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# Research Article
# Cholinergic Receptor Binding Profile of *Hypericum perforatum* L. and its Active Constituents

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## Abstract

**Background and Objective:** *Hypericum perforatum* L. (HP) is a popular herbal medicine with different pharmacological effects. This study investigated the possible cholinergic receptor affinities of HP extract and its three active constituents: hyperforin, hypericin and pseudohypericin. **Materials and Methods:** Radioactive compounds [3H]-N-methyl scopolamine used for muscarinic receptor binding studies in Chinese hamster ovary cells expressing human muscarinic receptor subtypes and [3H]-cytisine used for nicotinic receptor binding tests performed with mouse brains without a cerebellum. Muscarinic binding inhibition was observed with HP extract considerably for hM2 and hM5. **Results:** Hyperforin, hypericin and pseudohypericin showed a much lower affinity for muscarinic receptors at higher concentrations. The HP extract and its constituents did not produce any nicotinic receptor binding inhibition. **Conclusion:** These results suggested that post-junctional direct muscarinic receptor interaction may modulate some effects of HP extract and its constituents however different mechanisms apart from direct cholinergic receptor interaction might be considered for the pharmacological actions of hyperforin, hypericin and pseudohypericin.

Key words: *Hypericum perforatum* L., muscarinic receptors, nicotinic receptors, hyperforin, hypericin, pseudohypericin

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

*Hypericum perforatum* L. (HP or St. John's Wort, SJW) is a plant whose extract can be beneficial in the treatment of mild to moderate depression[1]. Additionally, studies revealed many other actions of HP in peripheral tissues[2,3], memory-enhancing effects[4], antiviral[5] and antibacterial activities[6]. Among many chemical constituents of HP, hyperforin as phloroglucinol, hypericin and pseudohypericin as naphthodianthrones and flavonoids attracted the most attention[7].

Previous findings pointed out possible signs of cholinergic system interaction of HP extracts and its ingredients, hypericin and hyperforin. Capasso *et al.*[2] showed that HP extract reduced the acetylcholine-induced contractions of the rat urinary bladder. The HP extract in high doses reduced the carbachol-induced smooth muscle responses of detrusor strips whereas lower doses slightly increased the carbachol-induced contractions *in vitro*. Active components; hypericin and hyperforin altered the carbachol-induced contractions of the urinary bladder at $10^{-8}$-$10^{-5}$ M concentrations[3]. The HP extract and hyperforin dose-dependently decreased gastric emptying in rats[8]. Kumar *et al.*[9] proposed a possible cholinergic mechanism in the central nervous system for the reversal of scopolamine-induced amnesia by orally administered Indian HP. Supporting evidence revealed that hyperforin acted as an antidementia agent and was involved in the memory-enhancing actions of the HP extract[4].

Direct interactions of muscarinic receptors with HP extract and some of its active ingredients have been studied. Butterweck and Schmidt[7] showed a modest affinity of hypericin for muscarinic cholinergic receptors (subtypes have not been studied) in the rat cerebral cortex. Hypericin only showed slight inhibition on muscarinic type-2 receptors (M2), whereas, significant inhibition on particular muscarinic receptor subtypes were obtained with HP ingredients, rutin and miquelianin[10]. Kumar *et al.*[11] revealed that there was no significant alteration in the [$^3$H] QNB binding on hippocampal synaptic membranes of rats following systemically administered Indian HP, indicating no significant regulation (±10%) in the expression density of M2 receptors.

Additional to muscarinic receptor-mediated actions, studies indicate possible nicotinic receptor interaction of the extract and its constituents. The HP extract altered the acetylcholine function at the post-synaptic level in the neuromuscular junction[12]. Additional to muscular type, neuronal type nicotinic receptors might play a role in the cognitive-enhancing actions of HP extract[4,13] since the stimulation of brain nicotinic receptors increase cognitive functions[14]. Regarding the direct nicotinic receptor interaction of HP and its constituents, none of the tested constituents had pronounced effects on nicotinic receptors[10] and a weak affinity of 1 μM hypericin had been discovered[15]. In this study, muscarinic receptor subtypes and nicotinic receptor affinities of HP extract and its three constituents; hyperforin, hypericin and pseudohypericin were investigated. The main reason to choose hyperforin, hypericin and pseudohypericin as constituents of *Hypericum perforatum* L. (HP) in this study was, that naphthodianthrones (hypericin and pseudohypericin) and phloroglucinols (hyperforin and adhyperforin) were considered as the most characteristic compounds of HP[7]. These three active ingredients show some pharmacokinetic differences regarding the half-lives and their peak plasma concentrations following oral ingestions[16]. Additionally, their pharmacological actions are different like the antidepressant action is mainly related to hyperforin, as well as the monoamine uptake and receptor binding profiles[7]. Hypericin is used for the standardization of HP extracts[17]. Therefore, in this study, these active ingredients with different pharmacokinetic and pharmacological properties were studied for cholinergic receptor binding affinities and compared with HP extract to clarify the possible involvement of direct cholinergic receptor interaction in the biological effects of HP.

## MATERIALS AND METHODS

**Study area:** The study was carried out at the Department of Neuroscience, Psychology, Drug Research and Child's Health, Section of Pharmaceutical and Nutraceutical Sciences, University of Florence, Italy from January, 2018 to March, 2019.

**Chemicals:** The HP extract, pseudohypericin; atropine sulfate and nicotine bitartrate were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Hypericin and hyperforin dicyclohexylamine salts were a gift from the Indena® company (20139, Milano, Italy). Radioactive compounds, [$^3$H]-NMS and Cytisine HCl, [3,5-$^3$H(N)]-with the specific activities of 70-87Ci (2590-3200GBq)/mmol and 15-40Ci (555-1480 GBq)/mmol, respectively, were obtained from Perkin-Elmer Life Science, Boston, MA. Hyperforin dicyclohexylamine salt, hypericin and pseudohypericin and HP extract were dissolved in DMSO ($10^{-2}$ M). Hyperforin dicyclohexylamine salt was diluted in DMSO: $H_2O$ = 25:75 ($10^{-5}$ M). Dilutions of tested compounds were prepared with PB for muscarinic binding and Tris-HCl buffer for nicotinic binding studies.

**Cell culture and membrane preparation:** Chinese hamster ovary (CHO) cells, expressing human muscarinic receptor subtypes (hM1-hM5) individually, provided by Prof. R. Maggio (University of L'Aquila, Italy), were kept in nitrogen, thawed at 37°C with 0.9 mL amount of cells and 60 µL of the antibiotic G418 sulfate (Geneticin®, Gibco™, Grand Island, NY, USA). Cells transferred into a growth medium containing Dulbecco's modified Eagle's medium (Sigma Aldrich, St. Louis, USA) and Ham's F12 w/o L-Glutamine (Biowest SAS, Nuaillé-France) in 1:1 mixture with 10% fetal bovine serum (FBS, GibcoTM, Grand Island, NY, USA), 1% Penicillin-Streptomycin solution (EuroClone, catalogue number: EC B3001D) and 1% L-glutamine solution 200 mM (100×) (EuroClone, catalogue number: ECB3000D).

Cells were incubated at 37°C in a 5% $CO_2$ humidified incubator. Cells were detached with trypsin and scraped then transferred into FBS containing tubes placed on ice. Supernatants were removed following centrifugation at 1200 rpm for 5 min. Each pellet was suspended with phosphate buffer (PB, 25 mM sodium phosphate and 5 mM $MgCl_2$, pH: 7.4) and homogenized with Ultra-Turrax® (IKA®, Germany) for 30 sec at setting 3 on ice. The pellet was sedimented at 12000 rpm for 15 min at 4°C. The supernatant was resuspended with PB, homogenized with Ultra-Turrax® and aliquoted to be stored at -80°C. About 50 µL aliquot was taken for protein quantification using Bio-Rad protein assay reagent (Bio-Rad Laboratories, Munich, Germany).

For nicotinic receptor binding studies, frozen mouse brains without cerebellum were slowly thawed and 30 mL $g^{-1}$ tissues were placed into cold 50 mM Tris-HCl buffer, containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$ (pH: 7.4). Following the homogenization with a glass-Teflon homogenizer on ice, homogenates were centrifuged at 40000 g for 10 min at 4°C. Pellets were resuspended in fresh buffer, centrifuged again and resuspended with fresh buffer. About 20 µL of the sample was used for protein assay using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, MA USA)[18].

**Radioligand binding assays:** Muscarinic receptor radioligand binding studies were performed in polypropylene 96-well plates (Sarstedt, Verona, Italy) filled with 175 µL of PB. About 25 µL of the tested compounds were added in different concentrations and then 25 µL of membrane preparations expressing muscarinic receptor subtypes (hM1-hM5) were added onto 25 µL (0.2 nM) of the radioactive compound [$^3$H]-N-methyl scopolamine ([$^3$H]-NMS). Microplates were incubated at room temperature on the shaker for 120 min. Final membrane concentrations inside the plates were 30 (hM1), 70 (hM2), 25 (hM3), 50 (hM4) and 25 µg mL$^{-1}$ (hM5). Free radioligand was separated from bound ligand by filtration through UniFilter GF/C plates (Perkin-Elmer Life Science, Boston, MA) using a FilterMate cell harvester (Perkin-Elmer Life Science, Boston, MA). Filters were washed with ice-cold buffer and 25 µL of scintillation liquid (MicroScint-20, Perkin-Elmer Life Science, Boston, MA) was added to totally dried filters. Radioactivity was counted by the TopCount NXT microplate scintillation counter (Perkin-Elmer Life Science, Boston, MA). Non-specific binding was determined with a $10^{-5}$ M concentration of atropine[19].

For nicotinic receptor binding, 400 µg mL$^{-1}$ of protein-containing samples and 0.75 nM final concentrations of [$^3$H]-cytisine were incubated with different concentrations of tested compounds and Tris-HCl buffer (1 mL final volume) for 75 min at 4°C. Bound fractions were separated from free fractions by filtration through Whatman GF/B glass fibre filters presoaked in polyethylenimine (PEI) by using a Brandel M-48R 48-well cell harvester (Brandel, Gaithersburg, MD, USA). Filters were rapidly rinsed three times with 3 mL of ice-cold 0.9% saline. The 6 mL of filter count scintillation cocktail (Perkin-Elmer Life Science, Boston, MA) was added to each vial and radioactivity was counted by Tri-Carb Liquid Scintillation Counter (Perkin-Elmer Life Science, Boston, MA). The $10^{-5}$ M concentration of nicotine bitartrate was used for non-specific binding[20]. Muscarinic and nicotinic receptor binding inhibition data were presented as $B/B_0$ (%) (transformed from the cpm counts of the tested compounds) and means of logarithmic concentrations±standard error of the mean of four to six experiments conducted in duplicate.

**Statistical analysis:** The inhibitory concentration was defined as B/B0 (%). Half inhibitory concentration ($IC_{50}$, defined as 50% inhibitory concentration) were calculated with nonlinear regression analysis (GraphPad Prism 5, La Jolla, CA) Data were expressed as Mean±SEM (n = 4-6, each performed in duplicate).

## RESULTS AND DISCUSSION

**Muscarinic receptor binding:** The HP extract showed significant inhibitions, particularly on hM2 and hM5 receptor subtypes, starting from 0.05 mg mL$^{-1}$ concentrations. The 0.1 mg mL$^{-1}$ HP extract showed ~70% inhibition for hM2 and ~45% for hM5. In higher doses (0.5 and 1 mg mL$^{-1}$), inhibitions were $\geq$60 and $\geq$70% for all subtypes. About 0.5 mg mL$^{-1}$ HP extract revealed ~80 and ~70% binding inhibitions for hM2 and hM5. The highest concentration of HP extract (1 mg mL$^{-1}$) showed 88 and 92% binding inhibitions for hM2 and hM5 (Table 1). Log $IC_{50}$±SEM of HP extract for inhibition of M1-M5 receptors, -0.4274±0.16, -1.403±0.05, -0.4860±0.08,

Table 1: Muscarinic receptor binding inhibitions for *Hypericum perforatum* extracts

| Muscarinic Receptor subtype | Total binding | 0.001 (mg mL$^{-1}$) | 0.005 (mg mL$^{-1}$) | 0.01 (mg mL$^{-1}$) | 0.05 (mg mL$^{-1}$) | 0.1 (mg mL$^{-1}$) | 0.5 (mg mL$^{-1}$) | 1 (mg mL$^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| hM1 | -0.10±1.2 | 0.52±1.2 | 13.71±2.9 | 6.76±6.0 | 10.35±5.0 | 24.46±4.0 | 56.29±3.3 | 69.81±1.0 |
| hM2 | 0.00±0.9 | 4.87±2.0 | 5.80±3.0 | 18.12±2.5 | 48.86±1.2 | 68.76±0.5 | 80.25±1.3 | 87.45±1.8 |
| hM3 | 0.29±2.0 | 2.15±1.0 | 3.25±0.9 | 6.95±2.4 | 15.20±1.4 | 27.25±4.1 | 60.07±2.6 | 79.30±1.6 |
| hM4 | 0.15±1.2 | 6.85±4.4 | 4.88±4.9 | 8.93±3.2 | 19.49±3.8 | 35.22±2.4 | 60.09±5.4 | 79.25±0.8 |
| hM5 | 0.24±1.2 | 3.99±3.7 | 5.93±3.2 | 16.81±1.8 | 27.15±0.7 | 46.64±2.6 | 70.34±2.6 | 91.97±1.1 |

Muscarinic receptor binding inhibitions for *Hypericum perforatum* extracts on membrane preparations expressing muscarinic receptor subtypes individually (hM1-hM5). Data were calculated as (100-B/$B_0$ (%)) and expressed as Mean±Standard error

Table 2: Muscarinic receptor binding inhibitions for hypericin, hyperforin and pseudohypericin

| Muscarinic receptor subtype | Total binding | $10^{-10}$ M | $10^{-9}$ M | $10^{-8}$ M | $10^{-7}$ M | $10^{-6}$ M | $10^{-5}$ M |
|---|---|---|---|---|---|---|---|
| **hM1** | | | | | | | |
| Hypericin | 0.06±1.4 | 12.73±7.3 | 4.16±4.3 | 6.37±1.9 | 0.76±2.7 | 20.02±8.6 | 42.30±7.3 |
| Hyperforin | 0.05±1.3 | 7.53±5.8 | 21.45±15.1 | 18.46±12.1 | 12.69±12.3 | 8.54±4.7 | 20.37±4.2 |
| Pseudohypericin | 0.10±2.1 | 5.17±7.9 | 16.10±14.4 | 21.22±10.7 | 13.99±14.1 | 26.23±13.0 | 24.09±6.4 |
| **hM2** | | | | | | | |
| Hypericin | 0.00±3.1 | 12.66±4.5 | 6.66±1.5 | -4.40±4.3 | 9.68±4.2 | 23.73±9.6 | 41.23±5.3 |
| Hyperforin | 0.01±1.2 | 7.02±2.5 | 7.58±6.6 | 7.89±4.6 | -1.10±4.2 | 15.67±4.8 | 31.91±2.1 |
| Pseudohypericin | 0.02±0.9 | -7.80±10.0 | 5.60±5.4 | 4.41±3.3 | 9.18±3.7 | 6.90±3.8 | 20.73±4.7 |
| **hM3** | | | | | | | |
| Hypericin | 0.01±2.1 | 0.27±8.5 | 3.08±6.4 | 1.95±5.4 | 0.60±4.9 | 3.63±8.9 | 36.21±5.4 |
| Hyperforin | 0.03±1.7 | 1.94±3.1 | 0.09±3.3 | 0.58±1.7 | -1.40±2.3 | -3.70±2.7 | 18.08±1.1 |
| Pseudohypericin | 0.01±4.2 | -8.50±3.3 | 4.04±3.7 | 5.29±1.8 | 0.39±3.8 | 2.64±3.7 | 20.86±3.8 |
| **hM4** | | | | | | | |
| Hypericin | 0.03±5.6 | 14.15±6.8 | 14.18±2.9 | 20.27±7.7 | 9.58±1.7 | 20.37±3.7 | 48.79±6.8 |
| Hyperforin | 0.02±2.3 | 5.42±11.1 | 12.00±15.1 | 7.35±6.4 | 4.67±14.7 | 7.55±5.5 | 32.49±3.9 |
| Pseudohypericin | 0.01±0.01 | -7.30±2.9 | 17.54±13.1 | 12.78±8.1 | -1.90±10.4 | 8.75±6.4 | 19.23±3.7 |
| **hM5** | | | | | | | |
| Hypericin | 0.04±3.1 | 4.55±5.5 | 2.96±7.7 | 19.71±12.9 | 0.88±4.3 | 5.96±3.9 | 43.98±5.7 |
| Hyperforin | 0.03±3.6 | 5.94±16 | -0.10±7.9 | 2.77±7.4 | 3.36±5.2 | 4.80±8.1 | 16.71±5.0 |
| Pseudohypericin | 0.01±2.2 | 6.23±0.3 | 4.33±2.4 | 12.50±7.4 | 8.26±1.9 | 12.10±5.7 | 32.27±6.6 |

Muscarinic receptor binding inhibitions for hypericin, hyperforin hypericin and pseudohypericin on membrane preparations expressing muscarinic receptor subtypes individually (hM1-hM5). Data were calculated as (100-B/$B_0$ (%)) and expressed as Mean±Standard error

-0.6855±0.09 and -0.8680±0.07 M, respectively (Fig. 1a-e). Previous studies observed that miquelianin and rutin constituents of HP showed ~55% inhibition on the hM2 and hM5 subtype suggesting that these constituents are likely to mediate hM2 and hM5 subtype inhibitions[10]. Studies performed on detrusor muscle strips or isolated rat stomach tissue showed that HP produce significant inhibition of the acetylcholine-induced bladder contractions (0.3-1 mg mL$^{-1}$)[2], carbachol-induced detrusor contractions (0.1 mg mL$^{-1}$)[3] and acetylcholine-induced stomach contractions (0.1-1 mg mL$^{-1}$)[8]. In line with previous findings, significant inhibition of hM3 and especially hM2 receptor subtype at 0.5 and 1 mg mL$^{-1}$ concentrations of the HP extract was observed ($\geq$80 and $\geq$60% for hM2 and hM3, respectively) in this study. The HP also alters the smooth muscle functions in electrical-field stimulation[2,8], which can be related to pre-junctional action. Together with previous data these results might indicate direct post-junctional cholinergic interaction of HP with M2 and M3 subtypes in relatively higher concentrations although pre-junctional modifications or post-junctional actions other than the muscarinic receptor interactions should also be considered.

Muscarinic cholinergic receptors belong to the G-protein coupled receptors (GPCR) family that naphthodianthrones and hyperforin showed to have an affinity to some of the GPCR. Hypericin ($10^{-6}$ M) has been shown to exert obvious binding inhibitions to dopamine type-3 and -4 receptors (70-80%), beta-1 and -2 adrenergic receptors (~90%). Pseudohypericin has a strong affinity to dopamine type-3 and -4 receptors (~80%)[10]. The lipophilic component of HP, hyperforin exerts strong inhibitions to dopamine type-1 and -5 receptors (~60%) and moderate affinity to $\mu$, $\delta$ and $\kappa$-opioid receptors with Ki: 400-1000 nM[7,21]. Pseudohypericin, in the same concentrations as hypericin (1 µM), only showed a weak inhibition (27%) of hM1 receptor binding whereas inhibition for other muscarinic receptor subtypes was lower than 10-15%. According to current findings, hypericin revealed minimal muscarinic receptor inhibitions at $10^{-6}$ M concentration which increased to ≈40-45% at $10^{-5}$ M concentration (Table 2). Log $IC_{50}$±SEM of hypericin for inhibition of M1-M5, -4.688±0.26, -4.805±0.15, -4.665±0.15, -4.670±0.35 and -4.732±0.17 M, respectively (Fig. 2a-e). Similarly, hyperforin did not produce any significant inhibition in concentrations lower than $10^{-5}$ M and binding inhibitions

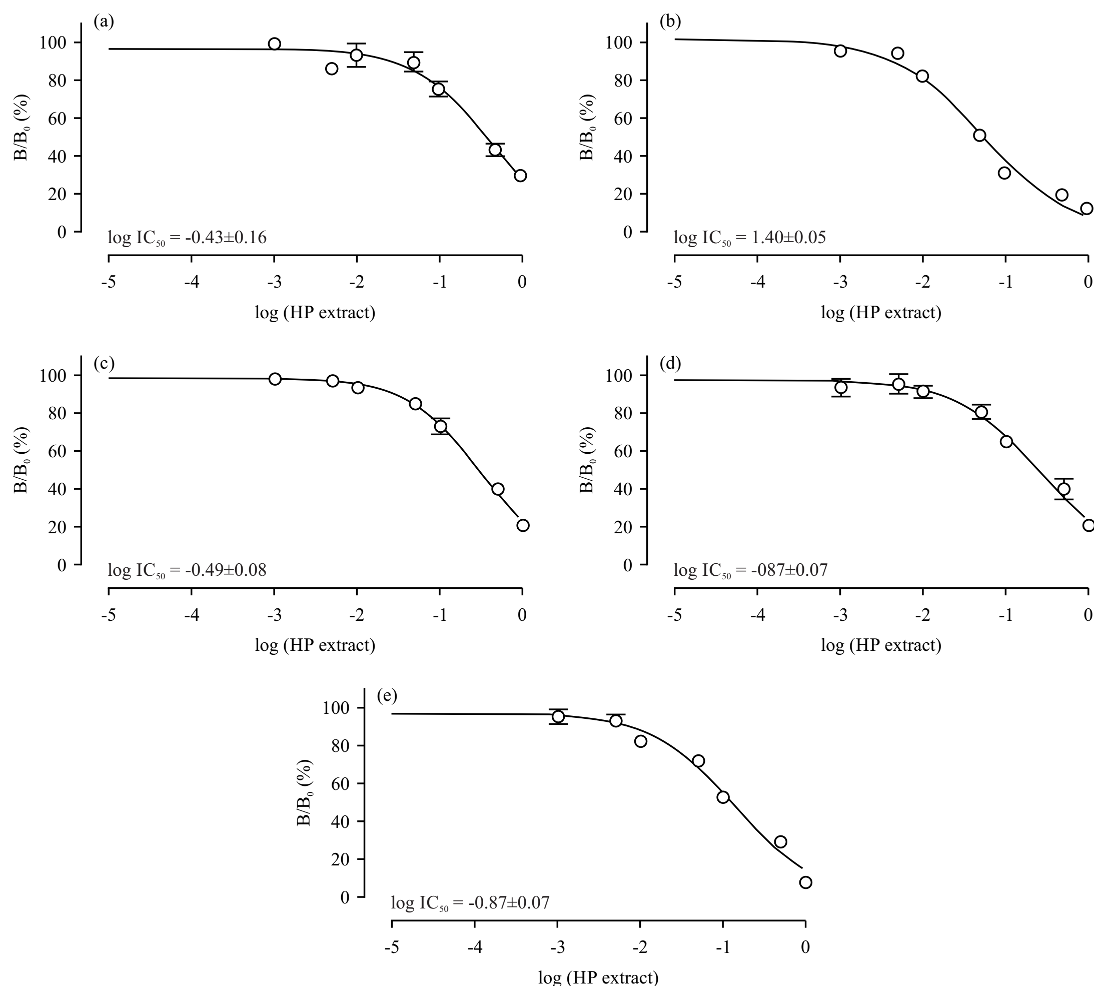


Fig. 1(a-e): Inhibition of [3H]NMS binding by *Hypericum perforatum* extracts on membrane preparations expressing muscarinic receptor subtypes, (a) hM1, (b) hM2, (c) hM3, (d) hM4 and (e) hM5

Atropine was used to define nonspecific binding. Data calculated as (100-B/$B_0$ (%)) and Inhibitor concentration 50 ($IC_{50}$). Data were expressed as Mean±Standard error of four to six experiments conducted in duplicate

were ~20-30% for all muscarinic receptor subtypes at $10^{-5}$ M concentration. Relatively higher inhibitions were observed for hM2 and hM4 (~30%) in the highest hyperforin concentration (Table 2). Log $IC_{50}$±SEM of hyperforin for inhibition of M1-M5, -9.596±0.90, -4.784±0.47, -4.752±0.16, -4.804±0.50 and -4.279±0.49 M, respectively (Fig. 3a-e). Pseudohypericin did not show a significant muscarinic receptor affinity, in concentrations lower than $10^{-5}$ M. In those concentration ranges, the highest inhibition was observed in hM1 receptor binding (~25%) in $10^{-6}$ M concentrations. In the highest tested concentration ($10^{-5}$ M) of pseudohypericin, minimal muscarinic receptor binding inhibitions were observed for hM1, hM2, hM3 and hM4 subtypes which were ~20-25% and ~30% for hM5 (Table 2). Log $IC_{50}$±SEM of pseudohypericin for inhibition of M1-M5, -4.047±1.05, -3.947±1.03, -4.334±0.20, -3.806±1.31 and -4.357±0.52 M, respectively (Fig. 4a-e). Increasing concentrations ($10^{-12}$-$10^{-6}$ M) of atropine showed significant and dose-dependent inhibitions on muscarinic receptor binding for all receptor subtypes (Fig. 1-4). This data showed a slight difference between the previous findings in which binding inhibition for hM1 receptor was not observed at the same concentrations tested and hypericin (0.1 μM) binding inhibitions approached 50% values. Taken together; three components of HP, hyperforin, hypericin and pseudohypericin, did not inhibit the binding to five subtypes of muscarinic acetylcholine receptors (hM1-hM5) in concentrations $10^{-10}$–$10^{-6}$ M ($\leq$25%, for all muscarinic receptor subtypes in $10^{-6}$ M) although hypericin inhibited binding between ≈36-48% for all muscarinic receptor subtypes in $10^{-5}$ M concentrations.

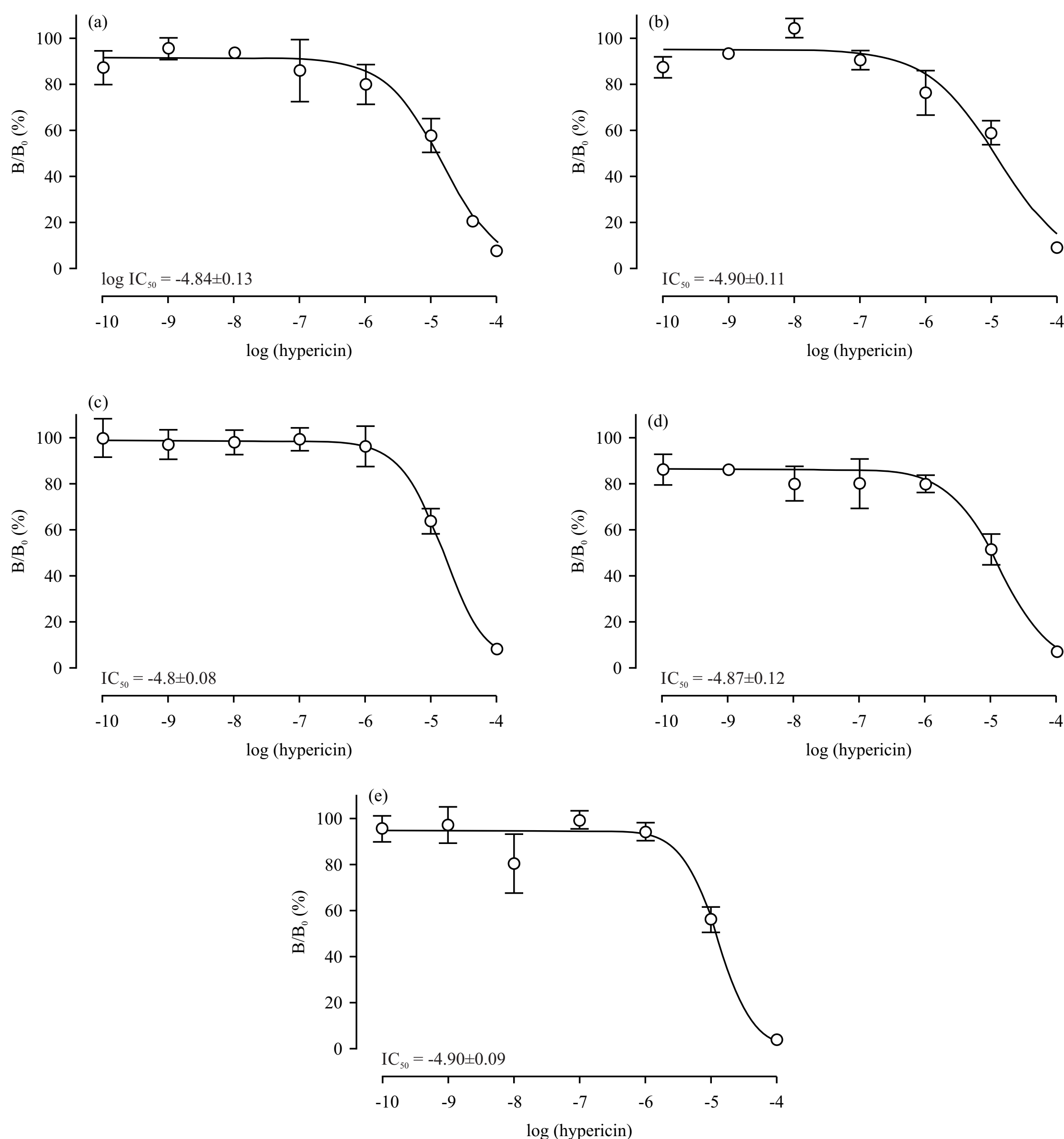


Fig. 2(a-e): Inhibition of [3H]NMS binding by hypericin on membrane preparations expressing muscarinic receptor subtypes, (a) hM1, (b) hM2, (c) hM3, (d) hM4 and (e) hM5

Atropine was used to define nonspecific binding. Data calculated as (100-$B/B_0$ (%)) and Inhibitor concentration 50 ($IC_{50}$). Data were expressed as Mean±Standard error of four to six experiments conducted in duplicate

Current findings suggested that direct post-junctional muscarinic receptor binding is unlikely to play a role for the tested three constituents (hyperforin, hypericin and pseudohypericin) in the alteration of smooth muscle contractions following HP administrations since hyperforin and hypericin have been shown to alter the carbachol-induced urinary bladder contractions significantly in $10^{-7}$ M concentrations[3] and in $10^{-6}$ M concentrations for acetylcholine-induced stomach contractions[8]. Hyperforin and hypericin modify the smooth muscle contractions in lower concentrations[3,8] although these results did not show any muscarinic receptor affinity in these concentrations.

Muscarinic type-2 receptors are located presynaptically in the brain and play role in acetylcholine (ACh) release. Hyperforin($10^{-5}$ M) increases the striatal and hippocampal ACh release in rats[22]. In this study, hyperforin ($10^{-5}$ M) showed ~30% inhibitions on hM2 receptors that which modulation of ACh release could be attributed. However, considering the

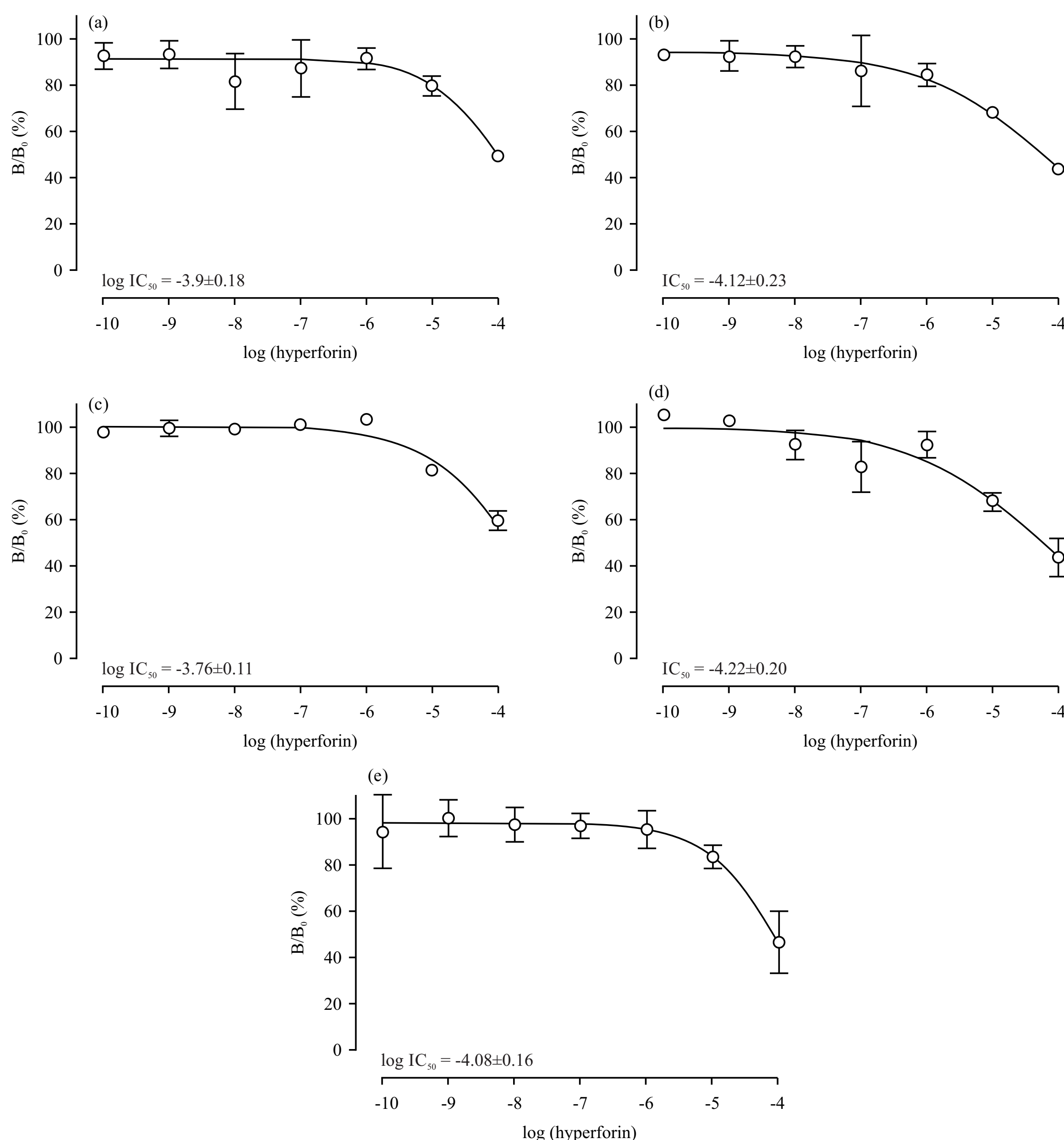


Fig. 3(a-e): Inhibition of [3H]NMS binding by hyperforin on membrane preparations expressing muscarinic receptor subtypes, (a) hM1, (b) hM2, (c) hM3, (d) hM4 and (e) hM5

Atropine was used to define nonspecific binding. Data calculated as (100-B/$B_0$ (%)) and Inhibitor concentration 50 ($IC_{50}$). Data were expressed as Mean±Standard error of four to six experiments conducted in duplicate

extracellular concentrations would be approximately 10% of the infused concentrations according to Buchholzer *et al.*[22], it is unlikely to attribute the acetylcholine release modifying effect to the hM2 binding of hyperforin since $10^{-6}$ M hyperforin inhibited the hM2 binding only at 15%. The action of hyperforin on sodium-dependent high-affinity choline uptake protein could play role in this biological action of hyperforin. This was also supported by a study concluding that hippocampal release of acetylcholine in the hippocampus occurs via an indirect mechanism which is calcium-dependent[12]. Additionally, modulation of free intracellular sodium concentrations and interaction with sodium-hydrogen exchangers[23] may also be involved.

**Nicotinic receptor binding:** Hypericin, pseudohypericin and hyperforin did not show any significant nicotinic receptor binding inhibition in [$^3$H]-cytisine studies, in the same way, HP extract (0.001-1 mg $mL^{-1}$) did not reveal any significant

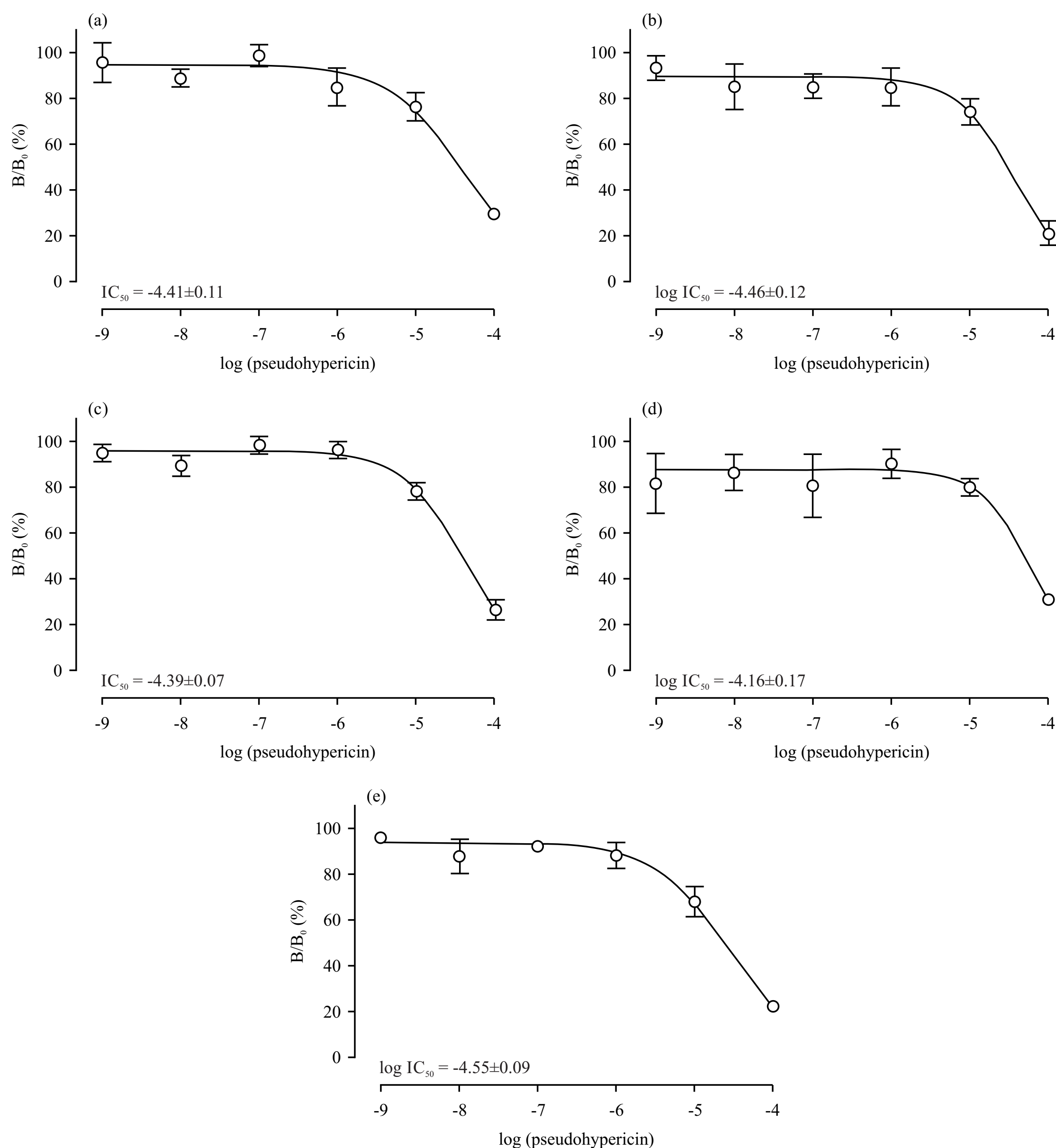


Fig. 4(a-e): Inhibition of [3H]NMS binding by pseudohypericin on membrane preparations expressing muscarinic receptor subtypes, (a) hM1, (b) hM2, (c) hM3, (d) hM4 and (e) hM5

Atropine was used to define nonspecific binding. Data calculated as (100-B/$B_0$ (%)) and Inhibitor concentration 50 ($IC_{50}$). Data were expressed as Mean$\pm$Standard error of four to six experiments conducted in duplicate

Table 3: Nicotinic receptor binding inhibitions for *Hypericum perforatum* extract

| Parameters | HP extract |
|---|---|
| Total binding | 0.04±1.6 |
| 0.001 (mg mL$^{-1}$) | 0.96±1.6 |
| 0.005 (mg mL$^{-1}$) | -3.30±1.9 |
| 0.01 (mg mL$^{-1}$) | 3.74±2.0 |
| 0.05 (mg mL$^{-1}$) | 1.52±2.5 |
| 0.1 (mg mL$^{-1}$) | -7.70±6.9 |
| 0.5 (mg mL$^{-1}$) | -7.40±11.0 |
| 1.0 (mg mL$^{-1}$) | 10.35±2.5 |

Nicotinic receptor binding inhibitions for *Hypericum perforatum* extract on membrane preparations expressing nicotinic receptors. Data were calculated as (100-B/$B_0$ (%)) and expressed as Mean$\pm$Standard error

nicotinic receptor binding inhibition (Table 3, 4, Fig. 5a-e). Increasing concentrations ($10^{-8}$-$10^{-4}$ M) of nicotine showed a significant and dose-dependent [$^3$H]-cytisine binding inhibition starting from $10^{-8}$ M (Fig. 5e) till the specific binding was completely erased at the maximum concentration used (log $IC_{50}\pm$SEM: -6.74$\pm$0.27). Following the previous reporting tested constituents of HP extract, this study did not show a neuronal type of nicotinic receptor binding inhibition of the three tested constituents as well as the HP extract[10,15].

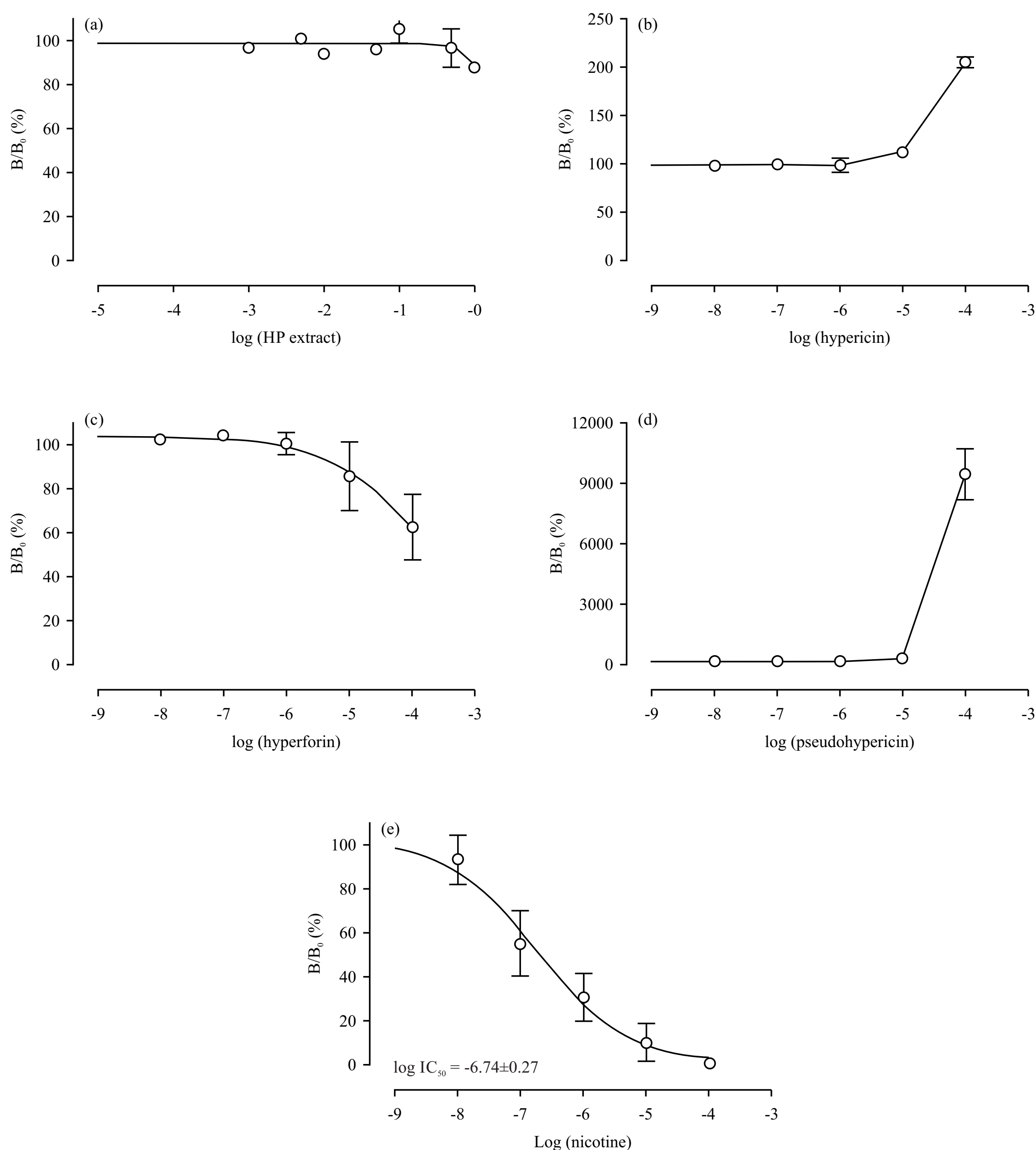


Fig. 5(a-e): Inhibition of [3H]-cytisine binding by (a) *Hypericum perforatum* extracts, (b) Hypericin, (c) Hyperforin, (d) Pseudohypericin on membrane preparations expressing the nicotinic receptor and (e) Nicotine was used to define nonspecific binding

Data calculated as (100-$B/B_0$ (%)) and Inhibitor concentration 50 ($IC_{50}$). Data were expressed as Mean±Standard error of four to six experiments conducted in duplicate

Table 4: Nicotinic receptor binding inhibitions for hypericin, hyperforin and pseudohypericin

| | Total binding | $10^{-8}$ M | $10^{-7}$ M | $10^{-6}$ M | $10^{-5}$ M |
|---|---|---|---|---|---|
| Pseudohypericin | 0.21±1.4 | 2.1±2.9 | -2.4±5.2 | -1.0±2.2 | ND |
| Hypericin | 0.78±0.6 | 2.14±0.6 | 0.85±2.5 | 1.76±7.2 | -11.8±4.0 |
| Hyperforin | -0.1±1.8 | -0.1±1.0 | -2.6±1.9 | 1.4±4.5 | 16.2±15.1 |

Nicotinic receptor binding inhibitions for pseudohypericin, hypericin and hyperforin on membrane preparations expressing nicotinic receptors. Data were calculated as (100-$B/B_0$ (%)) and expressed as Mean±Standard error

## CONCLUSION

The HP extract showed muscarinic receptor affinity, particularly hM2 and hM5, at some range. Higher than 60% binding inhibitions for HP extract on hM2 and hM3 point out a possible post-junctional muscarinic receptor interaction in the smooth muscle effects of this herb. Results revealed weak muscarinic receptor binding inhibitions for hyperforin, hypericin and pseudohypericin only in high concentrations ($10^{-5}$ M) claiming that these constituents of HP work independently from the muscarinic receptor interaction, but the other constituents may. However, pre-junctional site involvement and post-junctional mechanisms different from muscarinic receptor interaction are likely to play the main role in the actions of hyperforin and hypericin constituents of HP. Additionally, nicotinic receptors are unlikely to take part in the effects of this herb and its tested constituents. It looks like more than one receptor-mediated mechanism of action contributes to the effects of HP, hyperforin and hypericin and muscarinic receptor-binding only play a limited role in the biological functions of HP. This study provides novel findings by comparing the cholinergic receptor binding of the HP extract and its most active three constituents which contributes to the explanation of the receptor-mediated mechanism of some pharmacological actions of this herbal medicine.

## SIGNIFICANCE STATEMENT

This study represents the muscarinic (hM1-hM5) and nicotinic receptor binding characteristics of Hypericum perforatum extract and its highly biologically active three constituents: hyperforin, hypericin and pseudohypericin. In our study, our findings uniquely showed binding inhibition for some muscarinic receptor subtypes, hM2 and hM5 were observed with Hypericum perforatum extract. However, weak muscarinic receptor binding results for the studied three constituents of Hypericum perforatum point out that these constituents function via different mechanisms other than muscarinic receptors. Additionally, nicotinic receptors are unlikely to take part in the physiological effects of Hypericum perforatum extract and hyperforin, hypericin and pseudohypericin constituents.

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