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Kampo Medicine: Evaluation of the Pharmacological Activity of 121 Herbal Drugs on $GABA_A$ and $5\text{-}HT_{3A}$ Receptors

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Kampo medicine is a form of Japanese phytotherapy originating from traditional Chinese medicine (TCM). During the last several decades, much attention has been paid to the pharmacological effects of these medical plants and their constituents. However, in many cases, a systematic screening of Kampo remedies to determine pharmacologically relevant targets is still lacking. In this study, a broad screening of Kampo remedies was performed to look for pharmacologically relevant $5\text{-}HT_{3A}$ and $GABA_A$ receptor ligands. Several of the Kampo remedies are currently used for symptoms such as nausea, emesis, gastrointestinal motility disorders, anxiety, restlessness, or insomnia. Therefore, the pharmacological effects of 121 herbal drugs from Kampo medicine were analyzed as ethanol tinctures on heterologously expressed $5\text{-}HT_{3A}$ and $GABA_A$ receptors, due to the involvement of these receptors in such pathophysiological processes. The tinctures of *Lindera aggregata* (radix) and *Leonurus japonicus* (herba) were the most effective inhibitory compounds on the $5\text{-}HT_{3A}$ receptor. Further investigation of known ingredients in these compounds led to the identification of leonurine from *Leonurus* as a new natural $5\text{-}HT_{3A}$ receptor antagonist. Several potentiating herbs (e.g., *Magnolia officinalis* (cortex), *Syzygium aromaticum* (flos), and *Panax ginseng* (radix)) were also identified for the $GABA_A$ receptor, which are all traditionally used for their sedative or anxiolytic effects. A variety of tinctures with antagonistic effects *Salvia miltiorrhiza* (radix) were also detected. Therefore, this study reveals new insights into the pharmacological action of a broad spectrum of herbal drugs from Kampo, allowing for a better understanding of their physiological effects and clinical applications.

Keywords: Kampo, *Leonurus japonicus* (herba), *Panax ginseng* (radix), *Salvia miltiorrhiza* (radix), andrographolide, leonurine, $5\text{-}HT_{3A}$ receptor, $GABA_A$ receptor

INTRODUCTION

The $5\text{-}HT_{3A}$ and $GABA_A$ receptors are ionotropic receptors within the cys-loop superfamily of ligand-gated ion channels and therefore possess closely related structures. Both of these receptors share a pentameric structure, with each subunit consisting of four transmembrane domains (Connolly and Wafford, 2004). The physiological agonists of the receptors are gamma

aminobutyric acid (GABA) and serotonin (5-HT), respectively. There are many pharmacological similarities between the 5-HT_3 and $GABA_A$ receptors. For example, the plant-derived compound picrotoxin acts as a non-competitive antagonist of both receptors (Das and Dillon, 2005), and local anesthetics, such as lidocaine and procaine, can antagonize either receptor (Hara and Sata, 2007; Ueta et al., 2007). A number of $GABA_A$ receptor potentiators or agonists, such as propofol, methohexital, and pentobarbital, are capable of antagonizing the 5-HT_3 receptor (Olsen et al., 1991; Cestari et al., 1996; Barann et al., 2000, 2008). However, while the 5-HT_3 receptor displays a cationic selectivity, activation of the $GABA_A$ receptor triggers an influx of Cl^-, resulting in the hyperpolarization of the cell and reduced neuronal excitability. Because of this, the activation of $GABA_A$ receptors leads to sedative effects. Therefore, $GABA_A$ receptor potentiators and agonists are broadly used for restlessness and insomnia (Calcaterra and Barrow, 2014). For example, the allosteric $GABA_A$ receptor potentiator diazepam is commonly used for psychiatric disorders, including anxiety and epilepsy (Calcaterra and Barrow, 2014). The 5-HT_3 receptors are also involved in many pathophysiological processes, such as gastrointestinal motility disorders and the development of nausea and vomiting. Therefore, compounds that act on this receptor have broad clinical relevance (Doak and Sawynok, 1997; Gershon, 2004; Jeggo et al., 2005; Costedio et al., 2007). Specific 5-HT_3 receptor antagonists such as ondansetron are mainly used for the treatment of nausea for various conditions, including chemotherapy-induced nausea and vomiting (CINV) and nausea during the postoperative phase (PONV; Cubeddu et al., 1994; Gyermek, 1995).

Kampo is a form of traditional Japanese phytomedicine originating from traditional Chinese medicine (TCM). Typically, Kampo is administered as a mixture of various herbal drugs that have complementary physiological activities. Kampo is broadly used in alternative and complementary medicine and has also recently become popular in Western countries. Therefore, there is a general interest in understanding the underlying pharmacological mechanisms of these herbal drugs, which may also help to increase the impact of Kampo in Western medicine. Several previous reports have described the pharmacological actions of specific components of single plants (e.g., gingerol from *Zingiber officinalis*) or complex Kampo preparations consisting of multiple components, such as rikkunshito (Takeda et al., 2008; Tominaga et al., 2011; Herbrechter et al., 2015), on pharmacologically relevant targets, including G-protein-coupled receptors (GPCRs) and ion channels. During the last decades, screening of the pharmacological activity of plant extracts and subsequent trials to identify their active ingredients has led to a plethora of pharmacologically useful substances. Kampo medicine depends on a relatively limited number of 148 well-described mostly plant-derived ingredients (Watanabe et al., 2011). However, only a few attempts were made to establish a systematic and comprehensive screening of the action of all of the important Kampo or TCM preparations on specific drug targets, such as ion channels. Nevertheless, such investigations have led to the identification of new pharmacological tools, as demonstrated by the screening of 50 Chinese herbal plants, which led to the identification of bisandrographolide as the first natural TRPV4 activator (Smith et al., 2006).

Several of the Kampo remedies are used to treat symptoms such as nausea, emesis, gastrointestinal motility disorders, anxiety, restlessness, and insomnia. Therefore, the pharmacological effects of 121 herbal compounds from Kampo medicine were analyzed as ethanol tinctures on the heterologously expressed 5-HT_{3A} and $GABA_A$ receptors, due to their involvement in the above pathophysiological processes. We aimed to investigate if there is a correlation between the pharmacological action of the Kampo compounds on these receptors and the corresponding medical application. We further sought to establish an activity ranking and identify the most potent tinctures as well as new active ingredients.

MATERIALS AND METHODS

Tinctures and Substances

The ethanol tinctures of 121 Kampo remedies were obtained from Dr. Peter Lepke (Kronen Apotheke Wuppertal, Germany). Tinctures were made by extracting the plant material in ethanol (1:5 w/v). The tinctures used in this study are listed in Supplementary Table S1. All of the chemicals were purchased from either Sigma–Aldrich (5-HT hydrochloride, gamma-aminobutyric acid, tannic acid, schizandrin, schizandrin B, leonurine, boldine, berberine chloride, liquiritigenin, hesperetin, kaempferol, andrographolide, linderane, rosmarinic acid, 4-hydroxybenzaldehyd, chlorogenic acid, caffeic acid), Carl Roth (rutin) or PhytoLab (atractylenolide III).

Expression System

The expression plasmid pRc/CMV contained cDNA coding for the human 5-HT_{3A} protein (Invitrogen; Lankiewicz et al., 1998), and psGEM contained cDNA for the α1, β2, and γ2 $GABA_A$ receptor subunits (Sergeeva et al., 2010). cRNAs were prepared using the AmpliCap T7 high-yield message marker kit (Epicenter, Madison, WI, USA), following the manufacturer's protocol. Oocytes were obtained as previously described (Sherkheli et al., 2010), and 7–20 ng receptor coding cRNA was injected into the oocytes using an injection setup from WPI (Nanoliter 2000, Micro4). The injected oocytes were then stored in ND 96 (96.0 mM NaCl, 2.0 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5.0 mM HEPES, pH 7.2, 200 U/ml penicillin, 200 μg/ml streptomycin) at 14°C. Electrophysiological experiments were performed 1–5 days (5-HT_{3A}) or 2–3 days ($GABA_A$) after the cRNA-injections.

Electrophysiology

To investigate the heterologously expressed 5-HT_{3A} and $GABA_A$ receptors, the two-electrode voltage-clamp technique was used as described previously (Saras et al., 2008). All of the recordings were performed in a normal frog ringer's (NFR) buffer (115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 10 mM HEPES, pH 7.2) for the $GABA_A$ receptor. Currents were recorded at a holding potential of approximately −60 mV (5-HT_{3A} receptor) or −40 mV ($GABA_A$ receptors), using the software Cell Works 6.1.1. (NPI).

During the measurements, the oocytes were placed in a chamber with a constant and unidirectional ringer flow, allowing the washout of the tested substances and tinctures. The ringer flow was interrupted for the application of test substances and tinctures until the maximal response was transcended. To exclude solvent effects, the compounds were tested at the maximal applied concentration (0.1 Vol.-%). Neither ethanol nor DMSO exhibited a direct activation or modulatory effect on the 5-HT_{3A} and $GABA_A$ receptors at the examined concentration (data not shown). In addition, the action of the tinctures on non-injected oocytes was tested (1:1,000 dilution; $n = 3$). None of the tinctures exhibited a direct activating effect with currents >15 nA (Supplementary Table S2). The observed effects were negligible compared with typical agonist-induced currents (∼2 μA). The direct activating effects of the Kampo tinctures were examined using a 1:1,000 dilution. The diluted tinctures were applied to *Xenopus laevis* oocytes expressing either the 5-HT_{3A} or α1β2γ2 $GABA_A$ receptor and compared the evoked responses with the response from 5 μM 5-HT or 100 μM GABA, respectively ($n = 3$–8). For the investigation of the modulatory effects, the tinctures were applied in a 1:1,000-dilutuion witch contains the native agonists ($n = 3$–8). Concentrations of 10 μM GABA for the $GABA_A$ receptor and 5 μM 5-HT for the 5-HT_{3A} receptor were used. To identify the active compounds in tinctures with antagonistic and potentiating effects, the known ingredients of plants with previously identified potential bioactivity were tested at a concentration of 1 mM. This approach was successfully used to identify pharmacologically active plant ingredients in a previous study (Herbrechter et al., 2015).

Data Analysis

The currents of the tested substances/tinctures were normalized to the means of the reference compounds 5-HT and GABA for the 5-HT_{3A} and α1β2γ2 $GABA_A$ receptors, respectively. Sigmoidal regression analysis was performed using the 3- or 4-parameter Hill equation (SigmaPlot 8.0., SPSS) to fit concentration-effect curves and calculate the IC/EC_{50} values. Deviations are represented by the standard error of the mean (SEM). Significant differences were determined using Student's *t*-test (Excel 2010, Microsoft), and multiple comparisons were corrected using the Benjamini–Hochberg-correction ($^*p < 0.05$; $^{**}p < 0.005$; $^{***}p < 0.0005$).

RESULTS

The Effect of Kampo Tinctures on the 5-HT_{3A} and $GABA_A$ Receptors

First, the direct activating effects of the tinctures were investigated. The tincture of *Ligusticum striatum* (rhizoma) showed the strongest activation of the 5-HT_{3A} receptor, with more than 30% of the 5-HT-induced current (**Figure 1**; Supplementary Table S2). The strongest activator of the $GABA_A$ receptor was *Panax ginseng* (rhizoma; Ginseng white), with a mean current amplitude of more than 40% of the GABA-induced current. The strongest 12 direct activating tinctures are shown in **Figure 1**.

Next, the modulatory effects of the tinctures at a 1:1,000 dilution was evaluated. In these experiments, agonist concentrations of 10 μM GABA for the $GABA_A$ receptor and 5 μM 5-HT for the 5-HT_{3A} receptor were used. A variety of tinctures with modulatory action for both receptor types were identified. For the 5-HT_{3A} receptor, tinctures with significant antagonistic and potentiating effects were identified (13 tinctures with potentiating effects and 22 tinctures with inhibitory potential). The strongest potentiation was observed with *Chaenomeles speciosa* (fructus), which potentiated the 5-HT-induced response with only 37% (**Figure 2**). In contrast, eight tinctures [*Tetradium ruticarpum* (fructus), *Magnolia biondii* (flos), *Lonicera japonica* (caulis), *Paeonia* × *suffruticosa* (cortex), *Coptis chinensis* (radix), *Epimedium brevicornum* (herba), *Leonurus japonicus* (herba), and *Lindera aggregata* (radix)) exceeded 50% inhibition of the receptor, with a maximal inhibition of more than 98% by the *Lindera aggregata* (radix) tincture. In addition, the $GABA_A$ receptors were significantly potentiated by 14 tinctures and inhibited by 24 tinctures. The *Syzygium aromaticum* (flos), *Clematis armandii* (caulis), *Magnolia officinalis* (cortex), *Mentha canadensis* (herba), *Scutellaria baicalensis* (radix), and *Panax ginseng* (rhizoma; Ginseng red) tinctures exhibited the strongest potentiation. In particular, Ginseng red potentiated up to 135% (**Figure 3**). The most effective inhibitory tinctures were *Salvia miltiorrhiza* (radix), *Caesalpinia sappan* (lignum), *Terminalia chebula* (fructus), *Gentiana macrophylla* (radix), *Saposhnikovia divaricata* (radix), and *Schisandra chinensis* (fructus). The inhibition observed by the *Salvia miltiorrhiza* (radix) tincture exceeded an inhibition of 80% of the GABA-induced currents.

Investigation of Established Ingredients in the Active Kampo Tinctures

For several of the active tinctures, the active ingredients have already been identified. However, for many of the Kampo herbs that have been shown to act on the 5-HT_{3A} or $GABA_A$ receptor, the active ingredients are unknown. The investigated substances and their related plants are listed in **Table 1** and Supplementary Table S3.

The modulatory effects of these substances on both receptors are shown in **Figure 4**. Overall, alkaloid compounds were found to be the most effective antagonists. In particular, boldine [*Lindera aggregata* (radix)] and leonurine [*Leonurus japonicus* (herba)] each possessed a higher efficacy for the 5-HT_{3A} receptor than the $GABA_A$ receptor. These compounds were the most potent 5-HT_{3A} receptor antagonists in the screening (**Figure 4**). The alkaloid berberine inhibited both receptor types at approximately 60%. Another identified 5-HT_{3A} receptor antagonist is the flavonoid (-)-liquiritigenin (Herbrechter et al., 2015), which showed nearly no effect on $GABA_A$ receptors in this screen. All of the tested flavonoids inhibited 5-HT_{3A} receptors with higher efficacy than $GABA_A$ receptors. However, many of the substances showed inhibitory potential rather than potentiating effects on the receptors, with the exception of schizandrin B, which slightly potentiated the $GABA_A$ receptor. Within the phenolic compound category,

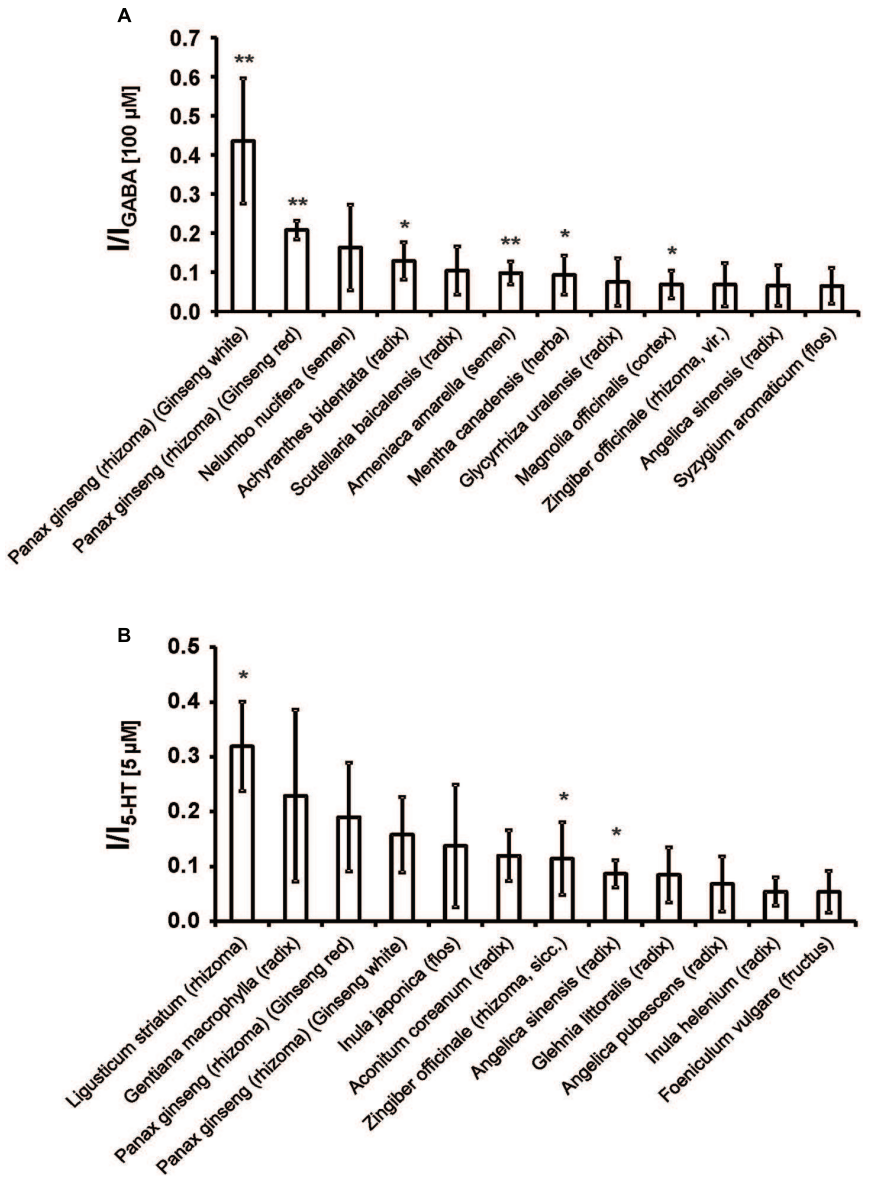

FIGURE 1 | The strongest 12 direct activating tinctures for the 5-HT$_{3A}$ (A) and GABA$_A$ receptors (B). The 121 tinctures were made from Kampo remedies via ethanol extraction (see section Tinctures and substances). A 1:1,000-dilution was applied to the oocytes and compared with agonist induced currents (5 µM 5-HT, 100 µM GABA). Error bars represent the SEM. Statistical significance was calculated based on the current evoked by ethanol (0.1 Vol.-%; *$p < 0.05$, **$p < 0.005$; $n = 3$–5).

tannic acid was identified as an antagonist of both receptor types, with an inhibition rate of more than 80%. Andrographolide and the lignane schizandrin were identified as weak GABA$_A$ receptor antagonists.

Concentration-Effect Curves of the Identified Ingredients with Potential Pharmaceutical Action

To quantify the antagonism of the identified active ingredients, concentration-effect curves were generated (**Figure 5**; Supplementary Table S4).

For the 5-HT$_{3A}$ receptor, boldine ($IC_{50} = 0.53 \pm 0.15$ µM) and leonurine ($IC_{50} = 2.17 \pm 0.15$ µM) were more potent inhibitors than tannic acid ($IC_{50} = 48.2 \pm 4.1$ µM) and schizandrin ($IC_{50} = 137 \pm 22$ µM). These four substances had approximately equal IC_{50} values for the GABA$_A$ receptor (∼100 µM, Supplementary Table S4), with the exception of schizandrin ($IC_{50} > 600$ µM). Andrographolide ($IC_{50} = 66.1 \pm 26.8$ µM) was found to be the most potent GABA$_A$ receptor antagonist. However, it also acted as a partial antagonist of the GABA$_A$ receptor, as even a concentration of 3 mM reduced the GABA-evoked current to 30% of the GABA

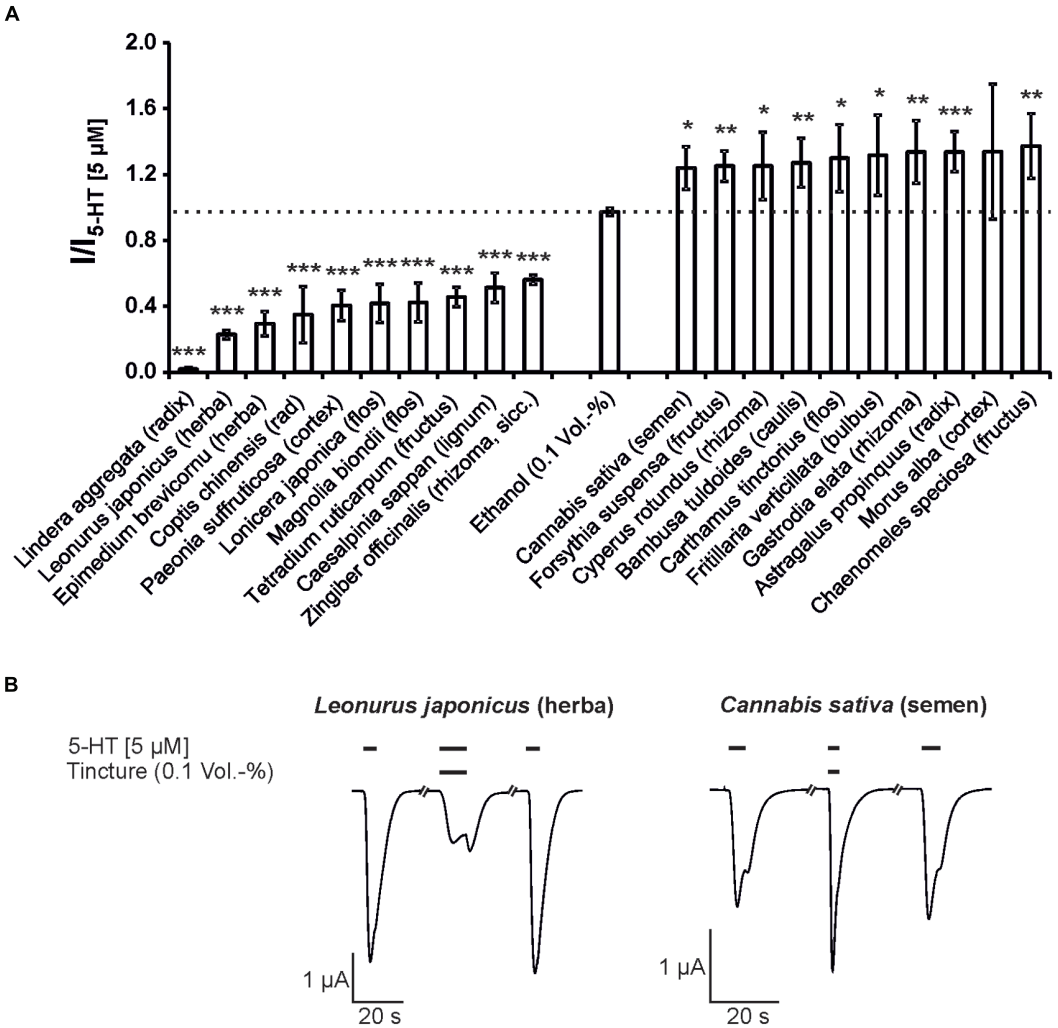

FIGURE 2 | The modulatory action of the strongest inhibitory and potentiating Kampo tinctures on the 5-HT$_{3A}$ receptor. (A) The tinctures with the 10 strongest antagonistic (left) and potentiating (right) effects are shown (5 µM 5-HT). Bars exceeding the dotted line represent potentiating effects. Statistical significance was calculated based on the reference solution containing 0.1 Vol.-% ethanol (equal to the max. ethanol concentration in the tinctures; $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$; $n = 3$–8). **(B)** Original registrations of the modulation observed by *Leonurus japonicus* (herba) and *Cannabis sativa* (semen) using a 1:1,000 dilution are shown. The effects were reversible following a 150-s washout with ringer's solution.

reference (**Figure 5B**). Leonurine shifted the EC_{50} for 5-HT at the 5-HT$_{3A}$ receptor from 2.5 µM determined in parallel under the same experimental conditions in our laboratory (Herbrechter et al., 2015) to 14.8 µM. While 30 µM leonurine almost completely blocked the evoked current at lower agonist concentrations, it was nearly ineffective at 5-HT concentrations ≥100 µM, suggesting a competitive antagonism of leonurine at the 5-HT$_{3A}$ receptors.

DISCUSSION

In this screening, two classical drug targets, the 5-HT$_3$ and GABA$_A$ receptors, were investigated. Several Kampo remedies have anxiolytic, sedative, antiemetic, or digestive effects, suggesting that these receptors may be the pharmacological targets of Kampo compounds. Therefore, 121 Kampo remedies were screened as ethanol extracts on heterologously expressed α1β2γ2 GABA$_A$ and homomeric 5-HT$_{3A}$ receptors, using the two-electrode voltage-clamp technique. As a result, several remedies that can modulate the 5-HT- or GABA-induced currents were found. An activity ranking of the activity of each plant preparation on these receptors was established. By testing single substances that are present in the active plant extracts, several new 5-HT$_{3A}$ blockers and GABA$_A$ receptor modulators were identified.

Pharmacologically Active Kampo Remedies: 5-HT$_{3A}$ Receptor

During the screening for compounds that are pharmacologically active at the 5-HT$_{3A}$ receptor, a number of remedies with

TABLE 1 | Investigated ingredients and their respective plants.

Substance	Kampo remedy	Reference
4-hydroxybenzaldehyd	*Lonicera japonica* (caulis)	Wang et al., 2013
andrographolide	*Andrographis paniculata* (herba)	Song et al., 2013
Atractylenolid III	*Atractylodes lancea* (rhizoma) *Atractylodis macrocephala* (rhizoma)	Shao et al., 2014
Berberine	*Coptis chinensis* (radix)	Lin et al., 2004
Boldine	*Lindera aggregata* (radix)	Han et al., 2008
Chlorogenic acid	*Inula helenium* (radix) *Lonicera japonica* (caulis)	Eberhard, 2003; Wang et al., 2014
Caffeic acid	*Inula helenium* (radix)	Wang et al., 2014
Hesperetin	*Citrus* × *aurantium* (fructus) *Citrus trifoliata* (fructus) *Citrus reticulata* (*pericarpium, vir.*) *Citrus reticulata* (pericarpium)	Zhao et al., 2015
Kaempferol	*Houttuynia cordata* (herba)	Lin et al., 2013
Leonurine	*Leonurus japonicus* (herba)	Chen and Kwan, 2001
Linderane	*Lindera aggregata* (radix)	Li et al., 2002
Liquiritigenin	*Glycyrrhiza uralensis* (radix)	Rauchensteiner et al., 2005; Kondo et al., 2007
Rosmarinic acid	*Salvia miltiorrhiza* (radix)	Adams et al., 2006; Huang et al., 2008
Rutin	*Citrus* × *aurantium* (fructus) *Citrus trifoliata* (fructus) *Citrus reticulata* (*pericarpium, vir.*) *Citrus reticulata* (pericarpium) *Morus alba* (folia)	Hunyadi et al., 2012; Zhao et al., 2015
Schizandrin B	*Schisandra chinensis* (fructus)	Pan et al., 2008
Schizandrin	*Schisandra chinensis* (fructus)	Panossian and Wikman, 2010
Tannic acid	*Syzygium aromaticum* (flos)	Bhowmik et al., 2012

antagonistic properties were identified. Several of these remedies, such as ginger [*Zingiber officinalis* (rhizoma)], have well-described antiemetic effects and are used for the treatment of nausea and gastrointestinal disorders (Ernst and Pittler, 2000; Haniadka et al., 2012; Ding et al., 2013). During our screen, rhizoma from *Zingiber off. sicc.* and *Zingiber off. vir.* exhibited antagonistic effects, and the effect of *Zingiber off. sicc.* (desiccated) was superior to that observed with the tincture of fresh rhizoma from *Zingiber off. vir*. This increased inhibition may be due to the manufacturing process of the tinctures, as a higher amount of dry matter was used for the *Zingiber off. sicc.* tincture, and this may have resulted in an increase in the concentration of the active ingredients within the tincture. Vanilloids from the gingerol and shogaol group of compounds, pungent substances of ginger, are well-known 5-HT_{3A} receptor antagonists. This may explain, in part, the observed inhibition of 5-HT-induced currents that have been reported previously (Walstab et al., 2013; Ziemba et al., 2015). In addition, terpenes within the essential oil of ginger, including geraniol, citronellol, linalool and galanolactone, were shown to antagonize the 5-HT_{3A} receptor (Huang et al., 1991; Ziemba et al., 2015). The antagonistic potential of *Panax ginseng* (radix) tinctures may be mediated by ginsenosides, which act in the pore region of the 5-HT_{3A} receptor, and its antiemetic properties have been previously described (Min et al., 2003; Kim et al., 2005; Lee et al., 2007). The *Magnolia biondii* (flos), *Mentha canadensis* (herba), *Glycyrrhiza uralensis* (radix) and *Syzygium aromaticum* (flos) tinctures, which are also reported to have antiemetic effects (World Health Organization, 2005; Bhowmik et al., 2012; Herbrechter et al., 2015), were among the top 20 inhibitory tinctures in the screening. *Syzygium aromaticum* (flos) contains the phenolic compounds eugenol, gallic acid, and tannins, as well as the flavonoid quercetin (Atawodi et al., 2011; Bhowmik et al., 2012). Instead of eugenol (the main flavoring ingredient in *Syzygium aromaticum*), which shows a negligible level of antagonism (Ziemba et al., 2015), tannic acid inhibits 5-HT-evoked currents with an IC_{50} value of approximately 50 μM (**Figure 5**). In addition to tannic acid, quercetin may contribute to the antagonistic effect of the clove tincture (Lee et al., 2008). *Glycyrrhiza uralensis* (radix) contains a structurally related flavonoid, liquiritigenin, which has been shown to antagonize the 5-HT_{3A} receptor in a previous study. This finding may help us to understand the antagonistic effect of this tincture (Herbrechter et al., 2015). Another flavonoid from licorice, named glabridin, was identified as a partial antagonist of the 5-HT_{3A}-receptor and a potentiator of $GABA_A$-receptors (Herbrechter et al., 2015; Hoffmann et al., 2016). The antagonistic effect of *Mentha canadensis* (herba) may be explained through the action of terpenes, which are known to inhibit the 5-HT_{3A} receptor. Menthol, a terpene from the essential oil of *Mentha canadensis* (herba), was recently characterized as a 5-HT_3 receptor antagonist (Walstab et al., 2014). In addition, *Coptis chinensis* rhizomes (Tjong et al., 2011) and *Lonicera japonica* (flos; Jung et al., 2014) may be useful for the treatment of gastrointestinal disorders through the action 5-HT_3 receptors, as discussed below.

The most potent inhibitory effects were observed for the tinctures of *Lindera aggregata* (radix) and *Leonurus japonicus* (herba; **Figure 2**; Supplementary Table S2) for which no connection to gastrointestinal disorders is described in the literature. By screening known constituents of these plants, boldine (*Lindera*) and leonurine (*Leonurus*) are found as potent inhibitors of the 5-HT_{3A} receptor with an IC_{50} between 0.5 and 2.3 μM (**Figures 5** and **6**). Boldine, which has been shown to ameliorate chemotherapy-induced nausea and vomiting as well as symptoms from irritable bowel syndrome, is an alkaloid from the aporphine class. Boldine has been previously shown to antagonize the 5-HT_{3A} receptor in a competitive manner in a luminescence-based cell assay (Walstab et al., 2014). The contribution of further ingredients, such as the related alkaloids norboldine, reticuline, and linderegatine, to the antagonistic effect of the *Lindera* tincture is likely (Han et al., 2008). Leonurine from *Leonurus* was shown to affect cardiac function via creatine kinase inhibition (Wang et al., 2004) and to also reduce platelet aggregation (Lin et al., 2007). These effects are in accordance with some of the traditional uses of *Leonurus*. The antagonistic effects of *Leonurus* and the identification of leonurine as a potent competitive 5-HT_{3A} receptor antagonist are reported here for the first time. Therefore, this study reveals new

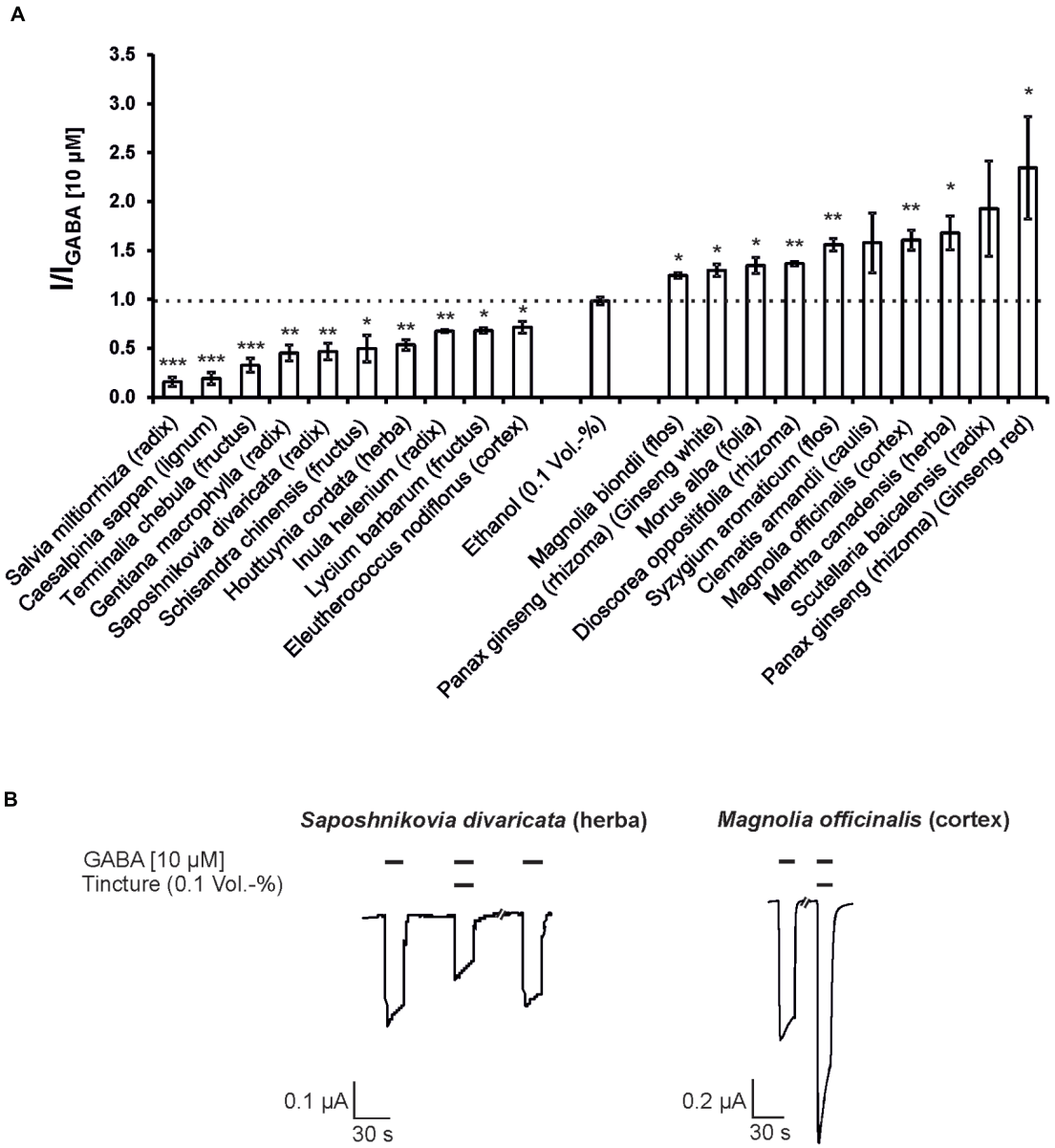

FIGURE 3 | The modulatory action of the strongest inhibitory and potentiating Kampo tinctures on the $GABA_A$ receptors. (A) The tinctures with the 10 strongest antagonistic (left) and potentiating (right) effects are shown (10 µM GABA). Statistical significance was calculated based on the reference solution containing 0.1 Vol.-% ethanol (equal to the max. ethanol concentration in the tinctures; $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$; $n = 3–8$). **(B)** Original registrations of the modulation observed via *Saposhnikovia divaricata* (herba) and *Magnolia officinalis* (cortex) with a 1:1,000 dilution are shown. The effects were reversible following a 150-s washout with ringer's solution.

insights into the pharmacological action of *Leonurus*. However, the traditional application of *Leonurus* is not correlated with the pharmacology of the 5-HT_3 receptor (Shang et al., 2014). Therefore, further studies examining if *Leonurus* can antagonize the 5-HT_{3A} receptor *in vivo* are needed.

Pharmacologically Relevant Kampo Remedies: $GABA_A$ Receptor

The $GABA_A$ receptors are inhibitory receptors and therefore reduce neuronal excitability. A variety of disorders are attributable to an imbalance of the GABAergic system. Therefore, benzodiazepines, such as diazepam, potentiate the GABA-induced responses and are used to treat disorders such as insomnia, restlessness, anxiety, and epilepsy (Calcaterra and Barrow, 2014).

In this screening, the potentiating activity of the tinctures correlated well with the medical use of the plants. For many of the top 10 potentiation tinctures, the active ingredients had been previously identified. The best potentiator, *Panax ginseng* (rhizoma; Ginseng red) is likely one of the most popular herbal

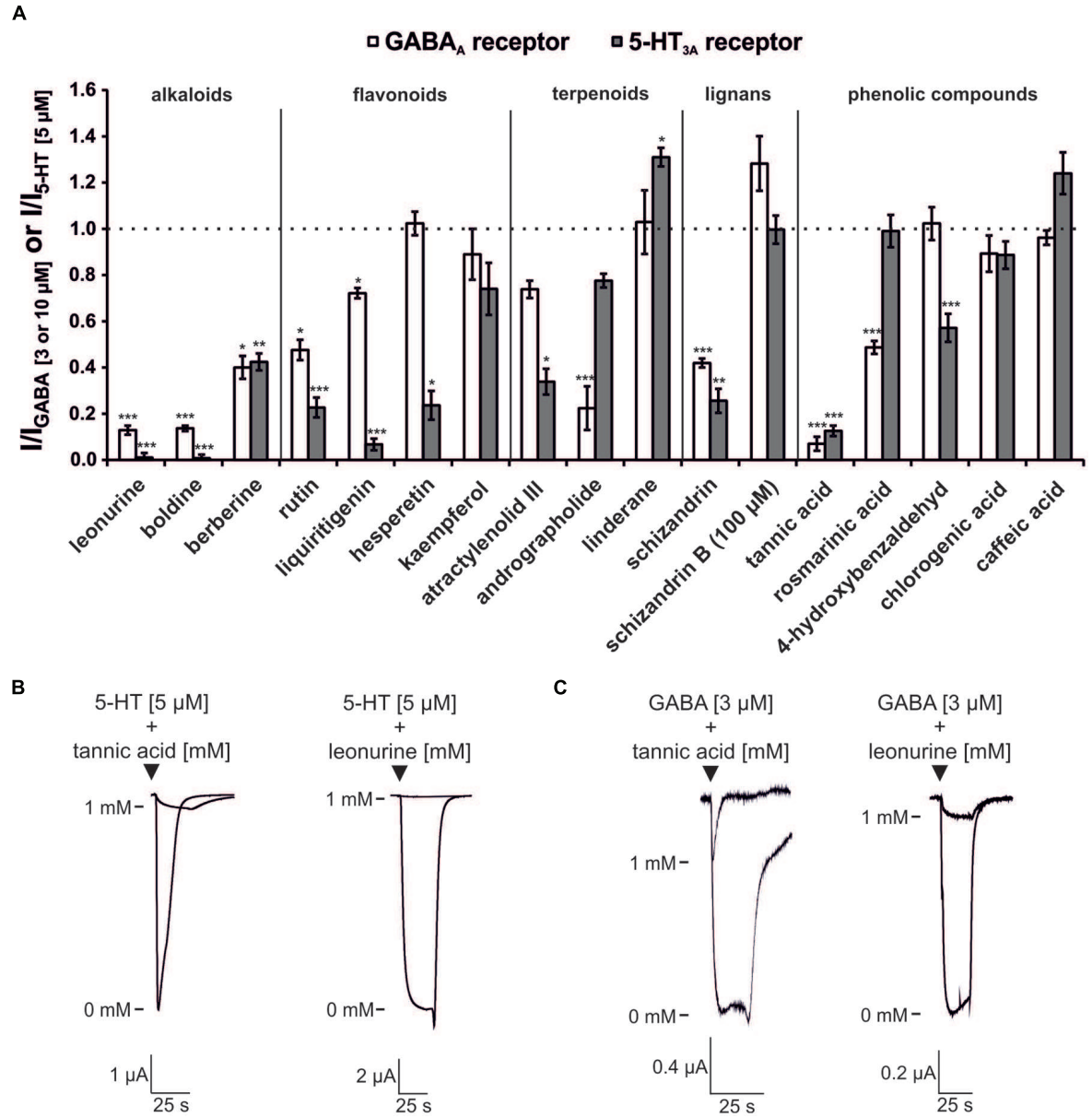

FIGURE 4 | Modulatory effects of the investigated ingredients on the 5-HT$_{3A}$ and GABA$_A$ receptors. (A) The bars represent the normalized current amplitude for the modulation of the 5-HT$_{3A}$ (gray bars) and GABA$_A$ (white bars) receptors. With the exception of schizandrin B, which was tested at 100 µM, all of the compounds were evaluated at a concentration of 1 mM. Bars exceeding the dotted line represent substances with potentiating effects. The substances are arranged based on their affiliation to classes of plant metabolites, as indicated by the vertical lines. Statistical differences were calculated based on the reference agonist, which contained an equal concentration of the solvent used to dissolve the test compounds (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$; $n = 4$–7). **(B)** Original registrations of the inhibition of the 5-HT$_{3A}$ receptor by tannic acid and leonurine. **(C)** Original registrations of the inhibition of the GABA$_A$ receptor by tannic acid and leonurine.

drugs. It is traditionally used for multiple purposes, including the treatment of anxiety and insomnia (Kim et al., 2005; Xiang et al., 2008; Lee et al., 2013). The ginsenosides (steroid glycosides) present in this compound may be responsible for the potentiation and hence the sedative and anxiolytic effects of ginseng, due to their potentiating effect on the GABA$_A$ receptor. The ginsenosides Rg$_3$ and Rc have been shown to potentiate the heterologously expressed GABA$_A$ receptor, and ginsenoside Rc also possesses agonistic properties toward the GABA$_A$ receptor (Choi et al., 2003; Lee et al., 2013). This may explain the observed direct activating effect of the extract (**Figure 1**). Two *Panax ginseng* (radix) tinctures were also tested, which differ in their manufacturing process. Whereas Ginseng red is desiccated directly after harvesting, Ginseng white is peeled and whitened prior to the desiccation process. Despite these manufacturing differences, both compounds were of the best nine potentiating

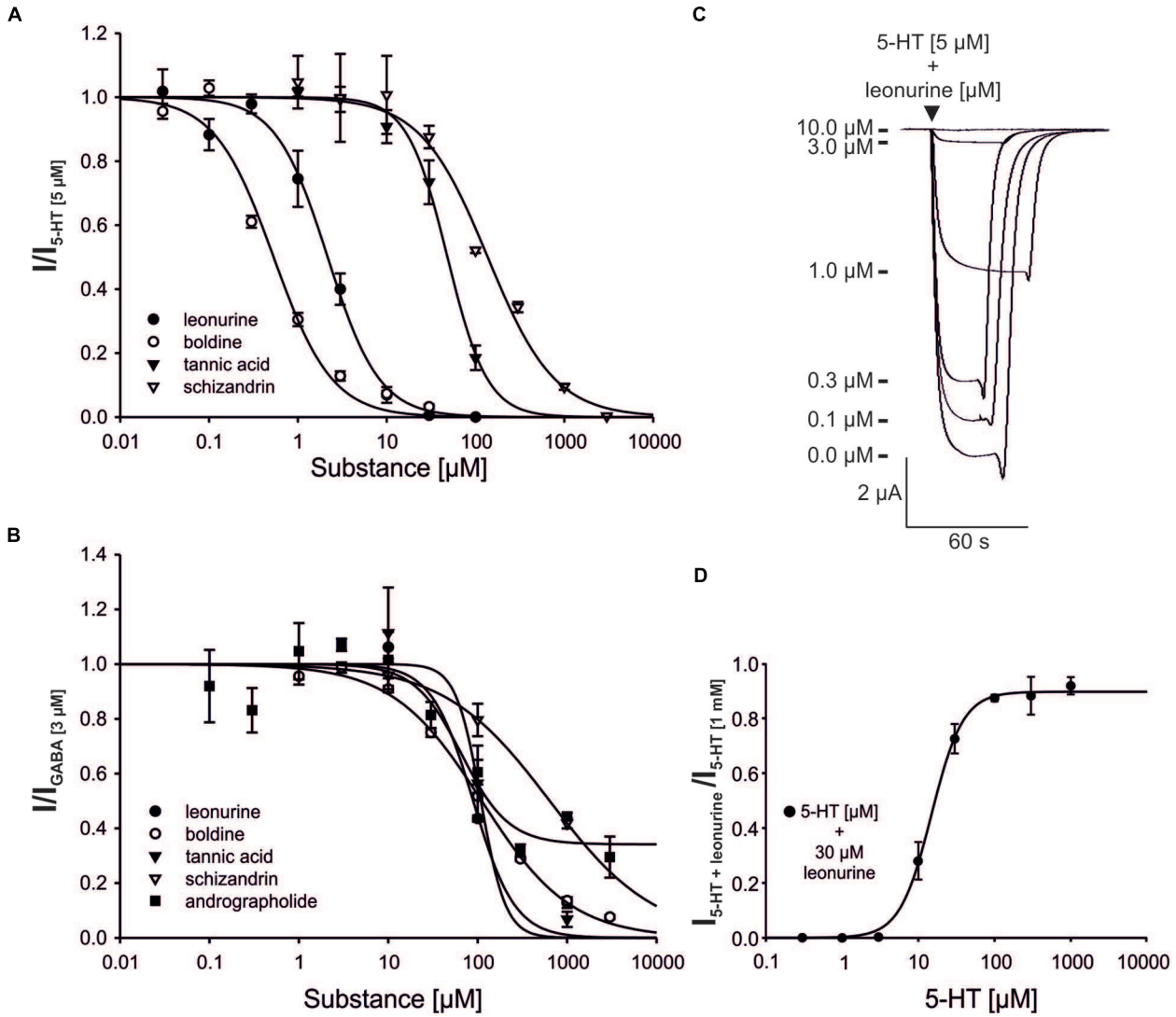

FIGURE 5 | Concentration-inhibition curves of select plant ingredients for the 5-HT$_{3A}$ (A) and GABA$_A$ (B) receptor, as well as the original registrations of leonurine for the 5-HT$_{3A}$ receptor (C) and the 5-HT concentration-effect curve in the presence of leonurine (D). (A,B) The agonist concentrations used in the experiment were 5 µM (5-HT) or 3 µM (GABA). Error bars represent the SEM (**A**: $n = 5$–7, **B**: $n = 4$–7). Andrographolide acts as a partial antagonist of the GABA$_A$ receptor. **(C)** Overlayed 5-HT$_{3A}$ receptor responses, modulated by different concentrations of leonurine (0.1–10 µM) are shown. **(D)** The concentration-effect curve of 5-HT at the 5-HT$_{3A}$ receptor in the presence of leonurine (30 µM) is shown. The determined EC$_{50}$ value was found to be 14.8 µM 5-HT ($n = 4$).

tinctures. However, Ginseng red is superior to Ginseng white due to its increased potentiation (135% instead of 30%; **Figure 3**; Supplementary Table S2).

The *Scutellaria baicalensis* (radix) tincture potentiated the GABA$_A$ receptor at a similar level to *Panax ginseng* (rhizoma; Ginseng red; **Figure 3**; Supplementary Table S2), and it was also previously linked to GABA$_A$ receptor-related effects. *Scutellaria baicalensis* (radix) possesses anticonvulsant, sedative and anxiolytic effects, which may be explained by the action of the flavone wogonin (Hui et al., 2002; Park et al., 2007). Wogonin was identified as a ligand for the benzodiazepine site of the GABA$_A$ receptor and was shown to potentiate GABA$_A$ receptors in electrophysiological assays (Hui et al., 2002). The observed anxiolytic effects of wogonin in mice were similar to that of diazepam, emphasizing the potency of wogonin (Hui et al., 2002). The *Mentha canadensis* (herba) and *Magnolia officinalis* (cortex) tinctures also exhibited a potentiating effect in the screening (**Figure 3**; Supplementary Table S2). The active components of *Magnolia officinalis* (cortex) are the lignans magnolol and its isomer honokiol, whose potentiation of the GABA$_A$ receptor is accountable for the anticonvulsant, antidepressive, and anxiolytic effects of Magnolia bark (Taferner et al., 2011; Alexeev et al., 2012). The potentiation of the GABA$_A$ receptor by the *Mentha* tincture likely occurs due to the action of menthol and the structurally related monoterpenoids, which were shown to potentiate GABA$_A$ receptors in a previous study (Hall et al.,

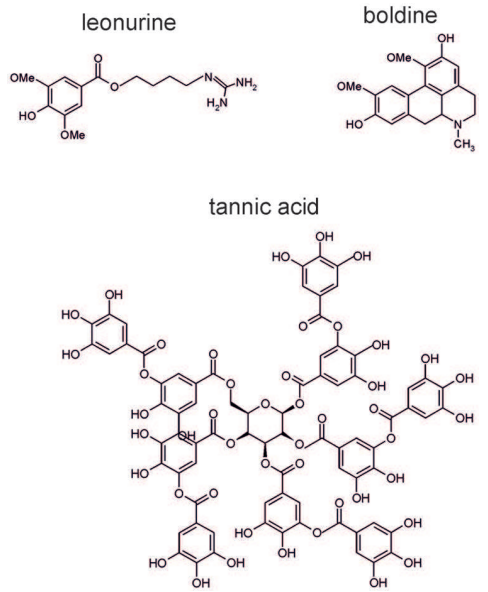

FIGURE 6 | Molecular structures of the alkaloids leonurine, boldine, and tannic acid.

2004). In addition, extracts of *Morus alba* displayed anxiolytic effects under different experimental paradigms, suggesting that this compound may also act on the $GABA_A$ receptor (Yadav et al., 2008). Our results support this idea, as *Morus alba* (folia) extracts belonged to the top 10 most potent $GABA_A$ potentiators. However, the active component in *Morus alba* (folia) extract is still unknown.

The sixth-strongest potentiation was observed by the clove tincture [*Syzygium aromaticum* (flos)], which has anti-inflammatory, antimicrobial, antioxidant, and anesthetic effects (Chaieb et al., 2007). *In vivo*, eugenol, the main ingredient of the clove tincture, exhibits a sedative effect in mice and rats (Guenette et al., 2006; Sharma et al., 2012) and has also been shown to potentiate the effects of the $GABA_A$ receptor *in vitro* (Aoshima and Hamamoto, 1999). Other potentiators were also identified, including carvacrol and thymol (Priestley et al., 2003; Kessler et al., 2014). In addition to eugenol, *Syzygium aromaticum* (flos) contains carvacrol and thymol (Priestley et al., 2003; Kessler et al., 2014), and all three are potentiators of the $GABA_A$ receptor. The mutual action of these compounds may explain the sedative effects of *Syzygium aromaticum* (flos).

Antagonism of the $GABA_A$ receptor is typically accompanied by stimulating effects at low doses but can cause seizures and anxiety at higher concentrations. Kampo remedies with antagonistic effects were detected, including the tinctures of *Salvia miltiorrhiza* (radix), *Terminalia chebula* (fructus), and *Schisandra chinensis* (fructus; **Figure 3**; Supplementary Table S2). However, we could not find an anxiogenic or seizure-inducing adverse side effect for these antagonistic tinctures in the literature. The *Schisandra chinensis* (fructus) tincture, which inhibited 50% of the GABA-induced current at a 1:1,000 dilution (**Figure 3**; Supplementary Table S2), possesses side effects such as restlessness and tension, when given at high doses (Eberhard, 2003). These effects may be explained, at least in part, by its antagonism of the GABA receptor. Nevertheless, *Schisandra chinensis* (fructus) is traditionally used for its hepatoprotective and sedative effects; the latter is based on the influence of the GABAergic and serotonergic systems, as the levels of their respective neurotransmitters can be altered in the brain (Ip et al., 1996; Wei et al., 2013; Zhang et al., 2014). The lignanoid schizandrin was identified as a weak inhibitor of the $GABA_A$ receptor (**Figures 4** and **5**; Supplementary Table S4), presumably acting in a non-competitive manner (Supplementary Figure S1). However, its contribution to the effect of the tincture remains hypothetically, with regard to the low potency.

The *Andrographis paniculata* (herba) tincture exhibited a weak antagonism (Supplementary Table S2). However, the tested labdane diterpenoid andrographolide, which possesses antiphlogistic, anticancer, and neuroprotective effects (Chan et al., 2010; Chun et al., 2010; Kou et al., 2014; Lin et al., 2014), is a partial antagonist of the $GABA_A$ receptor (**Figure 5**; Supplementary Table S4). Moreover, the action of andrographolide appears to be non-competitive in nature (Supplementary Figure S2). The andrographolide content of the *A. paniculata* leaves was approximately 1% of the dry weight (Chao and Lin, 2010). Hence, the concentration in the applied tincture can be approximated ($\approx$6 μM). Therefore, the antagonistic effect of the tincture is not solely due to the action of andrographolide.

The *Salvia miltiorrhiza* (radix) tincture exhibited the strongest antagonistic effect on the $GABA_A$ receptor, exceeding a blocking effect of 80% (**Figure 3**; Supplementary Table S2). This compound is traditionally used for the treatment of cardiovascular diseases and ischemia, and it inhibits platelet aggregation via the action of tanshinones (Han et al., 2008). One specific tanshinone, miltirone, was identified as a ligand at the benzodiazepine binding site of the $GABA_A$ receptor in the CNS, based on ligand binding assays (Lee et al., 1991). Nevertheless, miltirone failed to modulate the GABA-induced current of the $GABA_A$ receptor in a recombinant expression system (*Xenopus* oocytes) as well as in cultured rat hippocampal pyramidal cells (Mostallino et al., 2004). Hence, the involvement of miltirone in the observed inhibition of $GABA_A$ receptor responses by the *Salvia miltiorrhiza* tincture seems unlikely. Additionally, rosmarinic acid, a monoterpenoid from *Salvia miltiorrhiza* (Adams et al., 2006), only showed a slight inhibition of the $GABA_A$ receptor in the screening (**Figure 4**; Supplementary Table S3) and therefore does not account for the strong antagonistic effect of the tincture.

Salvia miltiorrhiza and *Astragalus propinquus* are compounds in a two-ingredient intermixture called myelophil. This mixture has been shown to reduce chemotherapy-correlated adverse side effects, including myelosuppression and anemia, from the cytostatic drug fluorouracil (Shin et al., 2008) and possesses no described side effects itself (Jung et al., 2009). Furthermore, myelophil reduced fatigue in patients suffering from chronic fatigue (Cho et al., 2009) and exhibited anti-amnesic effects in scopolamine-treated mice (Lee et al., 2014). Even if the latter is presumably caused by the increased expression of ERKs and mAChR1 (Lee et al., 2014), a contribution of the

inhibition of *Salvia miltiorrhiza* on the $GABA_A$ receptor to the anti-fatigue effects of myelophil is likely, due to the increased neuronal excitability resulting from $GABA_A$ receptor inhibition. In contrast to *Salvia miltiorrhiza*, *Astragalus propinquus* did not affect the $GABA_A$ receptor in this study (Supplementary Table S2).

CONCLUSION

We believe that a broad screening of the ethanol tinctures of herbal remedies via electrophysiological assays is a reliable method to obtain an overview of the pharmacological action of these compounds on clinically relevant receptors. In this study, a variety of tinctures were detected, whose pharmacological actions on the investigated receptors are in agreement with their traditional uses and physiological effects. For example, the antiemetic remedies *Zingiber officinalis* (rhizoma), *Panax ginseng* (radix), and *Syzygium aromaticum* (flos) were identified as antagonists of the $5\text{-}HT_{3A}$ receptor, and sedative drugs such as *Panax ginseng* (radix), *Scutellaria baicalensis* (radix), and *Mentha canadensis* (herba) were found as $GABA_A$ receptor potentiating tinctures. Furthermore, *Lindera aggregata* (radix) and *Leonurus japonicus* (herba) are identified as potent $5\text{-}HT_{3A}$ receptor antagonists. Boldin was recently identified as a potent $5\text{-}HT_{3A}$ receptor blocker (Walstab et al., 2014) and is likely the active component of *Lindera aggregata* (radix). In this study, leonurine was identified as a new natural $5\text{-}HT_{3A}$ receptor antagonist. It inhibited the $5\text{-}HT_{3A}$ receptor with a similar potency to boldine. Furthermore, tinctures that antagonize the $GABA_A$ receptor, were detected. E.g., *Salvia miltiorrhiza* (radix), which is part of a Kampo remedy called myelophil, blocks $GABA_A$ receptor responses with high efficacy. Myelophil was previously shown to ameliorate fatigue symptoms. Therefore, we hypothesized that *Salvia miltiorrhiza* (radix) may contribute to the anti-fatigue effects of myelophil due to the increased neuronal excitability accompanied by the antagonism of the $GABA_A$ receptor. Hence, electrophysiological screens are a helpful tool to understand the physiological effects of herbal drugs and enable the identification of new bioactive compounds, such as leonurine, for the development of new pharmaceutics or the more precise application of herbal remedies. The development of new pharmaceutical drugs out of the identified active ingredients of these herbs is a further possibility. The knowledge regarding the medicinal benefit of Kampo remedies has continued to evolve for centuries. This study can be thought of as an attempt to "translate" this traditional system into a contemporary, pharmacological system. Here, two classical drug targets were investigated, the $5\text{-}HT_3$ and $GABA_A$ receptors. However, there is still little knowledge with regard to the pharmacological action of many Kampo remedies on other clinically relevant targets, and therefore, there is great potential for future research.

AUTHOR CONTRIBUTIONS

KH, HH, MW, and GG conceived and designed the experiments; KH, RH, PZ, and LB performed the experiments; PL contributed materials; KH, RH, and GG wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphar.2016.00219

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