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Original Research

Bromophenols from *Symphyocladia latiuscula* (Harvey) Yamada as Novel Cholecystokinin 2 Receptor Antagonists

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Abstract

Background: Cholecystokinin (CCK) is one of the most abundant peptides in the central nervous system and is believed to function as a neurotransmitter as well as a gut hormone with an inverse correlation of its level to anxiety and depression. Therefore, CCK receptors (CCKRs) could be a relevant target for novel antidepressant therapy. **Methods**: *In silico* target prediction was first employed to predict the probability of the bromophenols interacting with key protein targets based on a model trained on known bioactivity data and chemical similarity considerations. Next, we tested the functional effect of natural bromophenols from *Symphyocladia latiuscula* on the CCK_2 receptor followed by a molecular docking simulation to predict interactions between a compound and the binding site of the target protein. **Results**: Results of cell-based functional G-protein coupled receptor (GPCR) assays demonstrate that bromophenols 2,3,6-tribromo-4,5-dihydroxybenzyl alcohol (**1**), 2,3,6-tribromo-4,5-dihydroxybenzyl methyl ether (**2**), and bis-(2,3,6-tribromo-4,5-dihydroxybenzyl) ether (**3**) are full CCK_2 antagonists. Molecular docking simulation of **1**–**3** with CCK_2 demonstrated strong binding by means of interaction with prime interacting residues: Arg356, Asn353, Val349, His376, Phe227, and Pro210. Simulation results predicted good binding scores and interactions with prime residues, such as the reference antagonist YM022. **Conclusions**: The results of this study suggest bromophenols **1**–**3** are CCK_2R antagonists that could be novel therapeutic agents for CCK_2R-related diseases, especially anxiety and depression.

Keywords: bromophenols; *Symphyocladia latiuscula*; cholecystokinin receptor; GPCRs; anxiety; depression

1. Introduction

L-Tryptophan (TRP) is one of the largest essential amino acids and an essential component in the biosynthesis of proteins, muscle, and enzymes, an essential substrate for the production of neurotransmitters and hormones. Acute TRP deficiency results in increased pain sensitivity, auditory startle, increased motor activity, and increased aggression, while chronic TRP deficiency causes ataxia, cognitive impairment and dysphoria with skin hyperpigmentation [1]. Kynurenine (KYN) and serotonin (5-HT) are derived from the TRP metabolic pathway. The kynurenine (KYN) pathway is involved in 95% of TRP degradation, resulting in de novo synthesis of nicotinamide adenine dinucleotide (NAD^+) [2]. The pathway begins with the conversion of TRP to KYN catalyzed by two enzymes, tryptophan 2,3-dioxygenase and indolamine 2,3-dioxygenase. Furthermore, depending upon the cell type, KYN is further metabolized through three distinct pathways. (1) Synthesis of 3-hydroxykynurenine (3-HK) in microglia by kynurenine 3-monooxygenase (KMO) and its metabolites 3-hydroxyanthranilic acid (3-HAA) and quinolinic acid (QUIN); (2) Synthesis of anthranilic acid in microglia by kynureninase; and (3) Synthesis of kynurenic acid (KYNA) in astrocytes by kynurenine aminotransferase (KAT) II [3]. QUIN is an endogenous glutamate *N*-methyl-d-aspartate (NMDA) receptor agonist and KYNA is a non-competitive antagonist of alpha-7 nicotinic acetylcholine receptor (α7nAchR). Hence, abnormalities in the KYN pathway have been implicated in the pathophysiology of depression.

Depression is a heterogeneous disorder, and the exact neuronal mechanisms driving it have not yet been discovered. Evidence also suggests that cholecystokinin (CCK) and its receptors play an important role in the pathogenesis of anxiety-related behaviors and depression [4]. Furthermore, the causal relationship between increased CCK-ergic neurotransmission in the socially defeated cortex and depressive-like symptoms strongly suggests the relevance of the CCKergic system as a novel target for antidepressant therapy [5,6]. Anxiety disorders are characterized by excessive fear, and Pavlovian threat conditioning is an adap-

tive mechanism by which organisms learn to avoid potential threats, thereby increasing their chances of survival [7]. The prefrontal cortex (PFC) is a central brain region in the pathogenesis of depression and it has been suggested that the ventromedial prefrontal cortex (vmPFC) plays an important role in the acquisition of Pavlovian threat conditioning [7]. Amygdaloid CCK_2 receptors appear to be involved in the expression of fear-enhanced startle, as fear-enhanced startle is blocked by systemic injections of CCK_2 antagonists [8]. Furthermore, injections of CCK-8 into the central nucleus of the amygdala increases arousal and fear in rats [9]. Therefore, CCK and its receptors could be one of the prime targets for antianxiety and antidepressant drugs.

Cholecystokinin (CCK) is a 33-amino-acid-long peptide hormone, first identified in the gastrointestinal (GI) tract, that regulates gut motility [10], pancreatic and gastric-acid secretion [11,12], and gall bladder contractions [13]. It is a secretagogue of insulin and pancreatic polypeptides in non-ruminants [14,15]. Also, it is one of the most abundant peptides in the central nervous system (CNS) that function as a neurotransmitter and has an inverse correlation of its level with depression and anxiety [16–20]. CCK levels are elevated in depression [21], and patients with high cerebrospinal fluid (CSF) CCK levels have more suicide attempts than those with low CSF CCK levels [16]. The receptor exists as two subtypes, CCK_1 and CCK_2, previously classified as CCK_A and CCK_B. CCK_1 is predominant in the GI system and has roles in gallbladder contraction and stimulation of pancreatic secretion [22,23]. CCK_2 is predominant in the CNS and has roles in satiety, memory, anxiety, osmotic stress, and neuropsychiatric disorders [24,25]. Several studies have shown that CCK peptides have anxiolytic effects in various experimental models of anxiety and can induce panic attacks in humans [26–28]. In addition, activation of CCK2 receptors in the brain can cause anxiety. Therefore, selective CCK2 receptor antagonists represent a unique class of anxiolytics [29]. Recently, much effort has been made in developing potent and specific CCK_2 receptor antagonists, which were classified as:

(a) benzodiazepine group: L-365,260, L-36 718 (devazepide), CI-988, YM022, Z-360, YF476 (netazepide), and YM022 [30–33];

(b) tryptophan dipeptide derivatives: PD-134,308 [28, 34];

(c) ureidoacetamides: RP-73,870 [35];

(d) pyrazolidinones - LY-288,513 [36].

Despite the progress in developing several CCK_2 receptor agonists/antagonists [12,37–46], none of them have reached the clinic, because of unfavorable, insufficient, and various biological effects discovered in clinical trials [39]. Only netazepide and Z-360 are currently under clinical development for the management of gastric neuroendocrine tumors and pancreatic cancer, respectively. Therefore, the discovery of selective CCK_2 receptor antagonists with suitable pharmacokinetic profiles is very important.

In drug discovery and development, halogenation plays a vital role in drug optimization by modulating lipophilicity and cell membrane solubility, increasing blood–brain barrier (BBB) permeability and the half-life, and improving membrane binding and CNS delivery of pharmaceutical drugs [47–50]. In recent years, the halogen bond has received a great deal of attention for hit-to-lead-to-candidate optimization to improve the drug-target binding affinity; as a result, 25% of the marketed drugs are halogenated [49]. Marine sources are predominant in bioactive, structurally diverse, and unique halogenated compounds, of which 45% are Bromo-metabolites [51]. These active metabolites have become structural models in developing synthetic derivatives with superior biological activity [52].

The red alga *Symphyocladia latiuscula* (Harvey) Yamada is rich in bromophenols with diverse biological activities, including anticancer [53], antibacterial [54], antifungal [55], antiviral [56], free-radical scavenging [57], aldose-reductase inhibitory [58], α-glucosidase inhibitory [59], and other properties [60–62]. Secondary metabolites from this alga contain a 2,3,6-tribromo-4,5-dihydroxybenzyl moiety with various substituents. Recently, we have reported anti-diabetic [63], anti-Alzheimer's disease [64], anti-tyrosinase [65], and anti-Parkinson's disease activity [66], of 2,3,6-tribromo-4,5-dihydroxybenzyl derivatives from *S. latiuscula*. The anti-Parkinson's disease activity of those derivatives were evaluated via their effect on human monoamine oxidase and dopamine receptors [66]. Monoaminergic neurotransmission is primarily based on G-protein coupled receptors (GPCRs) signaling. The aminergic system is dysregulated in anxiety disorders and major depressive disorder, and antidepressants act directly or indirectly through the GPCRS. Therefore, GPCRs are potential therapeutic targets for intervention in neurodegenerative diseases. However, apart from MAO and dopamine receptors, there are no reports on how natural bromophenols affect other GPCRs receptors.

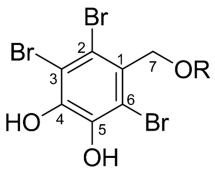
Therefore, our main objective in this study was to predict novel GPCRs targets via proteocheminformatics modelling (PCM) and characterize the functional effect of natural bromophenols on CCK_2 receptors for the management of CCK-mediated diseases, especially anxiety and depression.

2. Materials and Methods

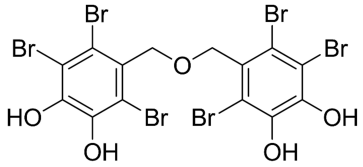
2.1 Chemicals and Reagents

We isolated three bromophenols, namely, 2,3,6-tribromo-4,5-dihydroxybenzyl alcohol (**1**), 2,3,6-tribromo-4,5-dihydroxybenzyl methyl ether (**2**), and bis-(2,3,6-tribromo-4,5-dihydroxybenzyl) ether (**3**) (Fig. 1), from the leafy thalli of *Symphyocladia latiuscula* (Harvey) Yamada and identified as described in our recent report [63]. We estimated the purity of the test compounds to be >98% as evidenced by proton and carbon NMR spectra. We obtained transfected Chinese hamster ovary (CHO) cells from

Eurofins Scientific (Le Bois I'Eveque, France). We obtained Hank's balanced salt solution (HBSS) buffer, Dulbecco's modified Eagle medium (DMEM) buffer, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer from Invitrogen (Carlsbad, CA, USA). We purchased reference drugs CCK-8s and YM022 from Sigma-Aldrich (St. Louis, MO, USA).



1: R = H; 2,3,6-tribromo-4,5-dihydroxybenzyl alcohol
2: R = CH_3; 2,3,6-tribromo-4,5-dihydroxybenzyl methyl ether



3: Bis-(2,3,6-tribromo-4,5-dihydroxybenzyl) ether)

Fig. 1. Structures of bromophenols isolated from *S. latiuscula*.

2.2 In Silico Prediction of Targets

Proteochemometric modeling (PCM) is a biological activity modeling technique based on the similarity of a set of ligands and a set of target proteins. It is built based on chemical descriptors that describe datasets of compounds and proteins of interest. Different interactions between a set of compounds and a set of targets can be described when describing the specific interaction between each compound and each target in the dataset. We did PCM as an *in-silico* way to predict potential protein targets of test bromophenols. The model was directed to chemically and biologically similar compounds (55,079 compounds active and inactive) against 99 human proteins (11,537 active pairs vs 43,542 inactive pairs). We selected 99 protein targets because these targets are considered 'critical nodes' in the biological network. Critical nodes need links that can crosstalk with other pathways, should be member of related proteins i.e., isoforms where two or more of the proteins in the group have unique biological roles and is highly regulated, either positively or negatively [67].

We evaluated the pattern that differentiates active and inactive complexes by using Parzen Rosenblatt Windows (PRW) [68,69] and predicted the activity of novel compounds against the 99 protein targets. We calculated chemical similarities using an Aitchison-Aitken kernel [70] and represented chemical structures as an ECFP_4 fingerprint [71]. Protein sequences were subjected to sequence alignment using MUSCLE [72], operated with the bio3d package [73], before we calculated the similarities between two protein sequences. (For full information on the model, see [74]).

2.3 Functional GPCRs Assay

We evaluated the functional effect of bromophenols **1**–**3** on cholecystokinin 2 (CCK_2) receptor in transfected CHO cells expressing human cloned CCK_2 receptors by measuring their effects on cAMP production using HTRF detection. In brief, we suspended stable transfectants (CHO-CCK_2) in HBSS buffer (Invitrogen, Carlsbad, CA, USA), supplemented with 20 mM hydroxyethyl piperazineethanesulfonic acid (HEPES) buffer and 500 μM 3-isobutyl-1-methylxanthine (IBMX), then distributed them into microplates at a density of 1×10^4 cells/well. Then, the plates were incubated for 30 min at 37 °C in the presence of HBSS (basal control), test bromophenols **1**–**3** (12.5, 25, 50, and 100 μM) or a reference agonist (CCK-8s). We prepared separate cells with 100 nM CCK-8s for stimulated control measurement. After incubation, cells were lysed, and a fluorescence acceptor (D_2-labeled cAMP) and fluorescence donor (anti-cAMP antibody with europium cryptate) were added. We measured a fluorescence transfer at $\lambda_{ex} = 337$ nm and $\lambda_{em} = 620$ and 665 nm, using a microplate reader (EnVision, PerkinElmer, Waltham, MA, USA) after 60 min of incubation at RT. The cAMP concentration was calculated as the ratio of the signal at 665 nm to that of 620 nm (i.e., by dividing the signal measured at 665 nm by that measured at 620 nm).

The agonist effect was expressed as a percentage of the control response to 100 nM CCK-8s. Similarly, for the antagonist effect, we evaluated the percent inhibition of the control response to CCK-8s 10 nM. We used CCK-8s as an agonist and YM022 as antagonists as reference drugs. We tested the standard reference drugs at several concentrations to generate a concentration-response curve from which either EC_{50} or IC_{50} value was calculated.

2.4 Homology Modeling

We obtained the primary sequence of the human CCK_2 receptor from UniProt (ID: P32239, GASR_HUMAN). The human adenosine (A_{2A}) receptor (PDB ID 3EML) has a higher similarity to CCK_2R [75]. Hence, we built the model on the template of the $A_{2A}R$ crystal structure from the RCSB protein data bank (PDB) using ID 5UIG with SWISS-MODEL. We refined the model using the 3Drefine server [76].

2.5 Statistics

The Kruskal-Wallis test and Dunn's multiple comparison test was performed to calculate statistical significance of the agonist/antagonist activity according to the treatment of bromophenols **1**–**3** using GraphPad Prism version 5.03 (GraphPad Prism, GraphPad Software Inc., San Diego, CA, USA). The significance levels were denoted as $*p < 0.05$.

Table 1. List of fifteen protein targets predicted from PCM modeling for bromophenols 1–3 with normalization values.

2,3,6-Tribromo-4,5-dihydrobenzyl alcohol (1)	2,3,6-Tribromo-4,5-dihydrobenzyl methyl ether (2)	Bis-(2,3,6-tribromo-4,5-dihydroxybenzyl) ether (3)
Serine/threonine-protein kinase mTOR (0.725)	Cholecystokinin 2 receptor (0.959)	Cholecystokinin 1 receptor (0.924)
Adenosine receptor A_{2A} (0.724)	Cholecystokinin 1 receptor (0.932)	Serine/threonine-protein kinase mTOR (0.920)
Beta-3 adrenergic receptor (0.708)	Serine/threonine-protein kinase mTOR (0.929)	PI3K-α isoform (0.901)
PI3K-α isoform (0.704)	Adenosine receptor A_{2A} (0.922)	Adenosine receptor A_{2A} (0.898)
Beta-2 adrenergic receptor (0.692)	PI3K-α isoform (0.912)	Cholecystokinin 2 receptor (0.896)
Adenosine receptor A_{2B} (0.683)	Adenosine receptor A_{2B} (0.909)	Adenosine receptor A_{2B} (0.883)
Beta-1 adrenergic receptor (0.674)	Substance-K receptor (0.908)	5-hydroxytryptamine receptor 1A (0.875)
5-hydroxytryptamine receptor 1A (0.664)	Substance-P receptor (0.908)	Muscarinic acetylcholine receptor M2 (0.874)
Mitogen-activated protein kinase 14 (0.662)	Endothelin-1 receptor (0.906)	Somatostatin receptor type 2 (0.873)
Dopamine D_4 receptor (0.659)	Vasopressin V_{1A} receptor (0.905)	Muscarinic acetylcholine receptor M3 (0.873)
Histamine H_1 receptor (0.657)	Somatostatin receptor type 2 (0.905)	Endothelin-1 receptor (0.873)
Muscarinic acetylcholine receptor M3 (0.655)	Vasopressin V_{1B} receptor (0.905)	5-hydroxytryptamine receptor 4 (0.872)
5-hydroxytryptamine receptor 1B (0.650)	Oxytocin receptor (0.904)	Vasopressin V_{1B} receptor (0.872)
Cholecystokinin 1 receptor (0.648)	Cannabinoid receptor 1 (0.903)	Vasopressin V_{1A} receptor (0.872)
Cholecystokinin 2 (0.641)	B1 bradykinin receptor (0.903)	Cannabinoid receptor 1 (0.872)

Note: Values in the bracket represent normalization values.

Table 2. Efficacy values (% stimulation and % inhibition) of bromophenols 1–3 and reference compounds at human cholecystokinin 2 receptor.

Compounds		Response at 100 µM % Stimulation [a]/(% Inhibition [b])		EC_{50} [c] (IC_{50} [d])
2,3,6-Tribromo-4,5-dihydrobenzyl alcohol (**1**)		–7.8 ± 1.41 (101.80 ± 0.42)		*ND* (27.44 ± 0.94)
2,3,6-Tribromo-4,5-dihydrobenzyl methyl ether (**2**)		–11.8 ± 1.70 (81.65 ± 9.40)		*ND* (72.81 ± 2.96)
Bis-(2,3,6-tribromo-4,5-dihydroxybenzyl) ether (**3**)		–8.05 ± 1.34 (104.35 ± 1.34) *		*ND* (21.01 ± 2.51)
CCK-8s		*ND*		1.6
YM022		*ND*		–0.49
Kruskal-Wallis test	*p* value	Dunn's multiple comparison test ($p < 0.05$)		
		1 vs 2	1 vs 3	2 vs 3
% Stimulation at 100 µM	0.0608	*NS*	*NS*	*NS*
% Inhibition at 100 µM	0.0273	*NS*	*NS*	*

ND: Not Determined.

NS: Not significant.

[a,b] % Stimulation and % inhibition of control agonist response at 100 μM of bromophenols, respectively.

[c] EC_{50}; half maximal effective concentration values of test compounds (μM) and reference agonist CC-8s (nM).

[d] IC_{50}; half maximal inhibitory concentration values of test compounds (μM) and reference antagonist YM-022 (nM).

* Represents significant difference at $p < 0.05$.

A level of $p < 0.05$ was considered statistically significant. Data in the figure and table are represented as mean ± SD and result from at least three independent experiments.

3. Results

3.1 In Silico Target Prediction

From PCM, we predicted the highest-ranked 15 potential protein targets for the three bromophenols. Table 1 lists the target proteins with the normalization rate. As shown in Table 1, most of the predicted target proteins (cholecystokinin receptor 1/2, adenosine receptor 2A/2B, 5HT1A receptor, and PI3K-α isoform) were the same for the three bromophenols. However, only bromophenol **2** had greater normalization values, and the ranking order of target proteins differed for each compound. Cholecystokinin type B/Gastrin receptor was predicted as a prime target for **2,** with the highest normalization value (0.959). The same CCK_2 receptor was predicted in a rank order of 5 and 14 with normalization value 0.896 and 0.648 for compounds **3** and **1**, respectively. Since bromophenol **2** is a major component in *S. latiuscula*, we then proceeded to validate the highest-ranked CCK_2prediction in GPCRs cell-based functional assays (Table 2).

3.2 Bromophenols as CCK_2R Antagonists

Following cell-based functional assays in stable transfectant (CHO-CCK_2), we characterized the functional effect of bromophenols **1–3** in modulating CCK_2 receptor

function by their potential to either stimulate or inhibit receptor activity. We tested a reference agonist CCK-8s and antagonist YM022 for comparison. The CCK_2 agonist or antagonist effect of 100 μM of **1–3** is shown in Table 2. As shown, **1–3** had a potent antagonist effect on CCK_2R with inhibition of the control agonist response by 101.80 ± 0.42, 81.65 ± 9.40, and 104.35 ± 1.34%, respectively. Statistically significant difference ($p < 0.05$) was found between the antagonist effect of bromophenols **2** and **3**. The agonist effect was negligible, as shown by the negative percent of simulation of the control agonist response.

Fig. 2 represents a concentration-dependent antagonist response of **1–3** and YM022 on CCK_2R along with their IC_{50} values. As shown there, bromophenols **1–3** inhibited a 50% agonist response of CCK-8s in CCK_2R at 27.44 ± 0.94, 72.81 ± 2.96, and 21.01 ± 2.51 μM, respectively. The reference antagonist YM022 had an IC_{50} value of 0.49 ± 0.01 nM.

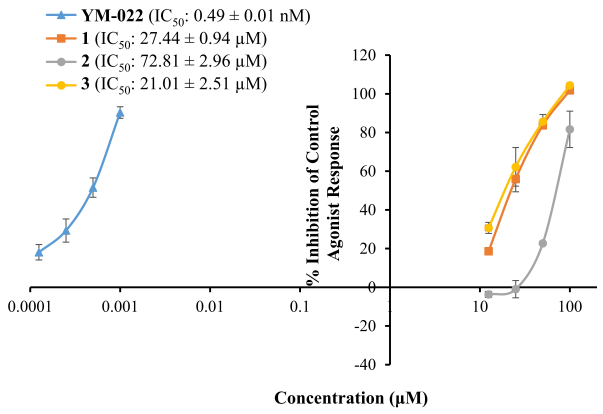


Fig. 2. Concentration-dependent percentage inhibition of control agonist response of bromophenols 1–3 and reference compound YM-022 on CCK_2 receptor. Bromophenols were tested at concentrations of 12.5, 25, 50, and 100 μM. Reference antagonist YM-022 was tested at 0.125, 0.25, 0.5 and 1.0 nM concentrations, respectively. The experiment was carried out in triplicates and inhibition values were expressed as the mean ± standard deviation (SD) (n = 3).

3.3 Molecular Docking Simulation

To explore the binding environment where ligands **1–3** interact with human CCK_2R, ligands were docked against the 3D model of CCK_2R using AutoDock 4.2 (Figs. 3,4); we analyzed the data based on interacting amino-acid residues and binding score (Table 3). Furthermore, we compared the docking results of test ligands with those of reference ligands. As shown in Table 3, **1–3** were predicted to bind with low binding scores (–5.45 kcal/mol to –7.38 kcal/mol), and ligand **3** had the lowest binding score (–7.38 kcal/mol) among the other test ligands. Ligands **1** and **2** formed two common H-bond interactions (Asn353 and Tyr380), as shown by the green broken lines in Fig. 4. Also, the hydrophobic interacting residues—Trp209 (π-π stacked, π-alkyl), Val138 (alkyl), Val349 (alkyl), Met134 (alkyl), His376 (π-alkyl)—observed for the binding of ligand **2** were in common with the ligand **1** binding. The only difference is that ligand **1** interacted with an additional residue, Phe227 (π-alkyl). However, **3** showed five H-bond interactions—Val194 with the C5 -OH group, Arg356 with the C4'-OH group, Pro210 with the C4' and C5'-OH groups, and Arg208 with C2 bromine atom—which were not observed for ligands **1** and **2**. The reference ligand YM022 had the lowest binding score (–9.74 kcal/mol) and displayed two H-bond interactions with His376 and Asn353.

4. Discussion

The aim of this study was to predict novel GPCRs targets of three natural bromophenols from *Symphyocladia latiuscula* via proteochemometric modeling (PCM), characterize the functional effect on CCK_2 receptors and predict the interactions between a compound and the binding site of the target protein for the management of CCK-mediated diseases, especially anxiety and depression. The pathophysiology of major depressive disorder and anxiety is complicated and not fully understood. Selective serotonin reuptake inhibitors (SSRIs), selective norepinephrine reuptake inhibitors, tricyclic antidepressants, and monoamine oxidase inhibitors are classic antidepressants used to treat depression. However, these drugs require a minimum of 2–4 weeks of continuous treatment to elicit therapeutic relief in depressed patients which leads to prolonged suffering and disability and increases suicide risk [6]. Due to the incomplete effectiveness of current treatment and the burdening neuroscience of fear-related behavior, however, compel the search for novel, more effective therapies [77]. In this regard, we gave a continuous effort to discover novel antidepressants from natural products.

Here, we focused on natural brominated compounds **1–3** from a red alga *S. latiuscula* to explore their functional effect on cholecystokinin 2 receptor based on our PCM prediction. We predicted a total of 15 target proteins for compounds **1–3,** and most of the predicted proteins were the same for the three compounds, with different rank orders following the normalization value (Table 1). The CCK_2 receptor was a prime protein target predicted for compound **2;** however, the rank order and normalization values were different for **1** and **3**. We attribute this difference to their structural differences. So, to verify the PCM prediction and get molecular insight into the test ligands–CCK_2 receptor interaction, we proceeded with the functional assay followed by molecular docking simulation.

The results of a functional assay conducted in the stable transfectant (CHO-CCK_2) indicated that bromophenols **1–3** are potent CCK_2 receptor antagonists. Bromophenol **1**

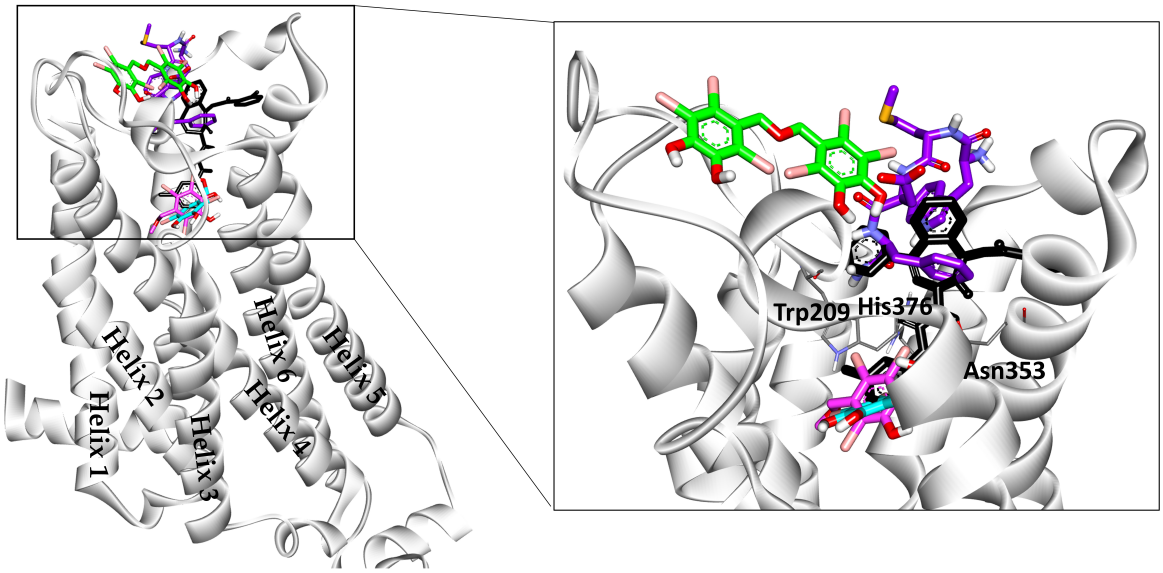


Fig. 3. Binding pose of test and reference ligands in the active site of CCK_2 receptor. Each ligand is depicted in different color. For instance, 2,3,6-tribromo-4,5-dihydrobenzyl alcohol (blue), 2,3,6-tribromo-4,5-dihydrobenzyl methyl ether (pink), bis-(2,3,6-tribromo-4,5-dihydroxybenzyl) ether (green), YM-022 (black), and CCK-4 (purple).

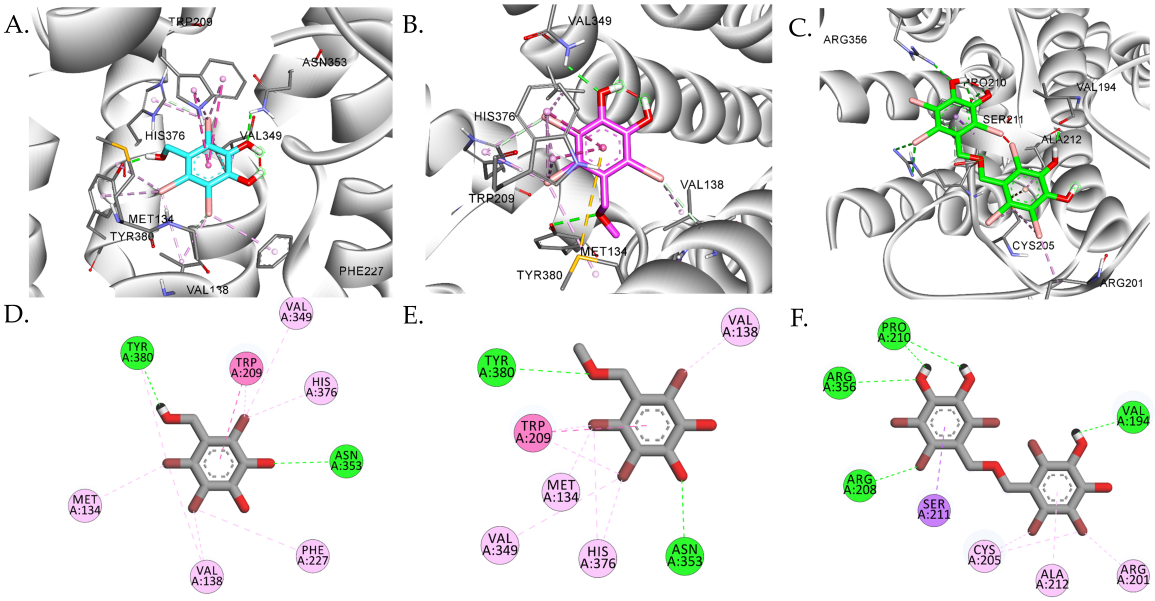


Fig. 4. 2D binding pose of test ligands in the active site of CCK_2 receptor. (A–C) Binding pose and (D–F) 2D-binding diagram of 2,3,6-tribromo-4,5-dihydrobenzyl alcohol, 2,3,6-tribromo-4,5-dihydrobenzyl methyl ether, and bis-(2,3,6-tribromo-4,5-dihydroxybenzyl) ether, respectively. Dotted lines with different colors represent different types of interactions. For instance, H-bond interactions are represented with green dotted lines and hydrophobic interactions with light-purple dotted lines.

and **3** are full CCK_2 antagonist that showed 100% antagonist effect at a 100 μM concentration, whereas the antagonist effect was approx. 82% for **2** at that concentration. All the tested bromophenols had no agonist effect, as indicated by the negative percentage agonist effect. The activity fashion of **1**–**3** was similar to our recent studies, where **1** and **3** showed better antidiabetic property by inhibiting protein tyrosine phosphate 1B and α-glucosidase enzyme activity, and by increasing insulin sensitivity and glucose uptake in a human liver-cancer cell line [63], anti-Alzheimer's disease activity via cholinesterase, self-induced $A\beta_{25-35}$ aggregation inhibition [64], anti-Parkinson's disease activity via the agonist effect on dopamine D_3/D_4 receptors [66], and anti-browning effect by inhibiting melanin content and intracellular tyrosinase levels in α-melanocyte-stimulating hormone-induced B16F10 melanoma cells [65].

Table 3. Binding affinity of compounds against cholecystokinin B receptor and reference ligands using AutoDock4.2.

Ligand	Binding Energy (kcal/mol)	Interacting residues	
		H-bond	Hydrophobic
2,3,6-Tribromo-4,5-dihydrobenzyl alcohol (**1**)	–5.45	Asn353, Tyr380	Trp209(π-π stacked, π-alkyl), Val349(alkyl), Val138(alkyl), Met134(alkyl), Phe227(π-alkyl), His376(π-alkyl), Tyr380(π-alkyl)
2,3,6-Tribromo-4,5-dihydrobenzyl methyl ether (**2**)	–5.54	Asn353, Tyr380	Trp209(π-π stacked, π-alkyl), Val138(alkyl), Val349(alkyl), Met134(alkyl), His376(π-alkyl)
Bis-(2,3,6-tribromo-4,5-dihydroxybenzyl) ether (**3**)	–7.38	Arg208, Arg356, Val194, Pro210	Ser211(Pi-Sigma), Arg201(Alkyl), Arg205(Alkyl), Ala212(Pi-Alkyl)
YM022 [a] (antagonist)	–9.74	His376, Asn353	Ala352(π-σ), Trp209(π-π stacked, π-alkyl), Met134(alkyl), Arg356(alkyl), Trp355(π-alkyl), His376(π-alkyl), Val349(π-alkyl), Pro210(π-alkyl), Ile372(π-alkyl)
CCK-4 [b] (agonist)	–6.76	Arg208, His376, Ala366, Asn115	Ile372(π-σ, π-alkyl), Pro201(π-alkyl), Ala352(π-alkyl)

[a] 1-(3-methylphenyl)-3-[(3*R*)-1-[2-(2-methylphenyl)-2-oxoethyl]-2-oxo-5-phenyl-3*H*-1,4-benzodiazepin-3-yl]urea.

[b] Tetragastrin; (3*S*)-3-[[(2*S*)-2-[[(2*S*)-2-amino-3-(1*H*-indol-3-yl)propanoyl]amino]-4-methylsulfanylbutanoyl]amino]-4-[[(2*S*)-1-amino-1-oxo-3-phenylpropan-2-yl]amino]-4-oxobutanoic acid.

All test compounds have a 2,3,6-tribromo-4,5-dihydroxybenzyl moiety in common, with different substituents at C-1. Bromophenol **1** has a hydroxymethyl group at C-1, **2** has a methyl ether, and **3** is a dimeric form, in which another 2,3,6-tribromo-4,5-dihydroxybenzyl moiety is attached at C-1 via a methyl ether linkage. Compared to the CCK_2R antagonist effect of **2**, bromophenols **1** and **3** had a better effect. However, the exact structure-activity relationship is uncertain, because we had only a few test bromophenols.

To get molecular insight on the promising antagonist effect of **1** and **3** over **2**, we did a molecular docking simulation. The CCK_2-binding score for **3** was the lowest (–7.38 kcal/mol) among the test bromophenols. Bromophenols **1** and **2** bound to the active site of CCK_2 receptor with a similar binding score (approx. –5.5 kcal/mol). Also, the interacting residues were almost common for **1** and **2** (Asn353, Tyr380 Trp209, Val349, Val138, Met134, His376, and Tyr380). The only difference is that the Br atom at C2 of **1** had an additional π-alkyl interaction with Phe227. Likewise, the most potent of them, **3**, had unique H-bond interactions with Arg208, Val194, Arg356, and Pro210 that were absent for the binding of **1** and **2**. The -OH group at C5, C4' and C5' and bromine atom at C2 of **3** had H-bond interactions with Val194, Arg356, Pro210, and Arg208, respectively. The reference antagonist YM022 and **3** had interacting residues Arg356 and Pro210 in common, whereas His376, Met134, and Trp20 are common for YM022, **1**, and **2**. Residues Arg356 (TM6) and Asn353 (TM6) are active site residues involved in CCK_2R antagonism for non-peptide antagonists [78], and bromophenols formed H-bond interactions with these residues. Two CCK receptor subtypes—CCK_1 and CCK_2 receptors—are 48% identical to each other and differ in terms of molecular structure, distribution, and affinity for the natural ligand (CCK and gastrin). Both subtypes share the same COOH-terminal pentapeptide amide sequence for receptor binding [79]. A study model conducted to discover 1,4-benzodiazepine allosteric ligands targeting CCK receptors suggested six prime amino-acid residues for the selectivity of subtype CCK_1: Asn98 (TM2), Thr117, Thr118 (TM3), Ile329, Phe330 (TM6), and Leu356 (TM7); those for CCK_2 were Thr111 (TM2), Val130, Ser131 (TM3), Val349, Tyr350 (TM6), and His376 (TM7). Asparagine residue is a conserved amino-acid residue in the TM6 of CCK receptor, and Asn353 is responsible for the H-bond interaction with the N4 of the benzodiazepine in CCK_2R [80]. Residues Thr111 (TM2) and His376 (TM7) were most important for binding CCK_2R selective ligands, and Asn353 (TM6) is an anchor residue with excellent enrichment and selectivity for CCK_2R [80]. Likewise, Val349 is a conserved amino-acid residue responsible for CCK_2R binding affinity for nonpeptide antagonists [81]. Our test ligands showed interactions with Asn353, Val349, and His376 and acted as CCK_2 receptor-specific antagonists.

Drugs modulate more than one target (six on average) to exert a therapeutic effect [82]. PCM is one of the quantitative bioactivity prediction techniques that predicts the potency or affinity for compound-target pairs [83], enables simultaneous modeling of chemical and biological information and thus compound's affinity and selectivity across a panel of targets [84]. Nonetheless, the effects of a compound at the cellular or the organism level are poorly understood because PCM cannot account for the interactions of a compound with other unrelated targets [85]. In the present study, though **2** was predicted with the highest normalization value for the CCK_2 receptor, it was the least active among the tested bromophenols in a functional assay. The reason behind this variation might be the ligand-receptor interaction and the ligand-receptor complex stability. This needs to be confirmed via molecular dynamics.

Since halogenation of drug molecules is a common strategy to improve ADME, membrane binding, permeation, and protein-ligand recognition [47,86–88], and addition of bromine and chlorine to peptide drugs improves CNS delivery by increasing BBB permeation [48]; these bromophenols **1**–**3** with excellent PPB and BBB values [64] could be developed into novel CNS drugs. Marine red algae are promising natural sources that contain around 90% of halogenated compounds [89]. Because of a wide array of biological activities of halogenated compounds and little halogen content in other natural products, structural modification of natural products by halogenation is emerging [49].

The sulfated octapeptide (CCK-8s) is the most abundant molecular form of CCK in the brain, with 400 pmol/g tissue content [90]. Earlier studies had reported an increase in behavioral arousal and fear in rats after CCK-8 injection into the central nucleus of the amygdala [91,92]. CCK_2 receptors have been strongly implicated in anxiety [93]. The systemic or intracerebral injection of the CCK-like peptides (CCK_2 or CCK_1/CCK_2 non-selective agonists) exhibited an anxiogenic-like effect in various animal models [94,95]. However, co-treatment with CCK_2 antagonist LY288513 blocked those effects; when administered alone, it attenuated anxious behavior in animals. Also, other highly selective CCK_2 receptor antagonists PD134308 and PD135158 were as effective as was diazepam to antagonize aversive behavior [96].

Food intake releases gastrin that binds to the CCK_2 receptor on enterochromaffin-like (ECL) cells and aids the synthesis and secretion of histamine, which stimulates parietal cells for acid secretion by proton pump via histamine H2-receptor and muscarinic M3-receptor [97]. Mental health is well-linked with the gut function [98–100] because of microbiota-gut-brain axis functions in a bidirectional manner in the regulation of depressive-like behaviors [101,102]. Therefore, these CCK_2 antagonist bromophenols can regulate depression-like behaviors.

5. Limitations and Future Directions

Our study highlights the *in vitro* CCK_2 antagonist effect of only three bromophenols in the red alga *Symphyocladia latiuscula* and predicts the binding mode. In silico molecular dynamics studies predicting the stability of ligand-receptor complexes are lacking. Penetration into the central nervous system and stability of the ligand-receptor complex remain to be studied *in vivo*. The small number of test compounds limited the structure-activity relationship. The effects of the tested bromophenols at the cellular or organismal level remain unknown because proteochemometric modeling cannot account for interactions between compounds and other unrelated targets.

In the future, a more detailed understanding of CCK_2 receptor signaling and regulation, especially using *in vivo* models, will be critical to ensure the activity of these natural bromophenols as anxiolytics and antidepressants.

6. Conclusions

Natural bromophenols 2,3,6-tribromo-4,5-dihydroxybenzyl alcohol (**1**), 2,3,6-tribromo-4,5-dihydroxybenzyl methyl ether (**2**), and bis-(2,3,6-tribromo-4,5-dihydroxybenzyl) ether (**3**) from *Symphyocladia latiuscula* are potent CCK_2 antagonists with good binding scores and interactions with prime residues at TM7 of the receptor in a similar manner to a reference antagonist YM022. This study suggests that bromophenols **1**–**3** are natural CCK_2 antagonists that could be novel therapeutic agents for CCK_2-related diseases, especially anxiety and depression, and provides a foundation for future studies to elucidate the molecular mechanism in animal models of anxiety and depression.

Author Contributions

PP participated in study design, isolation, treatment, and biochemical analysis, and drafted the manuscript. SEP and SHS performed the molecular docking studies. FMF performed PCM modeling. HAJ was involved in spectral analysis. JSC conceived the study, coordinated the study, and interpreted the data. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

The research reported here was conducted at Eurofins Cerep, France according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and was authorized from the Research Ministry to manipulate GMO, with a certificate number 28099.

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Conflict of Interest

The authors declare no conflict of interest. Pradeep Paudel is serving as one of the Guest editors of this journal. We declare that Pradeep Paudel had no involvement in the peer review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to Gernot Riedel.

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