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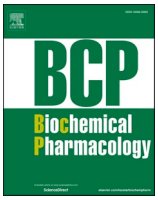

Selected cannabis terpenes synergize with THC to produce increased CB1 receptor activation

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ABSTRACT

The cannabis plant exerts its pharmaceutical activity primarily by the binding of cannabinoids to two G protein-coupled cannabinoid receptors, CB1 and CB2. The role that cannabis terpenes play in this activation has been considered and debated repeatedly, based on only limited experimental results. In the current study we used a controlled *in-vitro* heterologous expression system to quantify the activation of CB1 receptors by sixteen cannabis terpenes individually, by tetrahydrocannabinol (THC) alone and by THC-terpenes mixtures. The results demonstrate that all terpenes, when tested individually, activate CB1 receptors, at about 10–50% of the activation by THC alone. The combination of some of these terpenes with THC significantly increases the activity of the CB1 receptor, compared to THC alone. In some cases, several fold. Importantly, this amplification is evident at terpene to THC ratios similar to those in the cannabis plant, which reflect very low terpene concentrations. For some terpenes, the activation obtained by THC- terpene mixtures is notably greater than the sum of the activations by the individual components, suggesting a synergistic effect. Our results strongly support a modulatory effect of some of the terpenes on the interaction between THC and the CB1 receptor. As the most effective terpenes are not necessarily the most abundant ones in the cannabis plant, reaching "whole plant" or "full spectrum" composition is not necessarily an advantage. For enhanced therapeutic effects, desired compositions are attainable by enriching extracts with selected terpenes. These compositions adjust the treatment for various desired medicinal and personal needs.

1. Introduction

Cannabis is a multifaceted plant containing hundreds of different chemical compounds, including cannabinoids, terpenes and flavonoids. Phytocannabinoids unique to the cannabis plant have been the focus of cannabis research for mechanistic and therapeutic roles. Independently, the medical effects of terpenes have been studied for decades. Following Russo's publication on the subject [1], the focus has been extended to include terpenes among cannabis Active Pharmaceutical Ingredients (APIs). The potential therapeutic benefits related to cannabinoids-terpenes combinations were suggested to various physiological systems [1-6]. Consequently, a growing number of studies and caregiving instructions dealing with medical cannabis use terms such as "full spectrum", and "whole plant" (e.g., [7-9], suggesting that combinations of cannabis plant components, more specifically, compositions of selected chemovars, provide better treatment results compared to results of isolated cannabinoids. Russo [1] went even further, using the term "entourage effect" to suggest a synergistic cannabinoids-terpenes effect, which raised much debate [10]. Experimental confirmation of these suggested effects is quite limited.

Studying the pharmacological effect of even a single API in the endocannabinoids system (ECS) is complicated due to the multiple receptors involved in signal transductions in this system (e.g., [11,12]). Epidolex ® (cannabidiol (CBD)) and Dronabinol (a synthetic form of tetrahydrocannabinol (THC)) are good examples of such single APIs treatment. The picture is further complicated in the case of cannabis

Abbreviations: THC, tetrahydrocannabinol; CBD, cannabidiol; API, Active Pharmaceutical Ingredient; GIRK, G protein-activated inwardly rectifying K^+ channel; DR, dose-response; 2-AG, 2-Arachidonoylglycerol.

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Table 1
Summary of various terpene effects on CB1 receptor activation.

Compound	Response amplitude, normalized to 10 μM THC	Apparent EC_{50} (μM)	Apparent EC_{50} (μM)	
THC	1	2.3	2.3	
Compound	Response amplitude, normalized to 10 μM THC	Apparent EC_{50} with the addition of 10 μM terpene[1] (μM)	Apparent EC_{50} with the addition of terpene at terpene: THC ratio of 1:10 [1] (μM)	Effect of terpene at terpene: THC ratio of 1:10
α-pinene	0.29±0.03	0.64	2.5	no effect[3]
β-pinene	0.29±0.06	1.59	1.69	sum[4]
β-caryophyllene	N.D[2]	N.D[2]	1.98	no effect
bisabolol	N.D[2]	N.D[2]	2.88	no effect
borneol	0.18±0.03	0.59	0.49	synergistic
eucalyptol	0.41±0.04	1.78	2.45	no effect
geraniol	0.31±0.08	1.21	1.40	sum[4]
humulene	N.D[2]	N.D[2]	2.37	no effect
limonene	0.23±0.02	0.65	0.66	synergistic
linalool	0.18±0.02	1.12	1.42	sum
myrcene	0.26±0.07	1.10	1.86	no effect
nerolidol	N.D[2]	N.D[2]	2.01	no effect
ocimene	0.36±0.05	0.61	0.98	sum
sabinene	0.1±0.02	0.33	0.77	synergistic
terpineol	0.48±0.05	1.12	0.82	sum
terpinolene	0.45±0.02	0.82	1.92	no effect

[1] The THC concentration required to evoke 50% the response obtained by 10 μM THC. See Fig. 4A.
[2] Not determined because of the low solubility of the terpene at 10 μM.
[3] Effect was defined wherein a significant main effect of condition (i.e., CB1 response obtained by THC and terpene at 1:10 ratio > CB1 response obtained by THC alone, $p < 0.05$, two-ways ANOVA) was demonstrated (Table 5). Synergistic effect was defined when the DR curve obtained with the addition of terpene at 1:10 ratio was significantly higher than the calculated sum of the DR curves ($p < 0.05$, two-ways ANOVA).
[4] The DR curve obtained with the addition of terpene at 1:10 ratio was lower than the calculated sum of the DR curve of THC and the DR curve of the terpene in the corresponding concentrations.

Table 2
Reagents solubilities and reported concentrations in prior studies.

	Solubility[1]	Reported concentration[3]		
		LaVigne et al	Finlay et al	Santiago et al
THC	~10 μM		Up to ~30 μM	Up to ~10 μM
WIN		Up to ~30 μM		
α-pinene	16–37 μM		10 μM	Up to 100 μM
β-pinene	24–81 μM	Up to 1 mM	10 μM	Up to 100 μM
limonene	33–150 μM		10 μM	Up to 100 μM
myrcene	20–70 μM		10 μM	Up to 30 μM
ocimene	>10 μM[2]			
sabinene	18 μM			
terpinolene	28–70 μM			
borneol	1.7–3 mM			
eucalyptol	>1mM[2]			
geraniol	4.4–5.6 mM	Up to 1 mM		
linalool	4–10 mM	Up to 1 mM		Up to 100 μM
terpineol	2.4–12.8 mM			
β-caryophyllene	0.1–0.3 μM	Up to 1 mM	10 μM	Up to 100 μM
humulene	0.1–0.3 μM	Up to 1 mM		
bisabolol	6 μM			
nerolidol	~6μM[2]			

[1] Reported solubilities vary between sources, and comprise experimental data in some cases, and calculated solubilities in others. Data were taken from http://www.chemspider.com, from https://pubchem.ncbi.nlm.nih.gov and from [55-57].
[2] Estimated based on the solubility of similar terpenes.
[3] Reagents used in prior reports were dissolved in aqueous solutions containing salts ("physiological solutions") and/or residues of solvents used to form stock solutions, out of which the solutions to be tested were formed. The assumption made here is that, since the concentrations of those solutes are relatively small (under 1%), the solubility of the reagents in the reported solutions are similar to those in water.

preparations containing dozens of APIs, many of which may contribute to the effect of medical cannabis on ECS-controlled physiological functions. Interactions between various APIs and modulation of a given API effect by interactions with another API, may also occur.

The content of cannabis preparations notably varies as a function of genetic and agriculture variables, [13-16], as well as of processing parameters. The latter is mainly attributed to the production of cannabis extracts, wherein volatile terpenes are lost. As a result, the content of terpenes in cannabis extracts significantly differs from their content in the source plant [17-19].

Several studies have attempted to find a correlation between the terpene profiles of various chemovars and their impact on particular indications, e.g., anxiety [20,21] and pain [22]. Also related is the "sativa" vs. "indica" effect, mainly attributed to the chemovar/ product fit for energic activity vs. relaxation/sedation [23-25].

Several in vivo trials have compared the therapeutic effects of a single cannabinoid to those of an extract rich in the same cannabinoid [26,27], some of which demonstrate an increased therapeutic effect of the latter. Reaching conclusions for terpenes effects is, however, complicated by several factors: (i) Extracts contain additional (minor) cannabinoids having potential effects, which are difficult to distinguish from those of terpenes; (ii) *In vivo* trials involve many systems/receptors, complicating the interpretation of the results; and (iii) Many of these studies do not provide full analysis of the terpene content of the formulations used, nor the details of the method of manufacture of the extract. In that regard, testing multicomponent products in *in vitro* systems expressing a single receptor reduces the complexity and enables a direct assessment of terpene-cannabinoid interactions on the resultant activity of that single receptor. In order for these findings to be indicative, the composition of the tested formulations should be carefully selected.

Some in vivo and in vitro studies [28-30] failed to find evidence of any effect of terpenes on cannabinoids activity. Other studies have shown some effects, but at terpene to cannabinoid ratios significantly different from those occurring naturally in the plant [31]. Another study concluded that this effect is limited to cannabinoid-terpene compositions similar to those found in the plant [2].

The present study was designed to shed more light on the subject. Towards that goal, we examined the effect of sixteen terpenes and of THC-terpene mixtures, at terpene/cannabinoid ratios similar to those commonly found in cannabis plants, on the CB1 receptor activity using a heterologous expression system.

2. Materials and methods

2.1. Ethics statement

All experimental procedures used in this study were performed in accordance with relevant guidelines and regulations and were approved by the Hebrew University's Animal Care and Use Committee (Ethical approval number NS-11-12909-3).

2.2. Preparation of cRNA and Oocytes

cDNA plasmids of the two subunits of the G protein-activated

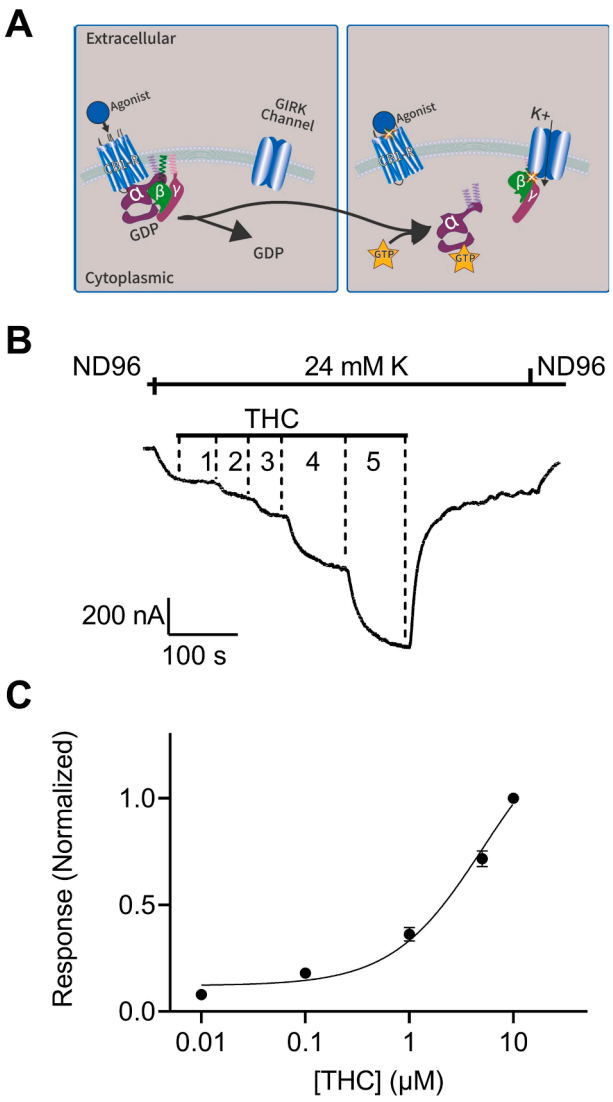

Fig. 1. Measurements of CB1 receptor induced GIRK current. **A**. The functional expression system. Binding of a ligand to the CB1 receptor activates G protein. The βγ subunits of the activated G protein bind to the GIRK channel and open it. **B**. Representative recording. Application of five THC concentrations lead to evolvement of GIRK currents that can be used as a measure for receptor activation. **C**. Dose response curve of CB1 receptor induced GIRK currents (each data point represents mean ± SEM from 11 oocytes). The solid lines here and below were generated by fitting Equation 1 to the data.

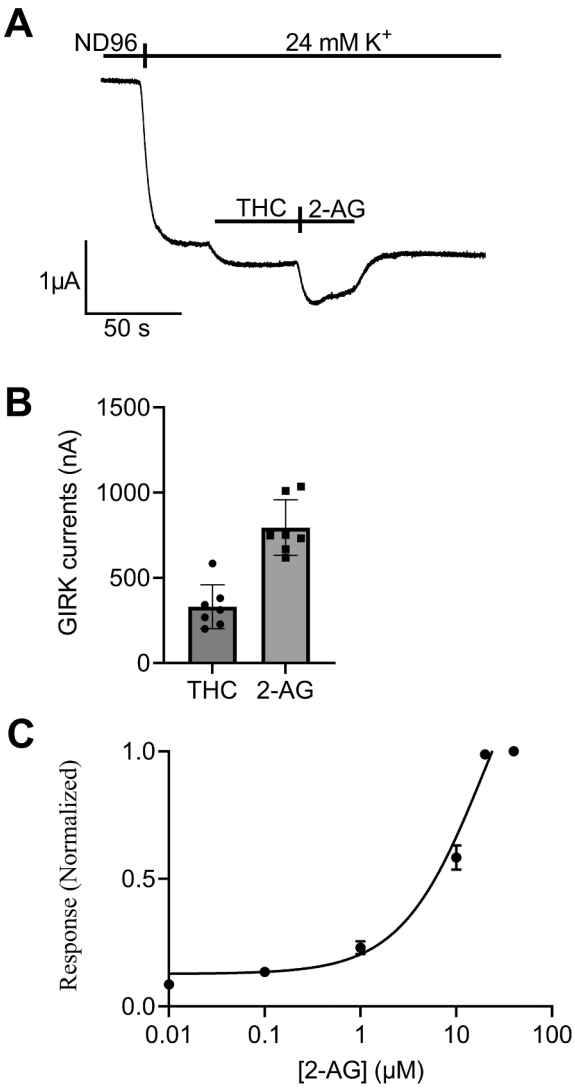

Fig. 2. THC acts as partial CB1 receptor agonist. **A**. A representative recording depicting the experimental procedure. Oocyte was voltage clamp at −80 mV at ND96 solution. Replacing the solution with a 24 mM K^+ solution evoked basal GIRK current. Application of 10 μM THC evoked CB1 receptor activated currents. Application of 10 μM of the full agonist 2-AG evoked an additional current. **B**. Collective results from 8 oocytes subjected to the same protocol. The average THC evoked current was 41% of the average current evoked by 2-AG from the same oocytes. **C**. Dose response curve of 2-AG induced GIRK current. Adapted, with permission, from [52].

inwardly rectifying K^+ channel (GIRK) (GIRK1 and GIRK2), the CB1 receptor, and the α subunit of the G-protein (Gαi3) were linearized with the appropriate restriction enzymes [32,33]. The linearized plasmids were transcribed in vitro using a standard procedure [34].

Oocytes were isolated from anesthetized (with 1 g/L MS-222) female adult X. laevis in a standard procedure and incubated in NDE96 solution composed of ND96 (in mM: 96 NaCl, 2 KCl, 1 CaCl2, 1 MgCl2, 5 Hepes, with pH adjusted to 7.5 with NaOH) with the addition of 2.5 mM Na^+ pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin (16). After their isolation, the oocytes were injected with the relevant cRNAs: cRNAs of CB1 receptor (2 ng) and GIRK1 and GIRK2 (200 pg each) were injected. In addition, cRNA of Gαi3 (1000 pg) was injected to decrease the basal GIRK current (I_K) and to improve the relative activation by the agonist [35].

2.3. Current Measurements

Currents were measured 3–5 days after cRNA injection and were recorded using the standard two-electrode voltage clamp technique (Axoclamp 2B amplifier, Axon Instruments, Foster City, CA). Each oocyte was placed in the recording bath containing ND96 solution and was impaled with two electrodes pulled from 1.5-mm Clark capillaries (Warner instruments, Hamden, CT). Both electrodes were filled with a 3 M KCl solution and the electrodes resistances was between 1 and 5 MΩ. The CB1 receptor-mediated GIRK currents were measured in 24 mM K^+ solution (in mM: 72 NaCl, 24 KCl, 1 CaCl2, 1 MgCl2, 5 Hepes, with pH adjusted to 7.5 with KOH) [32]. Ba^{+2} (1 mM) was used to block the currents in order to verify that the measured currents were indeed mediated by GIRK channels. All chemicals were purchased from Sigma (Rehovot, Israel) unless stated otherwise. pCLAMP10 software (Axon Instruments, Molecular Devices, San Jose, CA) was used for data acquisition and analysis.

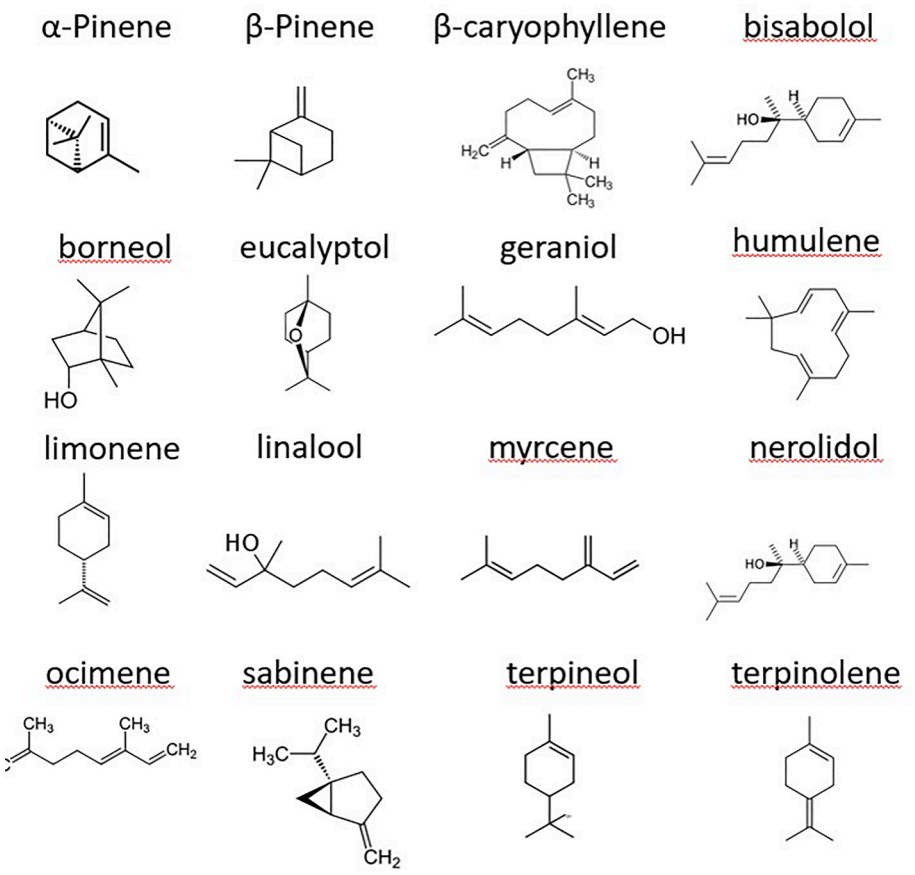

Fig. 3. 2D structures of the terpenes tested.

2.4. Solutions

THC was extracted from a THC-rich cannabis chemovar, using an authorized IGMP (Israeli Good Manufactory Practice) extraction process at Bazelet manufacturing plant (Or Akiva, Israel) and verified by a validated High Performance Liquid Chromatography (HPLC) analysis (HPLC Waters PDA 2996, equipped with a pump, autosampler, column-oven, and a Photodiode Array detector (PDA) detector). Purified terpenes were purchased from Vigon International Inc. (Pennsylvania, USA. α-pinene (natural, 98.2%), β-pinene (natural, 94%) limonene-D (natural, 99%), myrcene (natural, 95.5%), ocimene (Trans, natural, 69.3%) sabinene (natural, 76.67%), terpinolene (natural, 92.6%), borneol (natural, 59.9%), eucalyptol (natural, 100%), geraniol natural (97%), linalool (racemic mixture, 100%), terpineol (natural, 98%), β-caryophyllene (natural, 88.4%), humulene (natural, 91.6%), bisabolol (natural, 98.5%) and nerolidol (natural, 99%). Stock solutions were prepared containing 10 mM THC or 10 mM terpene (on pure basis) in DMSO, based on compound individual purity levels. Subsequent dilutions were made in 24 mM K^+ solution (see above). Similarly, a 10 mM stock solution in DMSO was prepared from 2-Arachidonoylglycerol (2-AG), purchased from Sigma (Rehovot, Israel).

2.5. Data analysis

The dose response curves were fitted by equation 1:

$$Y = \text{Bottom} + X^*(\text{Top-Bottom})/(EC_{50} + X), \quad (1)$$

where Y is the normalized response, X is the concentration of THC, Bottom and Top are the lowest and highest points of the curve and EC_{50} is the THC concentration that gives the half-maximal response. For all experiments, 10 μM was taken as the highest THC concentration, as dictated by solubility limit (see Table 2). Therefore, at the end of the recording from each oocyte, the response to 10 μM was measured as a reference value. The responses evoked in the same oocyte by either THC and\or terpenes measurements, were normalized to this reference value. Given that our dose–response curves did not reach saturation, we defined the THC concentration that evoked 50% of the response evoked by 10 μM THC, as the *apparent* EC_{50}.

2.6. Statistical evaluation

Statistical analysis was conducted using Statistical SPSS 20.0 software (IBM Corp., Armonk, N.Y.). One and Two-way ANOVA tests were used to evaluate the effects of terpenes on CB1 responses, and the effects of terpenes on the THC- derived CB1 responses. Post-hoc using Bonferroni adjustment for multiple comparisons were conducted to detect differences between groups. A one-way ANOVA was used to evaluate dose-dependent CB1 activity of the various terpenes (Fig. 4), Two-way ANOVA was used to evaluate terpenes effects on THC- derived CB1 responses (Figs. 7, 8 and 9), analyzing two main effects, of (1) Condition, i.e., the CB1 response obtained by application THC alone vs. the co-application of THC and a terpene, and (2) THC concentration levels (0.01, 0.1, 1 and 5 μM THC). An interaction between the main effects was also assessed. Data for the 10 μM THC was excluded from the analysis since this data point was used for normalization at each measurement (defined as 1). Another two-way ANOVA was used to assess possible synergistic effect results from terpene – THC interactions (Fig. 10). Therein the ANOVA analyzed the main effect of (1) Condition, i.e., the CB1 response obtained by co-application of THC and a terpene vs. a theoretical summation of the CB1 responses obtained by THC and the terpene alone, (2) THC concentration levels (0.01, 0.1, 1, 5 and 10 μM THC).

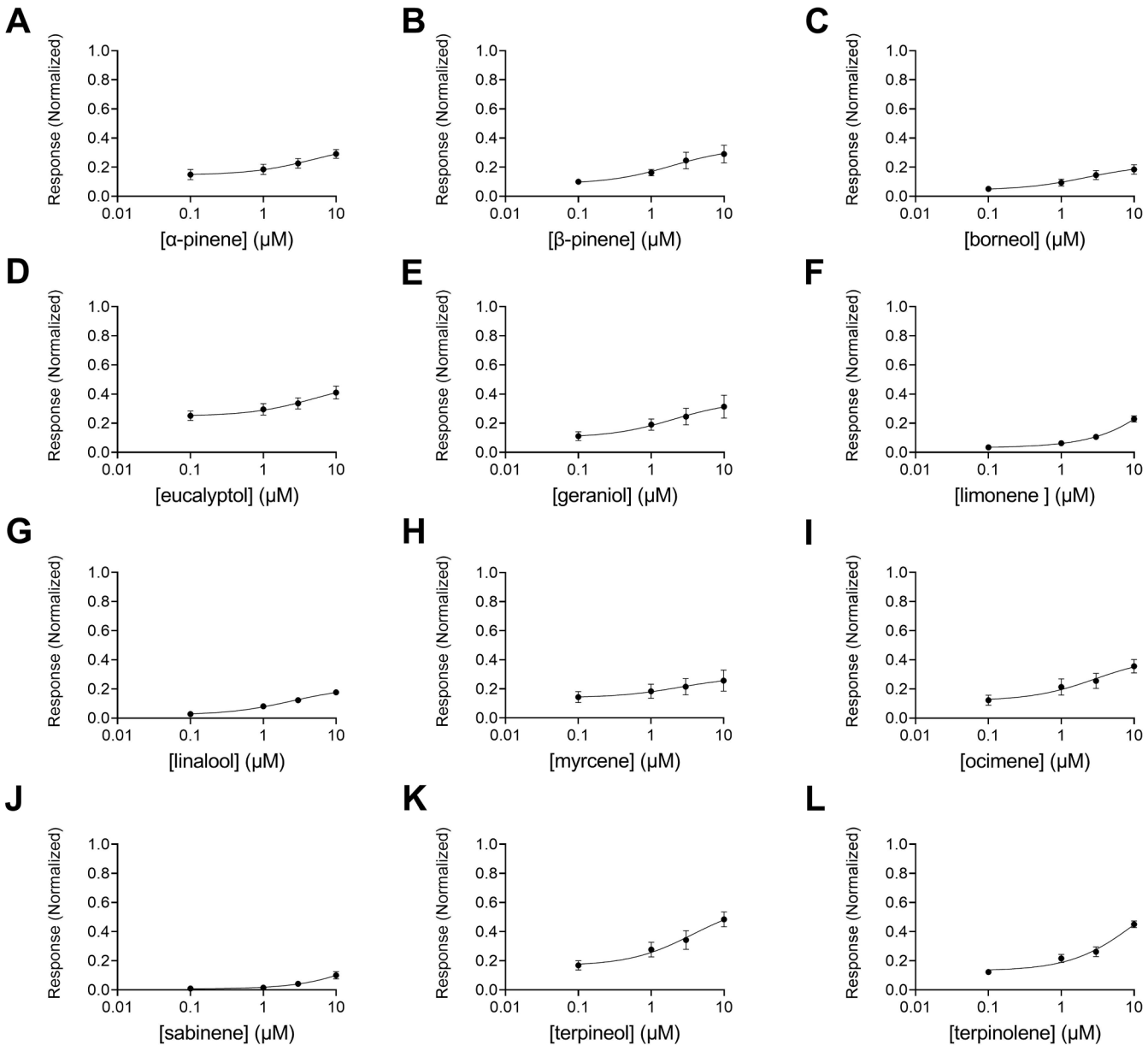

Fig. 4. Dose response curves of CB1 receptor activated GIRK currents by terpenes. Each graph (**A-L**) depicts the response to 4 terpene concentrations (each data point represents the mean ± SEM from 4 to 8 oocytes). Here and below, responses are normalized to the response evoked by 10 μM THC at the same oocyte. The solid lines here and below were generated by fitting Equation 1 to the data.

3. Results

We used the well-established Xenopus oocytes functional expression system [36-38] to test the possibility that the presence of various terpenes affects the activation of the CB1 receptor. To this end, Xenopus oocytes were injected with cRNAs of proteins involved in the pathway leading to activation of K^+ currents by CB1 receptor via βγ subunits of the G-proteins: The CB1 receptor, the two subunits of the GIRK channel (GIRK1 and GIRK2), and the Gαi3 subunit (Fig. 1A) [33].

First, the dependence of THC-induced K^+ current (I_{THC}) on THC concentration (dose–response, DR) was measured. Fig. 1B depicts the basic experimental protocol for five THC concentrations. The oocyte was voltage-clamped to–80 mV in a low K^+ (2 mM K^+) solution, ND96. Basal GIRK current (I_K) was developed upon replacement of the ND96 by the 24 mM K^+ solution. This current represents the basal activation of GIRK channel by endogenous βγ subunits present in the oocytes. Then, five different concentrations of THC were applied sequentially in ascending order giving rise to I_{THC}. This current was terminated upon washout of THC. Employing this basic experimental protocol, a full DR curve was constructed.

In order to compare between oocytes, I_{THC} at any particular THC concentration was normalized to I_{THC} obtained at a 10 μM THC, defined as the reference response, at this same oocyte.

Fig. 1C shows the average results from 10 oocytes showing a concentration-dependent activation of the GIRK channel by THC. From this DR curve an apparent EC_{50} of 2.3 μM was extracted for CB1 receptor activation by THC.

THC is known to act as a CB1 receptor partial agonist. Indeed, in our experimental system, 10 μM THC evoked ~41% of the maximal current evoked by 10 μM of CB1 receptor full agonist, the endocannabinoid 2-Arachidonoylglycerol (2-AG) (Fig. 2).

The effects of terpenes on CB1 receptor activation and on THC-induced CB1 receptor activation were next assessed. Sixteen cannabis terpenes were studied, including: α-pinene, β-pinene, limonene,

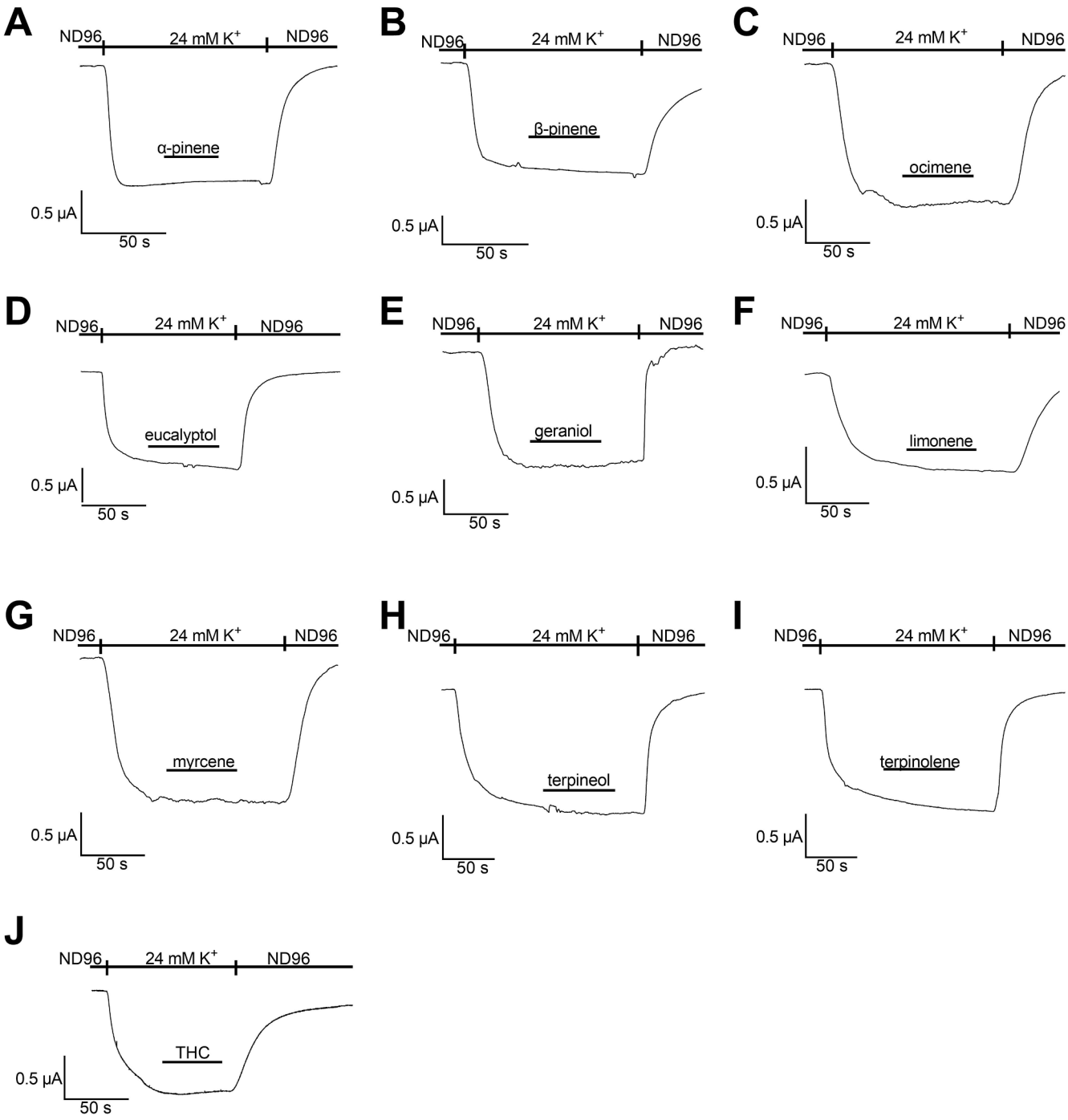

Fig. 5. Terpenes and THC do not affect GIRK currents in oocytes expressing the GIRK channel but not the CB1 receptor. **A-J.** Representative recordings. Oocyte were voltage clamped at −80 mV at ND96 solution. Replacing the solution with a 24 mM K^+ solution evoked basal GIRK current. In all cases, application of 10 μM of the indicated compound did not affect GIRK currents.

myrcene, ocimene, sabinene and terpinolene (monoterpenes, hydrocarbons consisting of two isoprene units, having the molecular formula of C10H16), borneol, eucalyptol, geraniol, linalool and terpineol, (monoterpenoids, oxygen-containing monoterpenes, C10H18O), β-caryophyllene and humulene (sesquiterpenes, hydrocarbons consisting of three isoprene units, C15H24), bisabolol and nerolidol (sesquiterpenoids, oxygen-containing sesquiterpenes, C15H26O). The chemical structures of the terpenes are presented in Fig. 3.

Terpene-derived CB1 activations are presented in Fig. 4, depicting DR curves of the various terpenes. Twelve out of the 16 terpenes were tested (β-caryophyllene, bisabolol, humulene and nerolidol were excluded from analysis as their solubility is below the tested concentration range; see Discussion). In order to estimate the potency of the terpenes as CB1 receptor agonists, the responses to each of the terpene in each experiment was normalized to the response evoked by 10 μM THC in the same oocyte, taken to be 1. As seen, CB1 receptor activity is detected for all terpenes, however, the magnitude varies notably among the various terpenes. The response to 10 μM terpene ranged between 10% and 48% of the response amplitude obtained by the reference 10 μM THC (Table 1). Activation amplitude >25% of the reference response is observed upon application of 10 μM α-pinene, β-pinene, eucalyptol, geraniol, myrcene, ocimene, terpineol and terpinolene.

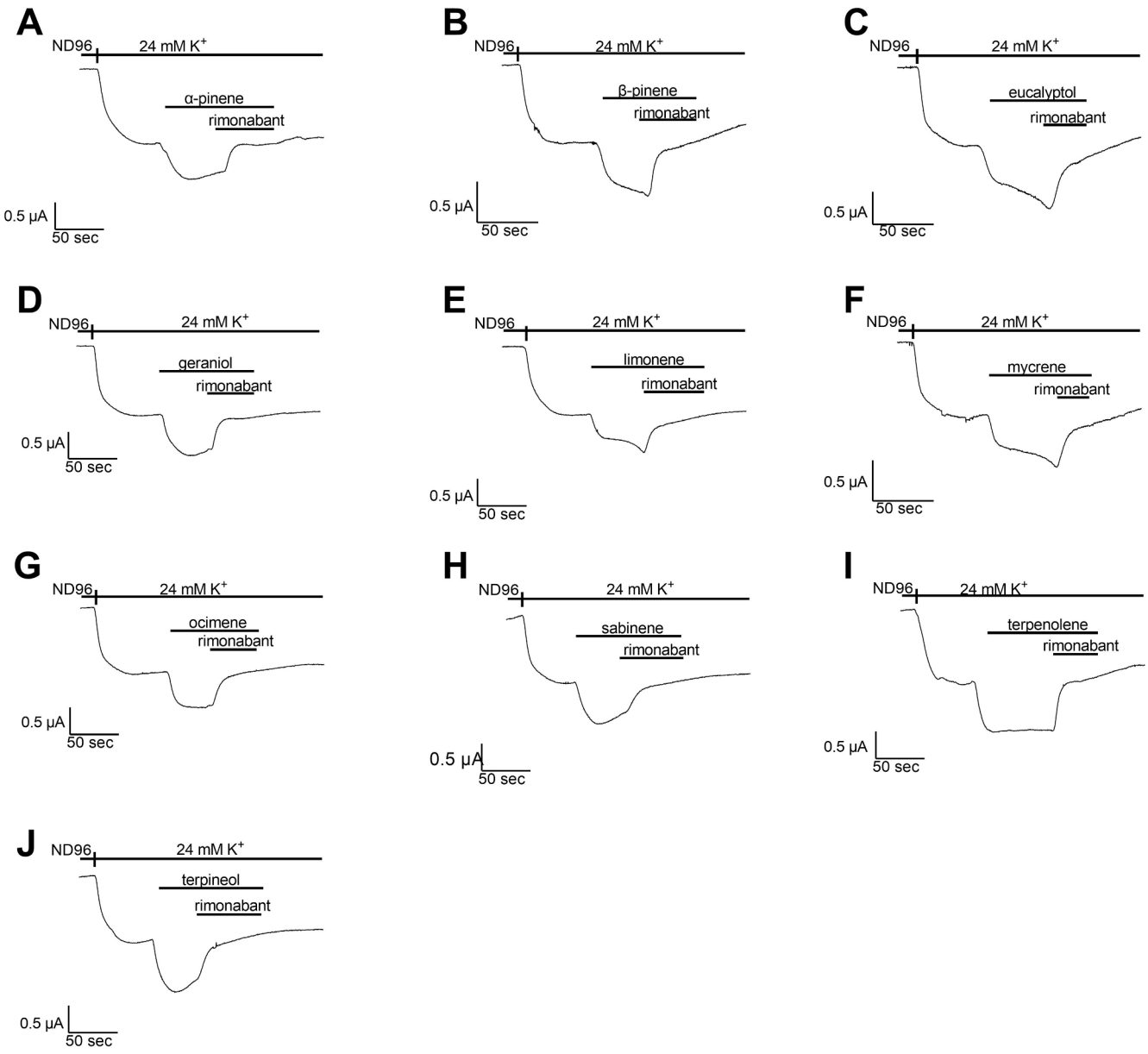

Fig. 6. Terpenes- evoked GIRK currents are mediated by CB1 receptor. **A-J.** Representative recordings. Oocyte were voltage clamped at −80 mV at ND96 solution. Replacing the solution with a 24 mM K^+ solution evoked basal GIRK current. Application of 10 μM of the indicated terpene evoked an additional GIRK current that was blocked by application of the CB1 receptor antagonist Rimonabant (1 μM).

Significant dose-dependent response ($p < 0.05$; one-way ANOVA) is detected for borneol, limonene, linalool, sabinene, terpineol and terpinolene. A trend is found for α-pinene, β-pinene, ocimene ($p = 0.05$) and eucalyptol ($p = 0.056$) (see Table 3).

We verified that THC and the various terpenes used in this study do not exert a CB1 receptor-independent effect on the GIRK channels. To do so, we measured the effect of THC and of the tested terpenes on oocytes expressing the GIRK channel but not the CB1 receptor. None of the compounds showed an effect on the GIRK current (see representative recordings in Fig. 5). Furthermore, application of the CB1 receptor antagonist Rimonabant diminished the terpene-evoked currents in CB1 receptor-expressing oocytes (see representative recordings in Fig. 6). These results demonstrate that terpenes- evoked GIRK currents in CB1 expressing oocytes were not due to direct activation of the coupled G protein, the GIRK channel or to any other CB1 receptor independent signaling pathway.

To study terpene effects on the THC-activated CB1 receptor response, the CB1 receptor activation by THC alone (Figs. 1C and 7, black symbols) was compared to its activation by the same THC concentrations in the presence of a10 μM terpene (Fig. 7, red symbols). Equation 1 was fitted to each one of the curves (see Materials and Methods). A significant effect of terpene- THC co-application (main effect of condition; i.e., THC > THC and terpene, $p < 0.05$, Two-way ANOVA) was found for twelve of the terpenes (Fig. 7. Statistics is detailed in Table 4). This increase in CB1 receptor activity was seen when THC was co-applied with α-pinene, β- pinene, borneol, eucalyptol, geraniol, limonene, linalool, myrcene, ocimene, sabinene, terpineol and terpinolene. The effect of terpene-THC co-application was similar across THC concentration levels in all terpenes except α-pinene, eucalyptol, myrcene and sabinene (for these terpenes, a significant interaction between main effect of condition and THC concentration levels was found, $p < 0.05$, Two-way ANOVA. Table 4). (β-caryophyllene, bisabolol, humulene and nerolidol were excluded from this analysis as their solubility is below the tested concentration range, see Table 2).

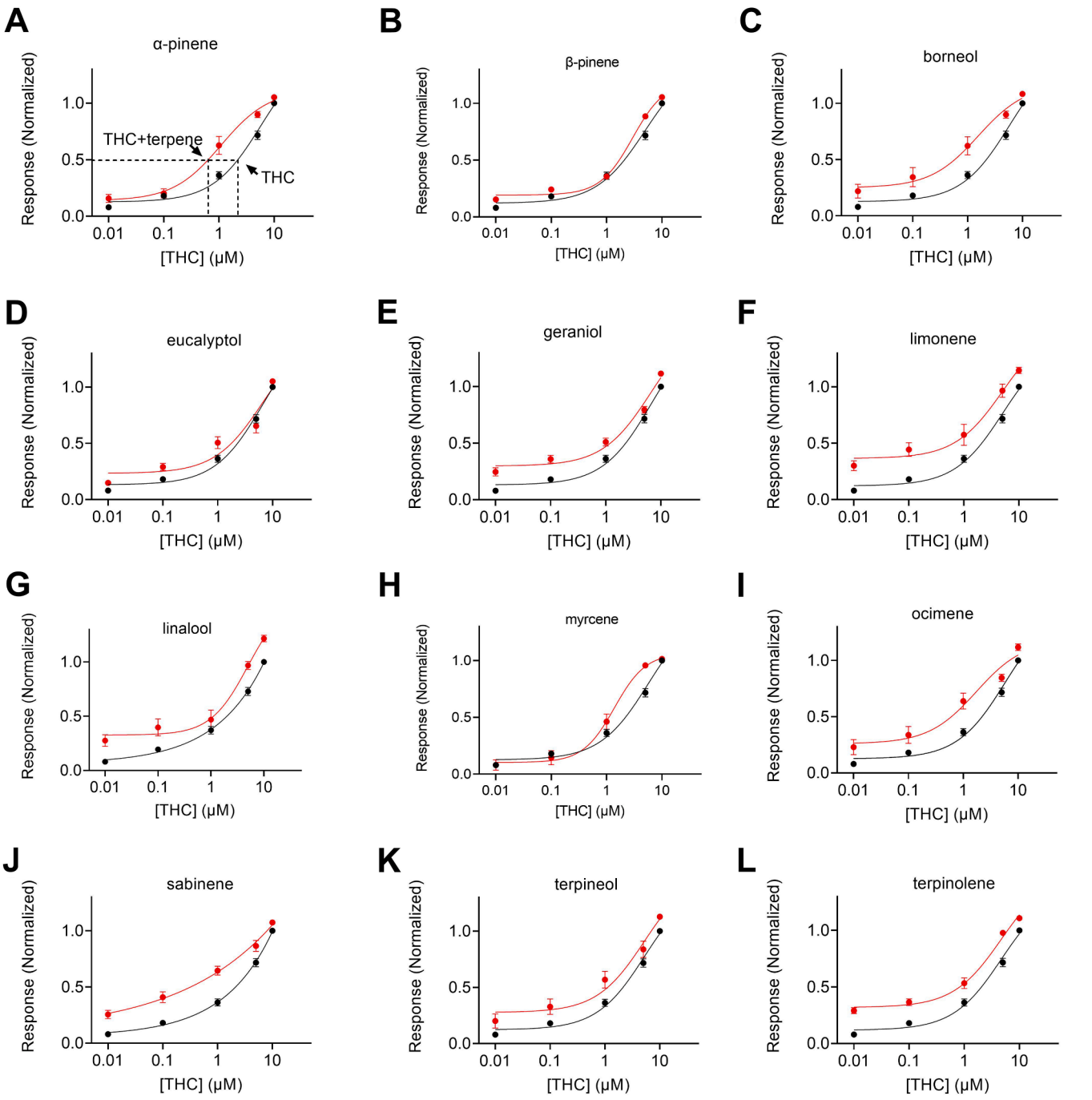

Fig. 7. Dose response curves of CB1 receptor activated GIRK currents following co-application of THC and 10 μM terpenes. **A-L.** Black symbols and lines represent activation of the receptor by THC alone (taken from Fig. 1). Red symbols and lines represent activation of the receptor by co-application of THC and 10 μM terpene (each data point represents mean ± SEM from 4 to 10 oocytes. The dashed lines in A depict the apparent EC_{50} obtained by THC and by THC co-applied with a terpene, shown in Table 1 (see Table 4 in for statistical data).

To further investigate these observations, we examined the effect of the terpenes at a terpene/THC weight/weight typical ratio found in the cannabis plant [39,40]. Hence, we repeated the experiments described above with the same THC concentrations, each supplemented with terpene at a weight/weight ratio of 1/10. Fig. 8 depicts eight DR curves in which the addition of the terpene significantly enhanced the activation of the CB1 receptor by THC (i.e., a significant main effect of condition (THC < THC and terpene, $p < 0.05$, Two-way ANOVA; DR curves for terpenes that did not show such an effect are shown in Fig. 9). The results demonstrate that the addition of borneol, geraniol, limonene, linalool, ocimene, sabinene and terpineol at this ratio significantly enhances the potency of THC in CB1 receptor activation. A significant, although weaker, effect was also demonstrated for application of THC with β- pinene. The effect of terpene-THC co-application was similar across THC concentration levels in all, but β- pinene and limonene (demonstrating significant interaction between main effect of condition and THC concentration levels, $p < 0.05$, Two-way ANOVA. See Table 5).

We then asked whether the enhancing effect observed in Fig. 8 might reflects accumulation of the effects of THC and of the terpene at CB1 receptor, or whether another mechanism may be involved. To this end we compared the DR curves in the presence of a terpene (Fig. 8, red symbols and lines) to theoretical DR curves that correspond to the sum

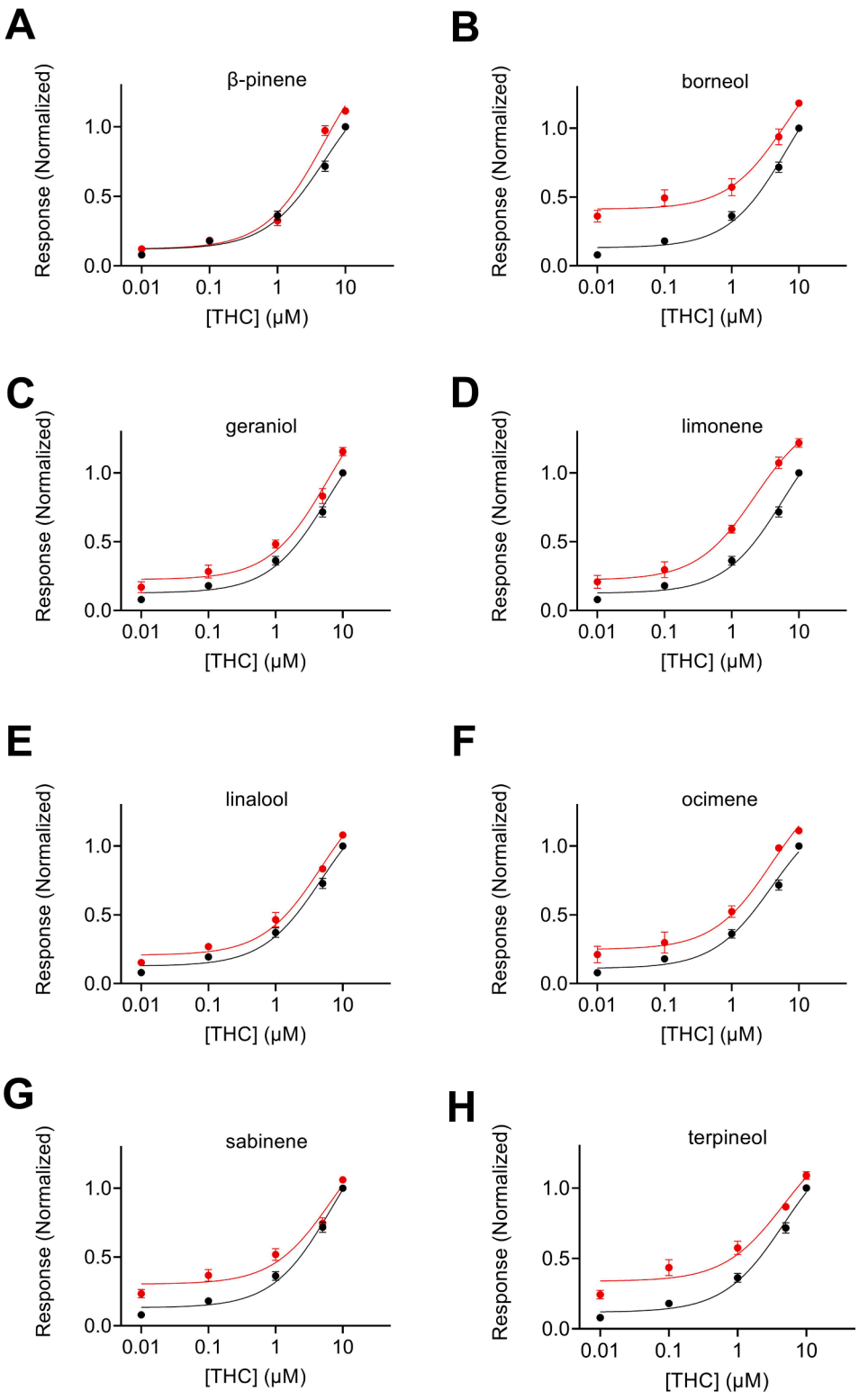

Fig. 8. Dose response curves of CB1 receptor activated GIRK currents following co-application of THC and terpenes at natural THC/ terpenes w/w ratio. **A-H**. Black symbols and lines represent activation of the receptor by THC alone (taken from Fig. 1). Red symbols and lines represent activation of the receptor by co-application of THC and terpene. The w/w ratio between THC and terpene was kept 10:1 throughout (each data point represents mean ± SEM from 6 to 14 oocytes). Only terpenes with significant modulatory effect, (i.e., significant main effect of condition, two-way ANOVA, $p < 0.05$) are present (see Table 5 for statistical data).

of the receptor activations by THC alone and by the terpene alone, at the corresponding concentrations (1/10 the concentration used for THC, as in Fig. 8). The results presented in Fig. 10 show that a mere summation of the effects can account for the increased activity of THC in the case of linalool, ocimene and terpineol (showing no significant main effect of condition). However, for borneol, limonene and sabinene the responses to the co-application of THC and a terpene were significantly higher than the response expected from a summation of their individual responses (significant main effect of condition, $p < 0.05$, Two-way ANOVA. See Table 6), suggesting a synergistic effect. Interestingly, the responses to the co-application of THC with β-pinene and geraniol, while larger than the response produced by THC alone, are lower than those expected by summations of their individual responses. (Fig. 10 and Table 6). This may suggest some complex interactions between these compounds and THC or between them and the CB1 receptor. Further study is needed to elucidate these interactions.

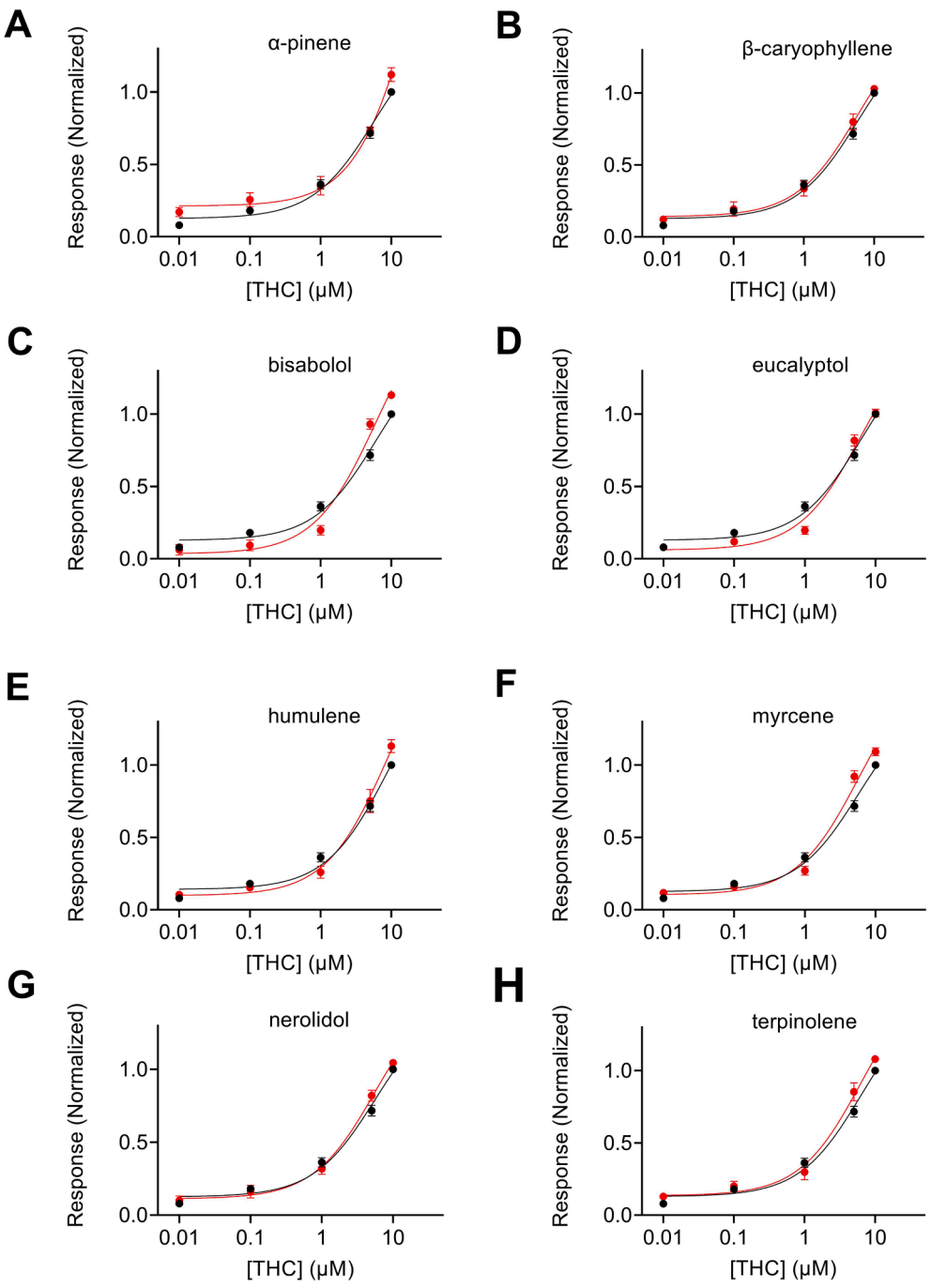

Fig. 9. Dose response curves of CB1 receptor activated GIRK currents following co-application of THC and terpene at natural THC/ terpenes w/w ratio. **A-H**. Black symbols and lines represent activation of the receptor by THC alone (taken from Fig. 1). Red symbols and lines represent activation of the receptor by co-application of THC and terpene. The w/w ratio between THC and terpene was kept 10:1 throughout (each data point represents mean ± SEM from 5 to 12 oocytes). Only terpenes with no significant modulatory effect, (i.e., significant main effect of condition, two-way ANOVA, $p > 0.05$) are present (see Table 5 in for statistical data).

4. Discussion

The current study evaluated the role of terpenes in CB1 receptor -mediated functions, studying terpenes as direct agonists of CB1 receptors and as modulators of THC agonism. The results are discussed in view of methodological considerations and in comparison to prior reports, elaborating on plausible mechanisms of action and important therapeutic implications.

Selection of the compositions to be tested. In order to optimally evaluate the role of terpenes on CB1 receptor and their modulation of THC-derived CB1 receptor activation, it is important to properly select (1) a representative set of diverse cannabis terpene; (2) a terpene to cannabinoid ratio representative of the natural cannabis plants, being in the order of 1/10 weight/ weight; and (3) concentrations within the solubility limits of the various reagents. The selections made here differ, at least partially, from those of prior studies, which may explain differences in findings.

Terpenes tested: Dozens of terpenes are found in the cannabis plant, the composition and content of which vary quite notably among different cannabis chemovars. In order to obtain more conclusive findings, the current study tested the effects of sixteen cannabis terpenes, including both major and minor terpenes. Previous studies [28,29,31] have tested only subsets of these terpene.

Terpene to cannabinoid ratios: Commonly marketed cannabis inflorescences generally contain about 5–25% THC. Total terpene concentrations in cannabis inflorescences were typically in the 1% range, however, due to selective breeding, this concentration was raised to about 3% in some inflorescences [39,40], reaching terpene to

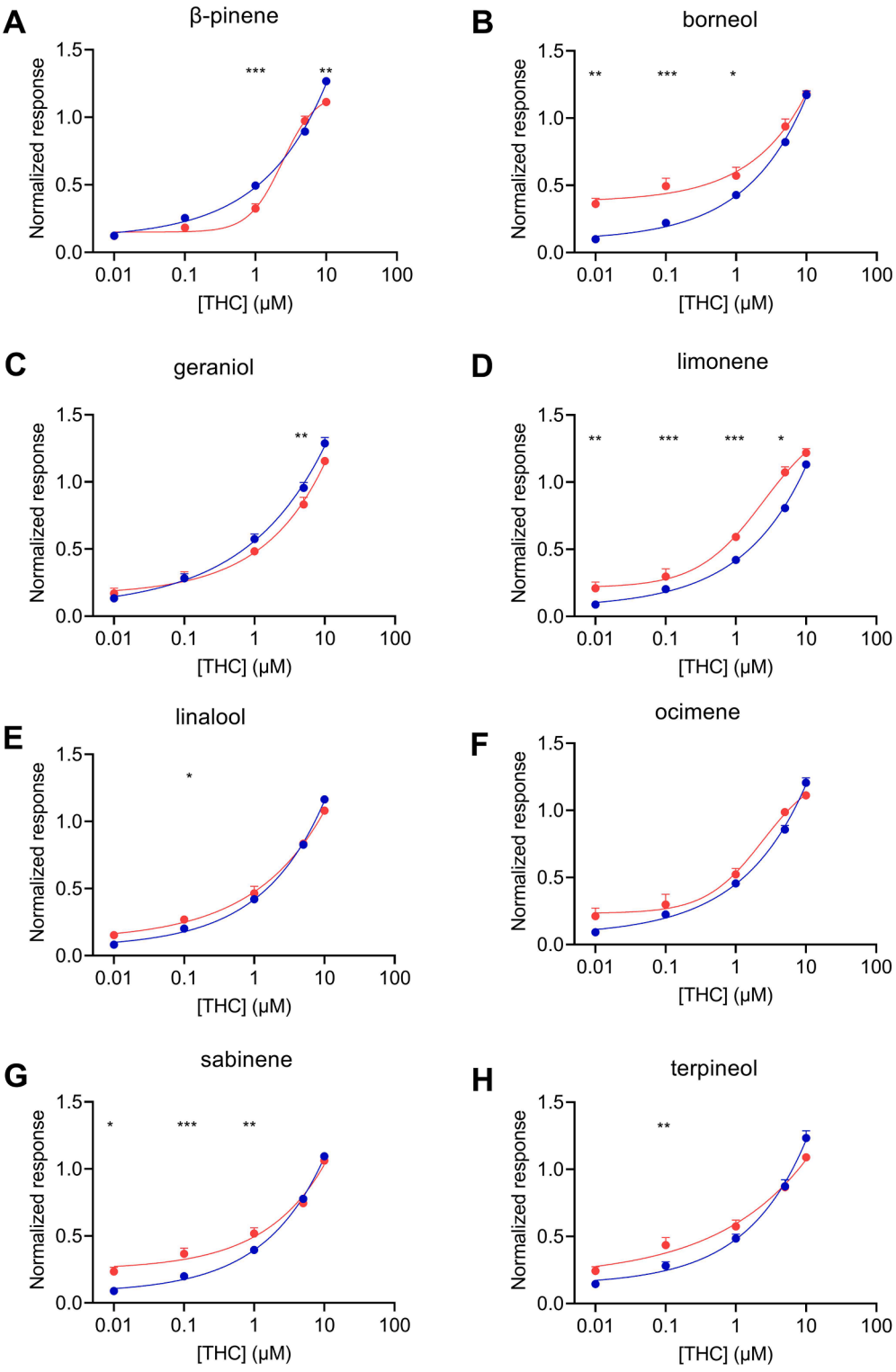

Fig. 10. Summation and synergistic modulatory effects of different terpenes. **A-H.** The blue symbols and lines are theoretical DR curves obtained by calculating the sum of (i) DR curves that describe the CB1 receptor activation by THC alone (Fig. 1) and (ii) the DR curves that describe the CB1 receptor activation by terpenes alone (Fig. 4). DR curves of the data presented in Fig. 8, obtained by co-application of THC and a terpene (red symbols and lines) are shown for comparison. *, $p < 0.05$; **, $p < 0.01$; ***; $p < 0.001$ (see Table 6 in for statistical data).

cannabinoid weight/weight ratios of up to about 1:10. Prior studies assessing modulation of THC activity at CB1 receptors by terpenes [28,29,31] have used terpene to THC ratios of 1:1 or higher, failing to represent the terpene to cannabinoid ratios which occur naturally in the cannabis plant.

Concentrations of reagents: The solubility in water of THC and of many of the terpenes tested (as well as in aqueous solutions containing salts – "physiological solutions") is low, a few milligrams per liter or less (Table 2). In-vitro tests referred to herein [28,29,31], have used concentrations of reagents of up to 1 mM. Specifically, while the concentrations of cannabinoids tested were within the solubility limit, those of the terpenes used were frequently outside this limit. This may question the conclusions derived from such studies. LaVigne et al. [31] for example, have presented dose responses at 5 μM to 1 mM for activation of CB1 receptors by linalool, geraniol, β-pinene, humulene and β-caryophyllene. However, β-pinene is soluble at up to about 50 μM while the solubility of humulene and β-caryophyllene is under 5 μM. Thus, the resulting dose–response curves seem to extend beyond real solubility. In

Table 3
Dose-dependent CB1 activity following application of the various terpenes. df, F and *p* values for the effect of terpenes' concentration levels (0.1, 1, 3 and 10 μM) on CB1 derived activity (One-way ANOVA). Data for β-caryophyllene, bisabolol, humulene and nerolidol were not included since their solubilities are below the tested range (see Discussion). Here and below, N is the number of independent oocytes used for each terpene. p values here and below are labeled as follows *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

	df	F	p	N
α-pinene	3,23	3.25	0.05	6
β-pinene	3,23	3.38	0.05	6
bisabolol	2,14	0.77	0.487	5
borneol	3,27	4.95	****0.01**	7
eucalyptol	3,23	2.97	0.056	6
geraniol	3,23	2.49	0.089	6
limonene	3,19	40.53	*****0.001**	5
linalool	3,145	19.68	*****0.001**	4
myrcene	3,23	0.76	0.528	6
nerolidol	2,14	1.60	0.241	5
ocimene	3,31	4.10	***0.05**	8
sabinene	3,15	7.09	****0.01**	4
terpineol	3,23	6.80	****0.01**	6
terpinolene	3,23	27.78	*****0.001**	6

the study presented by Finlay et al. [28], the trials at 10 μM, with α- and β-pinene, myrcene, limonene and possibly also with Mixture 3 were within attainable solubility. However, those using β-caryophyllene and Mixtures 1 and 2, were not. The study of Santiago et al. [29] tested myrcene (up to 30 μM), α- and β-pinene, β-caryophyllene, linalool and limonene (up to 100 μM each). Out of those, linalool is capable of reaching the desired concentration, and that could also be the case for myrcene. This is not the case for α and β-pinene, and particularly not for β-caryophyllene. On exceeding the solubility limit, there is room for the formation of colloids and for their effects [10].

CB1 activation by terpenes. CB1-dependent activations were demonstrated upon application of twelve cannabis terpenes tested; activation degrees ranged between 10 and 50% of the activation obtained using similar THC concentrations. A significant dose-dependent CB1 activity was detected for a subset of these terpenes. The role of terpenes in CB1 activation was recently supported by two in-vivo studies [31,41], demonstrating that the analgesic effect evident in the presence of selected terpenes was eliminated by introduction of CB1 antagonist [31] or in knockout mice [41].

CB1 activation by THC -terpenes mixtures. Our results further show that CB1 activation by THC in the presence of β-pinene, borneol, geraniol, limonene, linalool, ocimene, sabinene, and terpineol, significantly differs from the activation by THC in the absence of the terpene. This effect is found at terpene/THC weight/weight ratios as low as those

Table 4
Terpene effects on THC-activated CB1 responses: co-application of THC and 10 μM terpene. df, F, and *p* values for main effects ((1) condition; THC or THC + terpenes, (2) THC concentration levels; 0.01, 0.1, 1 and 5 μM) and their interaction, obtained by Two-way ANOVA. The two-way ANOVA compared the CB1 response obtained by THC and by THC at the same concentration co-applied with 10 μM terpene. Data for bisabolol, β-caryophyllene, humulene and nerolidol is not included, being outside solubility limits of these terpenes (see Table 2), N is the number of independent oocytes used for each terpene.

	Main effect of condition			Main effect of THC concentration levels			Interaction- condition * THC concentration levels			
	df	F	p	df	F	p	df	F	p	N
α-pinene	1,92	25.06	*****0.001**	3,92	165.52	*****0.001**	3,92	4.67	****0.005**	7
β-pinene	1,104	8.257	****0.005**	3,104	206.20	*****0.001**	3,104	2.03	0.114	10
borneol	1,112	72.60	*****0.001**	3,112	83.02	*****0.001**	3,112	0.73	0.533	5
eucalyptol	1,88	4.51	***0.037**	3,88	108.02	*****0.001**	3,88	5.54	****0.002**	6
geraniol	1,92	35.82	*****0.001**	3,92	144.87	*****0.001**	3,92	2.34	0.079	7
limonene	1,87	56.94	*****0.001**	3,87	90.03	*****0.001**	3,87	0.79	0.501	6
linalool	1,84	39.31	*****0.001**	3,84	105.97	*****0.001**	3,84	1.13	0.340	5
myrcene	1,88	6.13	***0.015**	4,88	181.53	*****0.001**	4,88	4.87	****0.004**	6
ocimene	1,88	35.39	*****0.001**	3,88	103.24	*****0.001**	3,88	2.37	0.076	6
sabinene	1,92	69.017	*****0.001**	3,92	136.13	*****0.001**	3,92	3.32	***0.023**	7
terpineol	1,80	20.77	*****0.001**	3,80	89.84	*****0.001**	3,80	1.14	0.335	4
terpinolene	1,92	80.85	*****0.001**	3,92	189.60	*****0.001**	3,92	0.45	0.714	7

Table 5
Terpene effects on THC-activated CB1 responses: co-application of THC and terpene at terpene/ THC w/w ration of 1/10. df, F, and *p* values for main effects ((1) condition; THC or THC + terpenes, (2) THC concentration levels; 0.01, 0.1, 1 and 5) and their interaction, obtained by Two-way ANOVA. The two-way ANOVA compared the CB1 response obtained by THC and by THC at the same concentration co-applied with a terpene at terpene/ THC w/w ration of 1/10, N is the number of independent oocytes used for each terpene.

	Main effect of condition			Main effect of THC concentration levels			Interaction- condition * THC concentration levels			
	df	F	p	p	F	df	N	p	F	df
α-pinene	1,96	1.69	0.196	*****0.001**	106.65	3,96	8	0.403	0.98	1,96
β-pinene	1,104	7.14	****0.009**	*****0.001**	248.71	3,104	10	*****0.001**	9.32	3,104
β-caryophyllene	1,84	0.68	0.794	*****0.001**	102.09	3,84	7	0.620	0.59	3,84
bisabolol	1,88	0.79	0.377	*****0.001**	181.36	3,88	6	*****0.001**	10.17	3,88
borneol	1,112	72.60	*****0.001**	*****0.001**	83.01	3,112	12	0.533	0.736	3,112
eucalyptol	1,84	2.34	0.130	*****0.001**	138.76	3,84	5	****0.006**	4.47	3,84
geraniol	1,96	15.22	*****0.001**	*****0.001**	130.63	3,96	8	0.985	0.05	3,96
humulene	1,96	1.04	0.311	*****0.001**	123.12	3,96	6	0.213	1.52	3,96
limonene	1,88	55.19	*****0.001**	*****0.001**	157.79	3,88	6	****0.008**	4.17	3,88
linalool	1,84	16.19	*****0.001**	*****0.001**	146.19	3,84	8	0.918	0.16	3,84
myrcene	1,108	1.24	0.267	*****0.001**	247.13	3,108	11	*****0.001**	9.18	3,108
nerolidol	1,104	0.05	0.811	*****0.001**	170.80	3,104	8	0.138	1.89	3,104
ocimene	1,96	35.42	*****0.001**	*****0.001**	116.50	3,96	9	0.356	1.09	3,96
sabinene	1,108	26.86	*****0.001**	*****0.001**	113.387	3,108	11	0.079	2.33	3,108
terpineol	1,120	57.41	*****0.001**	*****0.001**	122.68	3,120	14	0.465	0.85	3,120
terpinolene	1,100	1.15	0.286	*****0.001**	144.74	3,100	9	0.053	2.65	3,100

Table 6
Summation and synergistic modulatory effects of different terpenes. df, F and *p* values for main effects ((1) condition; THC-terpene co-application or the theoretical summation of their individual responses, (2) THC concentration levels;0.01, 0.1, 1, 5 and 10 μM) and their interaction, obtained by Two-way ANOVA. The two-way ANOVA compared the CB1 response obtained by co-administration of THC and terpene and the CB1 responses obtained by the theoretical summation of their individual responses, at the corresponding concentrations.

	Main effect of condition			Main effect of THC concentration level			Interaction- condition * THC concentration levels		
	df	F	p	df	F	p	df	F	p
β-pinene	13.85	1,80	***0.001	4,80	597.24	***0.001	4,80	3.54	***0.001
borneol	1,90	23.29	***0.001	4,90	109.15	***0.001	4,90	2.20	0.075
geraniol	1,70	5.68	*0.020	4,70	214.98	***0.001	4,70	1.56	0.194
limonene	1,60	55.84	***0.001	4,60	398.69	***0.001	4,60	1.66	0.174
linalool	1,65	1.84	0.180	4,65	460.54	***0.001	4,65	3.10	*0.022
ocimene	1,75	2.38	0.127	4,75	196.91	***0.001	4,75	1.67	0.1167
sabinene	1,85	11.51	***0.001	4,85	223.17	***0.001	4,85	5.37	***0.001
terpineol	1,100	1.71	0. 193	4,100	141.03	***0.001	4,100	3.55	*0.010

found in the cannabis plant.

Prior in vitro studies have not found such effects of terpenes in mixtures with THC [28,29]. Note, however, that they have focused on some common terpenes, some of which show limited activity in the present study as well (α-pinene, myrcene and β-caryophyllene). However, differences do exist in cases of β-pinene, limonene and linalool, showing significant effects in our study which were not shown in previous studies (β-pinene, limonene [28,29] and linalool [29]). The source for this discrepancy may be related to methodological considerations. Specifically, in our study the activity of the CB1 receptor was evaluated by measuring the increase in K^+ currents induced by the G protein activation and by the subsequent binding of released βγ subunits to the GIRK channel. Such measurements can easily detect currents in the order of tens of nano-amperes, which reflect the activation of a small number of channels. Possibly, such small currents cannot be reliably detected by membrane potential sensitive dyes as used by Santiago et al. [29]. The same might be true for the functional assay used by Finlay et al. [28] in measuring the inhibition in cAMP synthesis by Gi-inhibited adenylyl cyclase. Such measurements may not be sensitive enough to detect the subtle effects on receptor activation that we report here. In addition, Finlay et al. [28] reported that terpenes did not displace bound CB1 receptor radioligand, nor did they affect the displacement of this ligand by THC. Importantly, the methodology of Finlay et al. cannot identify cases wherein the terpene increased the binding of the THC to CB1 receptor, as suggested by the present study, nor the possibility that the terpenes tested affected the receptor by binding to a different allosteric site. Our observation that suggests a synergistic effect of THC and terpenes is compatible with such a mechanism, although further investigation is needed in order to examine this suggestion.

A possible additional explanation for the above-mentioned discrepancy may be that in the present study, the membrane potential of the cell was kept at −80 mV by voltage clamping the intact cell, while this parameter was not controlled in either one of these studies. Such difference may affect the observed activation of the receptor, as there is growing evidence to suggest that the affinity and potency of agonists toward many GPCRs, including the CB1 receptor, are controlled by membrane potential [36,42-52]. As −80 mV represents membrane potential that is typical to mammals' neurons, we suggest that our data reflects more accurately the physiological setting for the CB1 receptor in that regard.

Terpenes – THC synergism. A possible explanation for the increased CB1 receptor activation by THC in the presence of terpene is an accumulative effect, wherein both the terpene and THC contribute to the receptor activation. This option is tested in Fig. 10, which compares the activation found for THC-terpene mixtures to the calculated combined activation of each of the components alone at the corresponding concentration. It shows that accumulation cannot explain the results for most tested terpenes. In the cases of β-pinene and geraniol, the responses of the mixtures are lower than the sum of the contributions, while in the cases of limonene, borneol and sabinene, the responses are notably greater than the sum, suggesting a synergistic effect. To the best of our knowledge, this is the first demonstration of THC- terpene synergism in an in vitro controlled setting. Importantly, synergism here is found at terpene/THC ratios similar to those in the cannabis plant.

Terpene modulation of THC interaction with CB1 receptor. Prior studies have suggested various mechanisms for such synergism, including the role of terpenes in modulating THC permeability and absorption, increasing cerebral blood flow, improving THC transport through the blood–brain-barrier and activation of additional signaling pathways complementary to the ECS [1,5,53]. These mechanisms are not applicable to the synergism as found herein. The synergism demonstrated here suggests a modulatory effect of the terpene on the interaction between THC and CB1 receptor. This modulation increases the THC-derived CB1 receptor activation several fold (Table 1) and is found at very low terpene concentrations and at terpene/cannabinoids ratios similar to those in the natural cannabis plant.

It should be noted that modulation by terpenes also cannot be ruled out in cases wherein the activation by THC-terpene mixture is similar to that of summing up their individual contributions. However, modulation of THC by terpenes is not a general phenomenon, since no effect at all is observed with some of the tested terpenes. No obvious structural or chemical difference appears between the terpenes augmenting THC-derived signaling at CB1 and ones that do not. Addressing this issue will require further study.

Understanding the mechanisms of action at the basis for such modulation is of a great scientific interest and would guide the search for other terpenes and other additives with at least as great an effect. It may result from specific interactions with the receptor and/or with THC to improve availability for interaction. The interaction with the receptor may involve modulation of the membrane dynamics [5,31]. A related mechanism could be stabilizing the complex between the THC and the receptor via specific interactions with it.

THC – terpenes entourage effect? The term "entourage effect" was coined by Ben-Shabat et al. [54] to describe cases wherein co-existence of a compound having no ECS activity on its own, result in an increased ECS activation by a cannabinoid. Given that cannabis terpenes demonstrate both direct agonism at CB1 receptor and a modulatory effect on THC interaction at CB1 receptor, THC – terpenes effects are beyond the classical definition of entourage.

In the current study, we demonstrated terpene-derived CB1 receptor activation and terpene-derived amplification of THC activity at CB1 receptor by a subset of cannabis terpenes. Importantly, for some of those terpenes, a major amplification exists already at terpene to THC ratios similar to those in the cannabis plant, and at terpene concentrations as low as 0.001–0.01 μM.

While the term "entourage" might not fit here, this study demonstrates synergism in selected terpene-THC systems, indicating terpene-induced modulation of THC-CB1 receptor interactions. This finding motivates searching for such synergism in other receptor-cannabinoid-terpene systems as well.

The notable CB1 receptors activation and the desired synergism with THC were shown by only a fraction of the cannabis terpenes, many of which are not the most common ones. Thus, reaching "whole plant" or "full spectrum" composition is not necessarily an advantage. For enhanced therapeutic effects, medical cannabis should be rich in the terpenes most suitable for activation of receptors involved with the specific indication to be treated. Developing genetics rich in selected terpenes is doable, but requires major efforts and time. Enrichment of cannabis extracts with selected terpenes, sourced from cannabis or from other plants, is much easier and applicable to tablets and capsules produced from such extracts.

The use of selected terpenes may enable reducing the THC dose in some treatments, and as a result, potentially minimizing the THC-related adverse effects. This would also help in adjusting the treatment to more sensitive populations such as children and elderly. Enrichment with selected terpenes may allow for composition adjustment to personal needs and to changes during chronic use, such as for daytime versus for sleep.

Declaration of Competing Interest

NR, AME, DBZ and DHS are employees of the Bazelet group, a medical cannabis manufacturer in Israel. EMD, AD, MT and YBC declare no competing interests.

Data availability

Data will be made available on request.

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AUTHOR CONTRIBUTION

NR and AME designed the research, performed data analysis and wrote the manuscript. DBZ prepared the materials and contributed to research plan. DHS performed data analysis and contributed to manuscript preparation. EMD provided clinical practicable input. MT and AD performed the experiments. YBC designed the research, performed the experiments and the data analysis and wrote the manuscript. All authors reviewed and approved the final version of the manuscript.

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