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# Zebrafish-Based Screening of Antiseizure Plants Used in Traditional Chinese Medicine: *Magnolia officinalis* Extract and Its Constituents Magnolol and Honokiol Exhibit Potent Anticonvulsant Activity in a Therapy-Resistant Epilepsy Model

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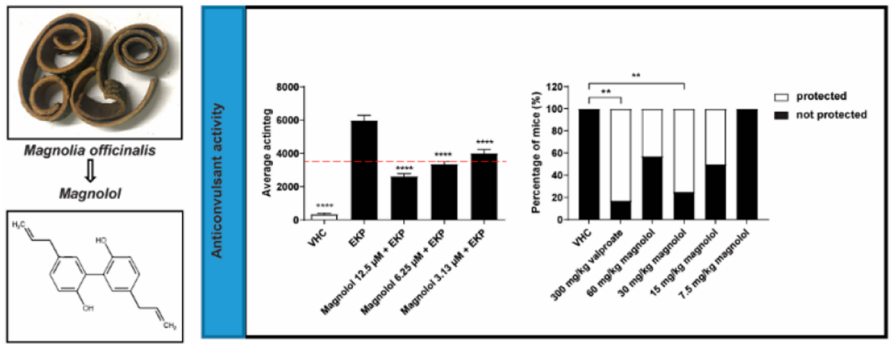


**ABSTRACT:** With the aim to discover interesting lead compounds that could be further developed into compounds active against pharmacoresistant epilepsies, we first collected 14 medicinal plants used in traditional Chinese medicine (TCM) against epilepsy. Of the six extracts that tested positive in a pentylenetetrazole (PTZ) behavioral zebrafish model, only the ethanol and acetone extracts from *Magnolia officinalis* (*M. officinalis*) also showed effective antiseizure activity in the ethylketopentenoate (EKP) zebrafish model. The EKP model is regarded as an interesting discovery platform to find mechanistically novel antiseizure drugs, as it responds poorly to a large number of marketed anti-epileptics. We then demonstrated that magnolol and honokiol, two major constituents of *M. officinalis*, displayed an effective behavioral and electrophysiological antiseizure activity in both the PTZ and the EKP models. Out of six structural analogues tested, only 4-*O*-methylhonokiol was active and to a lesser extent tetrahydromagnolol, whereas the other analogues (3,3′-dimethylbiphenyl, 2,2′-biphenol, 2-phenylphenol, and 3,3′,5,5′-tetra-*tert*-butyl-[1,1′-biphenyl]-2,2′-diol) were not consistently active in the aforementioned assays. Finally, magnolol was also active in the 6 Hz psychomotor mouse model, an acute therapy-resistant rodent model, thereby confirming the translation of the findings from zebrafish larvae to mice in the field of epilepsy. We also developed a fast and automated power spectral density (PSD) analysis of local field potential (LFP) recordings. The PSD results are in agreement with the visual analysis of LFP recordings using Clampfit software and manually counting the epileptiform events. Taken together, screening extracts of single plants employed in TCM, using a combination of zebrafish- and mouse-based assays, allowed us to identify allyl biphenol as a chemical scaffold for the future development of compounds with potential activity against therapy-resistant epilepsies.

**KEYWORDS:** *Zebrafish, Antiseizure drug discovery, Magnolol, Honokiol, Pharmacoresistant model, Antiseizure activity*

## INTRODUCTION

Epilepsy is a chronic and devastating brain disease characterized by an imbalance of excitatory and inhibitory processes, resulting in unpredictable, unprovoked, recurrent seizures.[1] So far, pharmacological intervention is the main treatment, since only few people are considered suitable for a ketogenic diet or surgical intervention.[2,3] Unfortunately, the marketed antiseizure drugs (ASDs) control seizures in only about 70% of the patients.[4] Moreover, a considerable number of patients also experience mild to moderately severe side effects.[5,6]

Before the advent of chemically synthesized compounds, medicinal plants have been used for centuries to treat epilepsy and control related syndromes in many countries around the world.[7] Consequently, plant extracts have been regarded as a

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Table 1. Antiseizure Activity of Medicinal Plant Extracts Using Zebrafish-Based Seizure Models[a]

| medicinal plant | plant part | PTZ model | | | EKP model | | |
|---|---|---|---|---|---|---|---|
| | | water extract (a) | ethanol extract (b) | acetone extract (c) | water extract (a) | ethanol extract (b) | acetone extract (c) |
| *Anemarrhena asphodeloides* Bunge. | rhizome | × | √ | √ | – | × | × |
| *Angelica pubescens* Maxim. | root | × | × | × | – | – | – |
| *Asarum heterotropoides* f. mandshuricum (Maxim.) Kitag. | rhizome and root | × | × | × | – | – | – |
| *Astragalus membranaceus* var. mongholicus (Bunge.) P.K.Hsiao. | root | × | × | × | – | – | – |
| *Bupleurum chinensis* DC. | root | × | × | √ | – | – | × |
| *Coptis chinensis* Franch | rhizome | × | × | × | – | – | – |
| *Gastrodia elata* Blume. | tuber | × | × | × | – | – | – |
| *Magnolia officinalis* Rehder & E.H.Wilson | bark | × | √ | √ | – | √ | √ |
| *Notopterygium incisum* K.C.Ting ex H.T. Chang. | rhizome and root | × | × | × | – | – | – |
| *Paris polyphylla* var. yunnanensis (Franch.) Hand.–Mazz. | rhizome | × | × | × | – | – | – |
| *Poria cocos* (Schw.) Wolf. | sclerotium | × | × | × | – | – | – |
| *Rehmannia glutinosa* (Gaertn.) DC. | tuberous root | × | × | × | – | – | – |
| *Scutellaria baicalensis* Georgi. | root | × | √ | × | – | × | – |
| *Nelumbo nucifera* Gaertn. | plumule | × | × | × | – | – | – |

[a]× Toxic effect or <40% reduction of seizure movement in comparison to PTZ/EKP control. √ ≥ 40% reduction of seizure movement in comparison to PTZ/EKP control. – Not tested in this model.

reliable source for the discovery of new ASDs.[8] This is illustrated by the recent finding that cannabidiol (CBD), a constituent of *Cannabis sativa*, demonstrated potent antiseizure activity in several animal models and in clinical practice.[8,9]

Traditional Chinese medicine (TCM) is one of the most widely practiced forms of botanical therapies in the world; it includes multiple recipes of medicinal plants against epilepsy and seizures.[10] Moreover, relying on the robust development of chromatographic and spectroscopic methods,[11] thousands of pure compounds have been purified and identified from TCM, facilitating the identification of active substances using a variety of *in vivo* and *in vitro* assays.

Relevant epilepsy and epileptic seizure models are crucial for the discovery of new ASDs. There are over a hundred models developed to date,[12] mainly grouped into seizure-induced and genetic models.[13] Rodents and zebrafish are the most frequently used animals in these models. Compared with rodent models, zebrafish models have advantages for large-scale drug screenings thanks to high fertility, fast embryonic development, small size, ease of maintenance and of drug administration.[14] Furthermore, zebrafish show high cell- and organ homologies to humans, and approximately 85% of human disease-related genes can be correlated to at least one zebrafish orthologue.[15]

To discover interesting lead compounds that could be further developed into compounds active against pharmacoresistant epilepsies, in this work, first neuroprotective and antiseizure medicinal plants were identified based on the Chinese Pharmacopoeia and data published in literature using search terms like "epilepsy", "seizure", "TCM", and "traditional Chinese medicine".[10,16−19] Then, extracts of the selected plant were tested for their antiseizure activity using a pentylenetetrazole (PTZ)-induced zebrafish locomotor model. PTZ-based animals models display generalized tonic-clonic seizures and epileptiform brain activity, and have been used extensively as a standard assay for the discovery of ASDs.[20,21] Active extracts were then further examined for their locomotor effect in an ethylketopentenoate (EKP)-induced zebrafish seizure model. Seizures elicited in zebrafish treated with EKP demonstrate a high level of resistance against commercially available ASD; hence, the EKP model is regarded as an interesting discovery platform to find mechanistically novel ASDs.[22]

We show that extracts of *Magnolia officinalis*, its main constituents magnolol and honokiol, as well as the structurally related allyl biphenolic methylhonokiol, exerted a potent and effective inhibition of PTZ- and EKP-induced locomotor and brain hyperactivity in zebrafish larvae. Moreover, magnolol was also active in the 6 Hz psychomotor mouse model, an acute therapy-resistant rodent seizure model.[23,24]

## RESULTS AND DISCUSSION

**Zebrafish-Based Screen of Antiseizure Medicinal Plant Extracts.** Fourteen medicinal plants (Table 1) used in TCM to treat seizures were collected. Their medicinal parts were ground into a powder and extracted in parallel with three different solvents of increasing polarity (acetone, ethanol, or water). A PTZ-induced locomotor assay in zebrafish was used as a first test for the antiseizure activity of all 42 extracts, administered to the medium. Extracts were prepared at concentrations ranging from 50 to 6.25 μg/mL, following a 2-fold serial dilution. An extract was considered effective if it reduced not less than 40% of PTZ-induced seizure movement, compared with the PTZ control group at its maximum tolerated concentration (MTC).

On the basis of this criterion, six plant extracts from four medicinal plants, i.e., *Anemarrhena asphodeloides* (*A. asphodeloides*), *Bupleurum chinensis* (*B. chinesis*), *M. officinalis* (see Figure 1 A−D), and *Scutellaria baicalensis* (*S. baicalensis*), were identified as effective (Table 1).

It is notable that most of these plants have been previously described to have neuroactivity and/or therapeutic effects. For instance, an extract of *A. asphodeloides* had a favorable effect in ischemia-induced brain injury in rats,[25] likely due to sarsasapogenin and possibly related compounds that possess neuroprotective activity.[26] Saikosaponin was shown to be responsible for the antiseizure activity of *B. chinensis* in a


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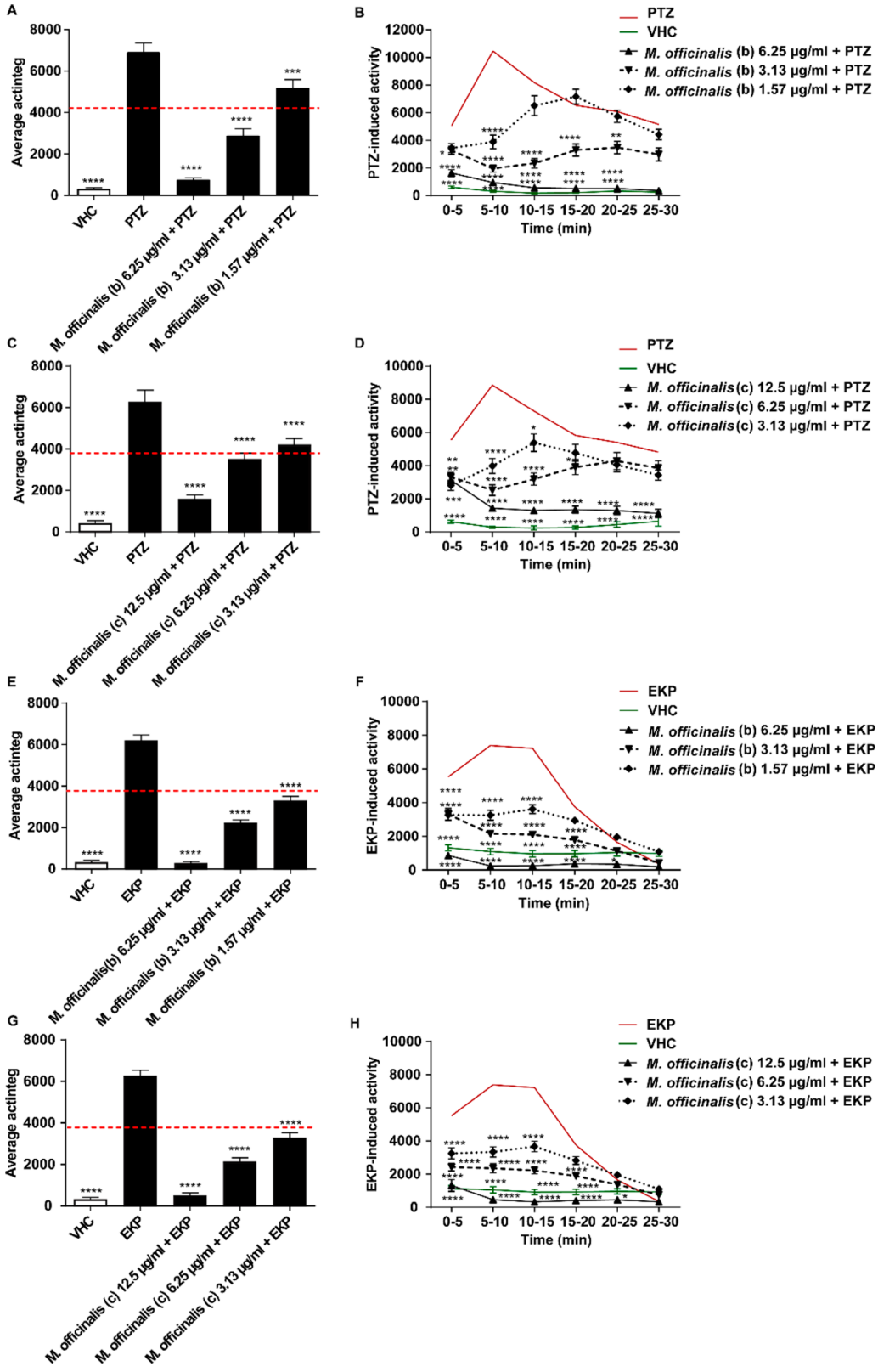


**Figure 1.** Behavioral antiseizure activity of *M. officinalis* ethanol and acetone extract in the PTZ/EKP zebrafish models. Behavioral antiseizure activity of the *M. officinalis* ethanol extract (b) and acetone extract (c) in the zebrafish (A−D) pentylenetetrazole (PTZ) seizure model and (E−H) ethylketopentenoate (EKP) seizure model, respectively, after 2 h of incubation. PTZ-induced seizure-like behavior is expressed as mean actinteg units/5 min (±SEM) during the (A, C) 30 min recording period and (B, D) over 5 min time intervals. EKP-induced seizure-like behavior is expressed as mean actinteg units/5 min (±SEM) during the (E, G) 20 min recording period and (F, H) over 5 min time intervals. (A, C, E, G) The red dashed line represents 60% of seizure-related movement of the PTZ/EKP control. Results were pooled from 3 independent experiments with 10 larvae per experiment. Statistical analysis: (A, C, E, G) one-way ANOVA with Dunnett's multiple comparison test and (B, D, F, H) two-way ANOVA with Bonferroni post-tests. Significance levels: $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, and $****p \leq 0.0001$. Abbreviation: VHC, vehicle.


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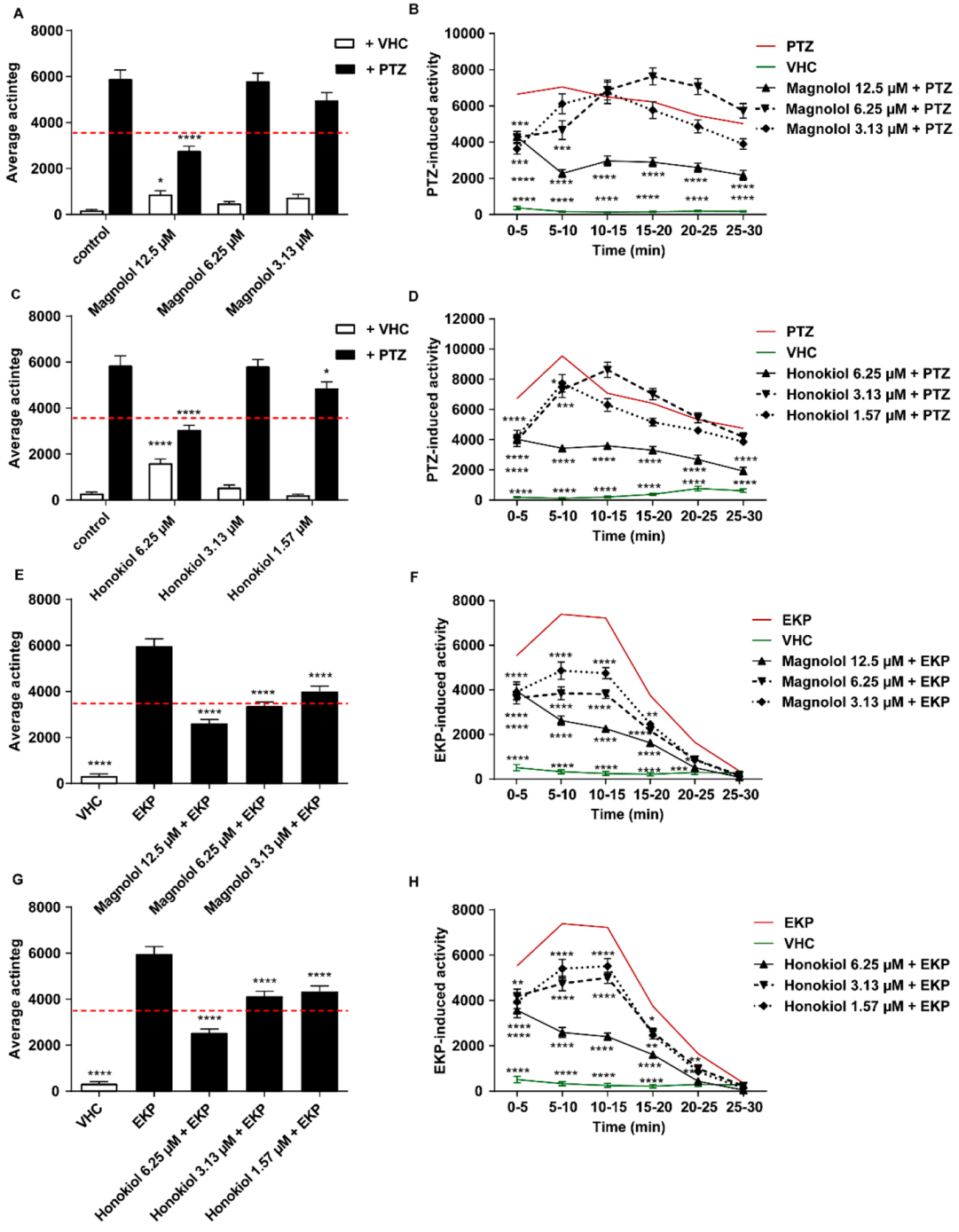


**Figure 2.** Behavioral antiseizure activity of magnolol and honokiol in the PTZ/EKP zebrafish models. Behavioral antiseizure activity of magnolol and honokiol in the (A−D) zebrafish pentylenetetrazole (PTZ) seizure model and (E−H) ethylketopentenoate (EKP) seizure model, respectively, after 2 h of incubation. PTZ-induced seizure-like behavior is expressed as mean actinteg units/5 min (±SEM) during the (A, C) 30 min recording period and (B, D) over 5 min time intervals. EKP-induced seizure-like behavior is expressed as mean actinteg units/5 min (±SEM) during the (E, G) 20 min recording period and (F, H) over 5 min time intervals. (A, C, E, G) The red dashed line represents 60% of seizure-related movement of the PTZ/EKP control. Results were pooled from 4 independent experiments with 10 larvae per experiment. Statistical analysis: (A, C, E, G) one-way ANOVA with Dunnett's multiple comparison test and (B, D, F, H) two-way ANOVA with Bonferroni posttests. Significance levels: $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, and $****p \leq 0.0001$. Abbreviation: VHC, vehicle.

variety of epilepsy models, as a result of the inhibition of the NMDA receptor current and the persistent sodium current (INaP).[27] Moreover, saikosaponin A also counteracted the inflammatory response and displayed the neuroprotective effects in traumatic brain injury rats.[28] Of interest, although the blood/brain barrier (BBB) permeability of saikosaponin might be restricted due to its hydrophilicity and its large molecular mass, several *in vitro* and *in vivo* studies demonstrated that saikosaponin can be converted into its lipophilic aglycon (i.e., saikogenin) by means of gastric fluid and the intestinal flora.[29,30] For *M. officinalis* and other Magnolia species, a broad range of therapeutic effects have been described, including anxiolytic, central nervous system (CNS) depressant, anti-inflammatory, anti-tumoral, and anti-


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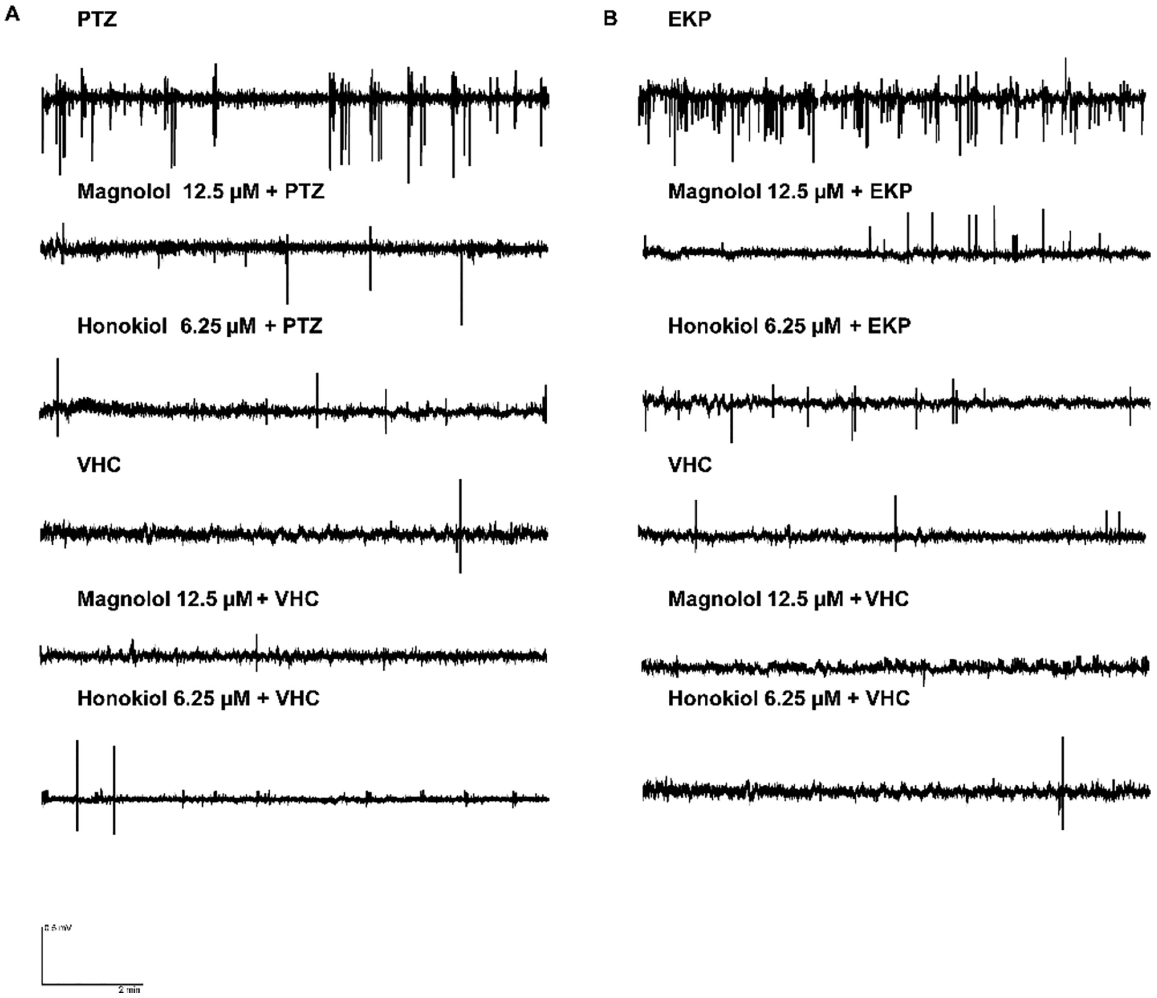


**Figure 3.** Representative local field potential recordings. 10 min noninvasive local field potential (LFP) recordings from the optic tectum of larvae pre-exposed to vehicle (VHC), pentylenetetrazole (PTZ), ethylketopentenoate (EKP), compound supplemented with PTZ/EKP, or compound supplemented with VHC. Larvae were incubated with 12.5 $\mu$M magnolol or 6.25 $\mu$M honokiol for 2 h.

epileptic.[31,32] The major constituents of this species are phenolic compounds, notably magnolol and honokiol, that make up to about 3−10% of their dry weight and were shown to be nontoxic.[33,34] Finally, in case of *S. baicalensis*, a flavone glucuronide called baicalin has been isolated that exhibited anticonvulsant and neuroprotective effects in a pilocarpine-induced epilepsy model in rats.[35] Interestingly, Zhang et al.[36] investigated the distribution of baicalin metabolites in various tissues of rats, and they found five metabolites in the brain, including methylbaicalein, a deglucuronidated and methylated aglycon. To what extent each of the metabolites contributes to the *in vivo* activity, however, is not known.

To find interesting hit compounds starting from TCM plant extracts that could be used against drug-resistant epilepsies, we then further tested the six effective plant extracts in the zebrafish EKP seizure model. In this case, only extracts of *M. officinalis* significantly inhibited EKP-triggered seizure behavior (see Table 1 and Figure 1 E−H) in an effective way (>40% reduction as compared to the EKP control group). Moreover, magnolol and honokiol, the biologically active constituents present in *M. officinalis* extracts, were chosen for further investigations.[37]

**Activity of Magnolol and Honokiol and Their Analogues in Zebrafish PTZ- and EKP-Seizure Models.** The antiseizure activity of magnolol and honokiol were tested in the PTZ and EKP model using a locomotor assay. A compound was considered effective if it reduced not less than 40% of PTZ/EKP-induced seizure movement compared with the PTZ/EKP control group at its maximum tolerated concentration (MTC). Both magnolol and honokiol significantly and effectively (>40% reduction) counteracted PTZ-provoked locomotor activity of larvae after 2 h of incubation but only at their MTC (Figure 2A−D). Lower concentrations were not active. Conversely, when tested in the EKP model, both compounds showed significant activity in a clear concentration-dependent manner (Figure 2E−H). Magnolol was more potent than honokiol, as it effectively inhibited the EKP-induced locomotor activity by more than 40% at its 1/2 MTC as well.

To confirm these results, the effects of magnolol and honokiol were investigated at their respective MTCs on the epileptiform brain discharges induced by PTZ and EKP. Brain activity was monitored by noninvasive local field potential (LFP) measurements, using an electrode positioned on the skin above the optic tectum. The recordings were scored by visual inspection using Clampfit software by counting the epileptiform events present, as described before by our group[20,38] and other groups.[39,40] As shown in Figures 3A and 4A and B, both compounds significantly reduced the frequency of epileptiform events, resulting in a decrease of


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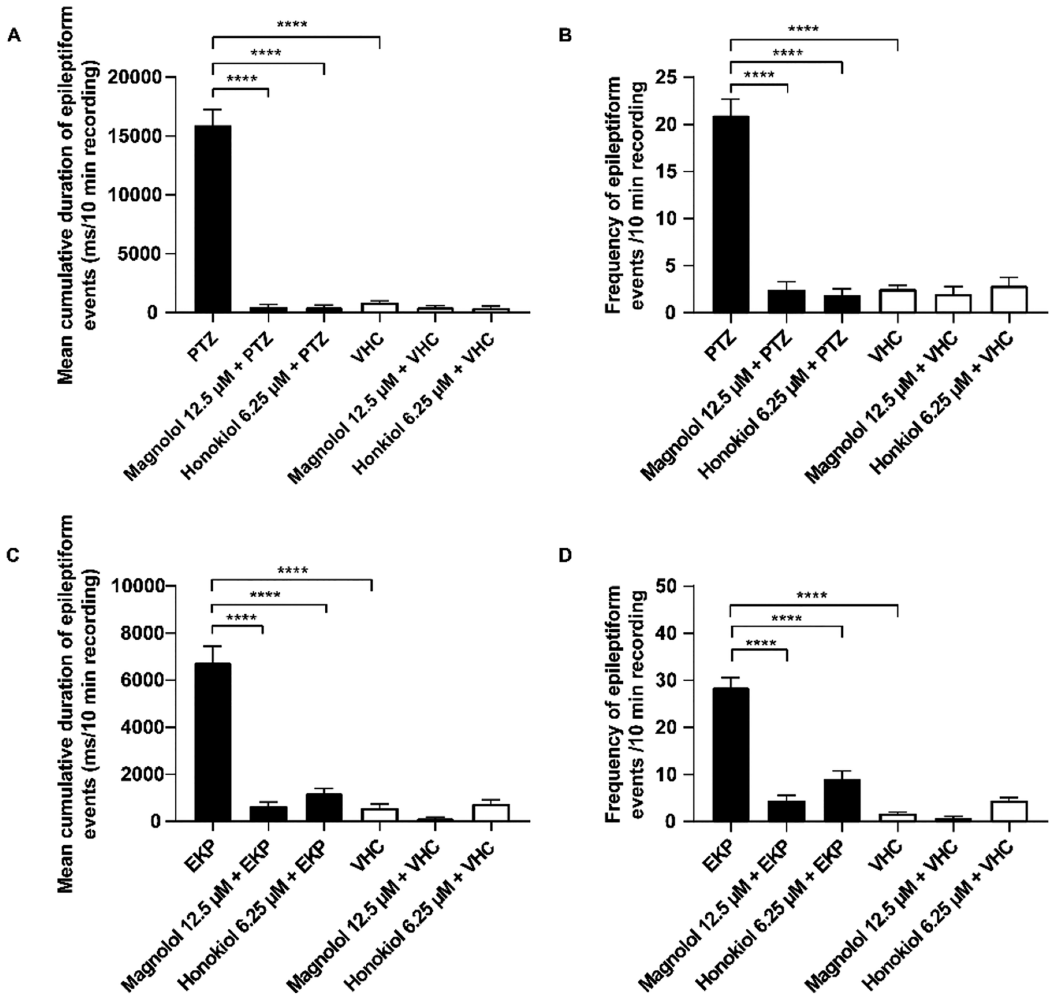


**Figure 4.** Electrophysiological antiseizure activity of magnolol and honokiol (visual analysis) in the PTZ/EKP zebrafish models. Noninvasive local field potential (LFP) recordings from the optic tectum of larvae pre-exposed to vehicle (VHC), pentylenetetrazole (PTZ), ethylketopentenoate (EKP), compound supplemented with PTZ/EKP, or compound supplemented with VHC. Larvae were incubated with 12.5 $\mu$M magnolol or 6.25 $\mu$M honokiol for 2 h. Epileptiform discharges are quantified by the (A, C) cumulative duration (mean ± SEM) and (B, D) number (mean ± SEM) of events per 10 min recording. Number of larvae per condition: $n$ = 31−33 for the VHC/PTZ/EKP group and $n$ = 15−17 for the magnolol/honokiol+PTZ/EKP/VHC group. Statistical analysis: one-way ANOVA with Dunnett's multiple comparison test. Significance levels: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, and ****$p \leq 0.0001$.

cumulative duration compared to PTZ-treated controls. A similar effect was observed in the EKP model (Figures 3B and 4C and D).

Somewhat surprisingly, we also found that magnolol and honokiol increased the locomotor activity in control conditions (i.e., in the absence of PTZ/EKP) (Figure 2A and C). However, a similar hyperactivity in control conditions has been observed also for other antiseizure compounds.[38,41] In addition, the electrophysiological analysis of brain activity in compound-treatment conditions did not reveal any abnormalities (Figures 3 and 4). This outcome suggests that magnolol and honokiol do not affect normal brain activity significantly and that the increased locomotor activity may be due to some undefined effect on the peripheral parts of the larval body.

Looking for a fast and reliable alternative to the visual analysis of LFP recordings, an automated method was developed that enabled us to quantify the increase in spectral power caused by the epileptic activity of multiple LFP recordings simultaneously. Power spectral density (PSD) computation has been used successfully before to analyze electrophysiological signals of the brain in rodents and patients.[42−44]

As shown in Figure 5A and C, the power spectral density estimates of signals in each LFP recording were determined per 10 Hz frequency band, ranging from 0 to 160 Hz using Welch's method. Next, the PSD was normalized against the VHC control and the data were plotted as mean (±SEM) PSD per 10 Hz. PTZ and EKP treatment induced a significantly higher LFP power in the frequency range between 30 and 130 Hz. Therefore, the PSDs were plotted as mean (±SEM) PSD per condition over the 30−130 Hz region (Figure 5B and D). As shown in Figure 5B and D, magnolol and honokiol had a major and statistically significant inhibitory effect on the PSD obtained after PTZ and EKP treatment, in line with the outcome of the visual analysis (Figure 4). These results therefore validate the PSD analysis as an accurate method to examine LFP recordings, dramatically increasing the possible data throughput.

Valproate and perampanel, used as positive controls in the PTZ and EKP models, respectively, exhibited a clear inhibitory


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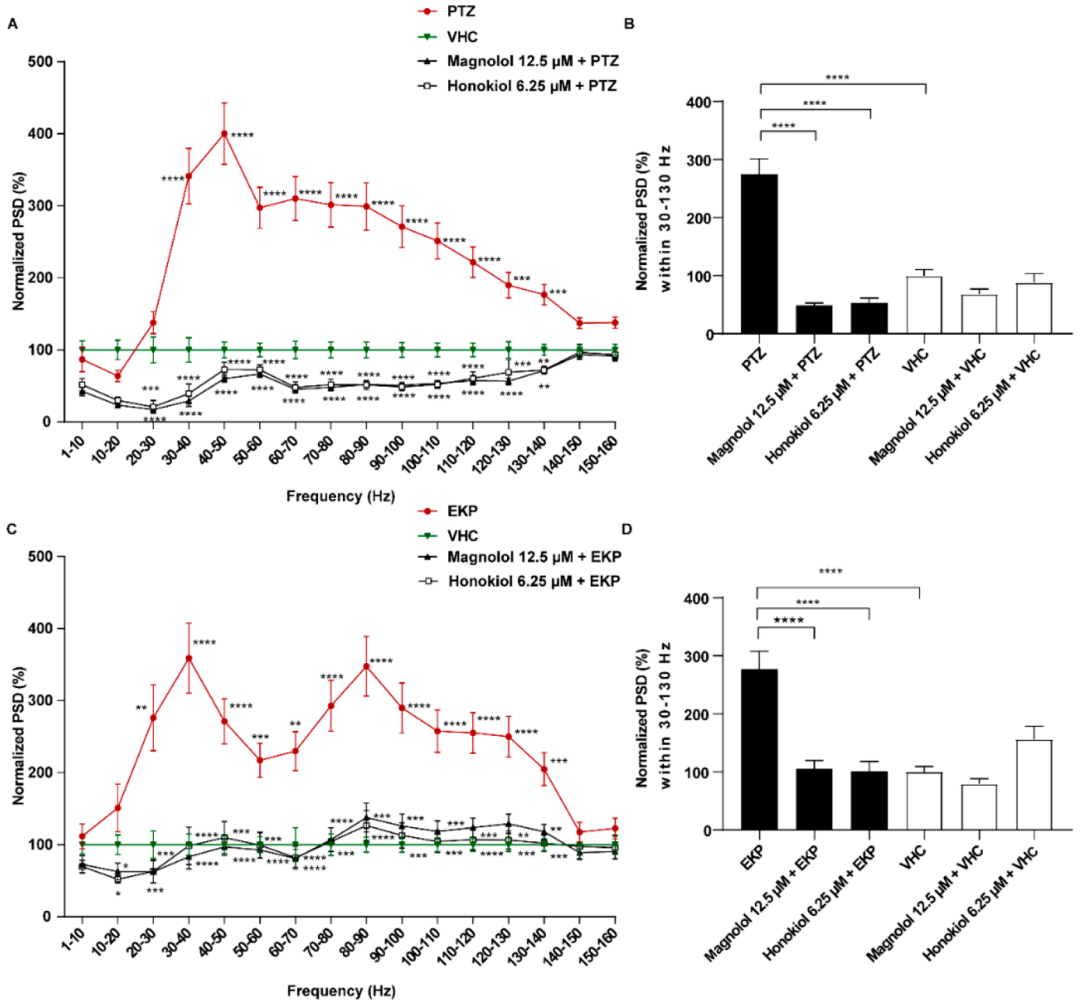


**Figure 5.** Electrophysiological antiseizure activity of magnolol and honokiol (PSD analysis) in the PTZ/EKP zebrafish models. The power spectral density (PSD) ranging from 0 to 160 is normalized against the (vehicle) VHC control, and the data are plotted as mean (±SEM) PSD (A, C) per 10 Hz and (B, D) per condition over the 30−130 Hz region. For the sake of clarity, the data of magnolol/honokiol+VHC are not shown in Figure 5A and C. Number of larvae per condition: $n = 31-33$ for the VHC/PTZ/EKP group and $n = 15-17$ for the magnolol/honokiol+PTZ/EKP/VHC group. Statistical analysis: two-way ANOVA with Bonferroni posttests, PTZ/EKP control in comparison to VHC group, (A, C) magnolol/honokiol +PTZ/EKP group in comparison to PTZ/EKP control, and (B, D) one-way ANOVA with Dunnett's multiple comparison test. (A−D) Outliers were identified via the Iterative Grubbs test ($\alpha = 0.1$). Significance levels: $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, and $^{****}p \leq 0.0001$.

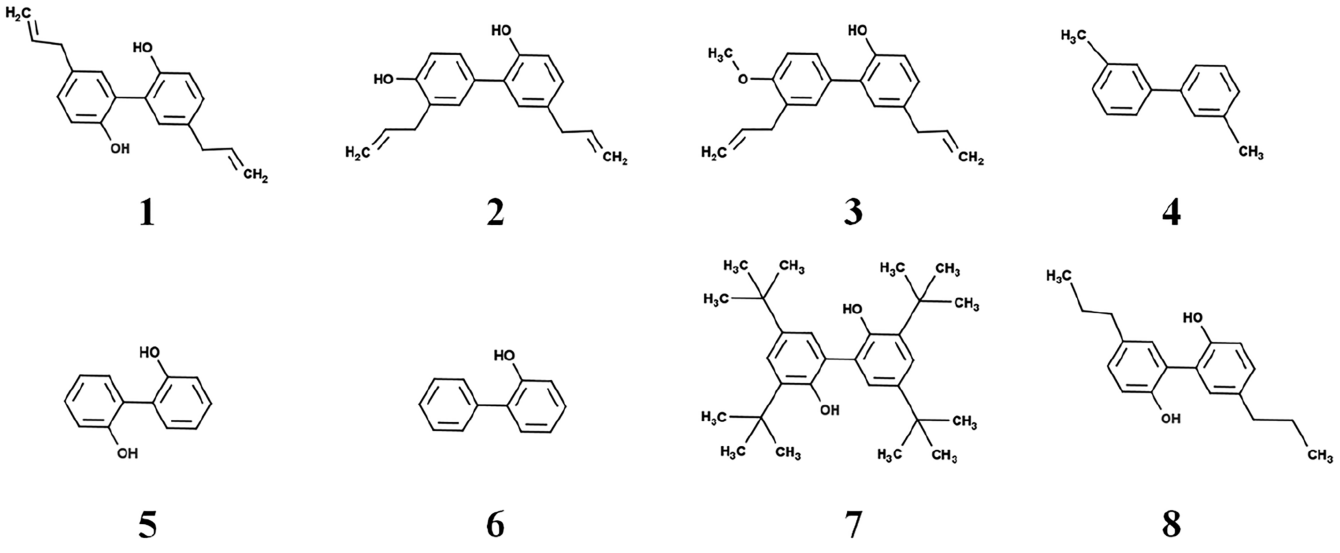


**Figure 6.** Structures of magnolol, honokiol, and their analogues.


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**Table 2. Physicochemical Properties and MTC of Magnolol, Honokiol, and Their Analogues**[a]

| | compound | MW | clog *P* | clog *D* (pH = 7.6) | PSA (pH = 7.6) | rotor | HBD (pH = 7.6) | HBA (pH = 7.6) | MTC ($\mu$M) |
|---|---|---|---|---|---|---|---|---|---|
| 1. | magnolol | 266.34 | 5.25 | 5.17 | 40.46 | 5 | 2 | 2 | 12.5 |
| 2. | honokiol | 266.34 | 5.25 | 5.17 | 40.46 | 5 | 2 | 2 | 6.25 |
| 3. | 4-*O*-methylhonokiol | 280.37 | 5.28 | 5.34 | 29.46 | 6 | 1 | 2 | 1.57 |
| 4. | 3,3′-dimethylbiphenyl | 182.27 | 4.67 | 4.65 | 0 | 1 | 0 | 0 | 50 |
| 5. | 2,2′-biphenol | 186.21 | 3.16 | 3.00 | 40.46 | 1 | 2 | 2 | 25 |
| 6. | 2-phenylphenol | 170.21 | 3.45 | 3.31 | 20.23 | 1 | 1 | 1 | 12.5 |
| 7. | 3,3′,5,5′-tetra-*tert*-butyl-[1,1′-biphenyl]-2,2′-diol | 410.64 | 9.67 | 9.19 | 40.46 | 5 | 2 | 2 | 6.25 |
| 8. | tetrahydromagnolol | 270.37 | 5.68 | 5.81 | 40.46 | 5 | 2 | 2 | 3.13 |

[a]MW: molecular weight. clog *P*: calculated partition coefficient. clog *D*: calculated distribution coefficient. PSA: polar surface area. Rotor: rotatable bonds. HBD: number of hydrogen bond donors. HBA: number of hydrogen bond acceptors. All physicochemical properties of compounds were calculated using ChemAxon software imbedded in MarvinSketch 20.2.0.

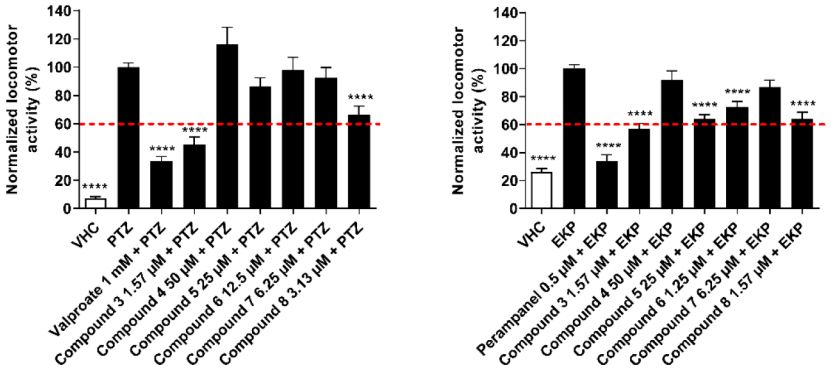


**Figure 7.** Behavioral antiseizure activity of structural analogues in the PTZ/EKP zebrafish models. Antiseizure activity of structural analogues in the zebrafish (A) pentylenetetrazole (PTZ) seizure model and (B) ethylketopentenoate (EKP) seizure model, respectively, after 2 h of incubation. (A) PTZ-induced seizure-like behavior within a 30 min recording normalized to PTZ control (mean ± SEM). (B) EKP-induced seizure-like behavior within a 20 min recording normalized to EKP control (mean ± SEM). (A, B) The red dashed line represents 60% of seizure-related movement of the PTZ/EKP control. Valproate and perampanel were used as positive controls for the PTZ and EKP model, respectively. Results were pooled from 3 independent experiments with 10 larvae per experiment. Statistical analysis: one-way ANOVA with Dunnett's multiple comparison test. Significance levels: $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, and $****p \leq 0.0001$. Abbreviation: VHC, vehicle.

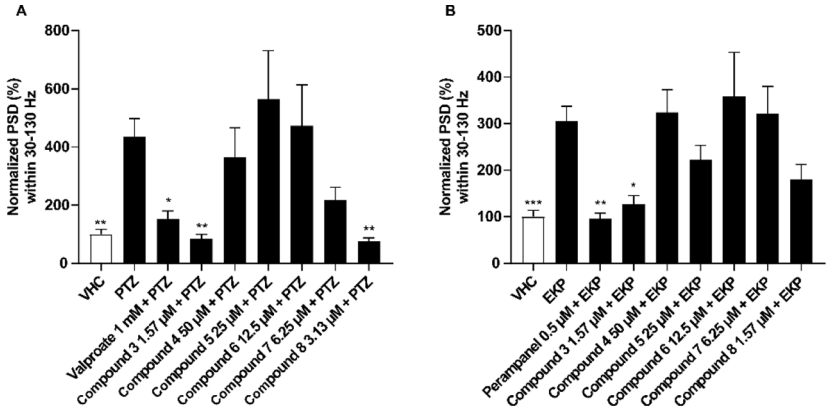


**Figure 8.** Electrophysiological antiseizure activity (PSD analysis) of structural analogues in the PTZ/EKP zebrafish models. (A and B) The power spectral density (PSD) ranging from 0 to 160 Hz is normalized against the (vehicle) VHC control, and the data are plotted as mean (±SEM) PSD per condition over the 30−130 Hz region. Valproate and perampanel were used as positive controls. Number of larvae per condition: $n = 16-19$ for the VHC/PTZ/EKP group, $n = 12-17$ for the valproate/perampanel+PTZ/EKP group, and $n = 10$ for analogues+PTZ/EKP. Statistical analysis: one-way ANOVA with Dunnett's multiple and outliers were identified via the Iterative Grubbs test ($\alpha = 0.1$). Significance levels: $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, and $****p \leq 0.0001$.

effect on the behavioral (Figure 7) and electrographical seizures (Figure 8, supplementary Figure 1A and D), as observed previously.[20,22]

Overall, our results demonstrate that magnolol and honokiol are among the main anticonvulsant constituents present in *M. officinalis* extracts. To start understanding the structure−activity relationship of these biphenolic structures, six commercially available analogues were examined at their MTCs for their inhibitory activity in both the PTZ- and EKP-zebrafish assays (Figure 6 and Table 2). Compound 7


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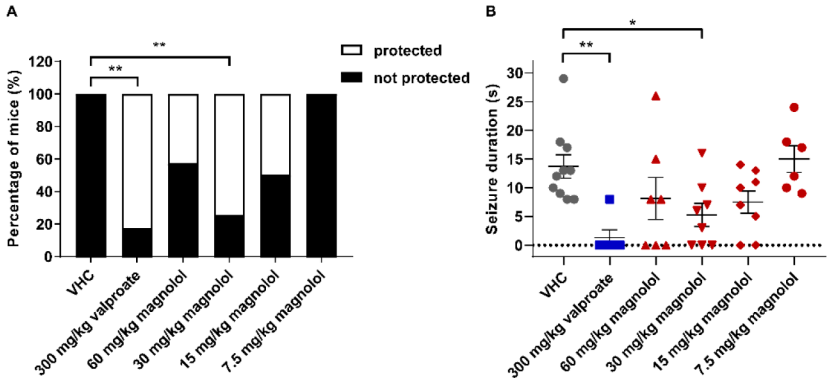


**Figure 9.** Antiseizure activity of magnolol in the mouse 6 Hz psychomotor seizure model. Psychomotor seizures were electrically induced (6 Hz, 0.2 ms rectangular pulse width, 3 s duration, 44 mA) through the cornea, 60 min after intraperitoneal injection of the vehicle (VHC, $n$ = 10), positive control valproate ($n$ = 6), or test compounds ($n$ = 6−8 per dose). (A) Number of mice protected against seizures are depicted and (B) defined by a seizure duration shorter than 8 s. Data are shown as the mean ± SEM. Statistical analysis: (A) Fisher's exact test, (B) one-way ANOVA with Dunnett's multiple comparison test, and (A, B) outliers were identified via the Iterative Grubbs test ($\alpha$ = 0.01). Significance levels: *$p \leq 0.05$ and **$p \leq 0.01$.

(Figure 6 and Table 2) precipitated in the VHC before reaching the MTC. In this case, the maximum soluble concentration (MSC) (6.25 $\mu$M) was used instead. Tetrahydromagnolol was tested at 1/2 MTC in case of the EKP assay, as this compound induced toxicity at its MTC when examined in the presence of EKP.

Methylhonokiol (compound 3) and tetrahydromagnolol (compound 8) exhibited a significant inhibitory effect on the PTZ-increased locomotor activity (Figure 7A); however, the latter did not effectively (<40% inhibition) reduce the PTZ-associated activity. In the EKP locomotor model, all analogues except for compound 4 (dimethylbiphenyl) and 7 (tetra-*tert*-butyl[biphenyl]diol) were significantly active, but only methylhonokiol (compound 3) inhibited the EKP-associated activity in an effective way (>40% reduction) (Figure 7B).

In general, this outcome is in agreement with the electrophysiological results (LFP recordings, PSD analysis). In the PTZ model, methylhonokiol (compound 3) was significantly active, but also tetrahydromagnolol (compound 8) exhibited similar inhibitory activity (Figure 8A, and supplementary Figure 1B and C). Conversely, in the EKP model, only methylhonokiol (compound 3) had an inhibitory effect on the epileptiform discharges. Tetrahydromagnolol (compound 8) exhibit some inhibitory (nonsignificant, $p$ = 0.15) activity (Figure 8B, and supplementary Figure 1E and F).

Taken together, only magnolol and honokiol and its methylated analogue (compound 3) had a substantial inhibitory effect on abnormal locomotor activity, especially epileptiform brain discharges that were induced by both PTZ and EKP. Tetrahydromagnolol was somewhat less active, whereas the other compounds were not consistently active.

As the BBB (blood/brain barrier) is not fully matured in 7 dpf larvae,[45] we can assume that all absorbed compounds penetrated into the central nervous system (CNS). Conversely, the inactivity of some of the compounds tested could be due to a lack of body absorption during larval exposure or after absorption due to a limited affinity for the protein targets concerned. Interestingly, recently hundreds of compounds reported to be active in zebrafish assays were chemically described, and it was found that zebrafish-absorbed compounds typically fulfill certain criteria: (a) MW ≤ 500, (b) clog $P \leq 5.3$, (c) HBD ≤ 3, (d) HBA ≤ 7, (d) PSA ≤ 124 A°, and (e) rotatable bonds ≤ 9.[46] From Table 2, it can be seen that all compounds used in this work comply with the requirements, except for compound 7 (log $P$ = 9.67). So it is anticipated that compound 7 was not active in our *in vivo* assays because of a lack of absorption in larval zebrafish.

Magnolol and honokiol have demonstrated positive allosteric modulatory effects on $\gamma$-aminobutyric acid type A ($GABA_A$) receptors.[32,47] In particular, honokiol exhibits a positive effect on the chloride current ($I_{GABA}$) through $GABA_A$ receptors of seven different subunit compositions, showing most activity on $\alpha_3\beta_2$, $\alpha_2\beta_2$, $\alpha_1\beta_2$, and $\alpha_1\beta_1$.[48] As compared to honokiol, its methyl-derivative (compound 3) was about twice as active at $\alpha_1\beta_2$ receptors as the $I_{GABA}$ modulator at 30 $\mu$M, whereas magnolol was equally active.[48]

According to the pharmacophore model established using the $\alpha_1\beta_2$ $GABA_A$ receptor, active honokiol derivatives exhibit hydrophobic regions (represented by the 2-propenyl substituents in honokiol), one acceptor (aromatic ring), and one donor region (hydroxy group).[48] Of interest, Fuchs et al. also found that an alkyl residual is essential for the action of biphenolics at $GABA_A$ receptors.[49] These data therefore suggest that compounds 4, 5, and 6 are not active in the PTZ assay, as they lack at least one of the pharmacophore characteristics.[48] Moreover, replacing the 2-propenyl (i.e., allyl) by a propyl substituent (as present in tetrahydromagnolol) modified the activity, indicating that the type of alkyl group is also of importance.

Furthermore, because PTZ prompts neuronal excitability predominantly by antagonizing GABAergic inhibition,[50] we conclude that potentiation of GABAergic transmission probably accounts for the antiseizure activity of the active allyl biphenolics observed in the PTZ model.

The EKP zebrafish model shows a poor response to several existing ASDs. For instance, out of 13 clinically tested antiepileptics, only the $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor inhibitor perampanel showed a clear inhibitory effect on both the EKP-triggered excessive behavior and brain activity,[22] whereas GABAergic compounds like tiagabine (which prevents GABA reuptake) and valproate (which inhibits GABA degradation) were inactive.[22] Thus, the positive allosteric modulatory effect on $GABA_A$ receptors exerted by these compounds is unlikely to account for their inhibitory effects observed in the EKP zebrafish model.


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However, allyl biphenolics can interact with other molecular targets as well, which might explain the activity observed in the EKP model. For instance, magnolol and honokiol have potent effects on cannabinoid (CB) receptors at low micromolar concentrations,[51−53] and CB receptors are therapeutic targets for epilepsy.[54,55] Furthermore, it was found that magnolol can weaken both glutamate- and NMDA-induced neurotoxicity, preventing the increasing $Ca^{2+}$ influx caused by glutamate[56] and exhibiting an affinity for the AMPA receptor.[57]

**Antiseizure Analysis of Magnolol in the Mouse 6 Hz Psychomotor Seizure Model.** Finally, we tested whether the antiseizure activity of magnolol, as detected in zebrafish models, translates to the 6 Hz (44 mA) psychomotor mouse model, a standard rodent model able to detect compounds with novel antiseizure mechanisms and with potential activity against drug-resistant seizures.[23,58] Seizures are characterized by stun, clonus, twitching of the vibrissae, and straub tail.

As shown in Figure 9A and B, VHC-injected mice showed a mean (±SEM) psychomotor seizure duration of 14 ± 2 s. Mice were regarded as protected when the seizure duration was shorter than 8 s.[59] Valproate-treated mice (positive control group) were nearly all protected (83%) and showed dramatically reduced seizure duration, in line with our previous results.[38] Magnolol at a dose of 30 mg/kg significantly decreased the mean seizure duration and protected 75% of the mice compared to the VHC group. Moreover, a dose-dependent relationship was found at doses between 30 mg/kg and 7.5 mg/kg magnolol (Figure 9A and B).

## CONCLUSION

Taken together, screening extracts of single plants employed in TCM using a combination of zebrafish and mouse seizure models allowed us to identify allyl biphenol as a chemical scaffold for future discovery of compounds possibly active in therapy-resistant epilepsies. The compounds are endowed with an interesting multitarget profile encompassing $GABA_A$, cannabinoid, and AMPA receptors. However, whether these or other unknown molecular targets play a key role is presently unexplored and warrants further investigation. Knowing the molecular target(s) is likely necessary for further lead optimization.

## METHODS

**Animals and Maintenance.** *Zebrafish.* Adult zebrafish (*Danio rerio*) of the AB strain were kept under standard husbandry conditions (28 °C, pH 6.5−7.8, 14/10 h light/dark cycle). Eggs and embryos were obtained following natural spawning and then sorted and raised in an embryo medium (0.3× Danieau's solution (DS): 1.5 mM HEPES, pH 7.6, 17.4 mM NaCl, 0.21 mM KCl, 0.12 mM $MgSO_4$, and 0.18 mM $Ca(NO_3)_2$) in a Peltier-cooled incubator (IPP 260, Memmert, Schwabach, Germany) at 28 °C after 5−7 days post-fertilization (dpf).

*Mice.* Male NMRI mice (provided by Charles River Laboratories) used in the experiments were 9 weeks old and weighed 18−20 g. Animals were housed in groups of 4 or 5 per cage and maintained under standard animal housing conditions at 22 °C with a 14/10 h light/dark cycle. Water and food were available *ad libitum*. The animals were fed a pellet diet and water *ad libitum* and were allowed to acclimate for 1 week before experiments were conducted.

All animal experiments were approved by the Ethics Committee of the University of Leuven (approval numbers 027/2017 and 023/2017) and by the Belgian Federal Department of Public Health, Food Safety, and Environment (approval number LA1210199).

**Plant Extract Preparation.** Dried medicinal plant material was purchased from Beijing Tong Ren Tang Pharmaceutical (BTP) Co. Ltd. (Beijing, China). The purchased plant materials were authenticated according to the Chinese Pharmacopeia (version 2010) by a local botanist. Vouchers of plant materials were deposited in our lab. The medicinal parts were ground, and the obtained powder was immersed in acetone, ethanol, or water at 1 g/10 mL, after which the suspension was sonicated 5× for 15 min over a 24 h period, followed by centrifugation at 3500 × *g* for 10 min. Then, 1 mL of the supernatant of each extract was transferred into 1.5 mL Eppendorf tubes for further evaporation using a Savant SpeedVac concentrator to acquire aqueous and ethanol extracts. The acetone extracts were dried by air blowing. The residues were dissolved in Milli-Q water or dimethyl sulfoxide (DMSO) for aqueous and organic extracts, respectively, at a final concentration of 40 mg/mL.

**Compounds Preparation.** Magnolol, honokiol, 3,3′-dimethylbiphenyl, 2,2′-biphenol, 2-phenylphenol, 3,3′,5,5′-tetra-*tert*-butyl-[1,1′-biphenyl]-2,2′-diol, and valproate were purchased from Sigma-Aldrich (analytical standard). The other compounds used were the following: 4-*O*-methylhonokiol (Enzo), tetrahydromagnolol (Cayman Chemical Company), and perampanel (Eisai). Compounds were dissolved in 100% DMSO at a concentration of 200 mM and stored at −20 C°. Stock solutions were diluted 100-fold in embryo medium before use (final DMSO concentration of 1% w/v).

All physicochemical properties of compounds (MW, clog *P*, clog *D*, PSA, rotor, HBD, and HBA) were calculated using the ChemAxon software imbedded in MarvinSketch 20.2.0 (ChemAxon, Hungary) requiring only 2D structural formula as input.

PTZ was purchased from Sigma-Aldrich (analytical standard). EKP was synthesized by the Laboratory of Organic Synthesis (Prof. Wim De Borggraeve, KU Leuven) according to the method described in ref 22. EKP was dissolved in 100% DMSO at a concentration of 800 mM and stored at −80 °C. PTZ was dissolved in an embryo medium at a concentration of 40 mM and freshly prepared before use.

**Toxicity Evaluation.** The maximum tolerated concentration (MTC) of the extracts and compounds was determined by a method described previously.[38] A dozen zebrafish larvae of 5 dpf were individually incubated in single wells of a 96-well plate and were treated separately with extracts or compounds at concentrations ranging from 6.25 to 50 μg/mL and from 3.13 to 200 μM, respectively, in 100 μL of VHC (1% DMSO). After 18 h, larvae were individually examined for their touch response, posture, edema, signs of necrosis, morphology, and swim bladder. The MTC was defined as the highest concentration at which an extract or compound did not exert any sign of toxicity in any of the larvae used. 3,3′,5,5′-Tetra-*tert*-butyl-[1,1′-biphenyl]-2,2′-diol (compound 7, Figure 6 and Table 2) was precipitated in the VHC before reaching the MTC. In this case, the maximum soluble concentration (MSC) (6.25 μM) was used for further testing the activity.

**Locomotor Activity Evaluation.** Zebrafish larvae of 5 dpf ($n$ = 10) were individually positioned in single wells of a 96-well plate, and incubated in 100 μL VHC or VHC supplemented with extract or compound for 2 h at 28 °C in the dark. Then, 100 μL of VHC, or VHC supplemented with PTZ (40 mM) or EKP (1 mM) was added, followed by placing the plates immediately in an enclosed tracking device (ZebraBox Viewpoint, France). Locomotion activity was expressed in "actinteg" units, and plotted per 5 min by ZebraLab software (Software Viewpoint, France). The total locomotor activity accumulated over the total tracking period of 30 min (PTZ model) or 20 min (EKP model) was calculated as well.

**Local Field Potential Recordings.** Epileptiform activities were measured by noninvasive local field potential (LFP) recording of the optic tectum (midbrain) of 7 dpf zebrafish larvae. The larvae were treated as described above. After incubation, 100 μL of VHC, or VHC supplemented with PTZ (40 mM) or EKP (1 mM), was added. After 15 min (PTZ model) or 8 min (EKP model), the larvae were immobilized in 2% low-melting-point agarose (Invitrogen) at room temperature (RT). A single glass electrode filled with artificial cerebrospinal fluid (ACSF) (124 mM NaCl, 2 mM KCl, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 1.25 mM $KH_2PO_4$, 26 mM $NaHCO_3$, and 10 mM glucose) was positioned on the skin above the optic tectum.


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Local field potential recordings were performed according to the method reported by Copmans et al.[38] and Zhang et al.[22]

Recordings were visually inspected using Clampfit 10.2 software (Molecular Devices Corporation, USA[60]). An electrical discharge was considered as epileptiform if it corresponded to a poly spiking event comprising at least 3 spikes with a minimum amplitude of 3 times the baseline amplitude and a duration of at least 100 ms.

In addition, a power spectral density (PSD) analysis of the recordings was performed using MatLab R2018 (MATrix LABoratory, USA) software.[61] In brief, the PSD of the signals were estimated using Welch's method of averaging the modified periodograms with a 512-point fast Fourier transform with 80% overlapping 100 sample (100 ms) long segments and a Hamming window. Then, the PSD estimate of each LFP recording was summed over each 10 Hz frequency band, ranging from 0 to 160 Hz. This analysis assumes that the epileptiform activity manifests as a high-power oscillation at certain frequencies. If epileptic activity occurs often throughout the recording then this will lead to an increased PSD estimate in the corresponding frequency band. Note that such a frequency-domain analysis, along with other time-domain signal characteristics, was successfully used to automatically detect and count epileptic events in the LFP recordings.[61] Next, the PSD estimates were normalized against the VHC control, and the data were plotted as mean (±SEM) PSD per 10 Hz and per condition over the 30−130 Hz region. Outliers were identified via the iterative Grubbs test ($\alpha$ = 0.1).

**Mouse 6 Hz Psychomotor Seizure Model.** The antiseizure activity of the compounds was investigated in the mouse 6 Hz (44 mA) psychomotor seizure model, as described previously.[38] NMRI mice (10 weeks old) weighing 27−31 g were randomly assigned to 1 of 6 groups, with every group consisting of at least 6 mice. The mice were injected intraperitoneally with valproate (300 mg/kg), magnolol (doses ranging from 7.5 to 60 mg/kg) dissolved in 200 $\mu$L VHC (DMSO:PEG200:saline, 0.25:0.25:0.5, v/v/v), or 200 $\mu$L of VHC (control). Then, 1 hour after injection, an ECT Unit 5780 stimulator (Ugo Basile, Comerio, Italy) was used for inducing psychomotor seizures through corneal electrical stimulation (6 Hz, 0.2 ms rectangular pulse width, 3 s duration, 44 mA). An ocular anesthetic (lidocaine, 0.5%) was applied to the cornea before stimulation. Psychomotor seizures characterized by stun, clonus, twitching of the vibrissae, and straub tail were scored and video-monitored. Seizure durations were determined by blinded video analysis to confirm or correct the initial observations. The mice were considered protected in case all four signs of psychomotor seizures were absent within 8 s after stimulus delivery.[59]

**Statistical Analysis.** GraphPad Prism 8 software (GraphPad Software, Inc., USA) was used for statistical analyses. For all analyses, differences between a treatment group and the equivalent control groups were considered statistically significant if the $p$ value was below 0.05 ($p < 0.05$), indicated by an asterisk (*). The $p$ values below 0.01, 0.001, and 0.0001 were marked by two (**), three (***), or four (****) asterisks, respectively.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschemneuro.9b00610.

Electrophysiological antiseizure activity (PSD analysis) of structural analogues in the PTZ/EKP zebrafish models; data are plotted as mean (±SEM) PSD per 10 Hz (PDF)

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### Author Contributions

J.L. performed the zebrafish and mice experiments, data analysis, and wrote the manuscript. D.C. was involved in the data analysis and experimental design. M.P. coordinated the mice experiments. B.H. designed the P.S.D. analysis software. W.L. was involved in the experimental design and the preparation of the manuscript and figures. P.dW. was responsible for the experimental design, data analysis, and preparation of the manuscript and figures. All authors edited and approved the final version of the manuscript.


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### Notes

The authors declare no competing financial interest.

## ■ ABBREVIATIONS

ACSF, artificial cerebrospinal fluid; AMPA, $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ASD, antiseizure drug; dpf, days post fertilization; BBB, blood/brain barrier; CB, cannabinoid; DMSO, dimethyl sulfoxide; EKP, ethyl-ketopentenoate; LFP, local field potential; *M. officinalis*, *Magnolia officinalis*; MTC, maximum tolerated concentration; PEG200, poly(ethylene glycol) MW 200; PSD, power spectral density; PTZ, pentylenetetrazole; RT, room temperature; TCM, traditional Chinese medicine; VHC, vehicle

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