Abstract: Phlorotannins are polyphenolic compounds in marine alga, especially the brown algae. Among numerous phlorotannins, dieckol and phlorofucofuroeckol-A (PFF-A) are the major ones and despite a wider biological activity profile, knowledge of the G protein-coupled receptor (GPCR) targets of these phlorotannins is lacking. This study explores prime GPCR targets of the two phlorotannins. In silico proteocheminformatics modeling predicted twenty major protein targets and in vitro functional assays showed a good agonist effect at the α2C adrenergic receptor (α2C AR) and an antagonist effect at the adenosine 2A receptor (A2A R), δ-opioid receptor (δ-OPR), glucagon-like peptide-1 receptor (GLP-1R), and 5-hydroxytryptamine 1A receptor (5-HT1A R) of both phlorotannins. Besides, dieckol showed an antagonist effect at the vasopressin 1A receptor (V1A R) and PFF-A showed a promising agonist effect at the cannabinoid 1 receptor and an antagonist effect at V1A R. In silico molecular docking simulation enabled us to investigate and identify distinct binding features of these phlorotannins to the target proteins. The docking results suggested that dieckol and PFF-A bind to the crystal structures of the proteins with good affinity involving key interacting amino acid residues comparable to reference ligands. Overall, the present study suggests α2C AR, A2A R, δ-OPR, GLP-1R, 5-HT1A R, CB1 R, and V1A R as prime receptor targets of dieckol and PFF-A.

Keywords: phlorotannins; GPCRs; agonist; antagonist; dieckol; PFF-A; molecular docking

1. Introduction

G protein-coupled receptors (GPCRs) are a family of membrane receptors that regulate human pathophysiology and are the leading target class for pharmaceuticals. At present, GPCRs mediate the effect of approximately one-third of the FDA-approved drugs [1–3]. However, these drugs target mainly biogenic amine receptors, which comprise around 30 members of the GPCR family [3]. There is, therefore, an immense potential within pharmaceuticals/natural products to exploit, considering the remaining family members for which no existing ligands have been identified.

In the traditional drug development process, the high-throughput screening (HTS) approach against drug targets of choice is the very first step to uncover new drugs, which has now been augmented by the in silico method to maximize the probability of novel leads discovery. Traditional Chinese medicine (TCM) is an important research object of network discovery.
(TCM herbs, targets, diseases, and syndromes) pharmacology, which aims to understand the network-based biological basis of complex diseases [4], and natural polyphenols are abundant in plant-based foods whose network proximity to disease proteins is predictive of the molecule’s known therapeutic effects [5].

Secondary metabolites from seaweeds have gained much interest in natural drug discovery, because the marine source is a huge reservoir of natural products with significant biological activities. In addition, secondary metabolites (carotenoids, polyphenols, and polysaccharides) with numerous biological activities make them a potential source of leads. Among marine organisms, marine alga, i.e., green algae (Chlorophyta), brown algae (Phaeophyta), and red algae (Rhodophyta), are rich sources of bioactive compounds with various biological activities. These macroalgae are well known by seaweeds and have been widely recognized as food, functional food, and potential drug sources for decades. Brown algae are the largest type of seaweed and so far, scientists have identified the therapeutic potential of brown algae-derived secondary metabolites (particularly phloroglucinol-based polyphenols, known as phlorotannins) including, but not limited to antioxidant [6,7], antimicrobial [8], anti-diabetic [9], anti-Alzheimer’s disease [10–12], anti-inflammatory [13], neuroprotective [14,15], anti-obesity [16], hepatoprotective [17], monoamine oxidase inhibitor [18], antihypertension [19] and anti-viral [20] activity. 

**Ecklonia stolonifera** OKA-MURA (**E. stolonifera**) is an edible brown alga of the Laminariaceae family that is widely distributed along the Eastern and Southern Korean coast and rich in phlorotannins [19,21]. Dieckol and phlorofucofuroeckol-A (PFF-A) are common phlorotannins in **E. stolonifera** and in our recent study, we had reported human monoamine oxidase (hMAO) inhibition, dopamine D_3R/D_4R receptor agonist effect, dopamine D_1/5-hydroxytryptamine 1A (5-HT_{1A})/neurokinin 1 (NK_1) receptor antagonist effect [22], and β-secretase and acetylcholinesterase inhibition by dieckol and PFF-A [10,11]. Nonetheless, other promising targets of these phlorotannins are yet to be identified.

Therefore, the main objectives of this study were: (a) predict prime protein targets of dieckol and PFF-A (Figure 1) via proteocheminformatics modeling (PCM), (b) validate the PCM prediction by evaluating the modulatory effect on predicted receptors via cell-based functional GPCRs assays, and (c) look at the specific binding interactions of test ligands and target receptors via molecular docking simulation.

![Chemical structures of dieckol and phlorofucofuroeckol-A.](image)

**Figure 1.** Chemical structures of dieckol and phlorofucofuroeckol-A.

### 2. Results

#### 2.1. In Silico Target Prediction

Proteocheminformatics (PCM) modeling is a quantitative bio-modeling technique that can predict the affinity and potency of a ligand against multiple different protein targets simultaneously by combining chemical and biological information from the ligand and related targets into a single machine learning model [23]. From in silico PCM modeling,
the highest-ranked twenty potential protein targets were predicted for the phlorotannins. Table 1 presents a list of the target proteins with an average score value.

**Table 1.** List of top 20 protein targets from proteocheminformatics modeling (PCM) prediction of dieckol and phlorofucofuroeckol-A, respectively.

| Rank | Dieckol          | Protein Name                  | Average Score | Phlorofucofuroeckol-A | Protein Name                  | Average Score |
|------|------------------|-------------------------------|---------------|-----------------------|-------------------------------|---------------|
| 1    | Vasopressin 1A receptor | 0.513                         | Vasopressin 1A receptor | 0.797                  |
| 2    | Vasopressin 1B receptor | 0.742                         |               |                       |                               |               |
| 3    | Oxytocin receptor | 0.737                         |               |                       |                               |               |
| 4    | B2 bradykinin receptor | 0.735                         |               |                       |                               |               |
| 5    | B1 bradykinin receptor | 0.727                         |               |                       |                               |               |
| 6    | Histamine H1 receptor | 0.721                         |               |                       |                               |               |
| 7    | Serotonin 1D receptor | 0.717                         |               |                       |                               |               |
| 8    | Type-1 angiotensin II receptor | 0.716 |               |                       |                               |               |
| 9    | Dopamine D2 receptor | 0.713                         |               |                       |                               |               |
| 10   | Cannabinoid receptor 1 | 0.711                         |               |                       |                               |               |
| 11   | Prostanoid EP3 receptor | 0.710                         |               |                       |                               |               |
| 12   | Rho-associated protein kinase 1 | 0.710 |               |                       |                               |               |
| 13   | Muscarinic acetylcholine receptor M3 | 0.709 |               |                       |                               |               |
| 14   | Cholecystokinin A receptor | 0.709                         |               |                       |                               |               |
| 15   | Serotonin 1A receptor | 0.706                         |               |                       |                               |               |
| 16   | Neurokinin 1 receptor | 0.706                         |               |                       |                               |               |
| 17   | Cysteinyl leukotriene receptor 1 | 0.706 |               |                       |                               |               |
| 18   | Alpha-1D adrenergic receptor | 0.705                         |               |                       |                               |               |
| 19   | Cholecystokinin B receptor | 0.704                         |               |                       |                               |               |
| 20   | Serotonin 1B receptor | 0.704                         |               |                       |                               |               |

As shown in the Table 1, the V_{1A} receptor was predicted as a top target for dieckol and PFF-A. For PFF-A, 5-hydroxytryptophan 1A (5-HT_{1A}R), 5-hydroxytryptophan 1B (5-HT_{1B}R), and cannabinoid 1 (CB_{1}R) receptors were among the predicted top twenty protein targets. Based on this prediction and reported biological activities of the phlorotannins in the literature, we proceeded to validate adenosine A_{2A} receptor (A_{2A}R), alpha-2A adrenergic receptor (α_{2A}AR), alpha-2C adrenergic receptor (α_{2C}AR), δ-opioid receptor (δ-OPR), CB_{1}R, free fatty acid receptor 1 (FFA_{1}R or GPR40), glucagon-like peptide-1 receptor (GLP-1), V_{1A}R, 5-HT_{1A}R, and 5-HT_{1B}R cell-based functional assays.

Firstly, the functional effect of dieckol and PFF-A was screened at a 100-µM concentration. As shown in Table 2, dieckol showed an agonist effect on α_{2C}AR (52.4 ± 4.24%) and V_{1A}R (106.73 ± 2.97%) and an antagonist effect on A_{2A}R (55.55 ± 4.03%), δ-OP (66.95 ± 0.92), CB_{1}R (158.75 ± 17.81%), and GLP-1R (101.0 ± 8.20%).

Likewise, PFF-A showed an agonist effect on α_{2C}AR (83.8 ± 0.07%) and CB_{1}R (113.8 ± 3.68%) and an antagonist effect on A_{2A}R (66.6 ± 2.26%), δ-OP (73.55 ± 5.44), and GLP-1R (105.7 ± 1.27%). These phlorotannins were either mild active or inactive at other tested protein targets as depicted by the negative and/or low value of % stimulation or inhibition (Table 2).

Based on the functional effect above 50% at 100 µM, the concentration-dependent effect was further tested and compared with the reference agonists and antagonists (Figures 2 and 3 and Tables 3 and 4) followed by molecular docking simulation. Molecular docking simulation of test ligands to the crystal structures of target proteins and comparison with the reference ligands results revealed the mechanism of ligand–target-protein interaction.
Table 2. Agonist and antagonist effect of 100 µM dieckol and phlorofucofuroeckol-A at several GPCRs.

| GPCRs                                      | Functional Effect at 100 µM Concentration | Dieckol | Phlorofucofuroeckol-A |
|--------------------------------------------|-------------------------------------------|---------|-----------------------|
| Adenosine A2A receptor (A2A)               | Agonist Effect                            | −0.1 ± 1.41 | 55.55 ± 4.03 |
|                                            | Antagonist Effect                          | −0.7 ± 0.57 | 66.6 ± 2.26 |
| Alpha-2A adrenergic receptor (α2AAR)       | Agonist Effect                            | 13.4 ± 19.87 | 46.15 ± 20.15 |
|                                            | Antagonist Effect                          | −0.5 ± 0.85 | 20.95 ± 1.77 |
| Alpha-2C adrenergic receptor (α2CAR)       | Agonist Effect                            | 52.4 ± 4.24 | −1.2 ± 6.08 |
|                                            | Antagonist Effect                          | 83.8 ± 0.07 | 19.2 ± 9.76 |
| δ-opioid receptor (δ-OPR)                  | Agonist Effect                            | −5.7 ± 0.14 | 66.95 ± 0.92 |
|                                            | Antagonist Effect                          | 14.7 ± 7.35 | 73.55 ± 5.44 |
| Cannabinoid receptor 1(CB1R)               | Agonist Effect                            | −23.3 ± 12.09 | 158.75 ± 17.18 |
|                                            | Antagonist Effect                          | 113.8 ± 3.68 | 21.35 ± 0.49 |
| Free fatty acid receptor 1 (FFA1R) (GPR40)| Agonist Effect                            | 0.2 ± 1.56 | 22.55 ± 5.44 |
|                                            | Antagonist Effect                          | −1.0 ± 0.07 | 30.15 ± 0.78 |
| Glucagon-like peptide-1 receptor (GLP-1)   | Agonist Effect                            | −16.3 ± 1.13 | 101 ± 8.20 |
|                                            | Antagonist Effect                          | −15.5 ± 2.55 | 105.7 ± 1.27 |
| Vasopressin 1A receptor (V1AR)             | Agonist Effect                            | 106.73 ± 2.97 | 57.77 ± 0.32 b |
|                                            | Antagonist Effect                          | 38.45 ± 7.14 a | 56.90 ± 5.37 b |
| 5-hydroxytryptophan 1A (5-HT1AR)          | Agonist Effect                            | 1.75 ± 0.64 a | 91.0 ± 3.11 |
|                                            | Antagonist Effect                          | 1.65 ± 0.49 a | 77.00 ± 11.03 |
| 5-hydroxytryptophan 1B (5-HT1BR)          | Agonist Effect                            | −7.3 ± 3.96 | −18.5 ± 2.69 |

*a* Value was extracted from our previous study [22]. *b* The test compound induces at least a 25% agonist effect at this concentration, which results in an apparent inhibition.

Figure 2. Dose-dependent agonist effect of dieckol and/or phlorofucofuroeckol-A on hα2C AR (A), hV1A R (B), and hCB1 (C) receptors.

Figure 3. Dose-dependent antagonist effect of dieckol and phlorofucofuroeckol-A against hA2A (A), δ-opioid (hδ-OP) (B), hGLP-1 (C), hV1A R (D), and h5-HT1AR (E) receptors.
Table 3. Concentration-dependent agonist effect of dieckol and phlorofucofuroeckol-A at several GPCRs.

| Compounds (µM) | Target GPCRs |       |       |       |       |       |       |       |       |       |       |
|---------------|--------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
|               | hA_{2A}R     | hα_{2A}AR | hα_{2C}AR | hδ-OPR | CB_{1}R | GPR40 | GLP-1 | hV_{1A}R | h5-HT_{1A}R |
| Dieckol       | 12.5         | –      | –      | –      | –      | –      | –      | –      | –      | –      | –      |
|               | 25           | –      | –      | –      | –      | –      | –      | –      | –      | –      | –      |
|               | 50           | –      | –      | –      | –      | –      | –      | –      | 50.40 ± 0.42 | –      | –      |
|               | 100          | –0.1 ± 1.41 | 13.4 ± 19.87 | 52.4 ± 4.24 | –5.7 ± 0.14 | –23.3 ± 12.09 | 0.2 ± 1.56 | –16.3 ± 1.13 | 106.73 ± 2.97 | 1.75 ± 0.64 c | –      |
|               | 150          | –      | –      | 73.0 ± 6.42 | –      | –      | –      | –      | 118.1 ± 2.83 | –      | –      |
|               | 200          | –      | –      | 74.77 ± 6.60 | –      | –      | –      | –      | –      | –      | –      |
|                | EC_{50} (µM) a | NA | NA | 98.80 ± 7.71 | NA | NA | NA | NA | 39.12 ± 2.12 | NA | – |
| PFF-A         | 12.5         | –      | –      | 22.03 ± 6.61 | –      | 46.7 ± 6.22 | –      | –      | –      | –      | –      |
|               | 25           | –      | –      | 55.0 ± 9.13 | –      | 80.3 ± 4.10 | –      | –      | –      | –      | –      |
|               | 50           | –      | –      | 62.27 ± 6.53 | –      | 96.45 ± 5.02 | –      | –      | –      | –      | –      |
|               | 100          | –0.7 ± 0.57 | –0.5 ± 0.85 | 83.8 ± 0.07 | 14.7 ± 7.35 | 113.8 ± 3.68 | –1.0 ± 0.07 | –15.5 ± 2.55 | 38.45 ± 7.14 c | 1.65 ± 0.49 c | –      |
|               | 150          | –      | –      | –      | –      | –      | –      | –      | –      | –      | –      |
|               | 200          | –      | –      | –      | –      | –      | –      | –      | –      | –      | –      |
|                | EC_{50} (µM) a | NA | NA | 23.67 ± 3.32 | NA | 13.42 ± 2.03 | NA | NA | NA | NA | – |
| Reference Drugs, EC_{50} (nM) b | 9.1      | 0.74 | 0.86 | 4.4  | 0.21  | 10000 | 0.049 | 0.72 | 2.5  | – |

a The 50% effective concentration (EC_{50}) values of compounds were expressed as mean ± SD, n = 3. b EC_{50} values of reference drugs (hA_{2A}R: 5’-N-ethylcarboxamidoadenosine (NECA), hα_{2A}AR: epinephrine bitartrate, hα_{2C}AR: epinephrine, hδ-OPR: DPDPE, CB_{1}R: CP 55940, GPR40: linoleic acid, GLP−1: GLP-1(7-37), hV_{1A}R: AVP, h5-HT_{1A}R: serotonin, h5-HT_{1B}R: serotonin). c Value was extracted from our previous study [22]. NA No activity. (−) Not tested.
Table 4. Concentration-dependent antagonist effects of dieckol and phlorofucofuroeckol-A at several GPCRs.

| Compounds (µM) | Target GPCRs | hA2AR | hα2AR | hα2C | hδ-OPR | CB1R | GPR40 | GLP-1 | hV1AR | h5-HT1AR | h5-HT1B |
|----------------|--------------|-------|-------|------|--------|------|-------|-------|-------|---------|---------|
| Dieckol        |              | 12.5  | –     | –    | –      | –    | –     | –     | –     | –       | –       |
|                |              | 25    | –     | –    | –      | –    | –     | –     | –     | –       | –       |
|                |              | 50    | –     | –    | 23.23 ± 4.04 | –    | –     | 53.80 ± 3.12 | 41.53 ± 7.39 | 54.7 ± 1.7 | –       |
|                |              | 100   | 56.0 ± 11.1 | 46.15 ± 20.15 | –1.20 ± 6.08 | 71.6 ± 17.85 | –1.20 ± 6.08 | 22.55 ± 5.44 | 101.0 ± 8.20 | 57.77 ± 0.32 | 91.0 ± 3.11 | –7.3 ± 3.96 |
|                |              | 150   | 62.43 ± 12.19 | –     | –     | –     | –     | –     | –     | 68.80 ± 0.85 | –       | –       |
|                |              | 200   | –     | –    | –      | –    | –     | –     | –     | –       | 100.9 ± 0.57 | –8.1 ± 0.85 |
| IC50 (µM) a    |              | 87.18 ± 2.63 | NA    | NA   | 80.46 ± 13.74 | NA   | NA   | 47.19 ± 2.46 | 82.71 ± 8.73 | 43.31 ± 3.22 | NA       |
| Dieckol        |              | 12.5  | –     | –    | –      | –    | –     | –     | –     | –       | –       |
|                |              | 25    | –     | –    | –      | –    | –     | –     | –     | –       | –       |
|                |              | 50    | –     | –    | 89.03 ± 0.70 | –    | –     | 83.87 ± 2.03 | 52.80 ± 8.09 | 85.55 ± 8.41 | –       |
|                |              | 100   | 92.2 ± 0.95 | 20.95 ± 1.77 | 19.2 ± 9.76 | 96.47 ± 0.84 | 21.35 ± 0.49 | 30.15 ± 0.78 | 105.7 ± 1.27 | 56.90 ± 5.37 | 77.00 ± 11.03 | –18.5 ± 2.69 |
|                |              | 150   | 99.93 ± 0.31 | –     | –     | –     | –     | –     | –     | 56.07 ± 4.72 | –       | –       |
|                |              | 200   | –     | –    | –      | –    | –     | –     | –     | –       | –35.5 ± 4.38 |
| PFF-A          |              | 12.5  | –     | –    | –      | –    | –     | –     | –     | –       | –       |
|                |              | 25    | –     | –    | –      | –    | –     | –     | –     | –       | –       |
|                |              | 50    | –     | –    | 4.67 ± 2.72 | –    | –     | 8.27 ± 2.03 | 52.80 ± 8.09 | 85.55 ± 8.41 | –       |
|                |              | 100   | 92.2 ± 0.95 | 20.95 ± 1.77 | 19.2 ± 9.76 | 96.47 ± 0.84 | 21.35 ± 0.49 | 30.15 ± 0.78 | 105.7 ± 1.27 | 56.90 ± 5.37 | 77.00 ± 11.03 | –18.5 ± 2.69 |
|                |              | 150   | 99.93 ± 0.31 | –     | –     | –     | –     | –     | –     | 56.07 ± 4.72 | –       | –       |
|                |              | 200   | –     | –    | –      | –    | –     | –     | –     | –       | –35.5 ± 4.38 |
| IC50 (µM) a    |              | <50   | NA    | NA   | <50   | NA   | NA   | 21.56 ± 2.16 | 42.25 ± 0.41 | 17.75 ± 3.42 | NA       |
| Reference Drugs, IC50 (nM) b |              | 0.41 | 17    | 22    | 9     | 77   | ND   | 4.6 | 1.9   | 4.4     | 23 |

a The 50% inhibition concentration (IC50) values of compounds were expressed as mean ± SD, n = 3. b IC50 values of reference drugs (hA2AR: ZM 241385, hα2AR: RX-821002, hα2C: rauwolscine, hδ-OPR: naltriben mesylate, CB1R: AM 281, GLP-1: exendin-3(9–39), hV1AR: [d(CH2)5, Tyr(Me)2]-AVP, h5-HT1AR: (S)-WAY-100635, h5-HT1B: GR55562). c The test compound induces at least 25% agonist effect at this concentration, which results in an apparent inhibition. NA No activity. (–) Not tested.
2.2. Dieckol and PFF-A as A2A R Antagonists

Dieckol inhibited the 3 nM epinephrine bitartrate response by 17.5%, 56.0%, and 62.43% at a concentration of 50, 100, and 150 µM, respectively, and yielded an IC50 value of 87.18 ± 2.63 µM (Table 3 and Figure 3A), while PFF-A inhibited the response of the reference agonist by 64.7%, 92.2%, and 99.93% at a concentration of 50, 100, and 150 µM, yielding an IC50 value < 50 µM (Figure 3A).

In the docking simulation, dieckol formed two H-bond interactions with Ile80 and Asp170 (Figure 4B) while four H-bond interactions (His278, Ala59, Ala81, Ser67) were observed for PFF-A (Figure 3C). The binding of reference ligands to the A2A R crystal structure showed the involvement of residues Phe168, Leu249, Asn253, and Met270. The total number of hydrophobic and electrostatic interactions involved in dieckol binding was greater than that of PFF-A binding (Table S2). Interestingly, only one interacting residue (Leu249) was in common with the reference ligand. However, PFF-A had two common interacting residues (Leu249 and Phe168) with reference antagonist ZM241385 (Table S1).

Figure 4. Molecular docking of dieckol and phlorofucofuroeckol-A in the active site of hA2A R (A), hα2C AR (D), and hδ-OPR (G) along with reported agonist (yellow stick) and antagonist (black stick). Detailed hA2A R–ligand (B) for dieckol and (C) for phlorofucofuroeckol-A, hα2C AR–ligand (E) for dieckol and (F) for phlorofucofuroeckol-A, and hδ-OPR–ligand interactions (H) for dieckol and (I) for phlorofucofuroeckol-A) on a 2D diagram.
2.3. *Dieckol* and *PFF-A* as $\alpha_{2C}$AR Agonists

Evaluation of the concentration-dependent agonist effect of phlorotannins (Table 3 and Figure 2A) at $\alpha_{2C}$AR depicted dieckol as a moderate agonist (EC$_{50}$: 98.80 ± 7.71 µM) and PFF-A as a good agonist (EC$_{50}$: 23.67 ± 3.32 µM). Even at a 25-µM concentration, PFF-A stimulated the effect of 1 µM epinephrine by 55%. The reference agonist epinephrine had an EC$_{50}$ value of 0.86 nM. To further support the functional effect and delineate the difference in activity between the two phlorotannins, a molecular docking simulation of test ligands and target protein was performed.

As shown in Figure 4D,E, dieckol interacted with Asn111, Ser108, Cys202, Asp206, and Gly203 via H-bond (Figure 2B). Similarly, PFF-A also displayed four H-bond interactions with Val414, Asp131, Ser401, and Gln413 (Figure 4F). H-bond interaction with Asp131 was a typical interaction observed for the reference agonist (epinephrine) and PFF-A, but absent in dieckol binding. Between two test ligands, hydrophobic interactions with Phe419, Tyr405, and Leu204 were common (Table S1).

2.4. *Dieckol* and *PFF-A* as $\delta$-OPR Antagonists

The dose-dependent antagonist effect at the $\delta$-opioid receptor depicted PFF-A as a potent natural antagonist. As shown in Table 4 and Figure 3B, even at the 50-µM concentration, PFF-A inhibited the effect of 25 nM [D—Pen2, D—Pen5]enkephalin (DPDPE) by 89.03 ± 0.70%, while the effect was 23.23 ± 4.04% for the same concentration of dieckol. Dieckol had an IC$_{50}$ value of 80.46 ± 13.74 µM, but the value was <50 µM for PFF-A. The reference antagonist naltriben mesylate had an IC$_{50}$ value of 9 nM. The binding of dieckol to the crystal structure of 4ej4 (Figure 4G,H) showed an involvement of three H-bond interactions (Asp128, Met132, Cys198) and numerous hydrophobic and electrostatic interactions (Met132 (Sulfur-O, $\pi$-alkyl), Lys108 ($\pi$-cation, $\pi$-Alkyl), Asp128 ($\pi$-anion), Val281 ($\pi$-sigma), Ile304 ($\pi$-sigma), Cys198 ($\pi$-sulfur), Ile277 ($\pi$-alkyl), and Val197 ($\pi$-alkyl)). Likewise, as shown in Figure 4I, PFF-A formed four H-bond interactions with Leu200, Lys214, Ile304, and Asp128 and five hydrophobic and electrostatic interactions with Asp128 ($\pi$-anion), Asp210 ($\pi$-Anion), Tyr129 ($\pi$-lone pair), Tyr308 ($\pi$-$\pi$ stacked), and Leu200 ($\pi$-alkyl).

The reference antagonist naltrindole showed an H-bond interaction with aspartic acid residue (Asp128) and numerous hydrophobic interactions with tryptophan residues - Trp284 ($\pi$-$\pi$T-shaped), Trp284 ($\pi$-alkyl), and Trp274 ($\pi$-alkyl). Only Asp128 was a common interacting residue among the test and reference ligands while Tyr308 was observed for PFF-A and reference ligand binding, but not for dieckol (Tables S1 and S2).

2.5. *PFF-A* as a CB$_1$R Agonist

Only PFF-A showed a full CB$_1$R agonist effect (113.8 ± 3.68%) at the 100-µM concentration. Therefore, the effect at lower concentrations was tested and, as shown in Table 3 and Figure 2C, PFF-A stimulated the effect of 10 nM CP 55940 by 46.7, 80.3, and 96.45% at 12.5, 25, and 50 µM, respectively. Hence, the log concentration vs. % simulation graph yielded an EC$_{50}$ value of 13.42 ± 2.03 µM. Reference agonist CP 55940 had an EC$_{50}$ value of 0.21 nM. To predict the binding affinity and characterize the binding mode of PFF-A and CB$_1$R, molecular docking simulation was performed (Figure 5A). As tabulated in Tables S3 and S4, PFF-A interacted with the active-state CB$_1$R (6kqi) by forming three H-bonds (Ser173, His178, and Met363) and numerous hydrophobic interactions—Phe177, Phe268, Trp279, Val196, Leu193, and Met363. Interactions with Ser173, Phe268, Phe177, Trp279, Val196, and Leu193 are a common observation in the binding of PFF-A and CP 55940 with the active-state CB$_1$R (6kqi). The reference antagonist taranabant interacted with the inactive-state CB$_1$R (5u09) by forming hydrogen-bond interactions with Ser173, Phe189, and Lys192 via the −CF$_3$ group. Likewise, other hydrophobic interactions involved in taranabant–5u09 binding were phenylalanine residues (Phe170, Phe174, Phe189, Phe268, and Phe379), Trp279, His178, Leu192, Leu193, Ile267, and Met363 (Figure 5B).
2.6. Dieckol and PFF-A as GLP-1R Antagonists

Results from the functional assay on mouse GLP-1 receptor-expressed βTC6 cells demonstrated dieckol and PFF-A as full antagonists of the GLP-1 receptor. At a concentration of 100 µM, both the compounds inhibited the effect of 0.3 nM GLP-1(7–37) by 100%. However, at the 25-µM concentration, PFF-A inhibited the reference agonist-response by 57.37% and dieckol by 21.23%. Additionally, a dose-dependent response curve yielded IC₅₀ values of 47.19 ± 2.46 and 21.56 ± 2.16 µM for dieckol and PFF-A, respectively (Table 4 and Figure 3C). The potency of PFF-A was two-fold higher than that of dieckol. The reference antagonist exendin-3(9–39) had an IC₅₀ value of 4.6 nM. From the molecular docking study, hydrogen-bond interactions with Ser352 and Thr355 (Table S3) and hydrophobic interactions with Leu354, Lys351, and Val405 (Table S4) were common observations in test ligands and reference antagonist NNC0640 binding with an inactive-state GLP-1R (5vex) (Figure 5C–E) in our molecular docking simulation. An unfavorable contact between dieckol and GLP-1R receptor was observed via the Asn407 residue.

2.7. Dieckol as Agonist and PFF-A as Antagonist of hV₁₆R

The agonist effect of dieckol at V₁₆R was first tested at 100 µM to compare with the effect of PFF-A that we reported earlier [22]. As tabulated in Table 2, dieckol at 100-µM concentration stimulated the percentage agonist effect of 1 µM arginine vasopressin (AVP) by 106.73 ± 2.97% and inhibited the percentage of control agonist response by 57.77 ± 0.32%. In the hV₁₆R antagonist assay, the 100-µM concentration of dieckol induced at least a 25% agonist effect. In comparison, PFF-A induced a 38.45 ± 7.14% stimulation and 56.90 ± 5.37% inhibition of the control agonist response at 100 µM. Furthermore, the
concentration-dependent dose–response curve depicted dieckol as a hV₁ₐR agonist (EC₅₀: 39.12 ± 2.12 µM) (Table 3 and Figure 2B) and PFF-A as an antagonist (IC₅₀: 42.25 ± 0.41 µM) (Table 4 and Figure 3D).

Molecular simulation of dieckol and PFF-A along with reference ligands to a crystal structure of hV₁ₐR predicted that both test ligands bind with high affinity (Figure 6A). Dieckol formed H-bond interactions with Gln131, Ala334, and Asp112, and hydrophobic interactions with Lys128, Met135, Trp204, Ala101, and Ala334 (Figure 6B). Reference agonist AVP formed H-bond interactions with Asp202 (Salt-bridge), Glu54, Asp112, and Ile330 and hydrophobic interactions with Trp204, Ile330, Ala101, Ala334, Val132, and Met135. This shows that dieckol and AVP have numerous residues in common that involve binding with the receptor.

Likewise, PFF-A bound to the hV₁ₐR via five H-bond interactions (Ser338, Cys203, Met135, Glu54, Ala101) and other hydrophobic interactions with Lys128, Met220, Phe189, Phe307, Val132, Val100, Ala101, Met135, Ala334, Ala205, and Val105 (Figure 6C). Three H-bond interactions with Gln131, Gln108, and Lys128, and hydrophobic interactions with Phe307, Trp204, Val132, Met135, Met220, Ala334, Ala205, Gln131, and Thr333 were observed for SR49059 binding. The docking result shows that, respective to their functional effect, dieckol and PFF-A interact with residues that were involved in the binding of the reference agonist and antagonist (Tables S5 and S6).

2.8. Dieckol and PFF-A as 5-HT₁ₐR Antagonists

An antagonist effect was observed for dieckol and PFF-A in a cell-based functional assay. At 100-µM concentration, dieckol and PFF-A inhibited the response of 30 nM serotonin by 91.0 ± 3.11% and 77.00 ± 11.03%, respectively (Table 2). A concentration-
dependent dose–response showed that dieckol and PFF-A inhibited the 50% response of 30 nM serotonin at 43.31 ± 3.22 and 17.75 ± 3.42 µM, respectively (Table 4 and Figure 3E). However, the agonist effect at 5-HT1A R was negligible for both the compounds when tested at the 100-µM concentration. As a result, the EC50 value was not determined. Reference drug serotonin had an EC50 value of 0.72 nM and antagonist GR55562 had an IC50 value of 4.4 nM.

Docking of test and reference ligands to the active site of 5-HT1A R demonstrated that aspartic acid residue Asp116 is one of the important binding residues (Figure 6D). Dieckol formed an H-bond interaction with Asp116, Thr200, Ser190, Asn386, and Tyr96 while PFF-A did with Thr188, Glu372, Tyr96, and Asn386 (Figure 6E,F). Reference ligands serotonin and WAY 100635 formed an H-bond interaction with Asp116 via a salt-bridge. Interactions with Thr200, Phe361, and Val117 were observed for test ligands and serotonin binding (Tables S5 and S6).

3. Discussion

Dieckol and PFF-A are phloroglucinol (1,3,5-trihydroxybenzene)-based polyphenols with a varied number of phloroglucinol units attached via dibenzofuran and dibenzodioxin linkages. Dieckol is a phloroglucinol hexamer and PFF-A is a phloroglucinol pentamer. A structure–activity relationship between phloroglucinol and its oligomers in our recent study [22] showed that more than three repeating phloroglucinol units are necessary for hMAOs inhibition and D3/D4 receptor agonist effect. Likewise, oligomerization of phloroglucinol with more than five repeating units is essential for the antagonist effect at D1, NK1, and 5-HT1A receptors. Here, although the monomer phloroglucinol is not included in the study, the pentamer (PFF-A) showed better activity than a hexamer (dieckol). An interesting observation in this study is that regardless of the receptors at which these two phlorotannins showed functional effects (except the hV1A R), PFF-A was two-fold more potent than dieckol. In contrary to the findings that the phenolic -OH groups attached to the benzene ring of polyphenols play a vital role in the antioxidant effect [24–26] and that an increase in the number of hydroxyl groups increases antioxidant activity, the functional effect of PFF-A at tested GPCRs was higher than that of dieckol despite having a lower number of hydroxyl groups. The possible reason underlying this might be the structure or orientation of PFF-A that enables it to reach the core active site cavity of receptors where it binds to conserved interacting residues leading to conformational change.

Adenosine is an endogenous autacoid that regulates cellular physiology via adenosine A1, A2A, A2B, and A3 receptors. These receptors are expressed in several cells and tissues throughout the body and play a crucial role in regulating the pathophysiology of the human body, suggesting a potential drug target. Of different adenosine receptor subtypes, A2A R is the main receptor subtype in the striatum colocalized with dopamine D2 receptor and it modulates motor function [27,28]. Activation of A2A R decreases the binding affinity of D2 R for agonists, implying A2A R antagonists as novel therapeutics for Parkinson’s disease [29]. At the synapse, A2A R facilitates glutamate release and potentiates NMDA receptor effects. It also stimulates glutamate release in astrocytes by inhibiting glutamate transporter-1 (GLT-1), and the level of A2A Rs in neurons and glia is significantly high in depression and Schizophrenia [30]. Hence, A2A Rs antagonists are effective as antidepressants and anti-anxiety agents. Here, dieckol and PFF-A showed an antagonist effect at hA2A R with IC50 values of 87.18 ± 2.63 and <50 µM, respectively. Furthermore, molecular docking simulation showed that dieckol and phlorofucofuroeckol-A strongly interact with the Phe168 residue, which is known as one of the important residues for ligand binding, via pi–pi interaction [31]. Structurally, dieckol and PFF-A are powerful radical scavengers [32] and as such, dieckol, in a recent study [33], protected dopaminergic neuronal cells by preventing α-synuclein aggregation via antioxidant mechanism. In a previous study [34], dieckol suppressed LPS-induced excessive microglial activation and protected neuronal cells by downregulating extracellular signal-regulated kinases, protein kinase B (PKB/Akt), and nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidase-mediated
pathways. Likewise, PFF-A inhibited glutamate-induced apoptotic PC12 cell death in a caspase-dependent manner [35].

Similarly, at α2C-AR, PFF-A showed a strong agonist effect and formed an H-bond with the Asp131 of α2C-AR, which is the conserved active site residue. Adrenergic receptors are targets for epinephrine and norepinephrine and are involved in maintaining homeostasis. Among several types of adrenergic receptors, highly expressed α2 adrenoceptors in astrocytes, and in glutamatergic and GABAergic neurons act by increasing intracellular Ca2+ levels [36]. The α2C-AR subtype mediates cold-induced vasoconstriction, inhibits dopamine release in basal ganglia [37], and serotonin in the mouse hippocampus [38]. Therefore, α2C-AR selective ligands have a therapeutic role in neuropsychiatric disorders [38] and α2C-AR agonists are implicated in the treatment of neuropathic pain [39–41].

Serotonin (5-hydroxytryptamine, 5-HT) is a monoamine neurotransmitter that plays a crucial role in physiological functions, and of a total of 14 subtypes of 5-HT receptors, the 5-HT1A receptor is a prominent target for the treatment of various neuropsychiatric and neurological disorders, prominently depression [42]. In the functional assay, dieckol and PFF-A showed a good antagonist effect at 5-HT1AR. Furthermore, they interacted with conserved aspartate residue (Asp116) of 5-HT1AR via H-bond and π–anion binding, respectively.

Vasopressin is an antidiuretic hormone that plays a vital role in the central nervous system (CNS) and peripheral nervous system (PNS). The vasopressin receptor is one of the promising targets for CNS drugs, and vasopressin antagonists represent a novel approach for the treatment of stress, mood, and behavioral disorders [43]. Likewise, as a peripheral role, V1AR is responsible for vasoconstriction, myocardial contractility, platelet aggregation, and uterine contraction [44]. Similarly, in a recent study [45], upregulated vasopressin 1 receptor (V1R) expression in hepatocytes of ischemia-reperfusion injury mouse model was identified and the V1R/Wnt/β-catenin/FoxO3a/Akt pathway was highlighted as vital for hepatoprotection.

Cannabinoid CB1 receptors are among the most abundant GPCRs in the brain and they modulate CNS activity [46]. Cannabinoid CB1 receptor agonist activation of the CB1 receptor leads to decreased levels in cellular cAMP via inhibition of adenylyl cyclase. Moreover, CB1 activation inhibits voltage-gated Ca2+ channels and activates K+ channels, and these overall intracellular signaling activities reduce cellular excitability [47]. Likewise, studies also indicate high expression levels of CB1R in various types of cancer [48,49]. Interestingly, a new study demonstrated a higher orexigenic effect of the CB1R agonist AM11101 than tetrahydrocannabinol [50]. This shows that CB1R agonists could be used as an appetite stimulant in underweight patients. In the present study, only PFF-A showed a promising agonist effect at CB1R with an EC50 of 13.42 ± 2.03 µM. Several reports on PFF-A show neuroprotective effects mainly via antioxidant mechanisms [14,35,51]. Likewise, a recent study suggested the ATF3-mediated pathway as a possible mechanism of PFF-A-induced apoptosis in human colorectal cancer cells [52]. However, it remains unclear whether the neuroprotective and anticancer effect of PFF-A is via CB1R agonist activity.

The human CB1 receptor is an important therapeutic target for obesity and obsessive disorders and the mechanism of its transition state (either active or inactive) is vital for understanding the regulatory action of the receptor [53]. A salt bridge between conserved Asp-Arg-Tyr (DRY) motif in the C-terminal region of transmembrane 3 (TM3) and transmembrane 6 (TM6) characterizes the active or inactive conformation of the rhodopsin-like GPCRs [54]. In an inactive conformation of CB1R, TM6 packs against TM3 and transmembrane 5 (TM5) and G protein-interacting residues—Phe200 (helix III) and Trp356 (helix VI) are obstructed [55]. The reference inverse agonist (taranabant) is bound to the inactive state crystal structure by forming an H-bond interaction between the NH of taranabant and the hydroxyl of Ser383 and the −CF3 group with Ser173, Phe189, and Lys192. This result corroborates the findings of a previous study [56] which concluded that a strong H-bond between the -NH group of taranabant and the hydroxyl of Ser383 was vital for superior affinity to CB1R. Likewise, the agonist CP55940 formed π–π interactions with Phe170 and Phe268, and two H-bond interactions with Ser173 and Ser383 in a similar
fashion, as reported earlier [56]. PFFA also formed a stable pi–pi interaction with Phe268 and an H-bond interaction with Ser173 of the active state crystal structure (6kqi), which could explain the agonist potency of PFFA in vitro.

Among the tested protein targets, CB_{1}R, GLP-1, and GPR40 are obesity/T2DM related GPCRs and in the functional assays, PFF-A showed a good agonist effect at CB_{1}R, while both the dieckol and PFF-A showed an antagonist effect at the GLP-1 receptor. Their effect at GPR40 was mild agonist. A gut-derived incretin hormone GLP-1 stimulates insulin and suppresses glucagon secretion, inhibits gastric emptying, and reduces appetite and food intake. In a previous study, intracerebroventricular injection of exendin (9–39), a specific GLP-1 antagonist, blocked the inhibitory effect of GLP-1 on food intake [57]. Hence, GLP-1 agonists represent a new class of antidiabetic agents [58]. In a recent study on the anti-diabetic effect in the zebrafish model [59], dieckol treatment reduced liver glucose-6-phosphate and phosphoenolpyruvate carboxykinase, and enhanced glucose transport and insulin sensitivity via protein kinase B (Akt) phosphorylation. It is of note that dieckol and PFF-A showed a good antagonist effect at GLP-1. Thus, the in vivo effects of these phlorotannins in GLP-1-mediated signaling are urgent.

In conclusion, the present study characterizes the receptors hA_{2A}R, hA_{2C}AR, hδ-OP, CB_{1}R, GLP-1, hV_{1}A R, and h5-HT_{1A}R as prime protein targets of dieckol and PFF-A. Moreover, the binding mechanism of test ligands with the target proteins strengthens the study and warrants further in vivo studies.

4. Materials and Methods

4.1. Chemicals and Reagents

A transfected Chinese hamster ovary (CHO), Hela, a murine interleukin-3 dependent pro-B (Ba/F3), PC12, and rat basophil leukemia cell lines were obtained from Eurofins Scientific (Eurofins-Cerep, Le Bois l’Éveque, France). Buffers—Dulbecco’s modified Eagle medium (DMEM) buffer, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, and Hank’s balanced salt solution (HBSS) buffer—were purchased from Invitrogen (Carlsbad, CA, USA). The reference agonists: 5′-N-ethylcarboxamidoadenosine (NECA), epinephrine bitartrate, epinephrine, DPDPE, CP 55940, linoleic acid, GLP-1(7–37, arginine vasopressin (AVP), and serotonin, and antagonists: ZM 241385, RX-821002, rauwolscine, naltriben mesylate, AM 281, exendin-3(9–39), [d(CH_{2})_{5}, Tyr (Me)_{2}]-AVP, (S)-WAY-100635, and GR55562) were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and reagents purchased from Merck and Fluka were of the highest available grade unless otherwise stated.

4.2. Isolation of Phlorotannins

Phlorotannins—dieckol and PFF-A were isolated from the ethyl acetate fraction of E. stolonifera ethanolic extract, as described previously [11,22].

4.3. In Silico Prediction of Targets

To predict potential protein targets for the phlorotannins, a proteocheminformatics modeling (PCM) in silico target prediction method was employed, as described recently [60]. For full information on the model, readers are further directed to a previous report [61].

4.4. Functional GPCR Assay

The functional assay using transfected cells expressing human cloned receptors, PC12 cells for adenosine A_{2A} receptor, rat basophil leukemia cells for human adrenergic alpha2A receptor, human delta opioid (δ-OP) receptor, CHO cells for adrenergic alpha2C receptor, human cannabinoid CB_{1}, and vasopressin (V_{1A}R), human embryonic kidney 293 (HEK-293) cells for free fatty acid receptor 1 (FFA_{1}R or GPR40), βTC6 cells for the glucagon-like peptide-1 receptor (GLP-1), Ba/F3 cells for serotonin (5-HT_{1A}), and Hela for 5-HT_{1B} receptors were carried out at Eurofins laboratory (Eurofins-Cerep, Le Bois l’Éveque, France).
The in-house assay protocol and experimental conditions are reported in our previous reports [15,22,62]. The functional effect of dieckol and PFF-A was characterized based on their modulation effect on cytosolic Ca\textsuperscript{2+} ion mobilization using a fluorimetric detection method or by measuring their effect on cAMP modulation using homogeneous time-resolved fluorescence (HTRF) detection.

4.5. Measurement of cAMP Level

Functional activity of phlorotannins over hA2A R, h\(\alpha_2C\) AR, hCB\(_1\) R, GLP-1R, and h5-HT\(_{1B}\) R was determined by measuring their effects on cAMP production by the HTRF detection method using transected cells expressing human cloned receptors.

4.5.1. Functional Activity over hA2A R

In brief, the PC12 cells were suspended in HBSS buffer (Invitrogen) complemented with 20 mM HEPES (pH 7.4), 0.2 U/mL ADA, and 100 \(\mu\)M rolipram, then distributed in microplates at a density of \(2 \times 10^3\) cells/well and preincubated for 5 min at room temperature (RT) in the presence of HBSS (basal control), the test compound, or the reference agonist or antagonist. For stimulated control measurement, separate assay wells contained 3 \(\mu\)M NECA. Following 10 min incubation at RT, the cells were lysed and the fluorescence acceptor (D2-labeled cAMP) and fluorescence donor (anti-cAMP antibody labeled with europium cryptate) were added. After 60 min at RT, the fluorescence transfer was measured at \(\lambda_{ex} = 337\) nm and \(\lambda_{em} = 620\) and 665 nm using an EnVision microplate reader EnSpire (PerkinElmer, Waltham, MA, USA). The cAMP concentration was determined by dividing the signal measured at 665 nm by that measured at 620 nm (ratio). Agonist result was expressed as a percent of the control response to 3 \(\mu\)M NECA while the antagonist effect as percent inhibition of the control response to 100 nM NECA. The standard reference agonist was NECA and the antagonist was ZM 241385, which were tested in each experiment at several concentrations to generate a concentration–response curve from which their EC\(_{50}\) and IC\(_{50}\) values were calculated.

4.5.2. Functional Activity over h\(\alpha_2C\) AR

Briefly, the transfected CHO cells suspended in HBSS buffer (Invitrogen) complemented with 20 mM HEPES (pH 7.4) and 500 \(\mu\)M IBMX were distributed in microplates at a density of \(10^4\) cells/well in the presence of either of the following: For agonist assay—HBSS (basal control), epinephrine 1 \(\mu\)M (stimulated control) or various concentrations (EC\(_{50}\) determination), or the test compounds. For antagonist assay—HBSS (stimulated controls), rauwolscine 10 \(\mu\)M (basal control) or various concentrations (IC\(_{50}\) determination), or the test compounds. The reference agonist epinephrine and the adenylyl cyclase activator NKH 477 were added at respective final concentrations of 100 nM and 5 \(\mu\)M. For basal control measurements, epinephrine was omitted from the wells containing 3 \(\mu\)M rauwolscine. After 10 min at 37 \(^\circ\)C, the cells were lysed and the fluorescence acceptor (D2-labeled cAMP) and fluorescence donor (anti-cAMP antibody labeled with europium cryptate) were added. After 60 min at RT, the fluorescence transfer was measured at \(\lambda_{ex} = 337\) nm and \(\lambda_{em} = 620\) and 665 nm using a microplate reader (Envision, Perkin Elmer). The concentration of cAMP was determined by dividing the measured signal at 665 nm by that measured at 620 nm (ratio). The agonist result are shown as a percent of the control response to 1 \(\mu\)M epinephrine and the antagonist result are expressed as a percent inhibition of the control response to 30 nM epinephrine. Epinephrine and rauwolscine were the standard reference drugs used in each experiment at different concentrations.

4.5.3. Functional Activity over hCB\(_1\) R

The transfected CHO cells were suspended in HBSS buffer (Invitrogen) complemented with 20 mM HEPES (pH 7.4). Then, the cells were distributed in microplates at a density of \(5 \times 10^3\) cells/well in the presence of either of the following: For agonist assay—HBSS (basal control), 30 nM CP 55940 (stimulated control) or various concentrations (EC\(_{50}\) determination).
determination), or the test compounds. For antagonist assay—HBSS (stimulated controls), 10 \( \mu M \) AM 281 (basal control) or various concentrations for IC\( _{50} \) determination, or the test compounds. Thereafter, the reference agonist CP 55940 and the adenylyl cyclase activator forskolin were added at respective final concentrations of 1 \( nM \) and 25 \( \mu M \). For basal control measurements, CP 55940 was excluded from the wells containing 10 \( \mu M \) AM 281. After 30 min of incubation at 37 \( ^\circ C \), the cells were lysed and the fluorescence acceptor (D2-labeled cAMP) and fluorescence donor (anti-cAMP antibody labeled with europium cryptate) were added. The fluorescence transfer was measured at \( \lambda_{ex} = 337 \text{ nm} \) and \( \lambda_{em} = 620 \text{ and } 665 \text{ nm} \) using an Envision microplate reader (PerkinElmer, Waltham, MA, USA) after 60 min at RT. The agonist results are expressed as a percent of the control response to 10 \( nM \) CP 55940 and the antagonist results are expressed as percent inhibition of the control response to 1 \( nM \) CP 55940. CP 55940 and AM 281 were standard reference drugs that were tested in each experiment.

4.5.4. Functional Activity over GLP-1\( R \)

The HBSS buffer (Invitrogen) complemented with 20 \( mM \) HEPES (pH 7.4) and 500 \( \mu M \) IBMX was used to suspend and distribute the \( \beta TC6 \) cells at a density of 1.5x10^4 cells/well. The plate was then incubated for 10 min at RT in the presence of HBSS (basal and stimulated control), the test compound, or the reference agonist and antagonist. In the agonist assay, separate assay wells containing 100 \( nM \) GLP-1(7–37) were prepared for the stimulated control measurement, while in the antagonist assay, the reference agonist GLP-1(7–37) was added at a final concentration of 0.3 \( nM \), and separate assay wells contained HBSS for basal control measurements. Following incubation, the cells were lysed and the fluorescence acceptor (D2-labeled cAMP) and fluorescence donor (anti-cAMP antibody labeled with europium cryptate) were added. After 60 min at room temperature, the fluorescence transfer was measured at \( \lambda_{ex} = 337 \text{ nm} \) and \( \lambda_{em} = 620 \text{ and } 665 \text{ nm} \) using an Envision microplate reader (PerkinElmer, Waltham, MA, USA). The results are expressed as either a percent of the control response to 100 \( nM \) GLP-1(7–37) or a percent inhibition of the control response to 0.3 \( nM \) GLP-1(7–37). The standard reference agonist was GLP-1(7–37) and the antagonist was exendin-3(9–39).

4.5.5. Functional Activity over 5-HT\( _{1B} \)R

Concisely, a plasmid containing the GPCR gene of interest (5-HT\( _{1B} \)) was transfected into Hela cells. The resulting stable transfectants were suspended in HBSS buffer (Invitrogen, Carlsbad, CA, USA) containing 20 \( mM \) HEPES (pH 7.4), 400 \( mM \) NaCl, 1 mg/mL glucose, and 500 \( \mu M \) IBMX and distributed in microplates at a density of 2 \( \times \) 10^4 cells/well. The plates were then incubated for 20 min at RT in the presence of either of the following: HBSS and 0.1% BSA (basal control), serotonin at 10 \( \mu M \) (stimulated control) or various concentrations for EC\( _{50} \) determination, or the test phlorotannins. Thereafter, the adenylyl cyclase activator NKH 477 (5 \( \mu M \)) was added and the plates were incubated at 37 \( ^\circ C \) for 20 min. Then, the cells were lysed and a fluorescence acceptor (D2-labeled cAMP) and fluorescence donor (anti-cAMP antibody with europium cryptate) were added following 60 min incubation at RT. After incubation, the fluorescence transfer was measured using an Envision microplate reader (PerkinElmer, Waltham, MA, USA) and the results are expressed as a percentage of the control response to 10 \( \mu M \) serotonin for the agonist effect and as percent inhibition of the control response to 100 \( nM \) serotonin.

4.6. Measurement of Intracellular \( [Ca^{2+}] \) Level

Functional activity of phlorotannins over human adrenergic \( \alpha_{2A} \) (h\( \alpha_{2A} \)), human \( \delta \)-opioid (h\( \delta \)-OP), free fatty acid receptor 1 (FFA\( _1 \)/GPR40), human vasopressin 1A (hV\( _1A \)), and human serotonin 1A (h5-HT\( _{1A} \)) receptors was assessed by measuring their effect on cytosolic \( Ca^{2+} \) ion mobilization at the transected cells expressing human cloned receptors using a fluorimetric detection method.
4.6.1. Functional Activity over hα2A AR

The rat basophil leukemia cells were distributed in microplates at a density of $1.1 \times 10^4$ cells/well after suspending in a HBSS buffer (Invitrogen) containing 20 mM HEPES. Then, the fluorescent probe (Fluo8, AAT Bioquest) mixed with probenecid in HBSS buffer (Invitrogen) complemented with 20 mM Heps (Millipore) (pH 7.4) was added into each well incubated for 60 min at 30 °C. Thereafter, the assay plates were positioned in a microplate reader (FlipR Tetra, Molecular Device) and we added test compounds, reference agonist/antagonist or HBSS buffer (basal control). Change in fluorescence intensity which varies proportionally to the free cytosolic Ca$^{2+}$ ion concentration was measured. For stimulated control measurements, separate assay wells containing 0.1 µM epinephrine bitartrate were prepared. The agonist effect was calculated as a % of control response to epinephrine bitartrate at 0.1 µM. Similarly, for the antagonist effect, % inhibition of the control response to epinephrine bitartrate at 3 nM was evaluated. Epinephrine bitartrate and RX-821002 were used as reference agonists and antagonists, respectively.

4.6.2. Functional Activity over hδ-OPR

At first, rat basophil leukemia cells were suspended in HBSS buffer (Invitrogen) complemented with 20 mM HEPES, and distributed in microplates at a density of 2.768 $\times 10^4$ cells/well. Thereafter, a mixture of fluorescent probe (Fluo8, AAT Bioquest) and probenecid in HBSS buffer (Invitrogen) complemented with 20 mM Hepes (Millipore) (pH 7.4) was added and plates were incubated for 60 min at 30 °C. Then, the assay plates were positioned in a FlipR Tetra microplate reader (Molecular Device, San Jose, CA, USA) for the addition of the test compound, reference agonist/antagonist, or HBSS buffer (basal control). Change in fluorescence intensity that varies proportionally to the free cytosolic Ca$^{2+}$ ion concentration was measured.

For stimulated control measurements, 1 µM DPDPE was added in separate assay wells. The results are expressed as a percent of the control response to DPDPE at 1 µM or a percent inhibition of the control response to DPDPE at 25 nM. The standard reference agonist and antagonist were DPDPE and naltriben mesylate, respectively.

4.6.3. Functional Activity over FFA1R/GPR40

In general, transfected HEK-293 cells suspended in DMEM buffer (Invitrogen) containing 1% FCSd were distributed in microplates at a density of $2.10^4$ cells/well. Then, the mixture of fluorescent probe (Fluo4 Direct, Invitrogen) and probenecid in HBSS buffer (Invitrogen) complemented with 20 mM Heps (Invitrogen) (pH 7.4) was added into each well and incubated for 60 min at 37 °C, followed by 15 min incubation at 22 °C. Thereafter, the assay plates were positioned in a CellLux microplate reader (PerkinElmer, Waltham, MA, USA) which was used for the addition of the following: For agonist assay—test compound, reference agonist, or HBSS buffer (basal control). Linoleic acid at 100 µM was added in separate assay wells for stimulated control measurement. For antagonist assay—test compound or HBSS buffer (basal and stimulated control), then, 5 min later, 20 µM linoleic acid. Agonist results are expressed as a percent of the control response to 100 µM linoleic acid while antagonist results are expressed as percent inhibition of the control response to 20 µM linoleic acid.

4.6.4. Functional Activity over hV1A R

Briefly, CHO-V1AR cells were separately suspended in DMEM buffer (Invitrogen, Carlsbad, CA, USA) complemented with 0.1% FCSd and distributed into microplates (4.5 $\times 10^4$ cells/well). Then, fluorescent probe (Fluo4, Invitrogen) mixed with probenecid in HBSS buffer (Invitrogen, Carlsbad, CA, USA) supplemented with 20 mM HEPES, pH 7.4 (Invitrogen) was added to each well, allowing to equilibrate with the cells for 60 min at 37 °C, then 15 min at 22 °C. Thereafter, the assay plates were positioned in a CellLux microplate reader (PerkinElmer, Waltham, MA, USA) and dieckol and PFF-A (12.5, 25, 50, 100, and/or 150 µM), reference agonist, or HBSS buffer (basal control) was added. For
stimulated control measurements, AVP at 1 µM was added in separate assay wells. The agonist effect on V1A R was calculated as a % of control response to 1 µM AVP. Similarly, for the antagonist effect, % inhibition of the control response to 10 nM AVP was evaluated. AVP and [d(CH2)5, Tyr (Me)2]-AVP were used as reference agonist and antagonist, respectively.

4.6.5. Functional Activity over h5-HT1A R

In brief, Ba/F3-5HT1A R cells were first suspended in HBSS buffer (Invitrogen, Carlsbad, CA, USA) complemented with 20 mM HEPES buffer (pH 7.4). Then, the cells were distributed into microplates at a density of 1 × 10⁶ cells/well. Subsequently, fluorescent probe (Fluo8, AAT Bioquest) mixed with probenecid in HBSS buffer (Invitrogen, Carlsbad, CA, USA) supplemented with 20 mM HEPES (Invitrogen) (pH 7.4) was added to each well, and the plates were incubated for 60 min at 37 °C. Thereafter, plates were fixed in a FlipR Tetra microplate reader (Molecular Device, San Jose, CA, USA) and dieckol and PFF-A (12.5, 25, 50, 100 and/or 150 µM), reference agonist, or HBSS buffer (basal control) was added. Fluorescence intensity was measured which varied in proportion to the free cytosolic Ca²⁺ ion concentration. Agonist effect on 5-HT1A R was calculated as a % of control response to 2.5 µM serotonin. Similarly, the percentage inhibition of the control response to 30 nM serotonin was calculated for the antagonist effect. Serotonin and (S)-WAY-100635 were used as reference agonists and antagonists, respectively.

4.7. Homology Modeling and Molecular Docking

The primary sequence of the human 5-HT1A R and human V1A R was obtained from UniProt (ID: P08908 and P37288, respectively). Based on the SWISS-MODEL, the 5-HT1B receptor (PDB: 5V54) was selected as a template for homology modeling of human 5-HT1A because it showed a good sequence similarity (0.42), sequence identity (42.97), and quaternary structure quality estimate (QSQE) (0.32) to this receptor. Similarly, µ-opioid receptor (PDB: 4DKL) was selected as a template for homology modeling of human V1A R, because it showed a good sequence similarity (0.32), sequence identity (24.54), and QSQE (0.19) to this receptor. The constructed model was refined using the ModRefiner server. Automated docking simulations were carried out with the AutoDock 4.2. program [63]. The structures of dieckol and PFF-A were generated and converted into 3D structures using Marvin Sketch (v17.1.30, ChemAxon, Budapest, Hungary). Structures of dieckol and PFF-A were energy-minimized using a molecular mechanics 2 (MM2) force field. X-ray crystallographic structures of GPCRs were obtained from the RCSB protein data bank (PDB) with respective PDB IDs—hA2A R (3eml) [31], hα2C AR (6kuw), hδ-OP (4ej4) [64], hCB1 R (6kqi) [65], and hGLP-1 [66]. The structures of reported agonists (5'-N-ethylcarboxamidoadenosine (NECA), epinephrine, DPI-287, CP 55940, PF-06882961, AVP, and serotonin, and antagonists (ZM241385, RS-79948, naltrindole, taranabant, NNC0640, SR49059, and WAY 100635) were downloaded from PubChem or PDB. For each ligand–protein complex, 10 docking poses were generated using the same grid parameters (size and center) and docking parameters (genetic algorithm and run options). The pose for the lowest binding energy was chosen for the final docking result. When the root-mean-square deviation (RMSD) value between our docking result and the original crystallographic structures of the protein was less than 0.15 nm, we considered our docking protocol to be valid and performed the simulation. Results were analyzed and visualized using Discovery Studio (v17.2, Accelrys, San Diego, CA, USA).

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/md19060326/s1, Table S1: Hydrogen bonding interaction residues between ligand–GPCRs (hA2A R, hα2C AR, and hDOP), Table S2: Hydrophobic and electrostatic interaction residues between ligand–GPCRs (hA2A R, hα2C AR, and hDOP), Table S3: Hydrogen, halogen, or electrostatic bonding interaction residues between ligand–GPCRs (hC12B R and hGLP-1), Table S4: Hydrophobic interaction residues between ligand–GPCRs (hC12B R and hGLP-1), Table S5: Hydrogen bonding interaction residues between ligand–GPCRs (hV1A R and h5-HT1A R), and Table S6: Hydrophobic and electrostatic interaction residues between ligand–GPCRs (hV1A R and h5-HT1A R).
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