



Muscarinic Acetylcholine Receptor Agonists and Antagonists Regulate Yeast to Hyphal form Transition in *Candida albicans*

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Abstract

Reversible transition from yeast to hypha in *Candida albicans* represents a good model system for studying cell differentiation. In this study, we have studied the effect of human muscarinic receptor antagonists, dicyclomine, solifenacin and flavoxate on *C. albicans* morphogenesis induced by muscarinic receptor agonists, acetylcholine and bethanechol. We show that muscarinic receptors antagonists inhibited yeast to hyphal form transition in a concentration dependent manner. Dicyclomine inhibited yeast to hyphal form transition induced by bethanechol and acetylcholine more than solifenacin and flavoxate. We are reporting that solifenacin and flavoxate have good binding with *C. albicans* Rrp9. Solifenacin and flavoxate formed hydrogen bond interactions with the residues GLU509 and ILE510 respectively in the active site region of Rrp9. Acetylcholine and bethanechol also bound with Rrp9 by forming hydrogen bond interactions. Acetylcholine formed hydrogen bond interaction with SER314 and SER323 while bethanechol formed hydrogen bond interaction with ASP384 and ASP506 at the active site of Rrp9. *C. albicans* Rrp9 may have a role in yeast to hyphal form morphogenesis and that muscarinic acetylcholine receptor antagonists may be repositioned as anti-virulence agents in *C. albicans*.

Keywords: Agonists; Antagonists; Muscarinic acetylcholine receptor; Morphogenesis; Molecular docking; Rrp9 protein

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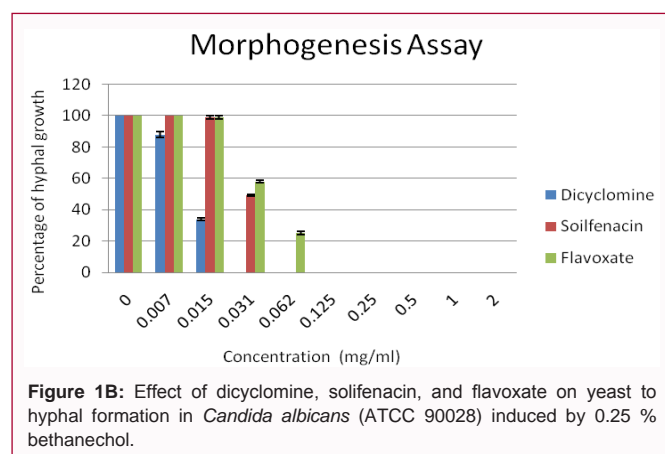
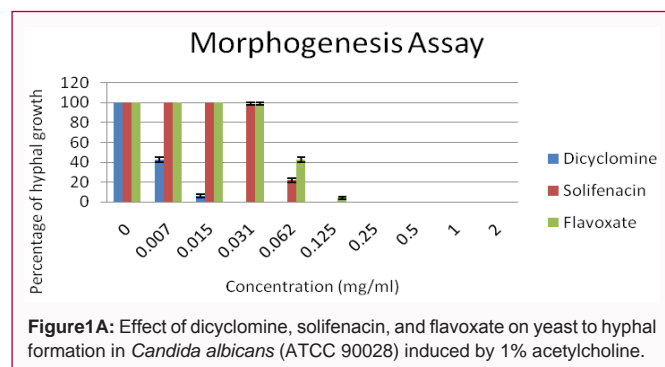
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Introduction

Human muscarinic acetylcholine receptors are typical G-protein coupled receptors involved in many physiological activities. Five classes of muscarinic receptors are known in humans. They are: M1, M2, M3, M4, and M5 [1-9]. When muscarinic M2 and M4 receptors are activated by agonist, they bind with G_i-protein to inhibit adenylyl cyclase activity. Whereas muscarinic M1, M3, and M5 receptors are coupled with G_q-protein to activate inositol phosphate pathway [10,11,12]. *Candida albicans* is a eukaryotic organism which processes many genes similar to humans [13]. G-protein coupled receptor system in *C. albicans* is simpler than human GPCRs. G-protein coupled receptors are known in *C. albicans*, such as Gpr1, Ste2, and Ste3. Gpr1 is a glucose sensor which has a role in morphogenesis [14]. Ste2 and Ste3 are pheromone receptors that are functional in mating and morphogenesis [15]. Yeast to hyphal form transition in *C. albicans* is a good model system for studying cell differentiation. In this article, we are reporting the potential role of muscarinic receptor agonists and antagonists on *C. albicans* morphogenesis. These drugs are commonly used in humans for therapeutic purposes. Acetylcholine is a neurotransmitter substance secreted from nerve cells to send signals to other cells. Acetylcholine stimulates both muscarinic acetylcholine receptors and nicotinic acetylcholine receptors [16]. In the central nervous system, acetylcholine functions in learning and memory. In the peripheral nervous system, acetylcholine functions as a muscle movement stimulator. Acetylcholine is often used as eye drops to cause constriction of the pupil during cataract surgery. Bethanechol is a muscarinic acetylcholine receptor agonist that directly stimulates muscarinic receptors. Bethanechol mimics the action of acetylcholine and has a longer duration of activity than acetylcholine because it is more resistant to cholinesterase mediated hydrolysis. It is used for the treatment of non obstructive urinary retention. Dicyclomine is a human muscarinic receptor antagonist which inhibits acetylcholine activity on smooth muscle by blocking muscarinic acetylcholine receptor activity. Dicyclomine can bind selectively with muscarinic M1 receptor and also it can target muscarinic M3 receptor [17,18,19]. It is an antispasmodic and an anticholinergic drug used for the treatment of stomach cramps. Solifenacin is an antimuscarinic

Table 1: Minimum Inhibitory Concentration (MIC) of muscarinic receptors antagonists, dicyclomine, solifenacin, and flavoxate against *Candida albicans* (ATCC 90028) morphogenesis induced by muscarinic receptor agonists, acetylcholine and bethanechol.

Sr.no.	Molecule name	MIC mg/ml	
		<i>Candida albicans</i> morphogenesis induced by	
		Acetylcholine	Bethanechol
1	Dicyclomine	0.007	0.015
2	Solifenacin	0.062	0.031
3	Flavoxate	0.062	0.062



receptor drug that binds to muscarinic M3 and M1 receptors [20]. This medicine is used for the treatment of Overactive Bladder (OAB) by blocking muscarinic receptor activity on bladder muscle. Flavoxate is the first drug approved by the Food and Drug Administration (FDA) and is used for the treatment of Overactive Bladder (OAB) [21]. It is a competitive muscarinic receptor antagonist and antispasmodic agent. Flavoxate is also used for the treatment of cystitis, urethritis, and prostatitis. In this study, we have tested the effect of human muscarinic receptor antagonists, dicyclomine, solifenacin and flavoxate on morphogenesis of *C. albicans* induced by acetylcholine and bethanechol. This study supports the hypothesis that *C. albicans* may have proteins like muscarinic receptors which play critical roles in *C. albicans* morphogenesis.

Materials and Methods

Chemicals and media

Acetylcholine chloride was purchased from TCI Chemicals (India) Pvt. Ltd. Dicyclomine hydrochloride injection form, Bethanechol chloride, Solifenacin succinate and Flavoxate hydrochloride tablet forms were purchased from local medical stores. Microtiter plates and

other media components were purchased from HiMEDIA Chemicals Ltd, Mumbai, India.

Culture of *Candida albicans*

C. albicans (ATCC 90028) was obtained from the Institute of Microbial Technology (IMTECH) Chandigarh, India. The culture was maintained on Yeast extract -Peptone -Dextrose (YPD) agar slant at 4°C and propagated by inoculating a single colony from the YPD agar plates (Yeast extract 1%, Peptone 2%, Dextrose 2% and Agar 2.5%) into 50 ml YPD broth in a 250 ml conical flask. Flasks were incubated overnight at 30°C at 100 rpm on an orbital shaking incubator. The cells were harvested by centrifugation at 2000 rpm and washed thrice with sterile 0.1 M Phosphate-Buffered Saline (PBS), pH 7.4 and the cell density was determined by a haemocytometer count. Cells were suspended in sterile PBS.

Morphogenesis assay

Yeast to hyphal form transition assay was done in 96-well microtiter plates [22]. *Candida albicans* cells stock was diluted to 1×10^6 cells/ml in 1% acetylcholine chloride and 0.25% bethanechol. Various concentrations of dicyclomine, solifenacin, and flavoxate were prepared in 1% acetylcholine and 0.25% bethanechol separately and ranged 2 mg/ml to 0.007 mg/ml and were added separately in each well. Wells without drugs were kept as a control. The final volume was kept at 200 μ l in each well. The microtiter plates were incubated at 37°C at 120 rpm shaking incubator for 2 hour. After incubation period, cells were observed microscopically by using inverted light microscope (Metzer, India). The concentration which inhibited hyphae formation by $\geq 50\%$ was compared to the control and was considered as the Minimum Inhibitory Concentration (MIC) for morphogenesis inhibition. All the experiments were done in triplicate.

Docking study

Ligands preparation: Solifenacin, flavoxate, acetylcholine and bethanechol structures were retrieved from Pubchem database in SDF format. The chemical structures followed by 2D structure cleaning, 3D optimization and viewing were done using MarvinView and saved in Mol2 files format. Solifenacin Mol2, flavoxate Mol2, acetylcholine Mol2 and bethanechol Mol2 files were converted to PDBQT formats in Autodock Tools version 1.5.6rc2 [23].

Molecular docking: The autodock tools package version 1.5.6rc2 was employed to generate docking input files. All the nonpolar hydrogens were merged and the water molecules were removed. For Docking, a grid spacing of 0.375 Å and 60×60×60 number of points was used. Before docking all the water molecules were removed from the protein structures followed by addition of hydrogen atoms to receptors and merging non-polar hydrogens. The predicted structure of *C. albicans* Rrp9 and the structures of the ligands, Solifenacin, flavoxate, acetylcholine, and bethanechol were converted to PDBQT formats [23]. Molecular docking studies of solifenacin, flavoxate, acetylcholine, and bethanechol with *C. albicans* Rrp9 were carried out by using AutoDock' suite as molecular-docking tool [24]. Default optimization parameters were done using Lamarckian Genetic Algorithm with a population size of 150 dockings. Autodock' tools generated sixty possible binding conformations, i.e. sixty runs for each docking by using Genetic Algorithm (GALS) Searches. The grid box used for specifying the search space was set at 60×60×60 centered on proteins with a default grid point spacing of 0.375 Å. Autogrid was used to obtain pre calculated grid maps. After completion of docking,

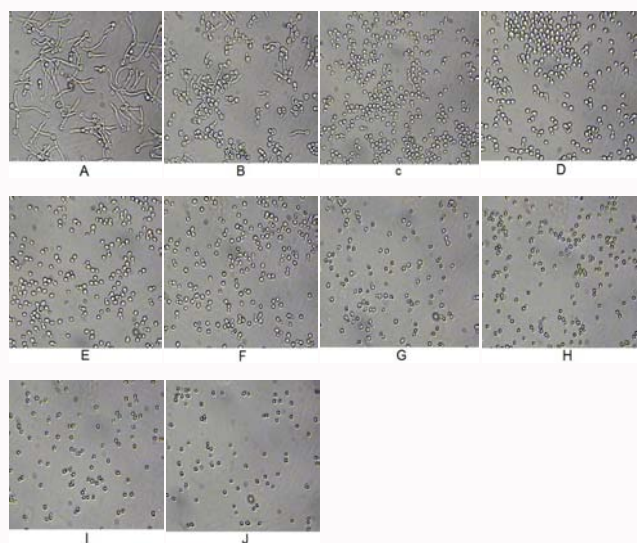


Figure 2A: Effect of dicyclomine on acetylcholine induced yeast to hyphal form transition in *Candida albicans* (ATCC 90028); A) Control; B) 0.007 mg/ml; C) 0.015 mg/ml; D) 0.031 mg/ml; E) 0.062 mg/ml; F) 0.125 mg/ml; G) 0.25 mg/ml; H) 0.5 mg/ml; I) 1 mg/ml; J) 2 mg/ml.

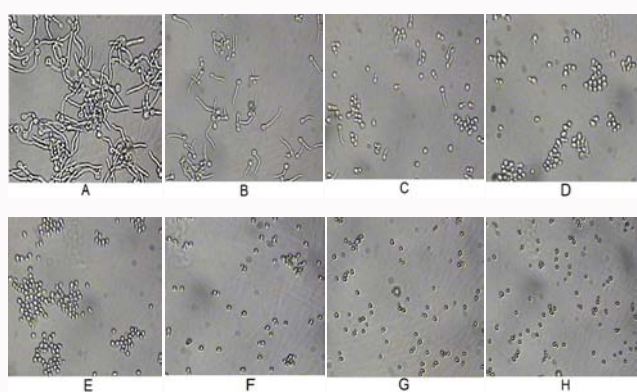


Figure 2B: Effect of solifenacin on acetylcholine induced yeast to hyphal form transition in *Candida albicans* (ATCC 90028); A) Control; B) 0.031 mg/ml; C) 0.062 mg/ml; D) 0.125 mg/ml; E) 0.25 mg/ml; F) 0.5 mg/ml; G) 1 mg/ml; H) 2 mg/ml.

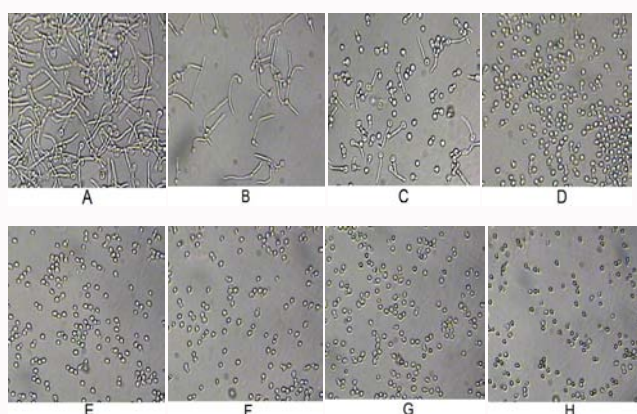


Figure 2C: Effect of flavoxate on acetylcholine induced yeast to hyphal form transition in *Candida albicans* (ATCC 90028); A) Control; B) 0.031 mg/ml; C) 0.062 mg/ml; D) 0.125 mg/ml; E) 0.25 mg/ml; F) 0.5 mg/ml; G) 1 mg/ml; H) 2 mg/ml.

most suitable conformation was chosen based on lowest docked energy. Selected conformations were analyzed by Autodock tool [23].

Analysis of molecular docking

The ligand-protein complex preparation between Solifenacin DLG, flavoxate DLG, acetylcholine DLG, and bethanechol DLG files and protein structure of Rrp9 in PDBQT format was done using AutoDock 1.5.6rc2. The structures of docking were saved in PDBQT format and analyzed for amino acid residues in the ligand binding sites of the protein structure associated with the binding interactions such as hydrogen bonding. This analysis was visualized by using Discovery Studio 4.1 Visualizer software [23].

Statistical analysis

Values in the control and treatment groups were compared using Student's t-test. A value of $p < 0.05$ was considered statistically significant.

Results

Muscarinic receptor antagonists inhibit yeast to hyphal form transition induced by acetylcholine and bethanechol in *Candida albicans*

The muscarinic receptor antagonists, dicyclomine, solifenacin and flavoxate inhibited yeast to hyphal form transition induced by 1% acetylcholine and 0.25% bethanechol in *C. albicans* (ATCC 90028) in a concentration-dependent manner. Dicyclomine showed best inhibition effect than solifenacin and flavoxate. Dicyclomine inhibited hyphal formation induced by 1% acetylcholine at 0.007mg/ml (Table 1) (Figures 1A and 2B). Solifenacin and flavoxate blocked yeast to hyphal formation induced by 1% acetylcholine at 0.062 mg/ml (Table 1) (Figures 1A,2B and 2C). Dicyclomine inhibited yeast to hyphal formation induced by 0.25 % bethanechol at 0.015 mg/ml (Table 1) (Figures 1B and 3A). However, solifenacin and flavoxate inhibited this transformation at 0.031 mg/ml and 0.062 mg/ml respectively (Table 1) (Figure 1B,3B and 3C).

Solifenacin, flavoxate, acetylcholine, and bethanechol bind with *Candida albicans* Rrp9

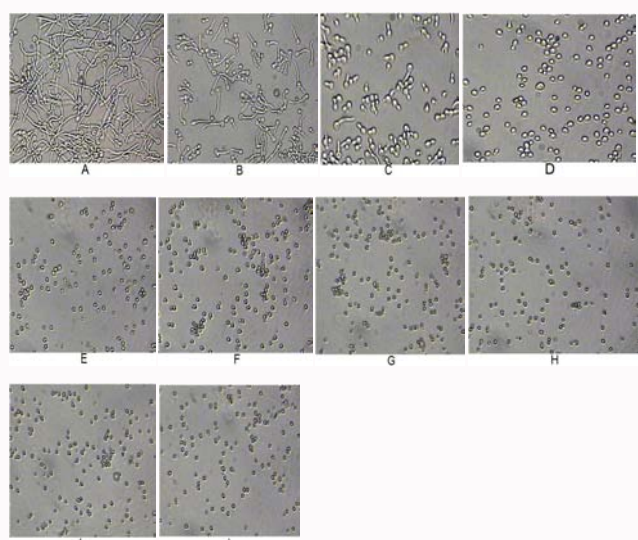
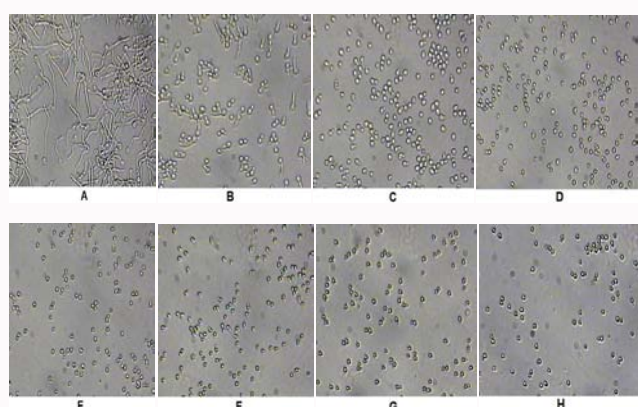
Solifenacin, flavoxate, acetylcholine, and bethanechol bound with *C. albicans* Rrp9 with binding energies of -11.33 Kcal/mol, -10.72 Kcal/mol, -4.75 Kcal/mol, and -5.27 Kcal/mol respectively (Table 2). Solifenacin and flavoxate bound with Rrp9 by forming hydrogen bond interactions with GLU509 and ILE510 amino acid residues respectively in the active site region (Table 2) (Figures 4A and 4B). Acetylcholine showed binding with Rrp9 by forming hydrogen bond interactions with SER314 and SER323 amino acid residues in the active site region (Table 2) (Figure 4C). Bethanechol also formed hydrogen bond interactions with the amino acids, ASP384 and ASP506 at the active site pocket of Rrp9 (Table 2) (Figure 4D).

Discussion

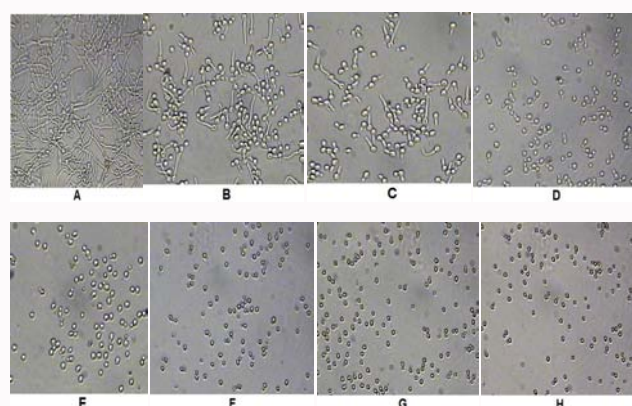
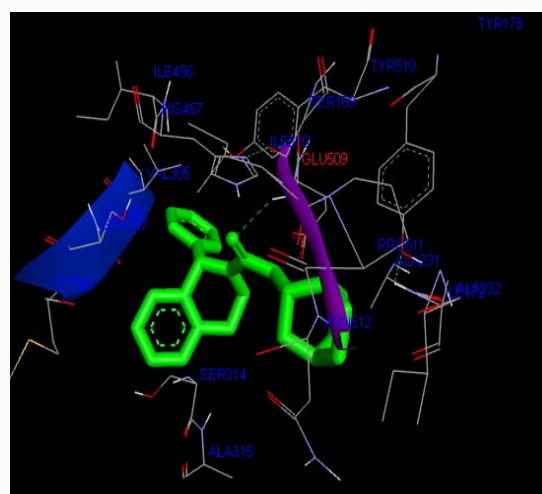
Receptor is defined pharmacologically as a protein that regulates a particular physiological process in response to signaling molecules [25]. Signaling molecules are either endogenous such as hormones, neurotransmitters or exogenous drugs. In pharmacology, agonist is described as a chemical substance that binds with the receptor causing activation and resultant intracellular changes while antagonist is a molecule that blocks the action of agonist on the same receptor. G-protein coupled receptors are a large family in mammals and mediate many biological processes. They may act as successful therapeutic targets for several diseases. Mechanisms of GPCR signaling in yeast

Table 2: Molecular interactions of Solifenacin, Flavoxate, Acetylcholine, and Bethanechol with *Candida albicans* Rrp9.

Ligands (Kcal/mol)	Run no. Electrostatic energy	Interacting residues	Interacting atoms (Amino acid .. Ligand)	H bond formed	Binding energy (Kcal/mol)	Electrostatic energy
Solifenacin	19	GLU509	OE1 ----- O1	1	-11.33	-0.02
Flavoxate	25	ILE510	HN2---O3	1	-10.72	-0.03
Bethanechol	1	ASP384	OD1---H16	2	-5.27	-0.24
		ASP506	OD1---H17			
Acetylcholine	15	SER314	HG ---- O2	2	-4.75	-0.32
		SER323	HG ---- O2			

**Figure 3A:** Effect of dicyclomine on bethanechol induced yeast to hyphal form transition in *Candida albicans* (ATCC 90028); A) Control; B) 0.007 mg/ml; C) 0.015 mg/ml; D) 0.031 mg/ml; E) 0.062 mg/ml; F) 0.125 mg/ml; G) 0.25 mg/ml; H) 0.5 mg/ml; I) 1 mg/ml; J) 2 mg/ml.**Figure 3B:** Effect of solifenacin on bethanechol induced yeast to hyphal form transition in *Candida albicans* (ATCC 90028); A) Control; B) 0.031 mg/ml; C) 0.062 mg/ml; D) 0.125 mg/ml; E) 0.25 mg/ml; F) 0.5 mg/ml; G) 1 mg/ml; H) 2 mg/ml.

and mammalian systems are similar and sometimes proteins may be exchangeable functionally [26]. Muscarinic acetylcholine receptors are typical G-Protein Coupled Receptors (GPCRs) involved in several biological activities. *C. albicans* Rrp9 is reported to exhibit identity and similarity with muscarinic M1 receptor [27]. The muscarinic M1 receptor antagonist, dicyclomine is reported to bind only with Rrp9 than *C. albicans* GPCR, Gpr1, Ste2, and Ste3 by forming hydrogen bond interaction on the active site region of Rrp9 [27]. In this study,

**Figure 3C:** Effect of flavoxate on bethanechol induced yeast to hyphal form transition in *Candida albicans* (ATCC 90028); A) Control; B) 0.031 mg/ml; C) 0.062 mg/ml; D) 0.125 mg/ml; E) 0.25 mg/ml; F) 0.5 mg/ml; G) 1 mg/ml; H) 2 mg/ml.**Figure 4A:** Molecular interactions of solifenacin with amino acid residues in the active site region of modeled structure of *Candida albicans* Rrp9 protein. The green dotted line represents the hydrogen bond formation and red letters show the amino acid residues involved in the bonding and solifenacin.

we tested the effect of muscarinic receptor antagonists, dicyclomine, solifenacin, and flavoxate against *C. albicans* morphogenesis induced by acetylcholine and bethanechol. We showed that dicyclomine, solifenacin and flavoxate can inhibit *C. albicans* morphogenesis induced by acetylcholine (Table 1) (Figures 1A,2A and 2C) and bethanechol (Table 1) (Figures 1B,3A and 3C) indicating that *C. albicans* may have a muscarinic receptor like protein. Dicyclomine inhibited yeast to hyphal formation induced by acetylcholine or bethanechol more than solifenacin and flavoxate. We have showed

that dicyclomine is more effective against *C. albicans* morphogenesis induced by acetylcholine and bethanechol than solifenacin and flavoxate (Table 1) (Figure 1A-B, 2A-C and 3A-C). The docking study revealed that solifenacin and flavoxate have good binding with Rrp9 (Table 2). Acetylcholine and bethanechol also could bind with Rrp9 (Table 2) (Figure 4C and 4D). Dicyclomine and solifenacin inhibited yeast to hyphal formation induced by acetylcholine and bethanechol may be by blocking the action of acetylcholine and bethanechol on the active site pocket of muscarinic receptor like protein in *C. albicans*. Dicyclomine was a better inhibitor of morphogenesis induced by acetylcholine or bethanechol compared to solifenacin and flavoxate. In humans, the antimuscarinic receptor action of flavoxate on smooth muscle is not fully understood. It is reported that flavoxate has moderate Ca^{+2} antagonistic activity rather than anticholinergic effect [28]. The effect of flavoxate against *C. albicans* morphogenesis may be due to the competition with acetylcholine and bethanechol in the active site region of protein like muscarinic receptor or due to the inhibition of calcium signaling pathway. Our study demonstrates the effect of muscarinic receptor agonists and antagonists on *C. albicans* morphogenesis. This is a first report of muscarinic receptor agonists and antagonists regulate the dimorphism in *C. albicans*. Our study suggests that Rrp9 protein may have a potential role in *C. albicans* morphogenesis and that muscarinic acetylcholine receptor antagonists could be as anti-Candida agents.

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