Original Article

Journal of Practical Biochemistry and Biophysics
Volume 1 Number 1, January - June 2016

In Silico Screening of Bioactive Molecules From *Elephantopus Scaber* Linn. for Binding with Cardiac Potassium Ion Channels, Kir2.1 and Kir3.1

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Abstract

The earlier work in the lab has reported the identification of bioactive components from the methanolic extract of *E. scaber* Linn. These components are evaluated *in silico* for their cardiotonic properties and the binding energy is compared with positive controls; Dronaderone and azimilide, both class III anti-arrhythmic drugs. The ligand, 2-amino-4-(4-phenylpiperazino)-1,3,5-triazine had exhibited efficient binding with Kir2.1 channel with a low binding energy of -10.14 kcal / mol; which was comparatively lower than Dronaderone (-9.06 kcal / mol). Similar result was obtained with the cardiac potassium ion channel, kir3.1, where the ligand ononin had better binding efficiency than the positive control, Azimilide with binding energies of -9.24 kcal / mol and -8.8 kcal / mol respectively. The present study has revealed 2-amino-4-(4-phenylpiperazino)-1,3,5-triazine and ononin with better cardiotonic property that are devoid of arrhythmic side-effects.

Keywords: Cardiotonic Agents; Anti-Arrhythmic Drug; *Elephantopus Scaber;* Cardiac Potassium Ion Channels Kir2.1 and Kir3.1, Autodock 4, Lipinski Rule of Five.

Introduction

Cardiotonic drugs helps in maintaining a healthier condition for every tissue in the body through improved blood circulation via efficient heart muscle contraction. Cardiotonic drugs create a positive inotropic action (ie, elevated myocardial contraction) which in turn increases the blood flow through left ventricle resulting in an overall improvement of cardiac output. Cardiotonic drugs are mainly used in cases of heart failure, atrial fibrillation, atrial flutter and paroxysmal atrial tachycardia. The commonly used cardiotonics include cardiac glycosides or digitalis glycosides, but, their prolonged usage often leads to deleterious side effects like headache, weakness, drowsiness, visual disturbances, nausea, vomiting, anorexia and arrhythmias [1].

Arrhythmias defined as the irregular rhythm of heart is often characterized by abnormal faster rates (tachycardia) or slower rates (bradycardia) [2]. Arrhythmias arise due to genetic mutations of cardiac ion channels or their supporting proteins or alterations in their level of expression. The symptoms associated with arrhythmia include tiredness, difficulty in breathing, light headedness, dizziness, fainting (syncope) and, occasionally, chest pain. However, asymptomatic conditions of arrhythmias are also reported in several patients [3].

Anti-arrhythmic agents treat cardiac arrhythmias by blocking cardiac ion channels like sodium, potassium, calcium or the adrenergic receptors. Class III anti-arrhythmic agents like azimilide, dofetilide, dronedarone, ibutilide, sotalol, terikalant, amiodarone and bretylium block cardiac potassium

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channels, by lengethening refractory period. But, continous usage leads to side effects like torsade de pointe, pro-arrhythmia, pulmonary fibrosis, hypothyroidism, Optic neuritis leading to blindness, fatigue etc. [4].

Medicinal plants have been emphasized since time immemorial as cardiotonic agents. Digitalis, a common cardiotonic drug has been isolated from the leaves of *Digitalis purpurea* and *Digitalis lanata* [1]. Several plants have been reported to possess cardiotonic property like *Sarothamnus scoparius, Fumaria officinalis, Zea mays, Crataegus monogyna* etc.. (http://www.botanical-online.com/remediesheartfailure.html). *Elephantopus scaber* too has been reported to be a cardiotonic agent [5]. In the present study, the cardiotonic property with emphasis on its anti-arrhythmic nature, of bioactive molecules identified from *E. scaber* is evaluated *in silico* by docking it with cardiac inward rectifier potassium channel, Kir2.1 and subunit of acetylcholine activated inward rectifier potassium channel, Kir3.1; as protein targets and were compared with positive controls dronedarone and Azimilide, both Class III antiarrhythmic drugs.

Materials and Methods

Softwares

Protein Data Bank (http://www.rcsb.org/pdb/); ChemSpider database (http://www.chemspider.com/); ChemSketch from Advanced Chemistry Development, Inc. (ACD/Labs); Molsoft online molecular property calculator http://molsoft.com/mprop/; Open Babel software version 2.3.2. (http://openbabel.org/ (accessed 20.03.2015)); Swiss-Pdb Viewer version 4.1.0 (http://www.expasy.org/spdbv/); Auto Dock 4.0 (http://autodock.scripps.edu/downloads); Cygwin64 Terminal (http://www.cygwin.com/); Discovery Studio 4.1 Client (http://accelrys.com/products/collaborative-science/biovia-discovery-studio/visualization-download.php/)

Ligand Preparation

Eleven molecules identified from the methanolic extract of *E. scaber* were selected as ligands for the study which included Methylumbelliferone, Hydroxydihydrobovolide, Lysine theophylline, Ononin, Alismorientol A, Lotaustralin, 2-amino-4-(4-phenylpiperazino)-1,3,5-triazine, Phytosphingosine, Chamazulene, Ethyl oleate and Piperine [6]. The structure of positive control, azimilide and dronedarone, were identified from ChemSpider database (http://www.chemspider.com/) and 3D structures of all these ligands were built with ChemSketch software developed by Advanced Chemistry Development, Inc. (ACD/Labs) and were subsequently converted to pdb format using the Open Babel software (Open Babel, version 2.3.2, http://openbabel.org (accessed 20.03.2015) for virtual screening.

Determination of Physico-Chemical Properties of the Ligands

The determination of molecular properties and drug likeness of the ligands is an important parameter to be looked upon and was determined using http://molsoft.com/mprop/. The percentage of absorption was calculated using equation: % ABS = 109 – (0.345 × TPSA) according to Zhao *et al* [7].

Preparation of Protein Structure

The protein crystal structure of cytoplasmic domains of the inward rectifier potassium channel, Kir2.1 (PDB ID: 1U4F) [8] and the acetylcholine-activated potassium channel, Kir3.1 (PDB ID: 1U4E) [8] was retrieved from Protein Data Bank (http://www.rcsb.org/pdb/) and subjected to protein optimization via removing all heteroatoms and energy minimized with Swiss-Pdb Viewer version 4.1.0 (http://www.expasy.org/spdbv/) [9].

Docking

The receptor-ligand interaction was performed with Autodock 4.0 which consists of two programs termed Autogrid and Autodock. Autogrid precalculates grid parameters like center of the grid, 3D search space and spacing between points whereas the latter performs docking of the molecules to the receptor, defined by these set of grids. A default grid spacing of 0.375 Å with its grid points set to 60 Å each in X, Y and Z coordinates was created with AutoDock 4.0 to analyze ligand-receptor interaction [10]. For Kir2.1, the grid box was centered on 42.305, 27.455 and 36.223 while, Kir3.1 was centered on 9.969, 38.489 and 27.193 based on the x,y and z coordinates of amino acid residues, Glu224 and Asp260 present on the active site of two proteins respectively [11]. The search was based on the Lamarckian genetic algorithm.

For each ligand, a docking experiment consisting of 10 simulations was performed using Cygwin64 Terminal and the analysis was based on binding free energies and the ligand molecules were then

ranked in the order of increasing docking efficiency. The outputs were then exported to Discovery Studio 4.1 Client for visual inspection of the binding modes and interactions of the ligands with amino acid residues in the active sites.

Results

Molecular Properties and Drug Likeness of the Ligands

In the present study 11 bioactive molecules from *Elephantopus scaber* were screened with cardiac potassium ion channels, Kir2.1 and Kir3.1 taken as specific protein targets. The ligand molecules were analyzed for its molecular properties, drug likeness and percentage of absorption, prior to docking (Table 1). All the ligands except lysine theophylline (HBD > 5) and ethyl oleate (Mollogp > 5) were found to follow Lipinski's rule of five. The drug likeness score of the ligand molecules were comparable with the positive controls Dronedarone and Azimilide.

Lipinski's rule of five helps in the selection of molecules that could be developed as drugs. The ligand molecules following Lipinski's rule is reported to have theoretically better absorption, permeability and oral bioavailability [12]. The rule states that the molecular weight (MW) of the ligand should be ≤ 500, the Hydrogen Bond Acceptor (HBA) and Donor (HBD) groups in the ligand should be ≤ 10 and 5 respectively and mol log P value; which is the partition coefficient of the component in water: octan-1-ol system ≤ 5. The hydrogen bonding between the ligand and the receptor is a vital factor determining drug permeability. The strong binding often results in poor absorption and poor permeability. The mol log P and mol log S value represents the lipophilicity and aqueous solubility of the ligands. Lipophilic drugs can be easily taken up by the surrounding tissues from gastro-intestinal tract whereas solubility determines the uptake of drug by blood from the site of administration.

Other parameters like Topological Polar Surfce Area (TPSA), % absorption, No: of stereo-centres and drug likeness score are also considered. TPSA defined as the sum of surfaces of polar atoms in a molecule, predicts the drug transport properties and cell permeation properties. The recommended TPSA values are < 140 A^0 and 90 A^0 for cell permeation and blood-brain barrier respectively. The percentage of absorption is calculated from TPSA value using equation: % ABS = 109 – (0.345 × TPSA). It is observed that with increase in TPSA value, the percentage of absorption was found to be decreased. Less no: of stereo-centres suggest that upon binding, the ligand undergoes only a slight conformational change. The drug likeness is a qualitative concept which predicts the likeness of a molecule to a drug. Overall, the bioactive molecules of *E. Scaber* exhibited the presence of relevant pharmacophoric groups in them.

Table 1: Physico-chemical properties of the ligands

S no:	Component	MW	HBA	HBD	MlogP	MLogS	MV	N-SC	DL	TPSA (A^0)^2	% ABS
1	Methylumbelliferone	176.05	3	1	1.81	-2.60	192.40	0	-0.43	38.26	95.8003
2	Hydroxyl dihydrobovolide	198.13	3	1	2.40	-1.36	246.39	1	-0.61	39.03	95.5347
3	Lysine theophylline	326.17	7	6	-2.80	-1.28	316.45	1	0.73	124.67	65.9889
4	Ononin	430.13	9	4	0.70	-4.54	403.49	5	-0.02	108.26	71.6503
5	Alismorientol A	272.20	4	4	1.61	-0.80	327.66	6	-0.59	62.21	87.5376
6	Lotaustralin	261.12	7	4	-1.94	-0.99	256.22	6	-0.12	96.40	75.742
7	2-amino-4-(4-phenylpiperazino)-1,3,5-triazine	256.14	3	2	1.59	-1.80	228.68	0	-0.22	57.45	89.1798
8	Phytosphingosine	317.29	4	5	3.51	-5.35	353.16	3	-1.59	70.25	84.7638
9	Chamazulene	184.13	0	0	4.76	-5.01	222.28	0	-1.16	0.00	109.00
10	Ethyl oleate	310.29	2	0	7.98	-6.67	388.60	0	-0.78	20.67	101.8689
11	Piperine	285.14	3	0	3.96	-4.88	328.92	0	-0.02	33.47	97.4529
12	Dronedarone	556.30	6	1	7.72	-10.46	578.80	0	1.02	74.61	83.2595
13	Azimilide	457.19	6	0	3.22	-3.27	468.51	0	1.72	58.24	88.9072

Receptor-Ligand Interaction

In the virtual screening, inhibition activity of the bioactive molecules towards the target proteins was analyzed by comparing with Dronedarone and Azimilide, known inhibitors of Kir2.1 and Kir3.1 respectively. The best possible binding modes of the bioactive molecules at the targeted protein's active sites were observed using Discovery Studio 4.1 Client and corresponding energy values were recorded.

Binding affinities of plant molecules were analyzed and ranked according to lower energies (Table 2). The interaction of components identified

Table 2: Binding energies of bioactive molecules from *Elephantopus scaber* towards the cardiac potassium ion channel, Kir2.1 and Kir3.1

Sl. No	Bioactive molecules	During ligand-Kir2.1 interaction.		During ligand-Kir3.1 interaction	
		Binding energy (kcal/mol)	Key protein ligands interaction	Binding energy (kcal/mol)	Key protein ligands interaction
Std	Dronedarone / azimilide	-9.06	ARG260, ASP259, MET301, ARG260, GLU224, ALA306, HIS226	-8.8	THR210, HIS222, THR257, ARG229, HIS272, ASP260, CYS271, ALA259
1	2-amino-4-(4-phenylpiperazino) -1,3,5-triazine	-10.14	GLU224, GLY300	-7.66	SER256, ASP275, VAL273, ASP252
2	Ononin	-8.55	HIS226, ARG260, ARG228,ALA225, GLU224, HIS226, GLU299, HIS226, ARG260, ALA306, HIS226	-9.24	GLN227, VAL253, SER256, THR257,PHE263, CYS271, VAL273, LEU251, ILE228, ASP260, GLN261, ARG229, ASP252, ALA259, GLU250, CYS271, VAL273, GLN227, GLN261
3	Hydroxydihydrobovolide	-8.13	ARG228,GLN230, ARG260,PHE262, HIS226, GLU299, ALA225, ALA306	-7.93	VAL253, PHE255, VAL273, ASP252
4	Lotaustralin	-7.05	GLN310,MET301, GLU224,THR308, GLN310, THR309	-7.61	HIS222,ALA226, GLN227,VAL253, SER256, THR257, VAL273, LEU262, ILE228, LEU251, ARG229, PHE263, PHE255
5	Phytosphingosine	-6.9	ASP259, ALA306, HIS226	-8.57	SER256, ALA259
6	Ethyl oleate	-6.88	ARG260,GLU299, ARG260,GLU299, ALA306, ALA225	-7.91	ASP252, VAL273, GLU250,VAL253, HIS272, PRO279, VAL273
7	Piperine	-6.72	GLU224	-8.0	PHE263,CYS271,VAL273, ASP252, ILE270, VAL253, CYS271, VAL273, ALA259
8	Alismorientol A	-6.57	ARG260,ARG312, HIS226, ARG260, THR309, HIS226	-7.27	VAL253, LEU251, ASP252, GLU250
9	Methylumbelliferone	-6.06	ARG312,ALA304, THR308,GLN310, ALA306, THR309	-5.94	VAL253, HIS272, VAL273, ILE228, ASP260, HIS272
10	Chamazulene	-5.5	GLU299, GLN310	-5.86	-
11	Lysine theophylline	-3.92	ARG228,ARG312, HIS226, ALA304, GLU224,THR308, GLN310,GLU303, THR309,GLU303, ALA225,GLN310	-3.54	VAL253,GLU250, ASP252, ILE228, ASP260, GLU250, PHE255

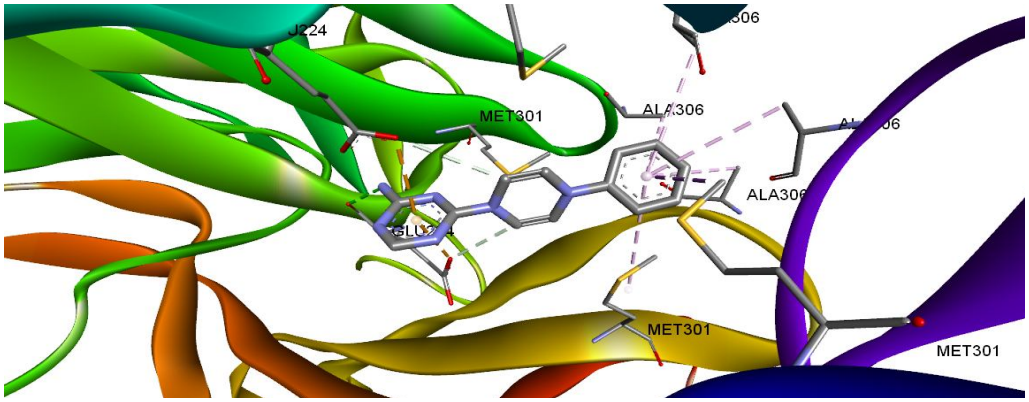

Fig. 1(A): The docked pose of 2-amino-4-(4-phenylpiperazino)-1,3,5-triazine at the active site of cardiac inward rectifier potassium ion channel Kir2.1, 1U4F. The key amino acids interacting with the ligand is labeled in the figure

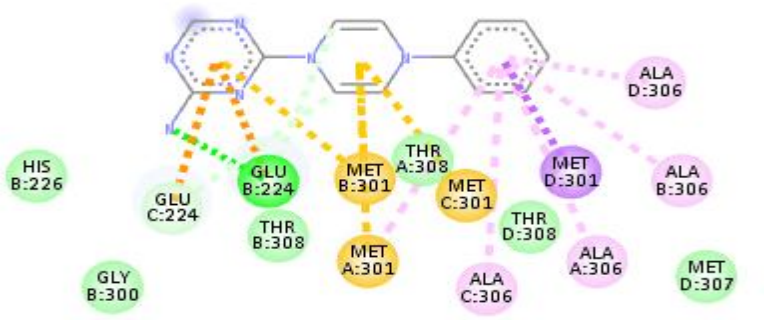

Fig. 1(B): Represents the protein-ligand interactions. Different bindings are shown in various colors. : Van der waals interaction, : Conventional hydrogen bond, : Carbon hydrogen bond, Pi- alkyl interaction, Pi-Sulfur, Pi-Antion and : Pi-Sigma.

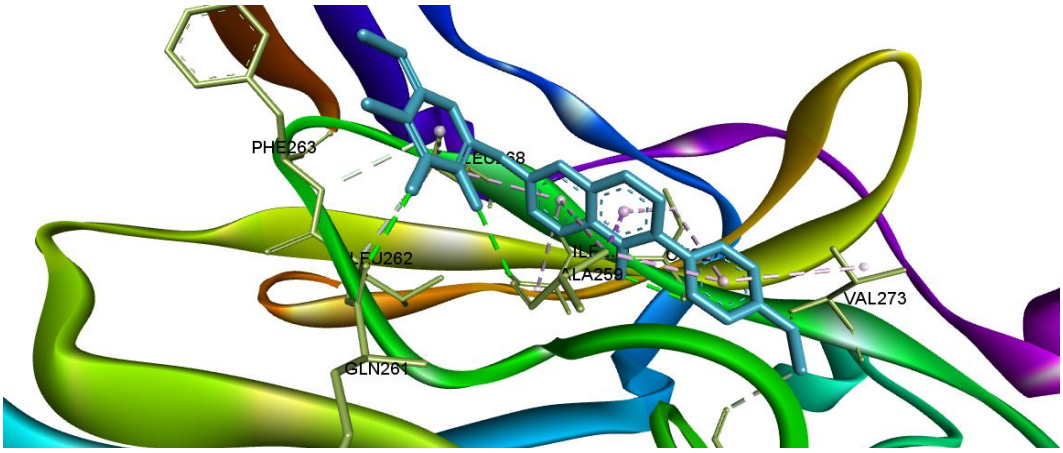

Fig. 2(A): The docked pose of ononin at the active site of G-protein-coupled inward rectifier potassium ion channel Kir3.1, 1U4E. The key amino acids interacting with the ligand is labeled in the figure

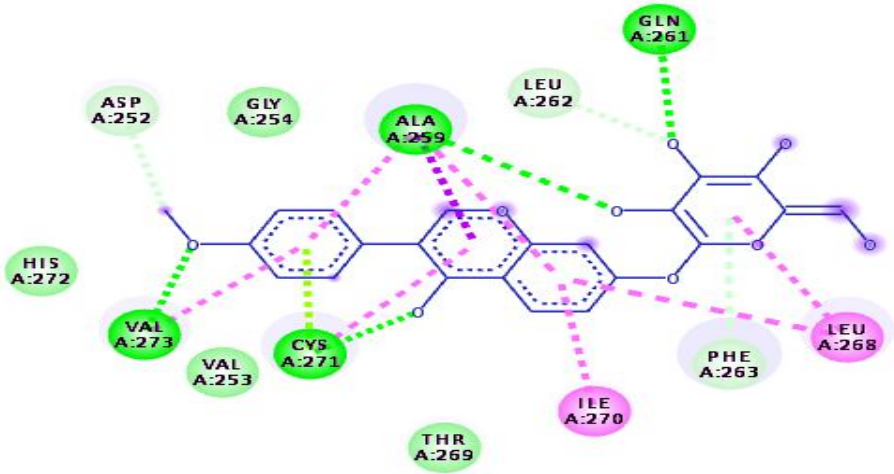

Fig. 2(B): Represents the protein-ligand interactions. Different bindings are shown in various colors. : Van der waals interaction, : Conventional hydrogen bond, : Carbon hydrogen bond, Pi- alkyl interaction, : Pi-Sigma, : Pi-Donor hydrogen bond and : Pi lone pair

from *E. scaber* with the cardiac potassium ion channel, kir2.1 revealed that 2-amino-4-(4-phenylpiperazino)-1,3,5-triazine (Figure 1A & B) had exhibited excellent binding than the positive control, Dronedarone with binding energies of -10.14 kcal / mol and -9.06 kcal / mol respectively. The components, ononin and hydroxydihydrobovolide have comparable binding energies with the positive control, Dronedarone for interaction with Kir2.1 (-8.55 kcal / mol and -8.13 kcal / mol). Similar result was obtained with the cardiac potassium ion channel, kir3.1, where the ligand ononin (Figure 2A and B) had better binding

efficiency than the positive control, Azimilide with binding energies of -9.24 kcal / mol and -8.8 kcal / mol respectively. Phytosphingosine and piperine with binding energies of -8.57 kcal / mol and -8.0 kcal / mol too exhibited better binding with the cardiac potassium ion channel, kir3.1.

Discussion

Kir 2.1 and Kir 3.1, components of the family of inwardly rectifying potassium ion channels, play a significant role in maintaining cardiac resting membrane potential, shape and duration of cardiac action potential curve and membrane excitability [8]. The impaired conductance of ions through these cardiac ion channels often leads to irregular heartbeat, either too fast or too slow, termed Cardiac arrhythmia. Several inward rectifier potassium channel blockers like Amiodarone, Azimilide and Chloroquine (Kir2.1 blockers) as well as Dronedarone Disopyramide, Flecainide (Kir3.1 blockers) have been identified as novel therapeutic agents for cardiac arrhythmias. They basically bind to these cardiac ion channels and prevent ventricular fibrillation and shortening of Action Potential Duration (APD) leading to therapeutic implications on various pathological conditions manifested by ventricular arrhythmia like myocardial ischemia, coronary heart Disease etc. [13].

Along with many of its beneficial effects, anti-arrhythmic drugs possess serious side effects that may even rise to more complicated rhythm disorders than ones being treated. Therefore, the search for ligand molecules which have cardiotonic properties and are devoid of arrhythmia has been an area of investigation. Ligands identified from the methanolic extract of *E. scaber,* a plant known to possess cardiotonic properties was screened with optimized and energy minimized 1U4F and 1U4E**;** cardiac inward rectifier potassium channel, Kir2.1 and Kir3.1 respectively. The protein optimization and energy minimization brings down the energy of macromolecules to a lower level as seen in the native cellular environment; by reducing the sterric clashes and bringing in more orientations that are similar to the theoretical true binding mode.

Upon docking, 2-amino-4-(4-phenylpiperazino)-1,3,5-triazine and ononin exhibited better binding efficiency than their corresponding positive controls. The binding of other ligands like Ononin, Hydroxydihydrobovolide and Lotaustralin (upon interaction with Kir 2.1) and phytosphingosine, piperine, hydroxydihydrobovolide and ethyl oleate (interaction with Kir 3.1) were comparable with positive controls. Binding energy is the amount of energy by which a ligand binds to the target protein. The lower the binding energy required by a ligand, greater will be its affinity towards the protein.

Prior to docking, the ligand molecules were analyzed for its molecular properties and violations of Lipinski rule of five. All ligands except lysine theophylline, (HBD > 5) and ethyl oleate (Mollogp > 5) were found to follow Lipinski's rule of five. The ligand molecules following Lipinski's rule is reported to have theoretically better absorption, permeability and oral bioavailability [12]. Topological Polar Surface Area (TPSA) values for the ligands were appropriate enough for efficient permeability through cellular plasma membrane. The value for water solubility (Mollog S) for the ligands ranged from -6.67 to -0.80. Overall, the ligands found in the bioactive fraction of *E. scaber* exhibited the presence of relevant pharmacophoric groups in them.

Arrhythmia, fluctuations in cardiac membrane potential is often due to the inflammations prevailing in the myocytes and interstitium (Chronic myocarditis). In fact, arrhythmia is reported to be the only clinical symptom in natural course of this disease. Myocardial inflammation which leads to micro- and macrovascular perfusion resulting in myocardial ischemia is reported to elevate the incidence of cardiac arrhythmogenicity [14]. The present study highlights its relevance, as bioactive molecules identified from *E. scaber*, reported to possess anti-inflammatory property [15] marked their efficiency as cardiotonic agents by inhibiting cardiac inward rectifier potassium ion channels Kir2.1 and Kir3.1 better than or comparable with the positive controls.

Conclusion

The present study has revealed the efficacy of 2-amino-4-(4-phenylpiperazino)-1,3,5-triazine, Ononin, Hydroxydihydrobovolide, Lotaustralin phytosphingosine, piperine, and ethyl oleate as cardiotonic agents that bind with cardiac potassium channels with high affinity. It was also clear, that these ligands exert cardiotonic property without posing much arrhythmic incidences. The physico-chemical properties of the ligands too indicate the presence of relevant pharmacophoric groups which can be further harnessed for drug development.

Acknowledegement

The acknowledge Department of Biotechnology

for the Grants received for the DBT BUILDER (No. BT/PR4800/INF/22/152/2012) for financial support

References

1. Drugs, I. Cardiotonics and Miscellaneous Inotropic Drugs. Appl. Ther. Clin. Use Drugs 2008.
2. Amin, A. S.; Tan, H. L.; Wilde, A. A. M. Cardiac ion channels in health and disease. Heart Rhythm. 2010; 7: 117–26.
3. Li, G. R.; Dong, M. Revisit of the Cardiac Inward Rectifier Potassium Current I K1. 2010; 95–102.
4. Homoud, M. K. Introduction to Antiarrhythmic Agents Anti Arrhythmic Agents Introduction to Antiarrhythmic Agents. Spring; 2008; 1–21.
5. Amrita, B. Nephroprotective Activity of Ehanolic Extract of Elephantophus Scaber Leaves on Albio Rats. ISSN 2230 – 8407. 2012; 3: 246–250.
6. Abhimannue, A. P.; Mohan, M. C.; B, P. K. Inhibition of Tumor Necrosis Factor-α and Interleukin-1â Production in Lipopolysaccharide-Stimulated Monocytes by Methanolic Extract of Elephantopus scaber Linn and Identification of Bioactive Components. Appl. Biochem. Biotechnol. 2016.
7. Zhao, Y. H.; Abraham, M. H.; Le, J.; Hersey, A.; Luscombe, C. N.; Beck, G.; Sherborne, B.; Cooper, I. Rate-limited steps of human oral absorption and QSAR studies. Pharm. Res. 2002; 19: 1446–57.
8. Pegan, S.; Arrabit, C.; Zhou, W.; Kwiatkowski, W.; Collins, A.; Slesinger, P. A.; Choe, S. Cytoplasmic domain structures of Kir2.1 and Kir3.1 show sites for modulating gating and rectification. Nat. Neurosci. 2005; 8: 279–87.
9. Guex, N.; Peitsch, M. C. SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. Electrophoresis. 1997; 18: 2714–23.
10. Morris, G. M.; Huey, R.; Lindstrom, W.; Sanner, M. F.; Belew, R. K.; Goodsell, D. S.; Olson, A. J. NIH Public Access. 2010; 30: 2785–2791.
11. Noujaim, S. F.; Stuckey, J. A.; Ponce-balbuena, D.; Ferrer-villada, T.; Lo, A.; Pandit, S.; Calvo, C. J.; Grzeda, K. R.; Berenfeld, O.; Sa, A. Specific residues of the cytoplasmic domains of cardiac inward rectifier potassium channels are effective antifibrillatory targets.
12. Lipinski, C. A.; Lombardo, F.; Dominy, B. W.; Feeney, P. J. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. 2001; 46: 3–26.
13. Tamargo, J.; Tamargo, J.; Caballero, R.; Caballero, R.; Gómez, R.; Gómez, R.; Valenzuela, C.; Valenzuela, C.; Delpón, E.; Delpón, E. Pharmacology of cardiac potassium channels. Cardiovasc. Res. 2004; 62: 9–33.
14. Klein, R. M.; Vester, E. G.; Brehm, M. U.; Dees, H.; Picard, F.; Niederacher, D.; Beckmann, M. W.; Strauer, B. E. [Inflammation of the myocardium as an arrhythmia trigger]. Zeitschrift fur Kardiol. 2000; 89 (Suppl 3): 24–35.
15. Kumar, B. P.; Abhimannue, A. P.; Mohan, M. C.; Jacob, J.; Babu, B. M. Research Article Inhibition of Lipoxygenase by Elephantopus Scaber Extractand Determication of its Inhibition Pattern. J. Adv. Sci. Res. 2015; 6: 1–5.