caulerchlorin (**4**) did not cause a pronounced change four binding affinities or binding site of compared to the effect of a bulkier moiety such as N acyl sphingosine in **5** (Figure 2). Consequently, whereas ligands **1**-**4** bound to the same region albeit in different pockets of the 3D41 protein target, ligand **5** bound to the opposite region of the protein target (Figure 1).

The relatively unchanged binding affinity in monomethyl caulerpinate, which contains a carbocyclic acid in place of a carboxylate ester in caulerpin, was expected. This is because carboxylic acid is known to have specific charge-charge interaction with its targets and is responsible for the binding of drugs to their targets with molecules incorporating this functional group called privileged molecules for protein binding (Hajduk et al., 2000). In addition, Bredael et al. (2022) also pointed out that carboxylic acid functionality especially can form hydrogen bonds. Indeed, 450 drugs in the market contain this functional group, underlining the importance of this functional group in protein-ligand interactions.

However, the moderately reduced binding affinity in caulersin, which contains a ketone in place of carboxylate ester, is debatable. In 2009, (Varadwaj, 2009) measured the Van Der Walls distance radii of ketone as 2.35 to 2.64 and carboxylic acid A as 2.75 A to 2.95 A and claimed that a ketone has stronger binding affinity than carboxylate in protein binding sites. They further supported their arguments by stating that the presence of extra oxygen near carbonyl in carboxylate weakened the binding of carboxylate to protein binding sites.

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Table 1. Binding affinity and interacting amino acids with PDB ID: 3D4I

Ligands	Binding affinity Hydrogen (Kcal/mol)	Interacting amino acids (3d4i)
Caulerpin (1)	-9.2	Pocket C3, Chain B Tyr381, Thr401, Trp402, Gly403, Asn420, Gly424, Me416, Pro426, Thr522, Lys523, Gly586 and Ile20.
Caulersin (2)	-8.7	Pocket C1, Chain B: Tyr48 Asp545 Val546 Tyr547 Lys554 Asn562 Trp563 Ala564 Trp627 Gly628 Trp629 Ser630 His740 Gly741 Tyr752
Caulerpisin (3)	-7.8	Pocket C2, Chain A: Thr351 Ser376 Asn377 Glu378 Glu379 Gly380 Tyr381 Thr401 Trp402 Glu403 Asn420 Gly424 Met425 Prp426 Thr522 Lys523 Phe524 Trp525 Tyr585 Gln586 Asp588 Lys589 Ile590 His592 Ala593
Cauler chlorin (4)	-9.3	Pocket C1, Chain B: Tyr48 Asp545 Val546 Tyr547 Lys554 Asn562 Trp563 Ala564 Trp627 Gly628 Trp629 His740 Gly741 Tyr752
Monomethyl caulerpinate (5)	-9.2	Pocket C3, Chain B: Tyr381 Thr401 Trp402 Glu403 Asn420 Gly424 Met425 Pro426 Glu521 Thr522 Lys523 Trp525 Tyr585 Gln586 Ile590
Teraxerole (6)	-7.9	Pocket C1, Chain B: Tyr48 Arg125 Asp545 Tyr547 Lys554 Asn562 Trp563 Ala564 Trp627 Trp629 Ser630 His740 Tyr752
β-sisterol (7)	-7.8	Pocket C2, Chain A: Met425 Glu452 Gln455 Pro475 Gly476 Leu477 Asp501 Leu504 Met509 Pro510 Ser511 Lys512 Leu514 Asp515 Trp525 Asp556 Thr557 Phe559
Linagliptin (8)	-9.0	Pocket C2, Chain A: Glu452 Arg453 Pro475 Gly476 Leu477 Asp501 Met509 Pro510 Ser511 Lys512 Ile529 Thr557 Val558 Phe559 Arg560 Thr565
Sitagliptin (9)	-8.4	Pocket C1, Chain B: Pro75 Gly476 Leu477 Asp501 Leu504 Met509 Pro510 Ser511 Lys512 Ile529 Thr557 Val558 Phe559 Arg560 Leu561 Asn562 Thr565
Recemosin B	-9.2	Pocket C1, Chain B: TYR48 ASP545 VAL546 TYR547 LYS554 ASN562 TRP563 ALA564 TRP627 GLY628 TRP629 GLY632 HIS740 GLY741 TYR752
Recemosin C	-8.2	Pocket C3; Chain B: TYR381 THR401 TRP402 ASN420 GLY424 MET425 PRO426 GLU521 THR522 LYS523 TRP525 TYR585 GLN586 GLY587 LYS589 ILE590

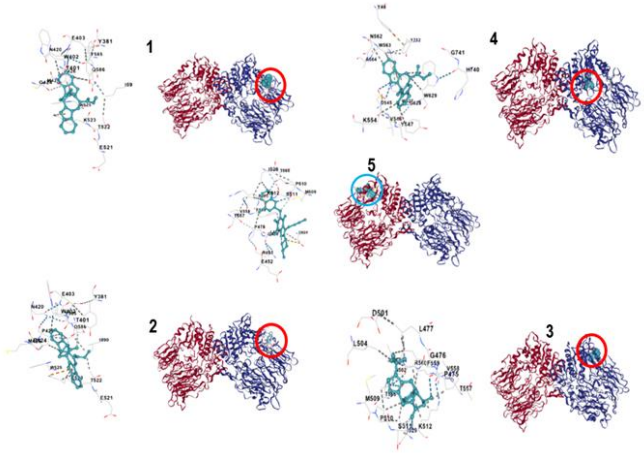

Figure 1. Different binding sites of caulerpin type compounds; (**1**) caulerpion, (**2**) caulersin, (**3**) monomethyl caulerpinate (**4**) caulerchlorin and caulerpiosin (**5**) to 3D4I.

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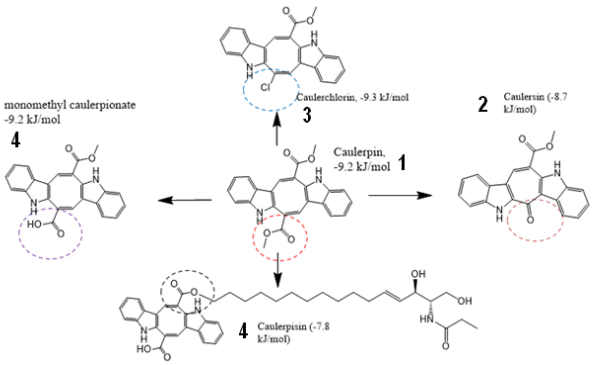

Figure 2. Different binding affinity of caulerpin typed molecules against 3D4I; caulerpin (**1**), caulersin (**2**), monomethyl caulerpinate (**3**), caulerchlorin (**4**).

Nevertheless, the existence of the electronegative oxygen adjacent to a carbonyl group in carboxylate such as in monomethyl caulerpinate and caulerpin may contribute to the electronic effects (donating or withdrawing electrons away from adjacent atoms or functional groups) that may participate in resonance and intrinsic inductive effects that contribute to an improved binding affinity. This is supported by the high electronegative nature of oxygen, which only comes second after fluorine, making it a good hydrogen bond acceptor from the H-bond donor as in the case of fluorine (Müller, 2007).

Molecular Modification

As for caulersin, the significantly reduced binding affinity of this compound compared to its caulerpin analogs is presumably related to the presence of a bulky N acyl sphingosine in the molecule. This long and bulky that might have weakened that binding affinity. Interestingly, however, the binding affinity increased with the presence of a halogen atom in place of a carboxylate ester in caulerchlorin. This result corroborates many reports on the improved effects of halogen on bioactivities. For example, (Xu, 2015) reported that halogen bonds have crucial roles both in improving drug-target binding affinity but also in tunning ADME/T profiles. In addition, (Molchanova, 2020)reported that the introduction of halogen (chlorine, bromine, and fluorine) improved the bioactivity of various pathogenic bacteria. Importantly, 13 FDA-approved drugs in 2021 contain fluorine and 14 in 2022 contain halogen (mostly bromine and fluorine) (Benedetto, 2022).

Armed with the information and the fact that all compounds had lipophilicity and toxicity issues (Table S1& S2 Supporting Information), fluorine and hydroxyl were introduced to the 7 hits compounds to improve their binding affinities and the ADMET profiles using a bioisosterism-like approach (Jayashree, 2022). Hence, instead of radically changing the molecules, a few substituents such as fluorine, hydroxyl, and/or ketone were introduced to caulerpin, teraxerole, and β-sisterol typed molecules and resulted in seven fluorinated analogs shown in Figure 3.

Molecular docking results on the fluorinated ligands against the 3D4I protein target using CB-dock 2 revealed both negative and positive effects of both the binding affinities of the 7 hit compounds (Table 2). Whereas caulerpin (**1**), caulerchlorin (**4**), and monomethyl caulerpinate (**5**) had binding affinities of (–9.2, –9.3 and –9.2) kcal/mol respectively, their fluorinated derivatives **1a**, **4a**, and **5a** showed slightly weaker binding affinities of (–9.0, –8.6 and –8.8) kcal/mmol respectively. In contrast, caulersin (**2**), caulerpsin (**3**), teraxerole (**6**) and β-sisterol exhibited binding affinities of (–8.7, –7.8, –7.9 and –7.8) kcal/mol while their fluorinated counterparts (**2a, 3a** and **6a**) had binding affinities of (–9.4, –8.4, –8.6 and –8.4) kcal/mol respectively.

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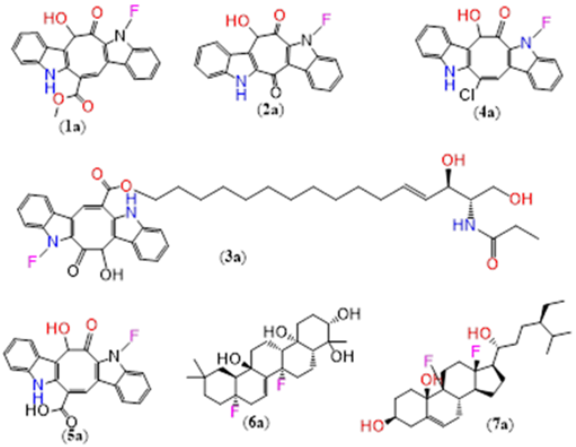

Figure 3. Seven Fluorinated compounds modified through bioisoterism approach; caulerflorin (**1a**), caulersiflorin (**2a**), caulersipiklorin (**3a**), caulerchloflorin (**4a**), cauleracidfluorin (**5a**), taraxeroflorin (**6a**) and □-sisteroflorin (**7a**).

Table 2. Molecular docking result of caulerbromin, cauleriodin and caulerflorin on 3D4I

Ligands	Binding affinity Hydrogen (Kcal/moll)	Interacting amino acids (3d4i)
Caulerfluorin (1a)	-9.0	Pocket C3, Chain B: Tyr381 Thr401 Trp402 Glu403 Asn420 Lys423 Gly424 Met425 Pro426 Glu521 Thr522 Lys523 Trp525 Tyr585 Gln586 Iel590
Caulersiflorin (2a)	-9.4	Pocket C4, Chain A: Pro475 Gly476 Met509 Pro510 Ser511 Lys512 Gle527 Ile529 Thr557 Val558 Phe559 Arg560 Leu561 Asn562 Ala564 Thr565
Caulerpisinflorin (3a)	-8.4	Pocket C2, Chain A: Met348 Ser349 Thr350 Thr351 Gly352 Ser376 Asn377 Glu378 Glu379 Gly380 Tyr381 Thr401 Trp402 Glu403 Asn420 Gly424 Met425 Pro426 Thr522 Lys523 Trp525 Tyr585 Gln586 Asp588 Lys589 Ile590
Caulerchloflorin (4a)	-8.6	Pocket C1, Chain B: Asp545 Val546 Tyr547 Lys554 Trp627 Gly628 Trp629 Ser630 Gly632 His740 Tyr752
MMC (5a)	-8.8	Pocket C3, Chain B: Tyr381 Thr401 Trp402 Gly424 Pro426 Glu521 Thr522 Lys523 Phe524 Trp525 Tyr585 Gln586 Gly587 Asp588 Lys589 Ile590
Teraxerofluorin (6a)	-8.6	Pocket C1, Chain B: Tyr48 Arg125 Asp545 Val546 Tyr547 Lys554 Asn562 Trp563 Ala564 Val575 Trp627 Trp629 Ser630 His740 Tyr752
β-sisterofluorin (7a)	-8.4	Pocket: C3, Chain B: Glu379 Tyr381 Thr401 Trp402 Glu403 Asn420 Glu421 Gly424 Met425 Prp426 Glu521 Thr522 Lys523 Trp525 Tyr585 Gln586 Gly587 Asp588 Lys589 Ile590
Recemofluorin B	-9.2	Pocket C1, Chain B: Tyr48 Asp545 Val546 Tyr547 Lys554 Asn562 Trp563 Aal564 Trp627 Gly628 Trp629 Gly632 His740 Gly741 Tyr752
Racemofluorin C	-9.0	Pocket C1, Chain B: Tyr48 Asp545 Val546 Tyr547 Lys554 Asn562 Ala564 Trp627 Gly628 Trp629 Gly632 His740 Gly741 Tyr752

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As mentioned before, this result confirmed previous findings on the effect of fluorine on bioactivity of many types of compounds (Benedetto Tiz et al., 2022; Xu et al., 2014). However, it is worth noting that the introduction of fluorine alone in the present study did not necessarily improve the binding affinities as much when it was introduced together with a hydroxyl group, indicating the importance of the hydroxyl group, presumably in improving the poor lipophilicity as explained later for the studied molecules. Indeed, the underappreciated role of a hydroxyl group for hydrophilicity of particular molecules has been explained before such as in the antibiotic roxithromycin case, where two hydroxyl groups in the molecule were shown to have a key role in modulating hydrophilicity and allowing membrane permeation of the antibiotic (Caron et al., 2019; Danelius et al., 2020).

The reason for the decreased binding affinities for fluorinated derivatives (**1a, 4a** and **5a)** apparently related to the position of fluorine in the molecules. It was described earlier by Bikowski (2006) who discovered that the bioactivity of corticosteroid improved significantly when fluorine was by introduced at position C9 of the molecule but had the opposite effect when it was replaced by a chlorine or other functional groups. Indeed, this phenomenon was observed for fluorinated b-sisterol that exhibited the strongest binding affinity value when one fluorine atom was introduced to position C9 of the molecule as in **7a** (-8.4 kcal/mol) than any other positions (-7.5 and -7.8 kcal/mol). As for the caulerpin type compounds, the effect of positioning of the fluorine atom in this type of molecule was less obvious. This is because the binding affinities of fluorinated caulerpin-typed molecules in the present study were affected not only by the introduction of fluorine but also by the presence of a hydroxyl group and to a certain degree by a ketone. Thus, future studies need to address this issue also if we are to optimize the bioactivity of potential DPP-4 inhibitors.

ADMET Evaluation

Unlike the effect on binding affinity, the halogenation of the hit compounds mainly positively affected the ADMET profiles of the ligands. Table S1 (Supporting Information) shows that except for caulersipin (**3**), all hit molecules and their fluorinated analogs had a high HI index between 83 and 97% (Table S1, Supporting Information). However, the values for Log SW of the non-fluorinated ligands were between -7.89 kcal/mol and -9.25 kcal/mol, and for Log Po/w between 3.70 and 4.92 (Table S2, Supporting Information). Because in SwissAdme scale, the value for the former was set not smaller than □6.0 and for the latter must be between 0 and 3, then all the hit compounds (**1**-**7**) and the DPP-4 inhibitors (**8**-**9**) were in the category of poor solubility and lipophilicity. Similarly, except for teraxerol (**6**) and b-sisterol (**7**), caulerflorin (**3**), and monomethyl caulirpinate (**5**) all other non-fluorinated ligands (**1**-**5**) and the DPP-4 inhibitors (**8**-**9**) exhibited hepatoxicity and only ligands **6** and **7** did not show AMES toxicity or related to mutagenicity (Table S1, Supporting Information). In contrast, the fluorinated derivatives showed improved ADMET profiles. Despite a slight variation (both slight increase and decrease) in HI index values between non-fluorinated and fluorinated ligands (Table S1, Supporting Information), all fluorinated compounds no longer showed AMES toxicity although ligands **1a-5a** still retained their hepatoxicity (Table S1, Supporting Information). In addition, the fluorinated ligands showed an increase in LogSw and a decrease in Log po/w values (Table S2, Supporting Information). The change significantly improved the solubility and lipophilicity of the fluorinated ligands especially compounds **2a**, **5a**-**7a**, changing it from poorly soluble to moderately soluble (Table S2, Supporting Information). Also, compounds **2a** (caulersiflorin) and compound **4a** were the only two compounds with the ability to penetrate the blood-brain barrier. Furthermore, all fluorinated compounds showed significant

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improvement in their drugability and satisfied the Lipinski rule of **5** with **1** (1V) or no violation (0V) of the rule.

Nevertheless, only four compounds showed a significant improvement in terms of binding affinities and ADMET profiles including compounds **2a, 4a**, **6a** and **7a** and were consdiered as the putative inhibitors of the DPP-4 enzyme in this study. Following the fluorination of the 7 hit compounds, the binding affinities of 4 of the compounds increased between□(□0.6 to □0.7) kcal/mol, suggesting the future potential for further improving their binding affinities. Also, although compounds **2a** and **4a** were predicted to retain their hepatoxicity (Tabel S1 & S1 Supporting Information), they no longer showed AMES toxicity. In addition, their solubility also increased from poorly soluble to moderately soluble particularly for compounds **2a**, **6a** and **7a** but not for compound **4a**. Furthermore, of these four ligands, only ligands **6a** and **7a** showed no AMES cytotoxicity and hepatocytotoxicity and only compound **2a** showed the ability to cross the blood-brain barrier. As such, compounds **2a, 6a,** and **7a** were considered as the most promising DPP-4 putative inhibitors.

Further analysis was done on the three putative inhibitors of the DPP-4 enzyme and the specific inhibitors of DPP-4 using protein-ligand interaction profiler (Adasme et al., 2021). The molecular interaction analysis showed two of the putative DPP-4 inhibitors, compounds 6a and 7a, bound to the protein target through hydrogen bonds and hydrophobic interactions similar to the specific inhibitors of DPP-4, sitagliptin (**8**) and linagliptin (**9**) (Table S1 Supporting Information, Figure 4). It is tempting to conclude that more diverse interactions shown by a molecule the stronger it binding affinity become. Indeed, it was the case for compound **2a**, having the highest binding affinity compared to other putative and known inhibitors because of the combinations between hydrogen bonds, hydrophobic interactions, and □□stacking. However, it was not true for sitagliptin (**8**) and linagliptin (**9**). Despite having less hydrogen bond and hydrophobic interaction than sitagliptin, compound **9** had much stronger binding affinities (-9.0 kcal/mol) than sitagliptin (-8.4 kcal/mol). This presumably related to the binding of functional groups in the ligands and amino acid residues of the proteins. As mentioned earlier, it was reported by (Varadwaj & Lahiri, 2009) that the Van Der Walls distance radii of the ketone was 2.35 to 2.64 A and carboxylic acid was 2.75 to 2.95A, therefore the ketone had much stronger binding affinity than the carboxylic acid functionality. Since protein and ligand interaction normally involves functional groups and have different conformational and topographical features, depending on the complexity of the ligand and protein, it is assumed that the relationship between the number of bonding interactions and binding affinities may be affected not only by the number of the interactions but also by functional groups or interacting proteins.

Table 3. Protein-Ligand Interaction between caulersiflorin (**2a**), teraxeroflorin (**B**) and b-sisteroflorin (**C**) and the protein target (PDB ID: 3D4I).

Ligands	Hydrogen Bond (Kcal/moll)	Hydrophobic Interactions	π stacking
Caulersiflorin	Tyr752, Tyr752	Asn562, Trp563 Trp629,	Trp627, Trp629
Teraxerofluorin	Tyr456, Gly553, Asp556, Arg560	Tyr547, Tyr547, Tyr547, Trp629	
β-sisterofluorin	Thr401, Met425, Thr522	Tyr381, Thr401, Pro426, Thr522, Lu523, Gly586, Lys589, Lys589, Ile590	
Sitagliptin	Lys512, Val558	Pro475, Pro510, Lys512, Phe559	
Linagliptin	Arg453	Pro475, Pro510, Lys512, Ile52	

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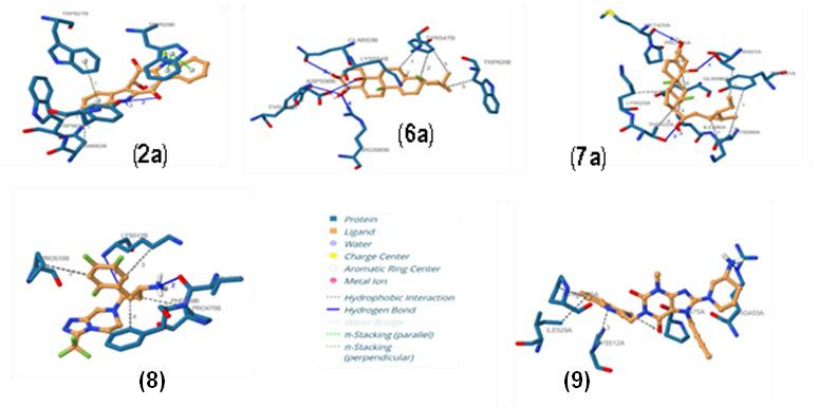

Figure 4. Protein-ligand interactions between caulerflorin (**2a**), teraxeroflorin (**6a**), betasisteroflorin (**7a**), sitagliptin (**8**), linagliptin (**9**), and the protein target (PDB ID: 3D4I).

Regarding the protein ligand interaction, the hydrogen bonding has been widely discussed for many years because of its many functions in protein ligand binding (Salentin et al., 2014; Sawada et al., 2010). Similarly, although to a less extend research has also focused on the importance of hydrophobic interactions and hydrogen bonds in stabilizing ligands at the binding site and in altering binding affinities and drug efficacy (Patil et al., 2020) and DNA drug-based design (Xiao et al., 2020). Furthermore, the effect of□□ stacking on bioactivity was proven crucial for the binding between the complex of the enzyme acetylcholinesterase and the Alzheimer's disease drug E2020 (Aricept) (Meyer et al., 2003). This is particularly relevant to compound **2a** which contain aromatic rings, the bis indole functionality (Table S1 & S2, Supporting Information). However, despite the improvements in its solubility, blood brain barrier, binding affinities and cytotoxicity particularly for AMES toxicity, compound **2a** still retained its hepatotoxicity. Similarly, despite being predicted to not have cytotoxic effects, improved binding affinities and solubility, compounds **6a** and **7a** cannot pass blood brain barrier which remain a work in progress for the current putative inhibitors of the DPP-4 (Table S1 & S2, Supporting Information). Nevertheless, their overall binding affinities and ADMET profiles were better than those of the known DPP-inhibitors such as linagliptin and sitagliptin particularly in terms of toxicity issue (Table S1 & S2, Supporting Information).

To date, diabetes remains a debilitating disease and an unmet medical need worldwide. The lack of medication and toxicity issues even for the last resort drug for antidiabetic drug such as metformin (Kanwal et al., 2022) suggest the urgency of the discovery of new drug for treating diabetic. As part of special delicacies in many coastal countries, it is no a surprising the green algae *Caulerpa* spp. have become attractive targets for bioactive compounds discovery ranging from anticancer to anti-inflammatory though antidiabetic drug lead discovery (Mehra, 2019). However, the green algae may contain a wide range of molecules, depending on the location and season. In addition, just because the green algae are edible doesn't mean that all the natural compounds, they have no toxicity issues. As shown in this study, despite their strong binding affinity towards 3D4I target protein, most of the caulerpin typed compounds had toxicity issues and need to be optimized if we are to discover safe antidiabetic drugs. Fortunately, it was also shown that by replacing certain substituents by the appropriate ones could optimize the hit compounds, suggesting the potential of further developing the putative DPP-4 inhibitors.

The three putative inhibitors of DPP-4 in this study have previously been reported to show a wide range of bioactivities mostly anticancer and also PTP1 inhibitory activity

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which is relevant to antidiabetic. Apart from anticancer, antiviral and anti-inflammatory, caulerpin and racemosin C have been reported as PT1B inhibitory (Yang et al., 2014). Thus, the present study confirmed the earlier results albeit against a different antidiabetic target and different number of screened molecules. However, despite their excellent potential activity against DPP-4, caulerpin typed compounds need be further optimized including compound **2a**, which still retained its hepatotoxic nature following a slight modification (Table S1 and S2, Supporting Information), if we are to find safe antidiabetic drugs. Nevertheless, the current improved after a slight modification suggested the possibility to achieve the goal in the future. The other two putative inhibitors of DPP-4 have also been reported as anticancer, phosphatase C inhibitors. In particular, □-sitosterol has also been reported as hepatoprotectant Azhaguraj et al. (2012), suggesting the potential of □-sitosterol (**6a**) as a new candidate for DPP-4 inhibitor. Although teraxeflorin has not been reported as hepatoprotectant it constantly showed no indication of AMES cytotoxicity and hepatotoxicity in addition to the improvement of its ADMET profile following a minor modification. Together, they showed the potential of compounds **2a, 6a** and **7a** as new inhibitors of the DPP-4 enzyme.

However, keeping in mind that supply for most of these compounds remain a serious problem in marine drug discovery (Lindequist, 2016), then one of the ways forwards is to conduct an optimized *in silico* study before *in vivo* study to validate and optimize the current putative DPP-4 inhibitors. This is important because until now supply for caulerpin remains very limited, yielding approximately 25% synthetic product (Li et al., 2018), which is presumably not practically and economically feasible. Although this also may open up opportunities to coastal people through seaweed mariculture or other biotechnological approaches (Lindequist, 2016), this would take long time in especially in developing countries. In addition, because algae could produce different compounds in different locations and environment, then the algae cultivation needs to be monitored regularly to ensure that the algae are producing the desirable compounds, which also would require another intensive research. Finally, the recent development in bioinformatics also offers various benefits for marine drug discovery and should be used to propel marine drug discovery. However, since the recent results were obtained through a prediction study *in silico*, further optimized chemical, in silico or clinical based trials should be done to confirm the present result.

CONCLUSION AND SUGGESTIONS

To sum up, three putative inhibitors of DPP-4 were identified from 30 known metabolites of *Caulerpa* spp. through molecular docking, ADMET studies using SwissAdme and pkCMS, protein ligand profiler and introduction of fluorine, ketone and hydroxyl to the hit compounds. The putative inhibitors of DPP-4 found in this study showed better binding affinities and ADMET profiles than the known inhibitors of the DPP-4 suggesting the potential of the putative DPP-4 inhibitors to be developed into potent and safe DPP-4 inhibitors. However, this prediction study needs to be further confirmed through optimized *in silico*, bioisoterism and *in vivo* and clinical based trials to confirm their antidiabetic activity.

Because of the urgent need of new and safe antidiabetic drugs, then an optimized in silico analysis should be done to confirm and optimize the current putative inhibitors of the DPP-4 enzyme before in vivo test. Also, since supply issue for caulerpin type compounds remains a bottleneck, cultivation of the green algae such as *Caulerpa racemosa* can be an alternative solution to provide a such bioactive compounds although further research on the appropriate environment for the alga to produce the desire compounds remained to be studied. Also, the development in computational technology should be further explored to

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propel the development of marine drug discovery.

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