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Modulation of the γ-aminobutric acid (GABA) system by *Passiflora incarnata* L.

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Abstract

Passiflora incarnata L. (Passifloraceae) is important in herbal medicine for treating anxiety or nervousness, GAD, symptoms of opiate withdrawal, insomnia, neuralgia, convulsion, spasmodic asthma, ADHD, palpitations, cardiac rhythm abnormalities, hypertension, sexual dysfunction, and menopause. However, the mechanism of action is still under discussion. Despite gaps in our understanding of neurophysiological processes, it is increasingly being recognized that dysfunction of the GABA system is implicated in many neuropsychiatric conditions, including anxiety and depressive disorders. Therefore, we investigated the *in vitro* effects of a dry extract of *Passiflora incarnata* (sole active ingredient in Pascoflair® 425mg) on the GABA system. The extract inhibited [³H]-GABA uptake into rat cortical synaptosomes but had no effect on GABA release and GABA transaminase activity. *Passiflora incarnata* inhibited concentration dependent the binding of [³H]- SR95531 to $GABA_A$-receptors and of [^{3}H]-CGP 54626 to $GABA_B$-receptors. Using the [^{35}S]-GTPγS binding assay *Passiflora* could be classified as an antagonist of the $GABA_B$ receptor. In contrast, the ethanol- and the benzodiazepine-site of the $GABA_A$-receptor were not affected by this extract.
In conclusion, we show the first evidence that numerous pharmacological effects of *Passiflora incarnata* are mediated via modulation of the GABA system including affinity to $GABA_A$ and $GABA_B$ receptors, and effects on GABA uptake.

Keywords

GABA, anxiety**,** insomnia, passion flower, *Passiflora incarnata* L., Pascoflair®

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Introduction

The genus Passiflora consists of 500 species that are mostly found in warm and tropical regions. The genus was first discovered by Spanish invaders in 1529. This plant has been used widely in herbal medicine in West India, Mexico, The Netherlands, South America, Italy and Argentina. *Passiflora incarnata* is the official species and monographed in several pharmacopoeias (e.g. Passiflorae Herba Pharm. Eur.). *Passiflora incarnata* contains C-glycosyl flavones such as vitexin, isovitexin, schaftoside, isoschaftoside and isovitexin-2-*O*glucoside phenols, glycosyl flavonoids and cyanogenic compounds (Wohlmuth et al. 2010).

Only a few human studies are available focusing on its potential role as an anxiolytic (Akhondzadeh et al. 2001, Miyasaka et al. 2007, Movafegh et al. 2008). In some experiments, it has potential effects for treatment of some diseases like anxiety, opiates withdrawal, insomnia, ADHD (attention-deficit hyperactivity disorder) and cancer (Sarris et al. 2001, Dhawan et al. 2004, Patel et al. 2009).

Very few pharmacological studies have been undertaken on the anxiolytic/sedative activity of *Passiflora incarnata*; most of these investigations have been carried out with different Passiflora species, such as *P. edulis* (Deng et al. 2010, Barbosa et al. 2008), *P. alata* (Barbosa et al. 2008), *P. coerulea* (Reginatto et al. 2006), or *P. quadrangularis* (de Castro et al. 2007) and with insufficient phytochemical characterization of the extracts.

The anxiolytic activity of an extract from *Passiflora actinia* has been shown to be exerted via the GABA receptor (Lolli et al. 2007), but the much more widely -used *Passiflora incarnata* has not been investigated in this context.

Grundmann et al. 2008, who investigated the same dry extract (sole active ingredient in Pascoflair® 425mg) which was used in this study, and Zhong et al. 2008 showed that *Passiflora incarnata* increased the time mice spent in the open arm of the elevated plus maze and they postulated that the effects were mediated via GABA receptors.

Although there are some reports speculating regarding the effects of *Passiflora incarnata* on the GABA system, investigations concerning the mechanism are still lacking.

Therefore, we hypothesized that the mode of action of the dry extract prepared from the flowers of *Passiflora incarnata* which is the sole active ingredient of the proprietary herbal drug Pascoflair® 425mg might be modulation via the GABA-system.

Overall, we show that this dry extract of *Passiflora incarnata* inhibited the binding of [^{3}H]- SR95531 to $GABA_A$-receptors and of [^{3}H]-CGP 54626 to $GABA_B$-receptors in a concentration dependent manner. Using the [^{35}S]-GTPγS binding assay *Passiflora incarnata* could be classified as an antagonist of the $GABA_B$ receptor. The *Passiflora incarnata* extract inhibited [^{3}H]-GABA uptake into rat cortical synaptosomes and showed no effect on GABA transaminase and GABA release.

Material and Methods

Botanical drug preparation

Passiflora incarnata dry extract (Passiflorae Herba Ph. Eur., DER = 5-7:1, extraction solvent: 50% ethanol (V/V)) was provided by Pascoe pharmazeutische Präparate GmbH (Giessen, Germany). This dry extract is the sole active ingredient of the proprietary herbal drug Pascoflair® 425 mg.

Dried extract was used in the pharmacological studies and was dissolved in a measured small amount of dimethlsulfoxide (DMSO) and diluted in the application solution immediately prior to testing. All test doses contained the same quantity of DMSO as did all control test solutions.

Chemicals used

[Butyryl-2,3-^{3}H]-SR 95531, [N-Methyl-^{3}H]-Ro-15-1788, [7,9-^{3}H]-Ro-15-4513 and GTP[γ-^{35}S] were from PerkinElmer (Massachusetts, USA). [^{3}H]CGP 54626 and SR 95531 hydrobromide were purchased from Biotrend Chemikalien GmbH (Cologne, Germany). Diazepam-ratiopharm® was from Ratiopharm GmbH (Ulm, Germany). GABA (γ-amino-n-butyric acid), (±) Baclofen, GABase were purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). Vigabatrin was from Sanofi-Aventis (Frankfurt, Germany).

In vitro pharmacological experiments

Binding assays

Male Sprague Dawley rats were sacrificed by decapitation. Rat brain (minus cerebellumand medulla oblongata, i.e. the hippocampus and cerebellum) tissue was homogenized at 4°C in 10 ml 10 mM Tris-HCl pH 7.4, 1 mM EDTA, and centrifuged for 15 min at 25,000xg. The pellet was finally resuspended in 50 mM Tris-HCl pH 7.4, 4 mM $MgCl_2$ and 1 mM EDTA, frozen in liquid nitrogen and stored at –80°C until usage. Binding experiments (GABA-/Benzodiazepine-/Ethanol-site, $GABA_B$-receptor) were performed as described (Heaulme et al. 1987, Mehta and Shank 1995, Asay and Boyd 2006).

The assay was terminated by transfer of the samples on GF/C filter plates, presoaked with 0.1% polyethyleneimine. Filters were washed four times with 200 µl of ice-cold 50 mM Tris-HCl pH 7.4 and filter-bound radioactivity was determined by a microplate reader (Microbeta, Wallac, Finnland).

Test compound data are presented specific ligand binding to the receptor. Specific binding is defined as the difference between the total binding and the non-specific binding determined in the presence of reference compound.

The IC_{50} values (concentration causing half-maximal inhibition of control specific binding) were determined by non-linear regression analysis of the competitive curves using the algorithm “sigmoidal dose-response” (GraphPadPrism, San Diego, USA). In case of ill-defined curves or algorithm-generated minima below the defined non-specific binding the IC_{50} value was determined by graphical extrapolation.

[^{35}S]-GTPγS-binding assays

[^{35}S]-GTPγS-binding assays were carried out as described (Bidlack und Parkhill, 2004): Membranes were preincubated with the respective effectors for 15 min, the incubation was initiated by the addition of 200 pM [^{35}S]-GTPγS (60 min incubation at 30°C in a final volume of 100 µl assay buffer).

The assay was terminated by transfer of the samples on GF/C filter plates. Filters were washed four times with 200 µl of ice-cold 50 mM Tris-HCl pH7.4 and bound radioactivity was determined by a microplate reader (Microbeta, Wallac, Finnland). From every data point the non-specific binding (binding of [^{35}S]-GTPγS in the presence of 10 µM GTPγS) was subtracted and the value normalized to the respective control signal of the respective plate.

EC_{50}- and IC_{50} values (concentration causing half-maximal stimulation/inhibition of control specific binding) were determined by non-linear regression analysis of the competitive curves using the algorithm "sigmoidal dose-response" (GraphPadPrism, San Diego, USA). In case of ill-defined curves the IC_{50} was determined by graphical exploration of the algorithm-generated curve.

GABA uptake experiments

Freshly dissected, rat cortex dissected from male Sprague-Dawley rats was immersed immediately in 10 volumes of ice-cold 0.32 M sucrose buffered with 10 mM HEPES pH 7.4 and homogenized. The resulting preparation was centrifuged at 900 x g for 10 minutes at 4 °C. The pellet was discarded and the supernatant was centrifuged again at 4 °C at 10.000 x g. The supernatant was discarded and the pellet was stored with 0.32 M sucrose/HEPES on ice until needed.

Assays were carried out in Farnebo buffer pH 7,4 (121 mM NaCl, 1,8 mM KCl, 1,3 mM $CaCl_2$, 1,2 mM $MgSO_4$, 25 mM $NaHCO_3$, 1,2 mM KH_2PO_4, 11 mM glucose, 0,57 mM ascorbic acid, saturated with 95% O_2/5% CO_2) containing the GABA transaminase inhibitor aminooxoacetic acid (100 µM). Briefly, 10 µl of drug solution, non-specific ligand or buffer, 180 µl Farnebo buffer and 50 µl of synaptosome preparation were added in each well of a 96 well filtration plate prewetted with Farnebo buffer (Millipore Multiscreen). The incubation proceeded for 10 minutes at room temperature after which 10 µl of [^{3}H] GABA (final concentration 100 nM) were added for a total volume of 250 µl. The incubation proceeded for 5 minutes at 37° C. Incubation was terminated by rapid filtration and washing with Farnebo buffer. Radioactivity remaining on the filters was counted with a liquid scintillation counter with an efficiency of about 50%. Specific binding is defined as total binding minus binding in the presence of 50 mM nipecotic acid (GABA uptake inhibitor). The results were statistically analyzed.

GABA release assays

Rat cortical slices (350 µm) were incubated with 3,3 µM [^{3}H]-glutamine in physiological buffer (121 mM NaCl, 1,8 mM KCl, 1,3 mM $CaCl_2$, 1,2 mM $MgSO_4$, 25

mM $NaHCO_3$, 1,2 mM KH_2PO4, 11 mM glucose, and 0,57 mM ascorbic acid, saturated with 95% O_2/5% CO_2, pH 7,4) for 45 min at 37° C. The slices were washed several times with buffer to remove non-specifically bound radioactivity.

The slices (n=4) were transferred into nylon mesh baskets and placed in 12 well plates with 3 ml buffer containing the test compounds and 1 mM nipecotic acid (GABA uptake inhibitor) per well. The plates were incubated for 10 min at room temperature. These wells were the basal fractions. The slices were than transferred into new plates with 3 ml stimulation buffer per well (buffer with 50 mM K^+, corresponding equimolar reduction of Na^+ to maintain osmolarity) containing the test substances and 1 mM nipecotic acid and the plates were incubated for 7,5 min. These wells were the stimulation fractions. The slices were removed and their wet weight was determined. The buffer samples were purified from the tritated metabolites of [^{3}H]-GABA by anion-exchange chromatography. The pH of the samples was adjusted to 4,0 with acetic acid and the diluted samples were applied to a DOWEX column equilibrated with sodium acetate. After washing the colums with 0,1% Triton X-100, GABA was eluted with 0,4 M Tris pH 7,5. GABA containing fractions were used for liquid scintillation counting.

Determination of GABA transaminase (GABA-T) activity

For the determination of GABA transaminase activity the rate of the reaction is determined by measuring the NADPH production at 340 nm at room temperature for 15 min within the linear range with a spectrophotometer in disposible PMMA cuvettes. GABase is a mixture of 4-aminobutyrate transaminase (E.C. 2.6.1.19) and succinate semialdehyde dehydrogenase (E.C. 1.2.1.16) from pseudomonas fluorescens.

One unit converts 1.0 µmole of γ-aminobutyric acid (GABA) to succinic semialdehyde and then to succinate per min with a stoichiometric reduction of 1,0 µmole of $NADP^+$ at pH 8,6 at 25°C.

Results

Binding of *Passiflora incarnata* extract to $GABA_A$ receptor

The specific $GABA_A$–receptor antagonist SR95531 competes for the binding of [^{3}H]-SR95531 to rat brain membranes in a concentration dependent manner (data not shown). The bound radioactivity in the presence of 100 µM SR95531 was defined as non-specific binding of the radioligand to rat brain membranes.

Passiflora incarnata extract competes for the binding of [^{3}H]-SR95531 to rat brain membranes in a concentration dependent manner (Fig. 1).

The IC_{50} value of *Passiflora incarnata* extract at this site was 101 µg/ml. Therefore, binding to the GABA-site of the $GABA_A$ receptor is a likely mode of action of *Passiflora incarnata* extract.

Figure 1: Competitive binding of *Passiflora incarnata* extract at the GABA-site of rat $GABA_A$ receptors. Competitive binding of *Passiflora incarnata* extract with [^{3}H]-SR95531 to rat brain membranes.

Data represent the mean specific binding ± S:E:M. of one experiment performed in duplicate. The results were confirmed in an independent second experiment.

Binding of *Passiflora incarnata* extract to $GABA_A$ receptor/benzodiazepine site

Diazepam competes with the binding of [^{3}H]-Ro-15-1788 (Flumazenil) - a highly specific antagonist of the benzodiazepine site of the $GABA_A$–receptor to rat brain membranes originating from cerebellum and hippocampus in a concentration-dependent manner. The competitive curve is shifted towards lower concentrations (to the left) in the presence of 10 µM GABA resulting in an about threefold decrease of the IC_{50} (data not shown). The bound radioactivity in the presence of 10 µM Diazepam was defined as non-specific binding of the radioligand to the membranes.

The IC_{50} values for competitive binding of *Passiflora incarnata* extract at the benzodiazepine site are very high (944 µg/ml). In addition, the binding to this site is not modulated by the presence of GABA as observed for the reference compound (Figure 2). Therefore, it is very unlikely that *Passiflora incarnata* extract acts via this binding site.

Figure 2: Competitive binding of *Passiflora incarnata* extract to the benzodiazepine site of $GABA_A$ receptors in rat cerebellum. Competitive binding of *Passiflora incarnata* extract with [^{3}H]-Ro-15-1788 binding to rat cerebellum membranes in the absence (■) or presence of 10 µM GABA (▲).

Data represent the mean specific binding ± S:E:M. of one experiment performed in duplicate. The results were confirmed in an independent second experiment and in two experiments with rat hippocampus membranes.

Binding of *Passiflora incarnata* extract to $GABA_A$ receptor/ethanol site

Ro-15-4513, the reference compound for the ethanol site, competes for the binding of [^{3}H]-Ro-15-4513 to rat brain membranes in a concentration-dependent manner. The IC_{50} values for competitive binding of *Passiflora incarnata* extract at the ethanol site was 512 µg/ml (data not shown). Therefore, it is very unlikely that *Passiflora incarnata* extract acts via this binding site.

Binding of *Passiflora incarnata* extract to $GABA_B$ receptor

Baclofen - a derivative of GABA that is a specific agonist of $GABA_B$ receptors - competes with the binding of [^{3}H]-CGP 54626 - a selective and potent $GABA_B$ receptor antagonist- to rat brain and rat hippocampus $GABA_B$ receptors in a concentration-dependent manner. The bound radioactivity in the presence of 1 mM Baclofen was defined as non-specific binding of the radioligand to the membranes.

Passiflora incarnata extract competes with the binding of [^{3}H]-CGP 54626 to rat brain and rat hippocampus $GABA_B$ receptors in a concentration-dependent manner the IC_{50} was 120 µg/ml (data not shown).

Binding of *Passiflora incarnata* extract to $GABA_B$ receptor ([^{35}S]-GTPγS-binding)

Baclofen - a derivative of GABA that is a specific agonist of $GABA_B$ receptors - stimulated the [^{35}S]-GTPγS binding to rat hippocampal membranes in a concentration-dependent manner (agonist mode). The selective and potent $GABA_B$ receptor antagonist CGP 54626 reduced the Baclofen-evoked signal in a concentration-dependent manner to the level of the basal signal (no effector added, antagonist mode).

Passiflora incarnata extract has a lower IC_{50} value (31 mg/ml) in the antagonist mode than in the agonist mode (115 mg/ml). Therefore *Passiflora incarnata* extract is an antagonist of the $GABA_B$ receptor (Figure 3).

Figure 3: Effect of *Passiflora incarnata* extract on rat hippocampal $GABA_B$-receptors. Concentration dependent effect of *Passiflora incarnata* extract on [^{35}S]-GTPγS-binding to rat hippocampal membranes.

Data represent the means ± SEM of one experiment carried out in duplicate and are normalized to 0% change. In the agonist mode (■) 0% change corresponds to the specific basal signal, in the antagonist mode 0% change corresponds to the maximal stimulation of the $GABA_B$ receptors by 100 µM baclofen (□). The results were confirmed in an independent second experiment.

GABA uptake and GABA release experiments

Up to 1000 µg/ml the *Passiflora incarnata* extract investigated in this study had no effect on potassium-evoked release of [^{3}H]-GABA synthesized from [^{3}H]-glutamine from rat cortical slices (data not shown).

The extract inhibited [^{3}H]-GABA uptake into rat cortical synaptosomes with an EC_{50} of 95.7 µg/ml (Figure 4).

Figure 4: Effects of a *Passiflora incarnata* extract on the GABA uptake

Radioactivity accumulated in the filters was normalized to the mean of control experiments. Raw data were statistically analysed and are expressed in the following data as mean ± CI_{95}, n=8.

Effects of *Passiflora incarnata* extract on GABA transaminase activity

The influence of the *Passiflora incarnata* extract on GABA transaminase activity was determined by measuring the NADPH production at 340 nm with a spectrophotometer. Vigabatrin (γ-vinyl GABA) an irreversible GABA transaminase inhibitor and effective antiepileptic was used as positive control. Vigabatrin significantly inhibited GABA transaminase with an EC_{50} of 55.5 mM. Incubation of GABA transaminase with five different concentrations of the passion flower dry extract (10, 50, 100, 250, 500 µg/ml) showed no effect on GABA transaminase.

Discussion

Extracts from passion flower (*Passiflora incarnata*) have been used to reduce anxiety and insomnia and there are numerous studies in mice and rats which demonstrate a reduced anxiety and stress with passion flower treatment (Dhawan et al. 2001; Miyasaka et al. 2007). In addition there is evidence that extracts from *Passiflora incarnata* may be helpful in the treatment of substance addictions as amphetamine, nicotine, marihuana and alcohol (Capasso und Sorrentino, 2005, Dhawan et al., 2002 and Dhawan and Sharma, 2003).

Although the anxiolytic activity of *Passiflora* species has been repeatedly evaluated in the last years, there is only limited information on the mechanism of action.

This study provides a comprehensive insight into the mode of action of a dry extract prepared from the flowers of *Passiflora incarnata* which is the sole active ingredient of the proprietary herbal drug Pascoflair® 425mg on the GABAergic system.

Preclinical studies have suggested that GABA levels may be decreased in animal models of depression, and clinical studies reported low plasma and CSF GABA levels in mood disorder patients. The *Passiflora incarnata* extract investigated in this study had no effect on potassium-evoked release of [^{3}H]-GABA synthesized from [^{3}H]-glutamine from rat cortical slices but the extract inhibited [^{3}H]-GABA uptake into rat cortical synaptosomes with a EC_{50} of 95,7 µg/ml. GABA transaminase (GABA-T), an enzyme target in the therapy of anxiety, epilepsy and related neurological disorders was not affected by *Passiflora incarnata* which is in accordance with already published results (Awad et al. 2007). Because preadministration of Flumazenil (Ro 15-1788), an antagonist of the benzodiazepine binding site of the $GABA_A$ receptor, attenuates the effects of *Passiflora incarnata in vivo* (Grundmann et al., 2008 and Medina et al., 1990)

it was assumed that *Passiflora incarnata* and Diazepam share the same pharmacology. Our study showed that the IC_{50} values for competitive binding of *Passiflora incarnata* extract at the benzodiazepine site are very high moreover the binding to this site is not modulated by the presence of GABA so it seems very unlikely that the mode of action of *Passiflora incarnata* extract includes binding to the benzodiazepine site. Similarly it is unlikely that it acts via the ethanol site. However, it is very likely that binding to the GABA-site of the $GABA_A$ receptor is one of the clinically relevant modes of action of *Passiflora incarnata* extract.

Another possible target might be the binding of *Passiflora incarnata* extract to the $GABA_B$ receptor. There is accumulating evidence that modulators of the $GABA_B$ receptor (a GPCR – G protein coupled receptor) might act as anxiolytic (Frankowska et al., 2007) and might be helpful in the treatment of substance addictions (Martin et al., 2009).

The *Passiflora incarnata* extract investigated in this study inhibited the binding of [^{3}H]-CGP 54626 to $GABA_B$-receptors in a concentration dependent manner. This could be verified using the [^{35}S]-GTPγS binding assay. It was found that *Passiflora incarnata* has a lower IC_{50} value in the antagonist mode than in the agonist mode. Therefore *Passiflora incarnata* needs to be classified as antagonist of the $GABA_B$ receptor. This opens possibilities for further investigations because $GABA_B$ antagonists may provide a pharmacological therapy for cognitive impairment (Helm et al. 2005).

Although the compounds responsible for the therapeutic activity of *Passiflora incarnata* are yet to be identified, this study provides novel evidence of the mechanism of action of a dry extract of *Passiflora incarnata* with respect to the GABAergic system.

Conflict of interest

Financial support for the analysis was provided by PASCOE pharmazeutische Präparate GmbH, the manufacturer of PASCOFLAIR® 425 mg. The sponsor had no influence on the conduct of the analysis.

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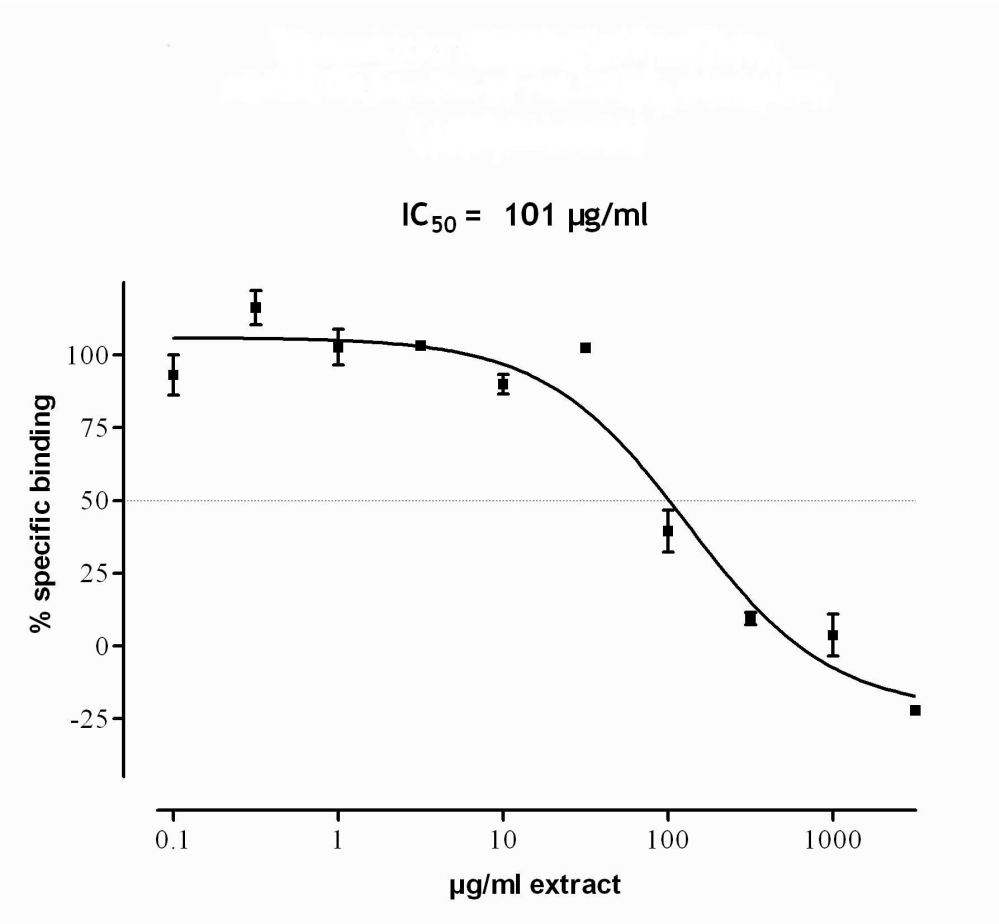

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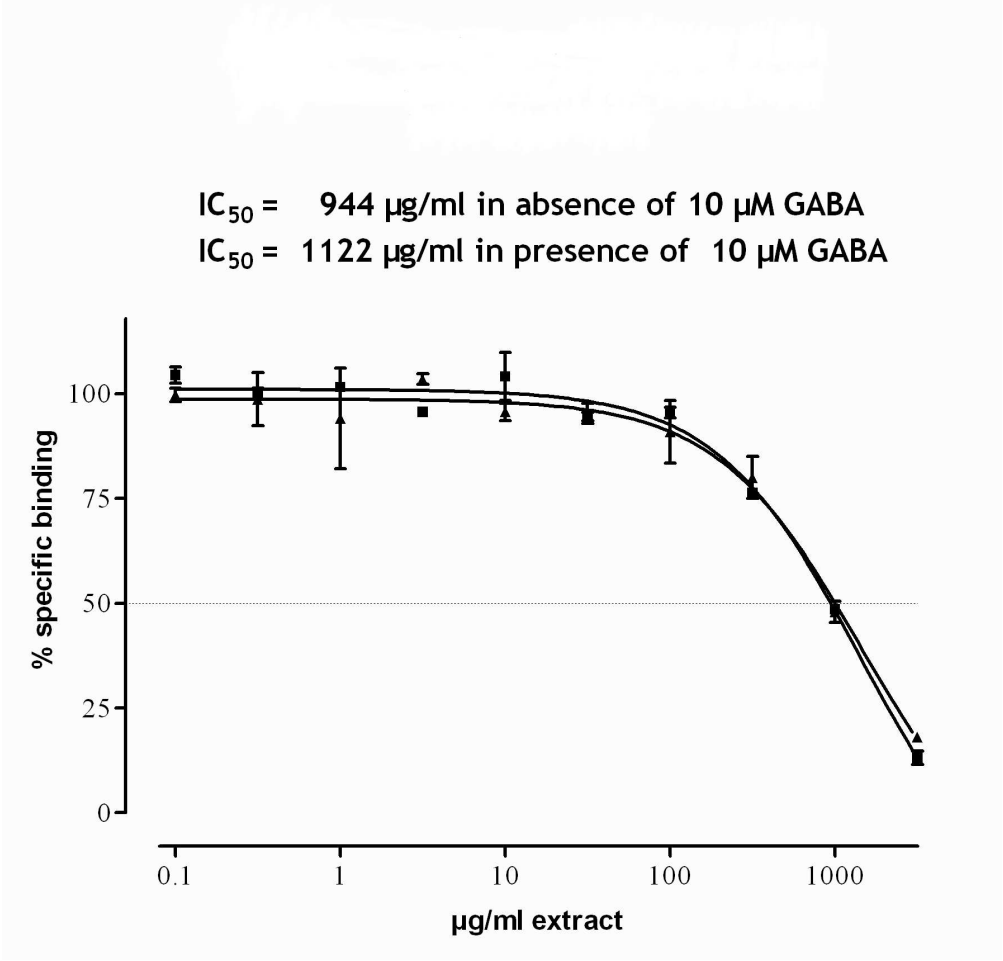

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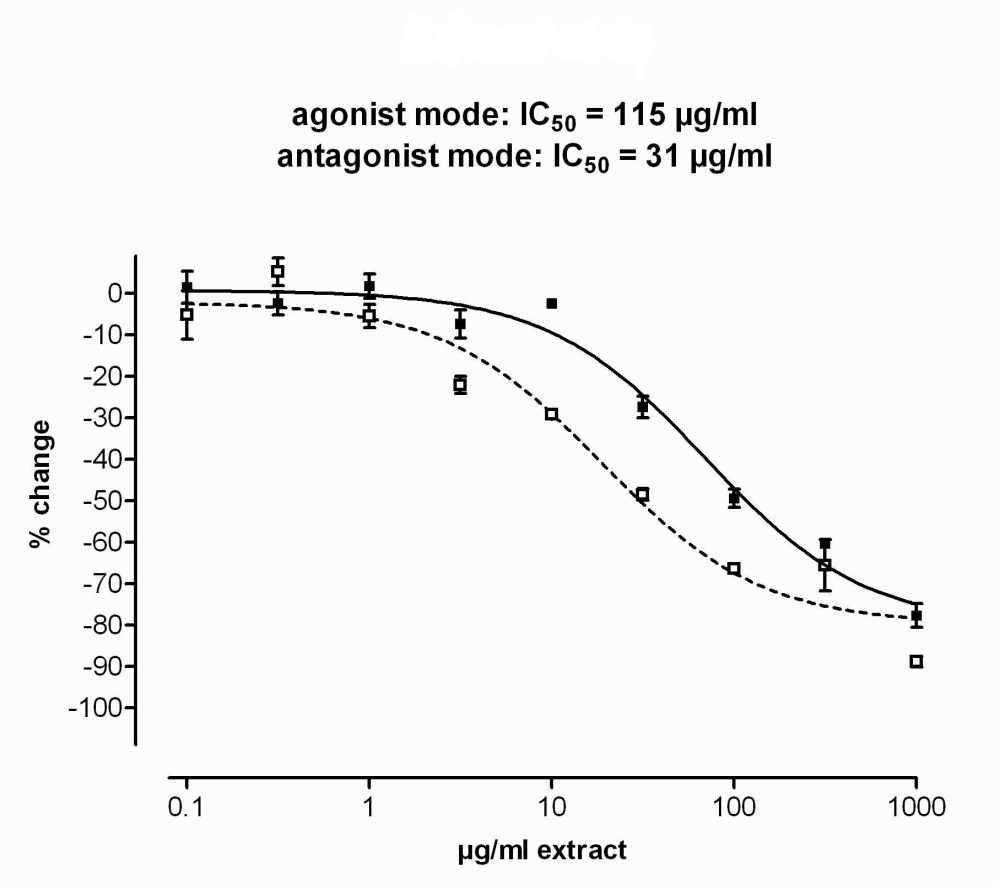
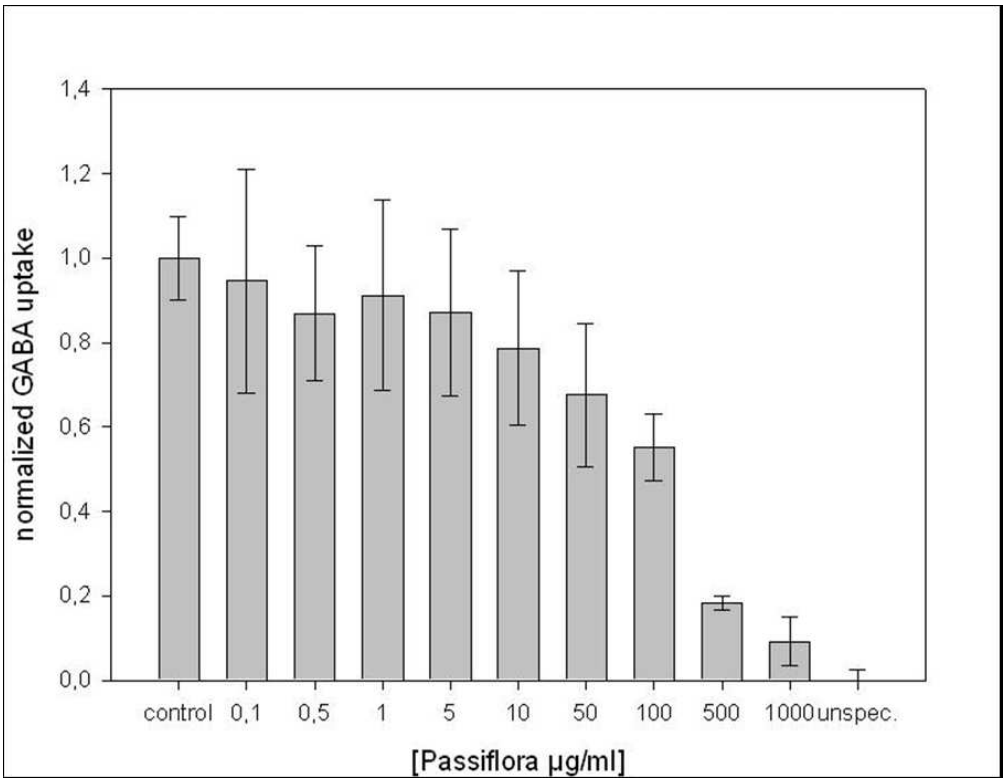


384x340mm (120 x 120 DPI)



153x118mm (150 x 150 DPI)