



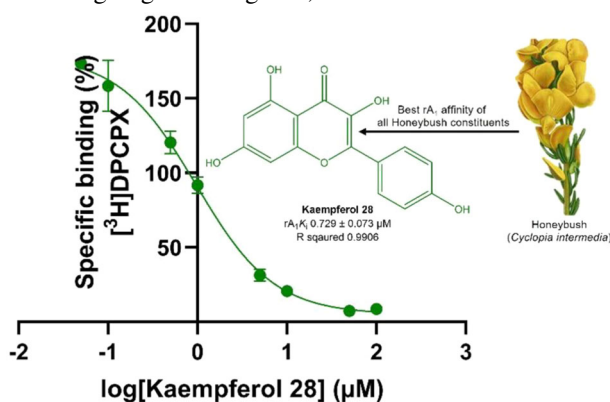
Evaluation of chemical constituents of Rooibos (*Aspalathus linearis*) and Honeybush (*Cyclopia intermedia*) as adenosine A₁/A_{2A} receptor ligands

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Abstract

Rooibos (*Aspalathus linearis*) and Honeybush (*Cyclopia intermedia*) are popular tisanes in South Africa and are of growing interest due to the wide variety of flavonoids and other phytochemicals they contain. Despite their history as herbal teas and traditional medicines, the chemical constituents of these tisanes have yet to be studied for their effects on adenosine receptors. A series of 30 commercially available chemical constituents of Rooibos and Honeybush were investigated via radioligand binding studies to determine their adenosine A₁ and A_{2A} receptor affinity at both rat and human subtypes in order to establish structure-activity relationships and identify novel adenosine receptor ligands. In addition, in silico evaluations of the 30 test compounds were also performed to predict their physiochemical and pharmacokinetic properties. The most promising chemical constituent was kaempferol (**28**) which showed sub-micromolar affinity towards the rat A₁ subtype ($rA_1K_i = 0.7287 \mu\text{M}$; $hA_1K_i = 9.88 \mu\text{M}$) and acted as an antagonist toward adenosine rA_1 receptors. Additionally, quercetin (**2**), chrysoeriol (**8**), luteolin (**9**), eriodictiol (**12**), and naringenin (**27**) also showed adenosine A₁ and/or A_{2A} receptor affinity. It was observed that a flavonol scaffold is preferred to flavone and flavanone scaffolds, and within the flavonols, C4'-OH substitution on ring B is preferred to C3',4'-diOH substitution. These phytochemicals, specifically kaempferol (**28**), may be considered lead-like and valuable in designing novel ligands, based on in vitro and in silico evaluation.



Keywords Rooibos · Honeybush · Adenosine A₁ receptor · Adenosine A_{2A} receptor · Structure-activity relationships.

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Introduction

Adenosine is involved in cellular activities, and thus, engaged in both physiologic and pathophysiologic processes. Four adenosine receptor (AR) subtypes, A₁, A_{2A}, A_{2B} and A₃ [1, 2] are members of the G protein-coupled receptor (GPCR) superfamily, which is also a therapeutic target for several pathologies [3]. A₁ and A₃ ARs mainly couple to inhibitory G-proteins, while A_{2A} and A_{2B} ARs couple to stimulatory G-proteins [4]. A₁ ARs are mostly found in the central nervous system (cerebral cortex, cerebellum, hippocampus, thalamus, brainstem and spinal cord) as well as some peripheral organs. A_{2A} ARs are concentrated in the striatum, nucleus accumbens, and olfactory tubercle [5–7]. The abundant yet distinct distribution of the A₁ and A_{2A} ARs make them suitable drug targets for neurological conditions, including Parkinson's and Alzheimer's disease [8–11], and depression [12].

Naturally occurring AR antagonists have been identified including the alkylxanthines, caffeine (1,3,7-trimethylxanthine) and theophylline (1,3-dimethylxanthine) [13–15]. However, due to the xanthine structures' weak photostability and low water solubility [16], there is an ongoing search for non-xanthine antagonists. Some of these non-xanthine scaffolds include flavonoids, which are phytochemicals and also found in tisanes like Rooibos and Honeybush [16].

A number of herbs have traditionally been used as herbal infusions or “teas” in South Africa. Two Cape fynbos plants, *Aspalathus linearis* (Brum.f) Dahlg. (family Fabaceae; tribe Crotalarieae), better known as Rooibos, and various species of *Cyclopia* Vent. (family Fabaceae; tribe Podalyrieae), commonly known as Honeybush, have enjoyed commercial success as herbal teas [17]. These teas are popular tisanes enjoyed for their taste and aroma and medicinal purposes [17, 18].

Aspalathus linearis, known as Rooibos, is a leguminous bush indigenous to the mountain areas of the Western Cape Province of South Africa. It is gaining popularity as an alternative beverage worldwide, mainly due to the absence of caffeine and its low tannin content [19]. Rooibos tea is widely known as a natural cognitive enhancer as it can maintain alertness and concentration in the short-term and may also be beneficial for long-term cognitive health [20, 21]. Traditional medicinal uses of Rooibos in South Africa include alleviating infantile colic, allergies, asthma, and dermatological problems [22]. It is asserted that Rooibos has a low toxicity profile with several health benefits [19, 23] which include antioxidant [24], antiaging [25], anticancer [26], anti-inflammatory [27], antimutagenic [28] and antidiabetic [29] properties.

Rooibos tea (aqueous extract) can either be prepared by infusion of oxidised (fermented) or “green” (unfermented)

plant leaves [30]. Various compounds have been isolated from such aqueous extracts [31], as well as from methanolic extracts [31, 32].

The short, woody shrub Honeybush (*Cyclopia intermedia*) is grown in the mountain slopes of the Langkloof district between the Eastern and Western Cape regions of South Africa. Honeybush tea is usually prepared by infusion of fermented leaves, stems, and flowers of the plant. During the fermentation process, the plant material changes colour from green to dark brown as the phenolic compounds are oxidized [18].

Aqueous extracts of both fermented and unfermented Honeybush have shown antimutagenic [33], antioxidant, immunomodulating [34, 35], lipolytic [36], bone resorption [37] and antidiabetic [38] activities. Honeybush is traditionally used as a restorative and as an expectorant in chronic catarrh and pulmonary tuberculosis [17].

In this pilot study, the chemical constituents of Rooibos and Honeybush (structures found in Appendix A, Table A1) were investigated for their potential A₁ and A_{2A} AR affinity and the possibility of identifying novel AR ligands. The results would give insight into structure-activity relationships toward gaining, or even losing, AR affinity, and previously unknown AR drug leads and scaffolds may be identified.

Results and Discussion

In silico prediction of physiochemical and pharmacokinetic properties

The physiochemical and pharmacokinetic properties of the test compounds **1–30** were computed via SwissADME, and the results are summarised in Appendix A, Tables A2–4 and Figs. A1–2.

For the purpose of this discussion, focus was placed on compounds **2, 8, 9, 12, 27** and **28** which showed promising in vitro rA₁ and/or rA_{2A} AR affinity. Table 1 summarises the results of the in silico predicted physiochemical properties, whereas Table 2 gives the drug-likeness and medicinal chemistry friendliness of selected chemical constituents found in Rooibos and/or Honeybush plants.

The drug-likeness properties of the compounds were assessed by rule-based filters from Lipinski [39], Ghose [40], Veber [41], Egan [42], and Muegge [43]. The violated rules can be observed in Appendix A, Table A4. None of the test compounds that show adenosine A₁ and/or A_{2A} receptor affinity (**2, 8, 9, 12, 27** & **28**) violated any of Lipinski's five rules which outline the physiochemical properties that support the likelihood of absorption upon oral administration [39].

Table 1 In silico predicted physiochemical properties of selected chemical constituents found in Rooibos and/or Honeybush plants

Compound	Physiochemical properties								Lipophilicity LogP _{o/w} (MLOGP) ^b	Water solubility	
	Chemical formula	Molecular weight (g/mol)	Fraction Csp ³	Number of rotatable bonds	Number of H-bond acceptors	Number of H-bond donors	Molar refractivity	TPSA (Å ²) ^a		LogS (ESOL) ^d	LogS (Ali) ^e
2	C ₁₅ H ₁₀ O ₇	302.24	0.00	1	7	5	78.03	131.36	−0.56	−3.16	−3.91
8	C ₁₆ H ₁₂ O ₆	300.26	0.06	2	6	3	80.48	100.13	0.22	−4.06	−4.87
9	C ₁₅ H ₁₀ O ₆	286.24	0.00	1	6	4	76.01	111.13	−0.03	−3.71	−4.51
12	C ₁₅ H ₁₂ O ₆	288.25	0.13	1	6	4	73.59	107.22	0.16	−3.26	−3.90
27	C ₁₅ H ₁₂ O ₅	272.25	0.13	1	5	3	71.57	86.99	0.71	−3.49	−3.99
28	C ₁₅ H ₁₀ O ₆	286.24	0.00	1	6	4	76.01	111.13	−0.03	−3.31	−3.86

^aTopological Polar Surface Area (TPSA, Å²)^bLogP_{o/w} (MLOGP)^cLogS scale: insoluble < −10 < poorly < −6 < moderately < −4 < soluble < −2 < very < 0 < highly^dLogS (ESOL)^eLogS (Ali)**Table 2** In silico predicted drug-likeness and medicinal chemistry friendliness properties of selected chemical constituents found in Rooibos and/or Honeybush plants

Compound	Drug-likeness						Medicinal chemistry friendliness		
	Number of violations						Number of violations		
	Lipinski ^a	Ghose ^b	Veber ^c	Egan ^d	Muegge ^e	Bioavailability score ^f	PAINS ^g	Brenk ^h	Lead-likeness ⁱ
2	0	0	0	0	0	0.55	1 (catechol)	1 (catechol)	0
8	0	0	0	0	0	0.55	0	0	0
9	0	0	0	0	0	0.55	1 (catechol)	1 (catechol)	0
12	0	0	0	0	0	0.55	1 (catechol)	1 (catechol)	0
27	0	0	0	0	0	0.55	0	0	0
28	0	0	0	0	0	0.55	0	0	0

^aLipinski: Molecular weight < 500, MLOGP < 4.15, N or O < 10, NH or OH < 5^bGhose: 160 < Molecular weight < 480, −0.4 < WLOGP < 5.6, 40 < Molar refractivity < 130, 20 < atoms < 70^cVeber: Number of rotatable bonds < 10, Topological Polar Surface Area (TPSA, Å²) < 140^dEgan: WLOGP < 5.88, TPSA < 131.6^eMuegge: 200 < Molecular weight < 600, −2 < XLOGP < 5, TPSA < 150, number of rings < 7, number of carbons > 4, number of heteroatoms > 1, number of rotatable bonds < 15, number of H-bond acceptors < 10, number of H-bond donors < 5^fThe probability that a compound will have > 10% bioavailability in rat or measurable Caco-2 permeability^gPAINS: pan assay interference structures^hBrenk: structural alertⁱLead-likeness: 250 < Molecular weight < 350, XLOGP3 < 3.5, number of rotatable bonds < 7

The WLOGP-versus-TPSA referential, also known as the BOILED-Egg model (Appendix A, Fig. A2), can be used to predict the passive human gastrointestinal absorption (HIA) and blood–brain-barrier (BBB) permeation [44]. Since A₁ and A_{2A} ARs are greatly expressed in the central nervous system, a drug that targets these receptors should be able to cross the BBB. Unfortunately, none of the test compounds that show promising affinity are predicted to cross the BBB but are expected to have high GI absorption. Yet, compounds **15**, **16**, **17**, **24** and **26** are expected to permeate the BBB. According to Daina & Zoete (2016) it is predicted that moderately polar (TPSA < 79 Å²) and relatively

lipophilic (MLogP 0.4–6.0) molecules have a high probability of entering the CNS, and thus, crossing the BBB.

It was also predicted that compounds **2**, **8**, **9**, **12**, **27** and **28** are soluble to moderately soluble in water. A soluble molecule makes drug development easier [45], and also influences GI absorption [46].

Two methods were employed to identify potentially problematic fragments in the present series. The first method identifies pan assay interference compounds (PAINS) and compounds having substructures that are likely to hinder biological assays and exhibit target promiscuity are identified [47]. The Brenk method identifies

fragments that may be potentially toxic, chemically reactive, metabolically unstable, or have poor pharmacokinetics [48]. Only three out of the six test compounds with affinity toward rA_1 and/or A_{2A} subtypes produced alerts; quercetin (**2**), luteolin (**9**) and eriodictiol (**12**), which all contain a catechol. It is seen as problematic since a catechol is rapidly oxidized to reactive quinones, which can damage biological macromolecules and interfere with membrane function. They can also produce toxic reactive oxygen species [49].

In vitro evaluation of adenosine A_1 and A_{2A} receptor affinity

Competitive binding assays: degree of binding affinity

The degree of binding affinity that the test compounds **1–30** showed towards rat A_1 and A_{2A} ARs was determined via radioligand binding studies in either duplicate (specific binding (%)) or triplicate (inhibition constant (K_i , μM)) and expressed as mean $\pm$ standard error of the mean (SEM). Test compounds were initially assessed at a high maximum concentration of 100 μM using rat whole brain membranes (rA_1) or rat striatal membranes (rA_{2A}). Only compounds **2**, **8**, **9**, **12**, **27** and **28**, which displayed specific binding values of $\leq 20\%$ at a maximum tested concentration of 100 μM , underwent full biological assays for determination of inhibition constant values (K_i , μM). These compounds were further investigated at human (h) A_1 and/or A_{2A} receptor subtypes. All other test compounds displayed specific binding values $> 20\%$; thus, no K_i values were determined. The A_1 and A_{2A} AR radioligand binding assays were validated with N^6 -cyclopentyladenosine (CPA, A_1 agonist), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, A_1 antagonist) and istradefylline (IST, A_{2A} antagonist) as reference compounds. Results are summarized in Table 3.

Rooibos (*Aspalathus linearis*)

Rooibos contains flavonoids – representing three flavonoid subclasses, namely, flavone, flavonol, and flavanone (Fig. 1) [32]. The health benefits of Rooibos are probably due to these polyphenols and major flavonoids [50].

The aqueous Rooibos leaves extracts mainly consist of two unique compounds, namely, the C-C-linked dihydrochalcone glucoside aspalathin (**1**) [51, 52] and the cyclic dihydrochalcone aspalalinin (Fig. 2) [53]. Only aspalathin (**1**) was evaluated in the present study but was devoid of rA_1 and A_{2A} AR affinity.

The flavonol quercetin (**2**) showed A_1 AR affinity ($rA_1K_i = 2.47 \mu\text{M}$ & $hA_1K_i = 3.38 \mu\text{M}$) (Fig. 3). The flavonol glycoside derivatives, isoquercitrin (**3**) (quercetin 3-O- β -D-glucopyranoside), rutin (**4**) (quercetin-3-O-

rutinoside) and its galactoside derivative hyperoside (**5**) (quercetin 3-O- β -D-galactopyranoside) showed no rA_1 or A_{2A} AR affinity. This may be due to the glycoside or galactoside groups present in these compounds. The flavonol quercetin (**2**) has previously been shown to possess affinity for the A_1 (inhibition constant (K_i) = 2.47 μM) and A_{2A} ($K_i = 6.99 \mu\text{M}$) ARs in the low micromolar range [54].

The flavones chrysoeriol (**8**: $rA_1K_i = 1.39 \mu\text{M}$; $rA_{2A}K_i = 3.94 \mu\text{M}$ and $hA_1K_i = 2.41 \mu\text{M}$; $hA_{2A}K_i = 7.99 \mu\text{M}$) and luteolin (**9**: $rA_1K_i = 2.32 \mu\text{M}$; $rA_{2A}K_i = 2.50$ and $hA_1K_i = 2.24 \mu\text{M}$; $hA_{2A}K_i = 13.1 \mu\text{M}$) showed promising affinity for both A_1 and A_{2A} AR subtypes for both species (Fig. 4). Luteolin (**9**) has previously showed affinity toward the rA_1 and rA_{2A} ARs (half maximal inhibitory concentration (rA_1IC_{50}) = 1.19 μM ; $rA_{2A}IC_{50}$ = 0.84 μM) [55]. Rooibos is also a source of nothofagin (3-dehydroxydihydrochalcone glucoside) and its flavone analogues, vitexin (**6**) (apigenin-8-C-glucoside), isovitexin (**7**) (apigenin-6-C-glucoside), as well as other flavones orientin (**10**) (luteolin-8-C-glucoside) and iso-orientin (**11**) (luteolin-6-C-glucoside) which showed no mentionable affinity. Again, the presence of a glycoside group may possibly hinder affinity.

The flavanone eriodictiol (**12**) and the flavan-3-ol catechin (**13**) are two other phytochemicals that have also been detected in Rooibos (Fig. 5) [19]. Albeit weak, eriodictiol (**12**) showed affinity towards A_1 and A_{2A} subtypes ($rA_1K_i = 55.3 \mu\text{M}$; $rA_{2A}K_i = 17.0 \mu\text{M}$ and $hA_1K_i = 10.7 \mu\text{M}$; $hA_{2A}K_i = 10.4 \mu\text{M}$).

Various phenolic acids are also present in Rooibos extracts and include caffeic acid (**14**), ferulic acid (**15**), *p*-coumaric acid (**16**), *p*-hydroxybenzoic acid (**17**), vanillic acid (**18**), protocatechuic acid (**19**) [52], and syringic acid (**20**) (Fig. 6) [56]. Although some of these constituents are predicted to cross the BBB, none of these acids showed affinity towards rA_1 and rA_{2A} receptors.

Honeybush (*Cyclopia intermedia*)

The two major phenolic constituents in the leaves of Honeybush are mangiferin (**21**) (a glycosyl xanthone) and the flavanone hesperetin (**23**) (Fig. 7) [18]. In the processed leaves and stems the xanthones isomangiferin (**22**), the isoflavone formononetin (**24**), the inositol (+)-pinitol (**25**) and the phenolic acid, tyrosol (**26**) were identified. None of these constituents showed affinity toward either the rA_1 or rA_{2A} ARs.

The flavanone, naringenin (**27**), found in Honeybush, showed affinity towards the rA_1 AR ($rA_1K_i = 31.9 \mu\text{M}$; $hA_1K_i = 14.1 \mu\text{M}$) (Fig. 8). The flavonol, kaempferol (**28**) - also found in Honeybush - showed the best affinity for the A_1 AR in the series ($rA_1K_i = 0.729 \mu\text{M}$; $hA_1K_i = 9.88 \mu\text{M}$). Compounds structurally related to kaempferol, such as kaempferide (**29**) and rhamnocitrin (**30**) – although not present

Table 3 Competition of test compounds for [³H]DPCPX binding at adenosine A₁ receptors and [³H]NECA binding at adenosine A_{2A} receptors

Compound	K _i value ± SEM (μM) ^a (Specific binding (%)) ^b	Selectivity index				GTP shift	
		hA ₁ vs 1 nM [³ H] DPCPX ^d	rA _{2A} vs 4 nM [³ H] NECA ^e	hA _{2A} vs 30 nM [³ H] NECA ^f	rA ₁ + 0.1 mM GTP vs 0.1 nM [³ H]DPCPX ^c	hA _{2A} K _i /hA ₁ K _i	hA _{2A} K _i /rA _{2A} K _i
1	(100) ^b	—	61 ^b	—	—	—	—
2	2.47 ± 0.64 ^a	3.38 ± 0.91 ^a	6.99 ± 0.89 ^a	—	1.49 ± 0.18 ^a	—	—
3	(88) ^b	—	(80)	—	—	2.83	0.60
4	(100) ^b	—	(98) ^b	—	—	—	—
5	(97) ^b	—	(78) ^b	—	—	—	—
6	(100) ^b	—	(100) ^b	—	—	—	—
7	(69) ^b	—	(79) ^b	—	—	—	—
8	1.39 ± 0.17 ^a	2.41 ± 1.33 ^a	3.94 ± 1.76 ^a	7.99 ± 0.63 ^a	1.29 ± 0.019 ^a	3.32	0.93
9	2.32 ± 0.10 ^a	2.28 ± 0.52 ^a	2.50 ± 1.27 ^a	13.1 ± 5.15 ^a	1.59 ± 0.15 ^a	5.74	0.69
10	(100) ^b	—	(100) ^b	—	—	—	—
11	(63) ^b	—	(73) ^b	—	—	—	—
12	55.3 ± 1.18 ^a	10.7 ± 3.91 ^a	17.0 ± 2.87 ^a	10.4 ± 5.38 ^a	26.4 ± 2.80 ^a	0.97	0.48
13	(42) ^b	—	(75) ^b	—	—	—	—
14	(87) ^b	—	(83) ^b	—	—	—	—
15	(88) ^b	—	(100) ^b	—	—	—	—
16	(92) ^b	—	(100) ^b	—	—	—	—
17	(73) ^b	—	(100) ^b	—	—	—	—
18	(100) ^b	—	(100) ^b	—	—	—	—
19	(98) ^b	—	(100) ^b	—	—	—	—
20	(96) ^b	—	(100) ^b	—	—	—	—
21	(89) ^b	—	(80) ^b	—	—	—	—
22	(100) ^b	—	(100) ^b	—	—	—	—
23	(52) ^b	—	(92) ^b	—	—	—	—
24	(98) ^b	—	(100) ^b	—	—	—	—
25	(100) ^b	—	(100) ^b	—	—	—	—
26	(90) ^b	—	(100) ^b	—	—	—	—
27	31.9 ± 1.70 ^a	14.1 ± 2.71 ^a	(8) ^b	—	20.7 ± 1.94 ^a	0.44	0.65
28	0.729 ± 0.073 ^a	9.88 ± 0.56 ^a	(39) ^b	—	0.73 ± 0.054 ^a	13.6	1.00
29	(41) ^b	—	(79) ^b	—	—	—	—
30	(69) ^b	—	(100) ^b	—	—	—	—
Reference standards							
CPA	0.0065 ± 0.0004 ^a	—	—	—	0.037 ± 0.002 ^a	—	5.62
	0.0059	—	—	—	0.035	—	

Table 3 (continued)

Compound	K_i value $\pm$ SEM (μM) ^a	(Specific binding (%)) ^b				Selectivity index				GTP shift	
		rA ₁ vs 0.1 nM [³ H] DPCPX ^c	hA ₁ vs 1 nM [³ H] DPCPX ^d	rA _{2A} vs 4 nM [³ H] NECA ^e	hA _{2A} vs 30 nM [³ H] NECA ^f	rA ₁ + 0.1 mM GTP vs 0.1 nM [³ H]DPCPX ^e	rA _{2A} K _i /rA ₁ K _i	hA _{2A} K _i /hA ₁ K _i	hA ₁ K _i /rA ₁ K _i	hA _{2A} K _i /rA _{2A} K _i	rA ₁ K _i + 0.1 mM GTP/rA ₁ K _i
DPCPX	0.0005 $\pm$ 0.0001 ^a 0.0006	0.004 $\pm$ 0.0002 ^a 0.003	0.004 $\pm$ 0.0002 ^a 0.003	0.149 $\pm$ 0.023 ^a 0.157	0.053 $\pm$ 0.005 ^a 0.129	0.0004 $\pm$ 0.00003 ^a 0.0005	298	13.3	8	0.36	0.80
IST	0.125 $\pm$ 0.006 ^a 0.230	0.741 $\pm$ 0.005 ^a 0.841	0.003 $\pm$ 0.001 ^a 0.002	0.003 $\pm$ 0.001 ^a 0.002	0.042 $\pm$ 0.005 ^a 0.026	—	0.024	0.057	5.93	14	—

^aInhibition constant (K_i , μM) is presented as the mean $\pm$ standard error of the mean (SEM) of experiments performed in triplicate^bSpecific binding (%) at maximum tested concentration of 100 μM is presented as the mean of experiments performed in duplicate^cDissociation constant (K_D): 0.36 nM^d K_D : 3.86 nM^e K_D : 15.3 nM^f K_D : 20.1 nM; Values without SEM are taken from the literature; r: rat; h: human; [³H]DPCPX: 8-cyclopentyl-1,3-dipropylxanthine, tritiated at dipropyl-2,3-positions, selective adenosine A₁ receptor antagonist; [³H]NECA: 5'-N-ethylcarboxamidoadenosine, tritiated at adenine-2,8 position, non-selective adenosine A₁, A_{2A}, and A₃ receptor agonist; GTP: guanosine-5'-triphosphate; CPA: N⁶-cyclopentyladenosine; DPCPX: 8-cyclopentyl-1,3-dipropylxanthine; IST: istradefylline

in Honeybush – was also tested; however, neither showed affinity toward rA₁ and A_{2A} ARs. Thus, it can be observed that the -OCH₃ group at position C4' (kaempferide) and position C7 (rhannocitrin), respectively, decreased affinity.

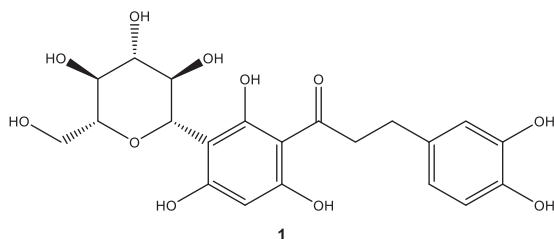
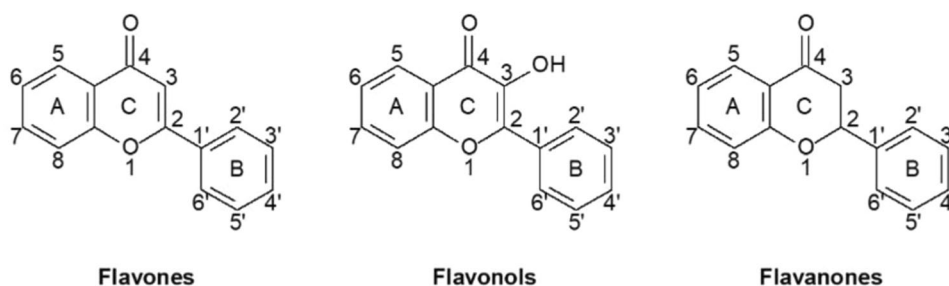
Structure-activity relationships

Structure-activity relationships (SAR) based on K_i values (μM) of test compounds **2**, **8**, **9**, **12**, **27** and **28** showed a coherent relationship between the constituents and rA₁ and rA_{2A} AR affinity (Fig. 9). The influence on AR affinity of polar (-OH) and/or non-polar (-OCH₃) groups, the number of these groups and their position on rings A, B and C of the flavones, flavonols and/or flavanones was explored. For the flavones, a comparison of chrysoeriol (**8**: C3'-OCH₃) to luteolin (**9**: C3'-OH) showed that C3'-OH substitution decreased rA₁ affinity. Although both substitution patterns led to dual AR affinity, it may be said that a C3'-OCH₃-C4'-OH substitution pattern (**8**) is preferred for rA₁ affinity and a catechol group (**9**) for rA_{2A} affinity. For the flavonols, a mono-hydroxy substituted compound, such as kaempferol (**28**), exhibited higher affinity than one which was 3',4'-dihydroxy substituted (for example quercetin (**2**)). Indicating that a catechol group (and potentially increased polarity), led to decreased rA₁ affinity. However, when the flavanones were compared, naringenin (**27**: C3'-OH) showed lower activity than its di-hydroxy substituted counterpart eriodictiol (**12**). From the limited data at hand, it seems that not only the number and position of the -OH or -OCH₃ group(s) influence affinity, but also the type of flavonoid. It was observed that the flavonols have the best affinity for the A₁/A_{2A} ARs, followed by the flavones and flavanones in decreasing order of affinity (flavonol>flavone>flavanones).

Functional binding assays: type of binding activity

A guanosine triphosphate (GTP) shift assay determined the type of binding affinity a test compound exhibits at the rat A₁ AR (agonist, antagonist, or partial agonist). The six compounds that showed promising rA₁ affinity (**2**, **8**, **9**, **12**, **27** and **28**) were selected to determine their functionality at the A₁ AR. These results are also summarized in Table 3.

GTP works by uncoupling the A₁ AR from its G-protein leading to a shift from a high to a low affinity state for agonists [57]. It is therefore possible to determine if a compound will act as an agonist, a partial agonist or an antagonist by comparing a test compound's binding curves (or K_i value) in the presence and absence of GTP [58]. With an antagonist, the binding curve is unaffected in the presence of GTP (as is the K_i value), and the antagonist test compound will have a GTP shift value of approximately 1, while an agonist test compound's binding curve will shift to the right in the presence of GTP (and the K_i value will increase) and have a GTP shift value greater than 1 [59, 60].

Fig. 1 Basic chemical structures of flavonoid subclasses**Fig. 2** Chemical structure of aspalathin (**1**)

The results obtained suggested that compounds **2**, **8**, **9**, **12**, **27** and **28** act as A_1 AR antagonists since there is no significant rightward shift of the binding curve (or change in the K_i values) in the presence of GTP and compounds have calculated GTP shifts of ≤ 1 (Fig. 10). Interestingly, GTP and endogenous adenosine binding interactions are controlled similarly by human and rat proteins in A_1 and A_{2A} AR binding studies [57]. As reference standards, CPA (A_1 agonist) and DPCPX (A_1 antagonist) were used to validate the GTP shift assays.

Correlation between species

The pK_i values of selected reference standards and test compounds (**2**, **8**, **9**, **12**, **27** and **28**) which showed affinity toward rat and human A_1 and/or A_{2A} receptor subtypes were compared, and thus, correlation coefficients were calculated. The R-squared value of the linear regression analysis is equal to the correlation coefficient (Appendix A, Fig. A3, 4).

Site-directed mutagenesis studies on several members of the G-protein coupled receptor superfamily exposed that a single amino acid can modulate ligand affinity, and thus, explain species differences [61, 62]. Predictably, the rat and human A_1 receptor subtype (R squared 0.88) showed better correlation than the A_{2A} AR subtype (R squared 0.81); since a 95% sequence homology at the amino-acid level between rA_1 and hA_1 receptor subtypes exists, compared to only 82% between rA_{2A} and hA_{2A} [63]. Prototypical xanthine derivatives DPCPX (selective A_1 receptor antagonist) and IST (selective A_{2A} receptor antagonist) used as reference standards were markedly less potent at the human than rat receptors (Table 3). DPCPX and IST were, respectively, eight and fourteen times more selective toward the rat than human A_1 and A_{2A} receptor subtype; indeed, the affinity of

xanthine-based antagonists is species-dependent. Therefore, it was suggested that different structural determinants may be responsible for binding of xanthine derivatives (such as DPCPX and IST) compared to other scaffolds.

Conclusions

Despite their history as herbal teas and traditional medicines, the commercially available chemical constituents of Rooibos and Honeybush plants were not investigated for their effects on A_1 and/or A_{2A} ARs prior to this pilot study which established structure-activity relationships and identified test compounds with lead-like properties. Overall, the chemical constituents of Rooibos and Honeybush plants did not show mentionable affinity toward either A_1 or A_{2A} receptor subtypes, except for compounds **2**, **8**, **9**, **12**, **27** and **28**. Of these compounds, the flavonol kaempferol (**28**) showed sub-micromolar AR affinity, making it the most promising test compound of the present evaluated natural products derived from either Rooibos or Honeybush. Additionally, it has drug- and lead-like properties and can be used as starting point for future studies. It was observed that a flavonol scaffold is preferred to flavone and flavanone scaffolds, and within the flavonol scaffold C4'-OH substitution on ring B is preferred to C3',4'-OH substitution. The observed structure-activity relationships may be used as reference to design a novel series of *para*-benzyl substituted flavonols which are structurally related to the parent compound kaempferol (**28**) in hopes of improving in vitro affinity and in silico physiochemical and pharmacokinetic properties (such as BBB permeation).

Materials and Methods

Chemistry

Test compounds **1–30** were not extracted from Rooibos and/or Honeybush plants, but purchased from manufacturers AmBeed or Sigma-Aldrich, and accompanied by a certificate of analysis, and thus, no further purification or analyses were needed.

Fig. 3 Chemical structures of quercetin (**2**), iso-quercetin (**3**), rutin (**4**) and hyperoside (**5**)

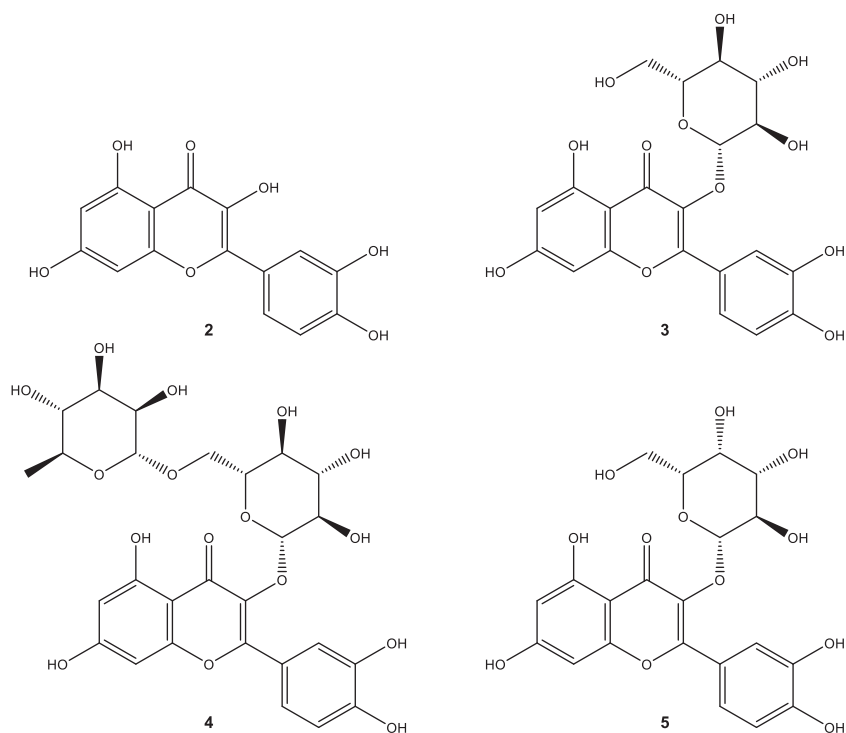


Fig. 4 Chemical structures of vitexin (**6**), isovitexin (**7**), chrysoeriol (**8**), luteolin (**9**), orientin (**10**) and isoorientin (**11**)

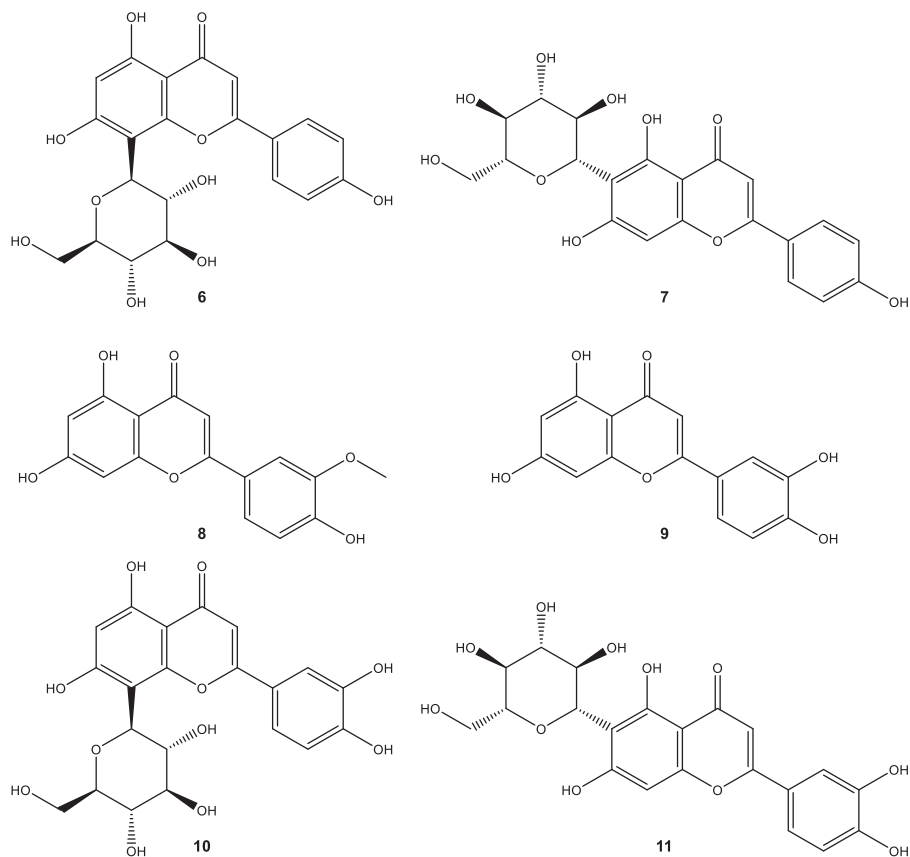


Fig. 5 Chemical structures of eriodyctiol (**12**) and catechin (**13**)

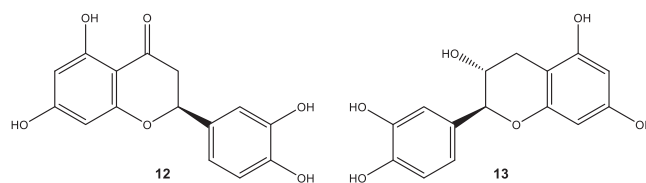


Fig. 6 Chemical structures of caffeic acid (**14**), ferulic acid (**15**), p-coumaric acid (**16**), p-hydroxybenzoic acid (**17**), vanillic acid (**18**), protocatechuic acid (**19**), and syringic acid (**20**)

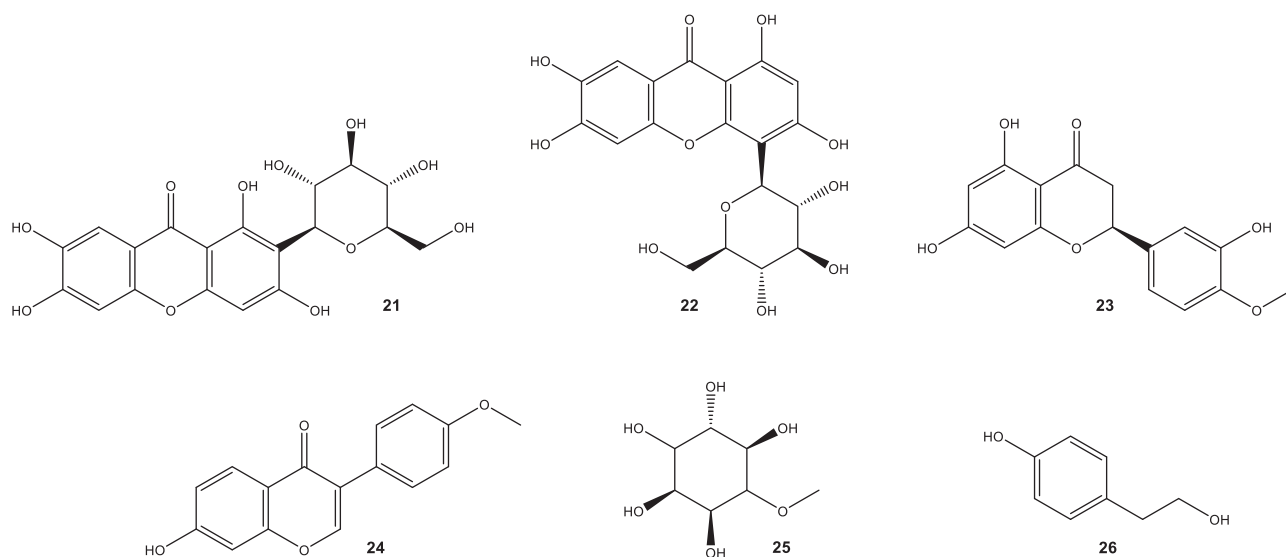
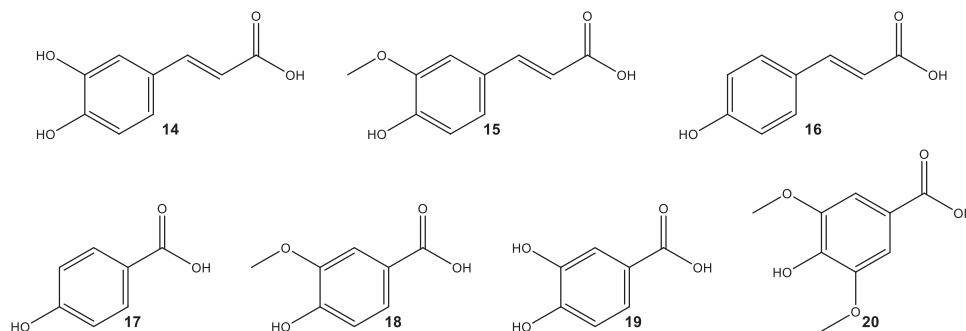


Fig. 7 Chemical structures of mangiferin (**21**), isomangiferin (**22**), hesperetin (**23**), formononetin (**24**), (+)-pinitol (**25**) and tyrosol (**26**)

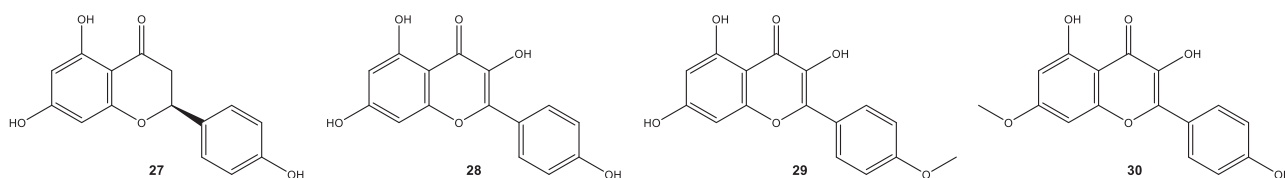
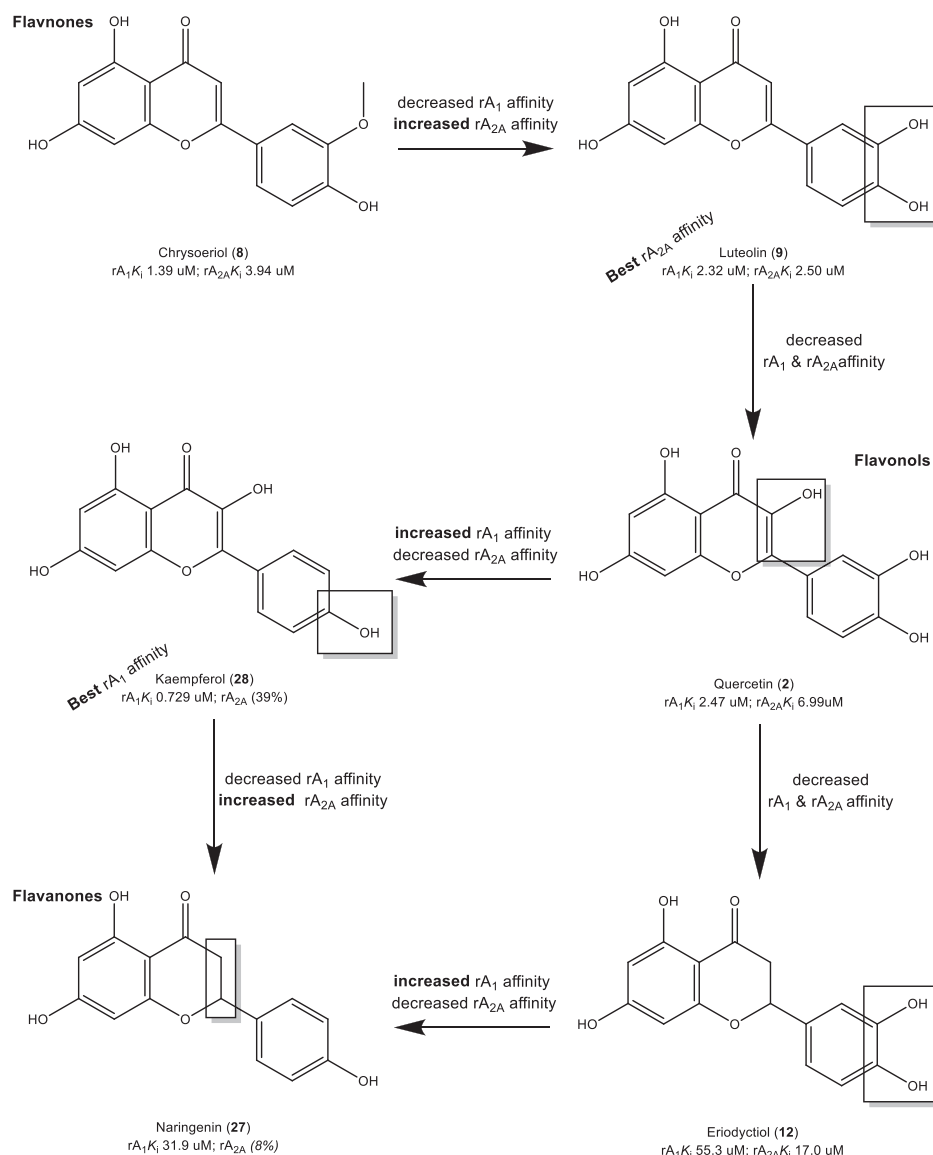


Fig. 8 Chemical structures of naringenin (**27**), kaempferol (**28**), kaempferide (**29**) and rhamnocitrin (**30**)

Fig. 9 Summary of structure-activity relationships of chemical constituents of Rooibos and Honeybush plants



In silico predictions of physiochemical and pharmacokinetic properties

SwissADME (<http://www.swissadme.ch>), a free web tool, was used to predict physiochemical and pharmacokinetic properties of test compounds **1–30** from their molecular structures using the most relevant computational methods [64].

In vitro evaluation of adenosine A1 and A2A receptor affinity and activity

All reagents and solvents were commercially available. This includes [3 H]-8-cyclopentyl-1,3-dipropylxanthine ([3 H] DPCPX; specific activity 120 Ci/mmol), 5'-N-[3 H]-ethyl-carboxamideadenosine ([3 H]NECA; specific activity 27.1 Ci/mmol) and filter count (liquid scintillation cocktail)

from PerkinElmer. Adenosine deaminase (5.9 mg protein/mL, 157 units/mg protein) from Sigma-Aldrich and Whatman GF/B 25 mm diameter filters from Merck. Residual radioactivity was measured with a Packard Tri-CARB 2810 TR liquid scintillation counter.

Membrane preparation

The North-West University Animal Care, Health and Safety Research Ethics Committee (NWU-AnimCare) approved the collection of tissue samples from adult male Sprague-Dawley rats for the radioligand binding studies (application number NWU-00035-10-A5). Rat brain membranes were prepared (whole brain and striatal membranes were pooled separately) and stored as described in literature [58]. Chinese hamster ovary (CHO) cells expressing the human A₁ or A_{2A} AR were kindly donated by Prof. K.N. Klotz from the

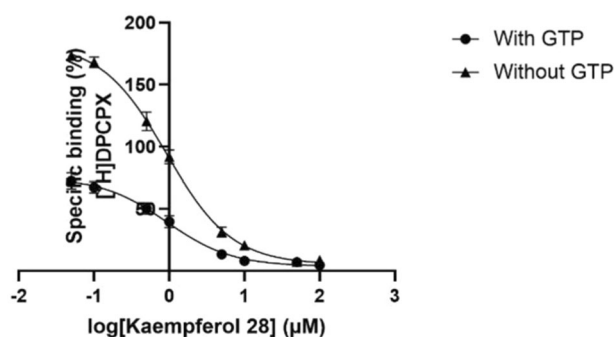


Fig. 10 The binding curve of kaempferol (**28**) is an example of an A_1 AR antagonist which is determined via a GTP shift assay (with 100 μ M GTP) in rat whole brain membranes expressing rA_1 ARs with [3 H]DPCPX as radioligand. **28** has a GTP shift of 1

University of Würzburg, Germany. CHO cells were grown adherently and maintained in Dulbecco's Modified Eagles Medium with nutrient mixture F12 (DMEM/F12) without nucleosides, containing 10% foetal bovine serum, penicillin (100 U/ml), streptomycin (100 μ g/ml), L-glutamine (2 mM) and Geneticin (G418, 0.2 mg/ml) at 37 °C in 5% CO_2 /95% air. Cells were split 2 or 3 times weekly at a ratio between 1:5 and 1:20, respectively. For radioligand binding studies, the culture medium was removed, cells were washed with phosphate-buffered saline (PBS) and frozen until preparation of membranes, upon which frozen cells in ice-cold hypotonic buffer (5 mM Tris/HCl, 2 mM EDTA, pH 7.4) was thawed again. The cell suspension was then homogenized on ice (Ultra-Turrax, 2 X $\times$ 15 s at full speed) and the homogenate was spun for 10 min (4 °C) at 1000 g. The supernatant was then centrifuged for 30 min at 100,000 g. The membrane pellet was resuspended in 50 mM Tris/HCl buffer pH 7.4, frozen in liquid nitrogen and stored at -80 °C. Protein content for rat and CHO cell membranes were determined using Bradford reagent and bovine serum albumin as reference standard [65].

Competitive binding studies

Rat (r): To determine rA_1 AR affinity, rat whole brain membranes (expressing A_1 ARs) and [3 H]DPCPX (selective A_1 antagonist) [66] were used, and for rA_{2A} affinity, rat striatal membranes (expressing A_{2A} ARs) and [3 H]NECA (non-selective A_1/A_{2A} antagonist) [67]. Each incubation of the rA_1 assay consisted of: (i) test compound (10 μ L), (ii) 0.1 nM [3 H]DPCPX (radioligand solution, 100 μ L) and (iii) 120 μ g rat whole brain membranes and 0.1 units/mL adenosine deaminase (membrane suspension, 890 μ L) [58, 66]. In turn, every incubation of the A_{2A} assay consisted of: (i) 120 μ g rat striatal membranes, 0.2 units/mL adenosine deaminase, 10 mM $MgCl_2$ (membrane suspension, 790 μ L), (ii) test compound (10 μ L), (iii) 50 nM N6-cyclopentyladenosine (CPA) (100 μ L) and (iv) 4 nM [3 H]

NECA (radioligand solution, 100 μ L) [58, 67]. The final volume of all incubations contained 1 mL of 50 mM Tris.HCl buffer (pH 7.7, 25 °C) and 1% DMSO [58]. Non-specific binding of [3 H]DPCPX and [3 H]NECA was defined as binding in the presence of 100 μ M CPA [58, 66, 67]. Specific binding was defined as the total binding minus the non-specific binding [58].

Human (h): To determine hA_1 AR affinity, CHO cell membranes expressing A_1 ARs and [3 H]DPCPX was used, and for hA_{2A} affinity, CHO cell membranes expressing A_{2A} ARs and [3 H]NECA [68]. Each incubation of the hA_1 or hA_{2A} assay consisted of: (i) test compound (2 μ L), (ii) 0.1 nM [3 H]DPCPX or 30 nM [3 H]NECA (radioligand solution, 20 μ L) and (iii) 20 μ g CHO cell membranes expressing hA_1 AR or 65 μ g CHO cell membranes expressing hA_{2A} AR and 0.2 units/mL adenosine deaminase (membrane suspension, 178 μ L). Samples were incubated for 1 h at 25 °C, filtered and washed three times with 4 mL ice-cold binding and filtered individually as described for conventional radioligand binding studies. Non-specific binding of [3 H]DPCPX and [3 H]NECA was defined as binding in the presence of 100 μ M CPA [58, 66, 67]. Specific binding was defined as the total binding minus the non-specific binding [58].

GTP shift assay: The guanosine triphosphate (GTP) shift assay resembles the [3 H]DPCPX radioligand binding assay; however, it requires the addition of 100 μ M GTP. Non-specific binding was defined as binding in the presence of 10 μ M DPCPX [69, 70].

Data analysis

The competitive binding studies' data analysis was done using Microsoft Excel and GraphPad Prism Software. Sigmoidal dose-response curves, from which half maximal inhibitory concentration (IC_{50}) values were calculated, were obtained by plotting the specific binding against the logarithm of the test or reference compounds' concentrations. Subsequently, the IC_{50} values were used to calculate the inhibition constant (K_i) values for the competitive inhibition of [3 H]DPCPX ($rK_d = 0.36$ nM [66]; $hK_d = 3.86$ nM [68]) against A_1 ARs and [3 H]NECA ($rK_d = 15.3$ nM [67]; $hK_d = 20.1$ nM [68]) against A_{2A} ARs by the test compounds using the Cheng-Prusoff equation [71]. Descriptive statistics were used to represent K_i values (μ M) as the mean $\pm$ standard error of the mean (SEM) of experiments performed in triplicate. Specific binding (%) values of the radioligand at a maximum tested concentration of 100 μ M were represented as the mean of experiments performed in duplicate. Where applicable, selectivity index (SI) values were calculated for the A_1 isoform as a ratio of $A_{2A}K_i$ and A_1K_i values of test compounds. GTP shifts were calculated by dividing the K_i values of compounds in the presence of

100 μ M GTP by the K_i values obtained in the absence of 100 μ M GTP [70].

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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