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Identification of Novel Drug Leads for Receptors Implicated in Migraine from Traditional Ayurvedic Herbs Using *in silico* and *in vitro* Methods

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Abstract

Background: Migraine is a chronic neurological disorder characterized by headaches along with several physiological and autonomic nervous system symptoms. Research suggests that migraine is a result of multi-gene mutation in combination with psycho-social and environmental factors.

Method: Mutated mammalian serotonin hydroxytryptamine receptor 2 (HTR2) implicated as factor causing migraine were retrieved from the National Centre for Biotechnology Information (NCBI), its 3D structure were determined by homology modelling. The 3D structures of phyto-compounds (from Ayurvedic herbs) were retrieved from various databases. The pharmacophore hypothesis was generated for the existing ligands and the phytocompounds were screened against the generated pharmocophoric hypothesis. Ligands were shortlisted based on their fitness score. The selected phytocompounds were screened against HTR2 receptor.

Results: The phytocompound having the best docking score and most interactions with the receptor are validated using receptor-ligand binding assay studies with HTR2 receptor in-vitro.

Conclusion: Phytocompounds selected as per receptor-ligand binding assay studies.

Keywords: Migraine; Serotonin; Ayurveda; Modeling; Pharmacophore; Docking; Rapid eye movement (REM); Binding assay

Background

The current manuscript is a continuation work of manuscript titled "Selecting the best ligand for Migraine Protein 5-hydroxytryptamine (serotonin) receptor 2A (HT2A) from the Compounds of *Valeriana wallichii*, *Asparagus racemosus* and *Acorus calamus*" by Somashekhar R, Bagchi P et al., 2014.

Rapid eye movement sleep behavior disorder (**RBD**) is a sleep disorder that involves abnormal behaviour during rapid eye movement (REM) sleep [1]. REM is the stage of sleep during which most vibrant dreaming occurs. The loss of motor inhibition leads to a wide spectrum of behavior during sleep. Migraine is a type of REM disorder [2]. Studies suggest that genetics, prenatal care with environment, combined with psychological and social factors are important causes of migraine. Research suggests that migraine is caused by actions of several mutated genes [3]. Migraine is the most frequent neurological disorder in the adult population worldwide. Headache is the primary clinical symptom and it has been associated with a hereditary or dependence of neurovascular reactions to cyclic changes in the central nervous system. Amongst the many neurotransmitters in the brain, the serotonergic (serotonin, 5-HT) system from the brainstem raphe nucleus has been most believably implicated in migraine pathophysiology [4]. The mammalian HT2 receptor is the main excitatory receptor subtype among the (G protein-coupled receptor) GPCRs for serotonin. HTR2 may also have an inhibitory effect [5,6] on certain areas such as the visual cortex and the orbitofrontal cortex. Serotonin (5-hydroxytryptamine (5-HT)1) is a major neurotransmitter that is involved in multiple physiological functions such as the control of endocrine secretion, motor behavior, mood, pain, sleep, thermoregulation, and appetite and is indicated as causal factor for several allied neuronal disorders [7,8].

Mutation in mammalian serotonin hydroxytryptamine receptor 2 (HTR2), implicated as factors causing migraine, is taken in this study [3,4]. The use of phytochemicals as novel, potential lead drug molecules for HTR2, a GPC receptor was tested *in silico* and *in vitro* in this study by matching the pharmacophoric features of a known ligand myristicin with the phytocompounds from Ayurvedic herbs (The psychotropic property of the herbs used in this work are based on practical studies carried out at Satsang Herbal Research Laboratory, Satsang, Deoghar, India) (Table 1). Myristicin is an agonist pertaining to HTR2 receptor [9]. It is a natural product isolated from parsley oil [10]. This volatile oil, myristicin, comprises a mixture of allylbenzene derivatives and terpines [11].

Methodology

Predicting the 3D structure of the receptors

The amino acid sequence of the HTR2 receptor was retrieved from the National Center for Biotechnology Information (NCBI). Using Basic Local Alignment and Search Tool (BLAST) search engine against Protein Data Bank (PDB) the homologous templates for the receptor was selected and their crystal structure was downloaded from PDB. Using these homologous templates, the 3D structure of the receptor was generated by modeler [12].

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3-O-Acetyl-a-boswellic acid	Phyllanthin	Rubiadin	Corosolic acid
Demethoxycurcumin	Eupalitin	18α-Glycrrhetinic acid	3,3',4,4'-Tetraahydroxy-2-Methoxychalcone
Lutein	Asiaticoside	Berberine chloride dihydrate	
14-Deoxy-11,12-didehydroandrographolide	Picroside I	Rutin	Hypophyllanthin
Mangiferin	Eupalitin-3-O-galactoside	18α-Glycrrhetinic acid	Crocetin dialdehyde
3-O-Acetyl-11-keto-β-boswellic acid	Agnuside	Bisdemethoxycurcumin	Ursolic acid
1,9-Dideoxyforskolin	Picroside II	Serratol	4-Hydroxyisoleucine
Marmelosin	Eclalbasaponin II	Glcyrrihizin ammonical hydrate	Curcumin
3-O-Acetyl-β-boswellic acid	Allylpyrocatechol 3,4 diacetate	Boeravinone B	Vasicinone
Diosgenin	Piperine	Sesamin	3'-Hydroxy,4'-methoxyglabridin
Methylgallate	Eicosyl caffeate	Guggulsterone	Caffeine
Apocynin	trans-Anethole	A-Boswellic acid	Vicine
Docosyl caffeate	Protocatechuic acid (3,4 dihydroxybenzoic acid)	Ahatavarin IV	Isoforskolin
Methyl eugenol	Epocatechin	Gymnemagenin	Campesterol
1'-Acetoxychavicol acetate	Aristolochic acid 1	Betaine	Vasicine
2',3'-Dehydrosalannol	Estragole	β-Sitesterol	Isoeugenol
3-O-Methylellagic acid	Artemisinin	β-Glucogallin	Capsaicin
4-Allylpyrocatechol	Psoralen	Betulinic acid	Vanillylacetone
7-O-Methylwogonin	Ferulic acid	Galangin	Isoformononetin
3,3'-Di-o-methyl ellagic acid	Atlantone	Caffeic acid	Caryophyllene
4'-O-β-D-Andrographolide	Punicalagin (α+β)	Stigmasterol	Wedelolactone
	Forskolin	Geraniol	Jujubogenin isomer of bacopasaponin C
Maslinic acid	Bacopasaponin C	Catechin	Bacopasaponin C
xylopyranoside	Pterostilbene	Stevioside	Cedrol
Andrograpanin	Furanoeudesma-1,3-diene	Geranyl acetate	Withanolide A
Methanol	Bacopaside II	Catechin-5-O-gallate	Kaempferol
I Deoxynojirimycin	Pyrogallol	Tetrahydrocurcumin	Chrysophanol
Apigenin	Formononetin	Harmalin	Withaferin A
Menthyl acetate	Bacopaside I	Chebulagic acid	11-keto-β-boswellic acid
Elemonic acid (α+β)	Piperyline	Trigonelline HCl	1,8-Cineole
Arjunetin	Gallic acid	Harmalol	Withanolide B
Neandrographolide	Bacoside A3	Chebulinic acid	L-Dopa
Elemonic acid (β)	Quercetin dihydrate	1,3,6-Trigalloyl-β-D-glucose	Cirsilineol
Arjunic acid	6-Gingerol	Harmine	Withanone
Negundoside	Bacoside A	Chlorogenic acid	Lupeol
Arjungenin	Rebaudioside A	Tribulosin	Cinnamic acid
Ellagic acid	8-Gingerol	Hexahydrocurcumin	Withanoside IV
Oleanolic acid	Bacosine	Colchicine	Luteolin
ar-Turmerone	Reserpine	Trigoneoside IV a	m-Coumaric acid
Embelin	10-Gingerol	Hydroxycitric acid Calcium	Withanoside V
1-Octacosanol	Bakuchicin	Salt	Licochalcone A
Eugenol	Rosamarinic acid	Corilagin	Deacylgymnemic acid
α-Asarone	Glabridin	3β-Taxaxerol	12-Deoxywithastramonolide
Panduratin-A	Bakuchiol	Hydroxycitric acid lactone	Lycopene
Epocatechin-3-gallate	Para methoxyethylcinnamte	Epigallocatechin 3-gallate	Asiatic acid
α-Asarone			
Source: Natural Remedies, Bangalore, India.			

Table 1: List of phyto-compounds from traditional ayurvedic herbs.

Model quality assessment

Modeller [12] generated five models. Using Structural Analysis and Verification Server (SAVES)'s PROCHECK Module, (this stereo-chemical check was applied to verify if the φ and ψ dihedral angles were in available regions of the Ramachandran plot) the best protein model was selected [13].

Phyto-compounds from traditional ayurvedic herbs

Ligand preparation

The 3d structures of the above phyto-compounds were downloaded from PubChem, a database of chemical molecules maintained by the NCBI and various other online databases.

Generating phase database

Now using Application→Phase→Generate Phase Database module of Maestro software phase database of the phyto-compounds was done [14].

Selection of ligands for HTR2 receptor

Ligand-based pharmacophore model was selected by extracting the common features of the three-dimensional structures of compounds

which are known to interact with the target protein (known ligand). Known ligands were loaded in the Maestro workspace and by using Applications→Phase→Create Hypothesis module pharmacophore features of the known ligands were noted [14].

Docking

Protein preparation: The modeler generated protein is not suitable for immediate use in docking or other molecular modeling calculations. By using Protein Preparation Wizard of Maestro9.1 the modeler generated protein was uploaded for optimization and energy minimization [14].

Binding site generation: The binding site position of the protein was determined by SiteMap module of Maestro [14].

Ligand preparation: The ligands were selected in Maestro workspace. Using ligprep, the ligands were minimised prepared for docking studies. LigPrep is tool to prepare high quality 3D structure for large number of molecules taking input as 2D or 3D structures and giving output as a single, low energy 3D structure [14].

Receptor grid generation: The receptor was loaded in workspace. Using Glide → Receptor Grid Generation the binding site region of the receptor was specified and the receptor was prepared for docking.

Glide docking: Using module Glide → Ligand Docking module of Maestro the receptor was docked with the selected ligands [14].

ADME screening

ADME is an acronym in pharmacokinetics and pharmacology for absorption, distribution, metabolism, and excretion. Using QikProp module the ADME properties of the above ligands was determined [14].

Generation of stable cell line expressing HTR2

Synthetic HTR2 gene (Geneart, Germany) was cloned into pcDNA3.1 vector (Invitrogen) between BamHI and XbaI sites. Clones were confirmed by sequencing. Human Embryonic Kidney (HEK 293) cell line (National Centre for Cell Sciences, Pune, India) were transfected with pcDNA3.1-HTR2 and grown in the presence of1mg/ml Geneticin (G418).Cells resistant to 1mg/ml G418 were selected, expanded and used for receptor ligand binding assay. The expression of HTR2 in stable cell line was confirmed by RT-PCR using specific primers.

Receptor-ligand binding assay studies

Binding efficacy of the phytocompounds colchicine and hypophyllanthanthin with the HTR2 receptor was tested *in-vitro* by measuring agonist stimulated calcium signalling using Fluo-4 Direct calcium assay kit (Invitrogen).5HT (Sigma), a known agonist of HTR2 was used as positive control for the test. 1×10^4 cells in 50 µl of DMEM (Dulbekos Modified Eagle's Medium)-10% FBS (Fetal Bovine Serum) were seeded in 96 well tissue culture plate and grown overnight at 37°C/5% CO_2 in humidified incubator. 50 µl of 2X Fluo-4 direct calcium reagent loading solution was added and incubated for 60 seconds at 37°C. Different concentrations of agonist were added in duplicate wells and incubated for 4 hours at 37°C. Fluorescence was measured with excitation at 494 nm and emission at 516 nm using Flurometer.

Results and Discussion

The HTR2 receptor was selected from the NCBI database (Table 2). The 3D structure of HTR2 receptor was modelled by Modeller [12].

Protein & NCBI Accession Number	Homologous Templates	Identity
HTR2 (NP_001159419)	**2VT4A:** Turkey Beta1 Adrenergic Receptor With Stabilising Mutations And Bound Cyanopindolol	E-value-8e-28
	2YOOA: Turkey Beta1 Adrenergic Receptor With Stabilising Mutations And Bound Partial Agonist Dobutamine	E-value-2e-28
	3SN6A: Crystal Structure Of The Beta2 Adrenergic Receptor-Gs Protein Complex	E-value-2e-26

Table 2: Proteins with the NCBI accession number and their template information.

	Number of residues in most favoured region	Number of residues in additional allowed region	Number of residues in generously allowed region	Number of residues in dis-allowed region
Model 1	314 (88.0%)	33 (9.2%)	7 (2.0%)	3 (0.8%)
Model 2	319 (89.4%)	32 (9.0%)	4 (1.1%)	2 (0.6%)
Model 3	331 (92.7%)	19 (5.3%)	2 (0.6%)	5 (1.4%) (selected)
Model 4	319 (89.4%)	31 (8.7%)	2 (0.6%)	5 (1.4%)
Model 5	328 (91.9%)	20 (5.6%)	3 (0.8%)	6 (1.7%)

Table 3: Values of 5HT2A protein obtained in favoured, allowed and disallowed region using Ramachandran Plot (SAVES server).

Identified ligands	Fitness score	Plant
Colchicine	2.121	*Colchicum autumnale*
Hypophyllanthanthin	1.713	*Phyllanthus amarus*

Table 4: Ligands which matches with the pharmacophore of Myristicin and their fitness score.

Predicting the 3D structure of HTR2 receptor

The 3d structure of HTR2 receptor was modeled since the crystal structure of HTR2 receptor was not available in the PDB. Using BLAST search against PDB templates or homologous proteins related to HTR2 were selected.

This best aligned template is taken for homology modeling studies by using modeler (Table 2). Ramachandran plot analysis of the best generated model gave 92.7% residues in the core region, 5.3% in allowed region, 0.6% in generously allowed region and 1.4% disallowed region (Table 3, Figures 1 and 2). This model was selected as the best model since it had most residues in the favoured region.

The three dimensional structure provides valuable insight into molecular function and also enables the protein–protein interaction to be analyzed.

Pharmacophore studies

Ligand-based pharmacophore models are selected by extracting the common features of the three-dimensional structures of the known ligands. To do this, possible conformers of compounds should be previously enumerated.

Then, we superpose our target compounds by overlapping the three-dimensional structures' common substructures as molecular graphs among the other parts of compounds. So, in this method, since we do not have to enumerate all the conformers of a compound, we usually save much computational time by ligand-based pharmacophore modeling [15].

The pharmacophore features of all known 5-HT ligands, were generated but none (except myristicin) of the phytocompounds showed any common feature with the pharmacophore of the known 5HT ligands.

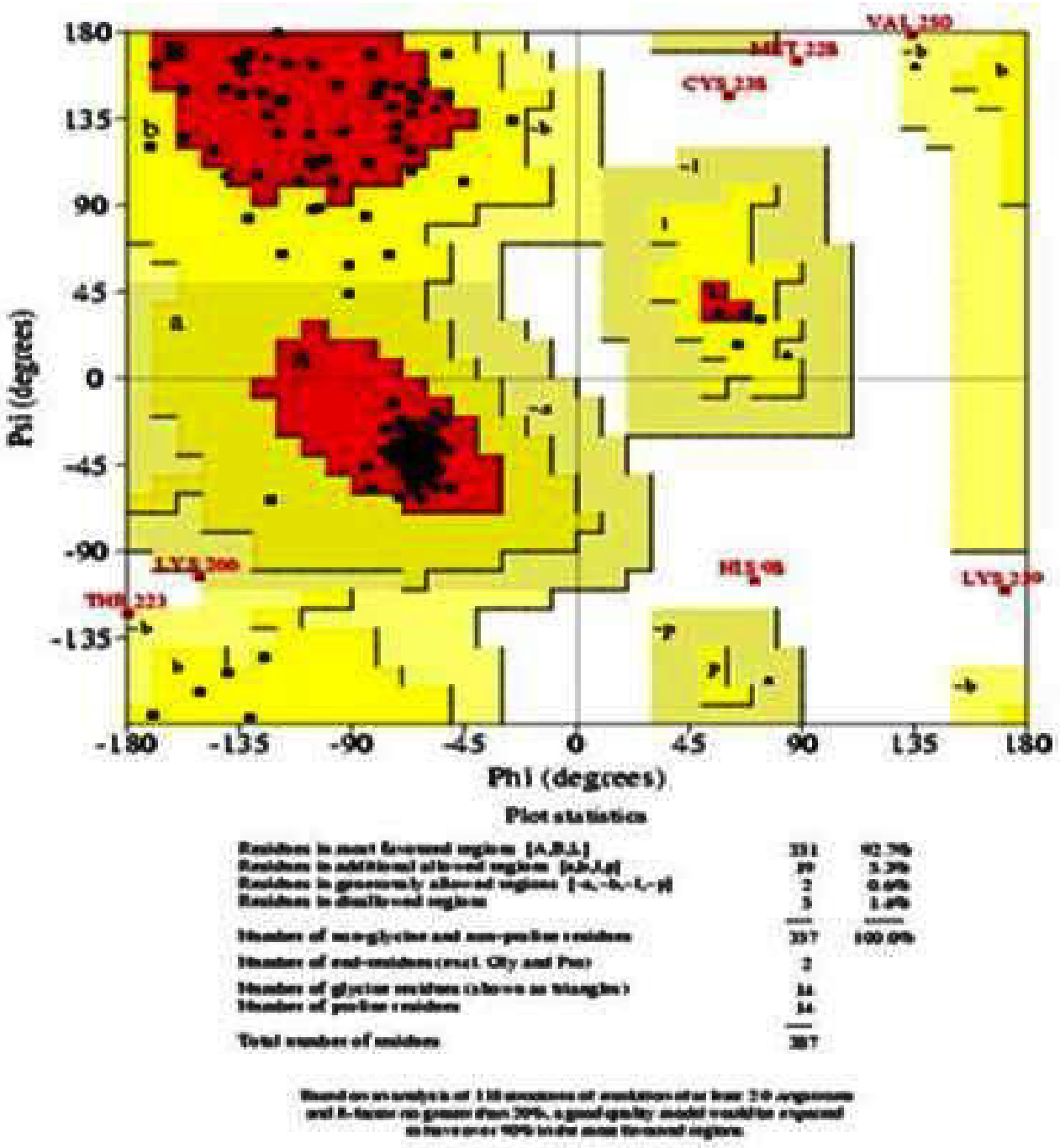


Figure 1: Ramachandran Plot of the selected best HTR2 model.

Myristicin

Myristicin is a known serotonin agonist, psychoactive drug, acting as an anticholinergic, and gets metabolised to 3-methoxy-4,5-methylenedioxyamphetamine (MMDA). Also, it has a weak monoamine oxidase inhibitor action and with elemicin that gets metabolised to an amphetamine-like compound which has hallucinogenic effects [16,17].

Myristicin was loaded in the Maestro workspace. Phase hypothesis gave pharmacophore features of Myristicin as A1, A2, A3, H4, H5 and R6 (Figure 3).

This pharmacophore features matched with the compounds in Table 4.

The above compounds were docked with HTR2 receptor.

Glide ligand docking

Sitemap module was used to determine the binding site residues of the HTR2 receptor and as per the output sitemap_site_2 with SiteScore 1.004 (2nd highest score) and size 231 was used to determine the binding site of the modelled protein.

Figure 2: 3d Structure of the selected best HTR2 model.

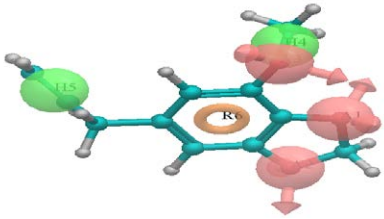


Figure 3: Pharmacophore features of Myristicin.

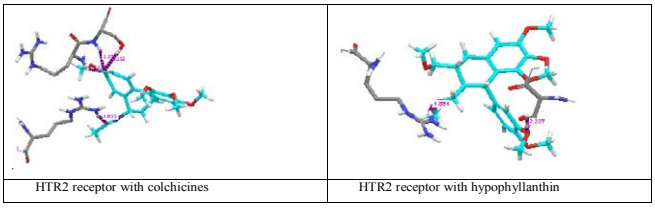


Figure 4: Docking results of HTR2 receptor with the colchicine and hypophyllanthin.

SER305, ALA306, CYS313, LYS316, PHE299, TYR303, VAL291, PRO293, ALA290, THR302, LEU298, ASN300, LYS320, GLU317, ASN376, ASP375, ALA372, LYS301, ASN318, ASP378, PRO321, TYR286, ILE174, TYR170, THR173, MET166, THR106, ARG89, LEU172, ILE168, THR169, MET166, THR106, PHE109, ILE165, ALA192, ALA195, ARG191, LYS107, LEU110, SER104.

The binding site region of the receptor was assigned and grid for the receptor was generated using Receptor Grid Generation module.

The HTR2 receptor was docked with colchicine and hypophyllanthin (Figure 4 and Table 5) [18,19].

ADME screening

ADME is an acronym for **a**bsorption, **d**istribution, **m**etabolism, and **e**xcretion. QikProp is a quick, accurate, easy-to-use ADME prediction program designed by Professor William L. Jorgensen. QikProp predicts physically significant descriptors and pharmaceutically relevant properties of molecules [14].

Ligand	Docking score/ glide g score	Doner	Distance in Å	Interaction
Colchicine	-7.191	ARG388 SER389 ARG185	2.207 2.250 1.633	ARG388(OH)...O(UNK) SER389(OH)...O(UNK) ARG185(OH)...O(UNK)
Hypophyllanthin	-6.303	THR386 ARG393	2.227 1.881	THR386(OH)...O(UNK) ARG393(OH)...O(UNK)

Table 5: Docking results of HTR2 receptor with colchicine and hypophyllanthin [19].

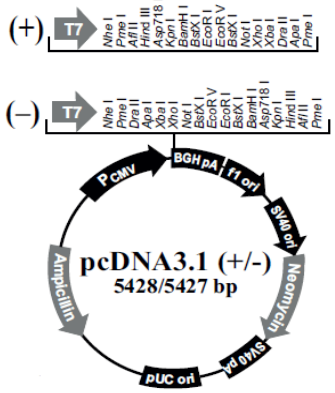


Figure 5: The figure summarizes the features of the pcDNA3.1(+) and pcDNA3.1(-) vectors. © invitrogen.

QikProp generated the following output (Tables 6 and 7) [19]:

Receptor-ligand binding assay studies

HEK cells were transfected with pcDNA3 containing HTR2 (Figures 5 and 6).

RT-PCR from total RNA isolated from stable cell line HEK- HTR2 confirmed the presence of HTR2 gene in the stable cell line. A band of 301bp was observed as expected (Figure 7).

Binding assay studies was done with HTR2 receptor with 5HT (known agonist for HTR2 receptor) [17], Colchicine and Hypophyllanthin using calcium assay buffer (Fluo-4 Direct reagent) [20].

Fluorometer reading at excitation at 494 nm and emission at 516 nm (done at Natural Remedies Pvt. Ltd., Bangalore, India) is given in Figure 8 and Table 8.

Conclusion

Phytocompounds colchicine and hypophyllanthin binds to HTR2 receptor and exhibits activity. Colchicine exhibits maximum activity at 10µm concentration and hypophyllanthin exhibits maximum activity at 100µm. Hence, the phytocompounds colchicine and hypophyllanthin proves agonists to HTR2 receptor and further *in vivo* and clinical trials should be done for establishing these as drugs for migraine and other neurological disorders.

Future Perspective

In this work the compounds colchicine and hypophyllanthin are already tested HTR2 agonist by *in silico* and *in vitro* methods. Currently

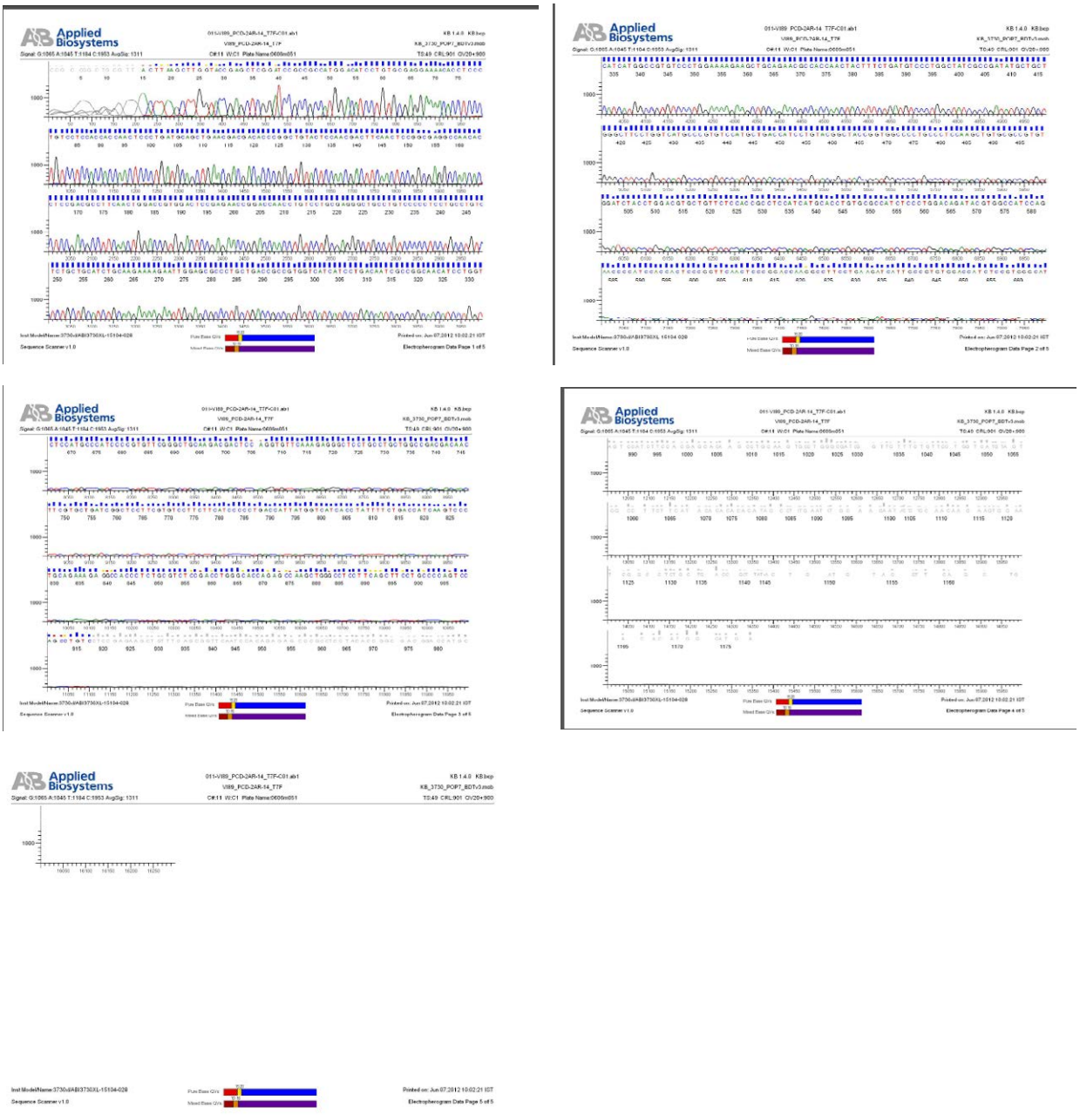


Figure 6: HEK DNA sequence p containing cDNA3 of HTR2.

Lead molecules	Molecular weight[a] (g/mol)	Molecular volume[b] (Å)	PSA[c]	HB[d] donors	HB[e] acceptors	Rotatable bonds[f]
Colchicine	399.443	1225.827	93.286	1.000	7.500	5.000
Hypophyllanthin	430.497	1319.288	54.254	0.000	7.150	7.000

A* indicates a violation of the 95% range.

[a]Molecular weight of the molecule.

[b]Total solvent-accessible volume in cubic angstroms using a probe with a radius of 1.4 Å.

[c]Van der Waals surface areas of polar nitrogen and oxygen atoms.

[d]Estimated number of hydrogen bonds that would be donated by the solute to water molecules in an aqueous solution. Values are averages taken over a number of configurations, so they can be non-integer.

[e] Estimated number of hydrogen bonds that would be accepted by the solute from water molecules in an aqueous solution. Values are averages taken over a number of configurations, so they can be non-integer.

[f] Number of rotatable bonds.

Table 6: Principal descriptors calculated by Qikprop simulation [19]. (Range 95% of Drugs).

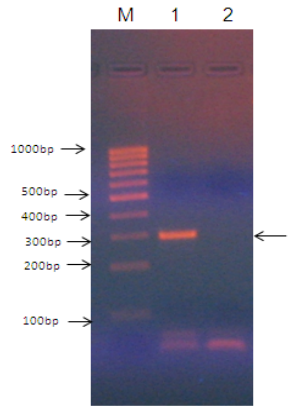
Lead molecule	QP log P(o/w)[a]	QP log S[b]	QP PCaco[c]	QP log HERG[d]	QP PMDCK[e]	% Human oral absorption[f]
Colchicine	2.545	-3.809	550	-3.180	483	91
Hypophyllanthin	3.160	-5.407	9906	-4.994	5899	100

A* indicates a violation of the 95% range.
An M indicates MW is outside training range.
[a]QP log P for octanol/water (-2.0, -6.5)
[b]Predicted aqueous solubility, log S. S in mol dm^{-3} is the concentration of the solute in a saturated solution that is in equilibrium with the crystalline solid (-6.5, -0.5)
[c]Apparent Caco-2 permeability (nm/s) (<25 poor, >500 great)
[d]log HERG, HERG K+channel blockage (concern below -5)
[e]Apparent MDCK permeability (nm/s) (<25 poor, >500 great)
[f]% Human oral absorption in GI (± 20%) (<25% is poor)

Table 7: Physiochemical descriptors calculated by Qikprop simulation [19]. (Range 95% of Drugs).

0.00001 mM	0.142958252	0.10027043	0.112376405
0.0001 mM	0.08932646	0.166483563	0.059114341
0.001 mM	0.233351644	0.068463196	0.148440801
0.01 mM	0.2227140201	0.073174596	0.067015972
0.1 mM	0.158761514	0.373278121	0.005936787
1 mM	0.133704471	0.068494887	0.0903617
	5HT	Colchicine	Hypophyllanthin

Table 8: Fluorometer reading of HTR2 receptor with ligands at different concentrations at excitation at 494 nm and emission at 516 nm.



M- 100bp DNA ladder; 1- HEK-5HT2AR Stable cell line; 2- HEK 293
Expected size of PCR product is 301bp

Figure 7: RT-PCR to check for expression of HTR2 gene in stable cell line.

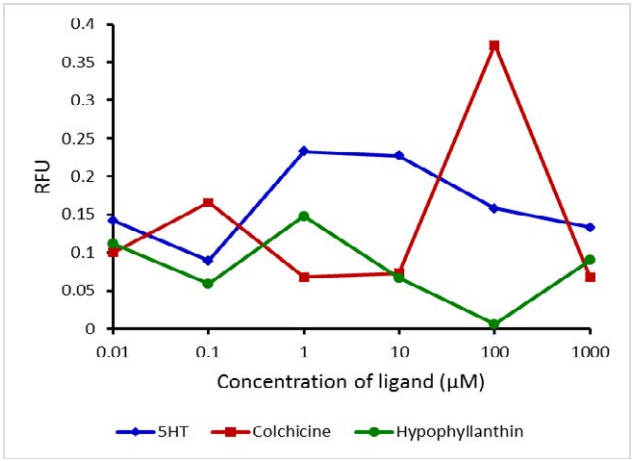


Figure 8: Calcium response to HTR2 receptor ligands (5HT agonist). Cells were stimulated with selected ligands. Measurements are given in fluorescent units (RFU) as maximum response minus the minimum response divided by the minimum response. Rank order ligand potency agreed with published results [20].

there is no animal model for migraine. Based on the present results, the authors would develop an animal model to test the compounds colchicine and hypophyllanthin to test *in vivo*.

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1) In silico work was performed at Department of Bioinformatics, SRM University, Chennai, India – which is being gratefully acknowledged.

2) Natural Remedies Pvt. Ltd., Bangalore, India.

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