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Interactions of *Magnolia* and *Ziziphus* extracts with selected central nervous system receptors

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ABSTRACT

Ethnopharmacological relevance: Magnolia officinalis Rehder and Wilson [Magnoliaceae] bark and *Ziziphus spinosa* (Buhge) Hu ex. Chen. [Fam. Rhamnaceae] seed have a history of use in traditional Asian medicine for mild anxiety, nervousness and sleep-related problems.
Aim of the study: To identify pharmacological targets, extracts of *Magnolia officinalis* (ME), *Ziziphus spinosa* (ZE), and a proprietary fixed combination (MZE) were tested for affinity with central nervous system receptors associated with relaxation and sleep.
Methods: In vitro radioligand binding and cellular functional assays were conducted on: adenosine A_1, dopamine (transporter, D_1, D_{2S}, D_3, $D_{4.4}$ and D_5), serotonin (transporter, 5-HT_{1A}, 5-HT_{1B}, 5-HT_{4e}, 5-HT_6 and 5-HT_7) and the GABA benzodiazepine receptor.
Results: Interactions were demonstrated with the adenosine A_1 receptor, dopamine transporter and dopamine D_5 receptor (antagonist activity), serotonin receptors (5-HT_{1B} and 5-HT_6 antagonist activity) and the GABA benzodiazepine receptor at a concentration of 100 μg/ml or lower. ME had an affinity with adenosine A_1 (K_i of 9.2 ± 1.1 μg/ml) and potentiated the GABA activated chloride current at the benzodiazepine subunits of the GABA receptor (maximum effect at 50 μg/ml). ME had a modest antagonist action with 5-HT_6 and ZE with the 5-HT_{1B} receptor.
Conclusion: The interactions in the receptor binding models are consistent with the traditional anxiolytic and sleep-inducing activities of *Magnolia officinalis* bark and *Ziziphus spinosa* seed.
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1. Introduction

Magnolia officinalis bark and *Ziziphus spinosa* seed have a history of use in traditional Asian medicine for mild anxiety, nervousness and sleep-related problems. Both are listed in the Pharmacopeia of the People's Republic of China (English Edition, 2005). Additionally, *Magnolia officinalis* bark is listed in the Japanese Pharmacopeia XIV (English Edition, 2001). Extracts of both botanicals have demonstrated activity in rodent studies. *Magnolia* extracts, in traditional combinations with other herbs, have demonstrated anti-depressant effects in the tail suspension test, the forced swimming test as well as anxiolytic activity in the elevated plus-maze assay in mice (Kuribara et al., 2000; Luo et al., 2000). The activity in the plus-maze assay was traced to a constituent in *Magnolia* bark; namely honokiol (Kuribara et al., 2000). An extract of *Ziziphus spinosa* demonstrated anxiolytic activity in the elevated plus-maze assay and the black and white test in mice. Furthermore, the extract demonstrated a sedative effect in prolonging hexobarbital-induced sleeping time in mice (Peng et al., 2000). Activity guided fractionation identified spinosin as an active constituent of *Ziziphus spinosa* (Li and Bi, 2006). Spinosin augmented pentobarbital-induced sleep, increasing sleep time and reducing sleep latency in mice (Wang et al., 2008).

Extracts of *Magnolia officinalis* bark and *Ziziphus spinosa* seed were combined in a proprietary product called Seditol® (MZE) that is produced by Next Pharmaceuticals. MZE is characterized as containing a minimum of 2.7% honokiol and 0.1% spinosin. MZE is marketed as a dietary supplement in the USA for the improvement of sleep difficulties associated with restlessness, stress or anxiety. Seditol was subjectively assessed for tolerability and benefit in 295 volunteers with mild to moderate sleep difficulties in an uncontrolled, open-label study. The participants took the product for a minimum of 2 weeks and over 80% subjectively reported that Seditol helped them to relax, assisted in a restful sleep and was effective in reducing fatigue due to lack of sleep (unpublished).

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Table 1
Summary and general experimental conditions of BDZ and transporter receptor-binding assays.

Receptor	Reference compound	Ligand	Conc. (nM)	Incubation	Reference
BDZ	Diazepam (3 μM)	[3H]flunitrazepam	0.4	60 min, 4 °C	Speth et al. (1979)
DA transporter	BTCP (10 μM)	[3H]BTCP	4	120 min, 4 °C	Pristupa et al. (1994)
5-HT transporter	Imipramine (10 μM)	[3H]imipramine	2	60 min, 22 °C	Tatsumi et al. (1999)

Table 2
Summary and general experimental conditions of dopamine and serotonin cellular functional assays.

Receptor	Reference compound agonist effect	Stimulus agonistic effect (control)	Reference compound antagonist effect	Stimulus antagonistic effect (nM)	Incubation	Reference
D_1	Dopamine	None (10 μM dopamine)	SCH 23390	Dopamine (300)	30 min, 22 °C	Zhou et al. (1990)
D_{2S}	Dopamine	None (100 nM dopamine)	(+)Butaclamol	Dopamine (30)	20 min, 37 °C	Missale et al. (1998)
D_3	Dopamine	None (30 nM dopamine)	(+)Butaclamol	Dopamine (10)	10 min, 37 °C	Missale et al. (1998)
$D_{4.4}$	Dopamine	None (300 nM dopamine)	Clozapine	Dopamine (100)	10 min, 37 °C	Missale et al. (1998)
D_5	Dopamine	None (1 μM dopamine)	SCH 23390	Dopamine (50)	30 min, 22 °C	Sunahara et al. (1991)
5-HT_{1A}	8-OH-DPAT	None (1 nM 8-OH-DPAT)	WAY 100635	8-OH-DPAT (10)	15 min, 22 °C	Newman-Tancredi et al. (2001)
5-HT_{1B}	Serotonin	None (10 μM serotonin)	Methiothepin	Serotonin (100)	30 min, 37 °C	Giles et al. (1996)
5-HT_{4E}	Serotonin	None (1 μM serotonin)	GR 113808	Serotonin (30)	30 min, 22 °C	Mialet et al. (2000)
5-HT_6	Serotonin	None (10 μM serotonin)	Methiothepin	Serotonin (100)	45 min, 37 °C	Kohen et al. (1996)
5-HT_7	Serotonin	None (10 μM serotonin)	Mesulergine	Serotonin (100)	45 min, 37 °C	Adham et al. (1998)

In order to better understand the pharmacological targets for ME and ZE extracts and their combination MZE, the preparations were tested for binding affinity with a number of central nervous system receptors associated with relaxation and sleep. Adenosine is a neurotransmitter known to play an important role in the initiation of sleep (Basheer et al., 2004). Dopamine plays an important role in mood and internal reward systems (Runyon and Carroll, 2006). Serotonin (5-hydroxytryptamine, 5-HT) is involved in a number of physiological functions including sleep, appetite, pain perception, and sexual activity (Tatarczynska et al., 2004). Gamma-aminobutyric acid (GABA) modulates arousal and attention, anxiety and sleep (Khom et al., 2006). The selected receptor/receptor subtypes for testing were adenosine A_1, dopamine (transporter, D_1, D_{2S}, D_3, $D_{4.4}$ and D_5), serotonin (transporter, 5-HT_{1A}, 5-HT_{1B}, 5-HT_{4e}, 5-HT_6 and 5-HT_7) and the GABA benzodiazepine receptor. This work expands on previous receptor binding studies on extracts of *Magnolia officinalis* (Squires et al., 1999; Ai et al., 2001; Li and Bi, 2006) and *Ziziphus spinosa* (Liao et al., 1995).

2. Materials and methods

2.1. Test material

Seditol (MZE) is a proprietary blend of a patented extract of the bark of *Magnolia officinalis* Rehder and Wilson [Magnoliaceae] (ME) and an extract of the seeds of *Ziziphus spinosa* (Buhge) Hu ex. Chen. (syn. *Ziziphus jujube* var. *spinosa* (Bunge) Hu ex HF Chow) [Fam. Rhamnaceae] (ZE). ME is produced with carbon dioxide, and is the subject of two US patents (Nos. 6,582,735 and 6,814,987) describing composition and methods of preventing, treating in managing sleeplessness, restlessness and weight gain due to stress or lack of sleep. ZE is an ethanol:water extract with a ratio of 4–6:1. MZE is characterized as containing a minimum of 2.7% honokiol, an active constituent of *Magnolia officinalis* bark and 0.1% spinosin, a chemical marker of quality for *Ziziphus spinosa* seeds. The raw plant materials were identified and supplied by the manufacturer's contacts in the People's Republic of China. Voucher specimens of the crude botanicals are deposited at the extract manufacturers' facilities. Samples of the individual extracts (ME and ZE) and their blend (MZE) are retained by the manufacturer and by the authors.

2.2. Radioligand binding assays

2.2.1. Adenosine binding assays

The binding assays were conducted as described elsewhere (Müller et al., 2002). In short, the extracts were investigated in radioligand binding assays at A_1 adenosine receptors of rat brain cortical membranes using the A_1-selective radioligand [^{3}H]2-chloro-N^6-cyclopentyladenosine ([^{3}H]CCPA). The extracts were dissolved in dimethyl sulfoxide (DMSO) and a final concentration of 2.5% DMSO was used in the assays. Membranes were preincubated with 0.2 IU/mg protein of adenosine deaminase in order to remove endogenous adenosine. Radioligand binding to rat brain cortical membranes was carried out in Tris–HCl buffer 50 mM, pH 7.4. Assays were performed by incubating the mixtures at 23 °C for 90 min. Nonspecific binding was defined using 10 μM of 2-chloroadenosine (CADO). [^{3}H]CCPA was used in a final concentration of 1 nM. Protein (ca. 50 μg per well containing a final volume of 0.2 ml) was added to start the reaction. Incubations were terminated by rapid filtration using a Brandel 96-channel cell harvester (Brandel, Gaithersburg, MD, USA) through Packard 96-well GF/B-glass fibre filter plates. Filters were rinsed three times with 0.2 ml of ice-cold Tris–HCl buffer 50 mM, pH 7.4, each. Radioactivity of the wet 96-well filter plates was counted after 9 h of preincubation with 40 μl of Microscint-20 scintillation cocktail (Packard Bioscience). All experiments were performed in triplicate.

Data were analyzed using GraphPad PRISM® Version 4.0 (San Diego, CA, USA). For nonlinear regression analysis, the Cheng-Prusoff equation and K_D-values of 0.2 nM for [^{3}H]CCPA, 8 nM for [^{3}H]MSX-2, 0.41 nM for [^{3}H]PSB-603 and 4.9 nM for [^{3}H]PSB-11 were used to calculate K_i-values from IC_{50}-values.

2.2.2. Dopamine, serotonin and GABA central binding

In vitro binding assays were performed using the general procedures shown in Tables 1 and 2. All receptors were human cloned, except for BZD and 5-HT_{1B}, which were endogenous to the rat cerebral cortex and the Chinese hamster ovary (CHO) cells, respectively.

The extracts were dissolved in DMSO at a concentration of 30 mg/ml and then diluted in water or saline for initial screening at a concentration of 100 μg/ml. Selected assays were repeated at a concentration of 10 μg/ml. Each determination was performed in

Table 3
Effects of ME, ZE and their combination MZE on radioligand binding.

Receptor	ME	ZE	MZE
DA transporter	Interference (39)	–	64 (11)
D_1		Antagonist: 14	–
D_{2S}		–	–
D_3			–
$D_{4.4}$			–
D_5	Antagonist: 66 (21)	–	Antagonist: 21
5-HT transporter	Interference	13	–
5-HT_{1A}	–	–	–
5-HT_{1B}	Agonist: 102	Antagonist: 23	Antagonist: 29
5-HT_{4E}			–
5-HT_6	Antagonist: 48 (15)	–	Antagonist: 20
5-HT_7			–
BZD	61	–	–

The results are expressed as a percent inhibition of control specific binding with a concentration of 100 μg/ml (mean values; $n = 2$). Tests at a concentration of 10 μg/ml are included in parenthesis. (–) Indicates an inhibition of less than 10%. Blank squares indicate that the test was not run.

duplicate. In each experiment, the respective reference compound was tested at a minimum of eight concentrations in duplicate to obtain a competition curve in order to validate the experiment.

For the receptor binding studies, the specific radioligand binding to the receptors was defined as the difference between total binding and nonspecific binding determined in the presence of an excess of unlabeled ligand. Results are expressed as a percent of control specific binding and as a percent inhibition of control specific binding obtained in the presence of the tested extracts.

For the cellular functional assays, the measured reaction product was the second messenger cyclic adenosine monophosphate (cAMP). The specific radioligand binding to the receptors was defined as the difference between total response and measured specific agonist response determined in the presence of an excess of ligand. Results are expressed as a percent of control specific response or as a percent inhibition of control specific agonist response obtained in the presence of the tested extracts. The conditions for specific assays are detailed in Table 2.

2.2.3. $GABA_A$ binding assays

The binding assays were conducted as described elsewhere (Khom et al., 2006). Briefly, the experiments were conducted with $GABA_A$ receptors expressed from *Xenopus laevis*. Stage V–VI oocytes were prepared and cRNA injected as previously described. Female *Xenopus laevis* (NASCO, USA) were anesthetized by exposing them for 15 min to a 0.2% MS-222 (methanesulfonate salt of 3-aminobenzoic acid ethyl ester; Sandoz, Germany) solution before surgically removing parts of the ovaries. Follicle membranes from isolated oocytes were enzymatically digested with 2 mg/ml collagenase (Type 1A, Sigma, Germany). Synthesis of capped run-off poly(A^+) cRNA transcripts was obtained from linearized cDNA templates (pCMV vector). One day after enzymatic isolation, the oocytes were injected with 50 nl of DEPC-treated water (diethylpyrocarbonate, Sigma, Germany) containing the different rat cRNAs at a concentration of approximately 300–3000 pg/nl/subunit. The amount of cRNA was determined by means of a NanoDrop ND-1000 (Kisker-Biotech, Steinfurt, Germany). To ensure expression of the gamma2S subunit cRNAs encoding for α1, β2 and γ2S subunits were mixed in a ratio of 1:1:10. Oocytes were stored at 18 °C in ND96 solution. Voltage clamp measurements were performed on the 1st and 2nd days after cRNA injection. Electrophysiological experiments were performed by the two-microelectrode voltage clamp method making use of a TURBO TEC 01C amplifier (npi electronic GmbH, Tamm, Germany) at a holding potential of −70 mV. The bath solution contained 90 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 5 mM HEPES (pH 7.4).

GABA and ME were applied by means of an automated fast perfusion system (Baburin et al., 2006) To elicit I_{GABA}, the chamber was perfused with 120 μl of GABA-containing solution at volume rates of 300 μl/s. ME was solved in DMSO at a concentration of 10 mg/ml and then diluted in bath solution to 10 μg/ml, 50 μg/ml and 100 μg/ml. Indicated concentrations of ME were co-applied with GABA EC_{3-10} (effective concentration of GABA that induces 3–10% of maximal GABA-evoked current). Percent potentiation of I_{GABA} by ME was calculated using formula: $(R/C - 1) \times 100\%$, where R is the amplitude of the chloride current evoked by co-application of control GABA EC_{3-10} and the indicated concentration of ME, and C is the amplitude of the chloride current evoked by application of control GABA EC_{3-10} alone. Bar graphs were built using Origin software (OriginLab Corporation, USA). Statistical significance was calculated using unpaired Student's t-test with a confidence interval of $P < 0.05$.

3. Results

The receptor binding results for selected receptors associated with the neurotransmitters dopamine, serotonin and GABA are summarized in Table 3. Results of interactions with the adenosine A_1 receptor are depicted in Fig. 1 and the results of interactions with the GABA benzodiazepine receptor are depicted in Fig. 2.

3.1. Adenosine

ME exhibited 48 ± 15% inhibition of radioligand binding at 100 μg/ml and a K_i of 9.2 ± 1.1 μg/ml (Fig. 1). MZE had a small interaction with the adenosine A_1 receptor (14 ± 1% inhibition of radioligand binding at 100 μg/ml). This effect was due to ME as ZE was not active when tested individually at this concentration. Further testing investigated the nature of the interaction between ME and the adenosine receptor. ME at a concentration of 10 μg/ml completely inhibited forskolin-stimulated cAMP accumulation in CHO cells expressing the adenosine A_1 receptor. As this result was considered likely to be an artifact, the functional properties of ME were investigated in GTP-shift experiments using rat cortex membrane preparation containing adenosine A_1 receptors. ME did not cause a GTP-shift in this experiment.

3.2. Dopamine

The selected receptor/receptor subtypes associated with dopamine included the transporter, D_1, D_{2S}, D_3, $D_{4.4}$ and D_5. ME

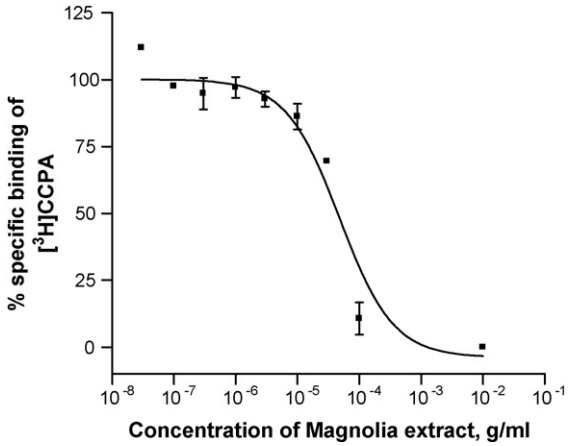

Fig. 1. Interaction of ME with adenosine A_1. Competition curve of ME with rat adenosine A_1 receptor with K_i-value of 9.2 μg/ml. Data points represent means of two independent experiments including standard errors of the mean.

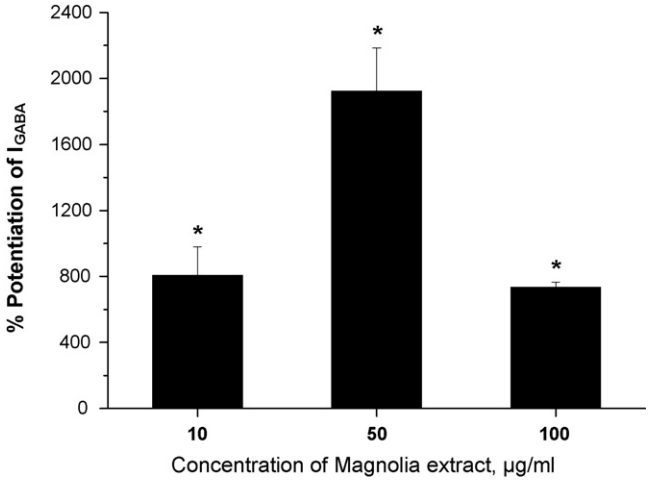

Fig. 2. Interaction of ME with $GABA_A$. Potentiation of the chloride current through $GABA_A$ receptors composed of α1, β2 and γ2S subunits by 10, 50 or 100 μg/ml of sample M. The *Y*-axis indicates the increase compared to the control current in %. Data are given as mean ± SEM from three different experiments. *Statistical significance from zero ($P < 0.05$).

and MZE bound with the dopamine transporter and the D_5 receptor (Table 3). ME inhibited binding to the DA transporter by 39% at 10 μg/ml with no results being determined at 100 μg/ml due to interference in the test system. ZE was not active at 100 μg/ml in this system. MZE inhibited binding to the DA transporter by 64% at 100 μg/ml and 11% at 10 μg/ml.

ME interacted with the D_5 receptor causing a 66% inhibition at 100 μg/ml and 21% inhibition at 10 μg/ml. ZE was not active at 100 μg/ml in this system. MZE interacted modestly with the D_5 receptor with 21% inhibition of control at 100 μg/ml.

3.3. Serotonin

The selected receptor/receptor subtypes associated with serotonin included the transporter, 5-HT_{1A}, 5-HT_{1B}, 5-HT_{4e}, 5-HT_6 and 5-HT_7. MZE bound modestly as an antagonist to the 5-HT_{1B} and 5-HT_6 receptors (Table 3). ZE had an antagonist effect with the 5-HT_{1B} receptor with 23% inhibition of control binding at 100 μg/ml. MZE displayed 29% inhibition of control at that concentration. ME had no antagonist effect at 100 μg/ml and instead had an agonist effect, with 102% inhibition of control at 100 μg/ml. However ME appeared not to interfere with the activity of ZE when the two were combined in MZE.

ME displayed an antagonist effect with the 5-HT_6 receptor with 48% inhibition of control at 100 μg/ml and 15% inhibition of control at 10 μg/ml. This effect was also seen with MZE, which had an antagonist effect with 20% inhibition of control at 100 μg/ml.

3.4. GABA

ME had a strong interaction with the GABA central benzodiazepine receptor at a concentration of 100 μg/ml, causing a 61% inhibition at that concentration. Neither ZE nor MZE had a significant interaction with the receptor at that concentration. The activity of ME was eliminated when the concentration was reduced 10-fold to 10 μg/ml (Table 3). This explains why there was no activity demonstrated with MZE. In a separate experiment, the *Magnolia* extract strongly potentiated the GABA activated chloride current at the benzodiazepine subunits of the GABA receptor in a bell-shaped dose–response curve with maximum effect at 50 μg/ml (Fig. 2).

4. Discussion

In the present series of assays, interactions with the adenosine A_1 receptor, dopamine transporter and dopamine D_5 receptor (antagonist activity), serotonin receptors (5-HT_{1B} and 5-HT_6 antagonist activity) and the GABA benzodiazepine receptor were demonstrated. MZE demonstrated an interaction with the adenosine A_1 receptor that appears to be due to the constituent ME. Adenosine A_1 is known to play an important role in the initiation of sleep. During the day, adenosine accumulates in neurons and in the evening it is released into the nerve synapses where it prepares the body to enter into a relaxed state (Basheer et al., 2004). ME was tested for adenosine A_1 agonist activity. The rationale for this is that it was hypothesized that the *Magnolia* extract might act in a similar means to an extract of Valerian root (*Valeriana officinalis* L). Previously it has been reported that a Valerian extract counteracted functional central arousal caused by the oral administration of caffeine, a well known adenosine antagonist (Schellenberg et al., 2004). Results with the Valerian extract indicate that it is a partial agonist of adenosine A_1 (Müller et al., 2002). The GTP-shift experiments with ME indicate that it may be either a partial agonist in analogy with the Valerian extract, or an antagonist.

Dopamine is a neurotransmitter that plays an important role in mood. Dopamine is increased as an internal reward system leading to feelings associated with pleasure and contentment. Dopamine transporter inhibitors (in this case MZE) increase the level of dopamine in neural synapses increasing and prolonging the action of dopamine. The dopamine transporter is a target for the development of pharmacotherapies for a number of central disorders including attention deficit hyperactivity disorder (ADHD), obesity, depression, and stimulant abuse (Runyon and Carroll, 2006). D_1 and D_5 receptor antagonism appear to play a role in the development of fear or anxiety (Inoue et al., 2005). MZE and ME displayed D_5 antagonistic activity. In addition, weak binding at the 100 μg/ml level indicated potential D_1 antagonistic for ZE.

Serotonin (5-HT) is a neurotransmitter involved in a number of physiological functions including sleep, appetite, pain perception, and sexual activity. Several pathological states such as migraine, depression, and anxiety have been linked to the serotonergic system. To date, there are 14 known serotonin receptor subtypes through which serotonin exerts its actions. One receptor in particular, known as 5-HT_{1B}, plays a crucial role in regulating serotonin transmission in the brain. Recent studies have suggested a role for the 5-HT_{1B} receptor in depression, as well as in obsessive-compulsive disorder, drug addiction, anxiety, aggression and sleep. 5-HT_{1B} antagonists reduce the latency to onset of anxiolytic behavior and play a role in stress regulation with activity comparable to diazepam (Tatarczynska et al., 2004). MZE exhibited a mild 5-HT_{1B} antagonist activity that appears to be due to the presence of ZE. ME tested individually had the opposite effect. Functional assays and competitive binding curves would be the next step to better understand the activity of each extract and their combination on this particular receptor subtype. MZE also displayed a modest antagonist effect with the 5-HT_6 receptor. This activity appears to be due to the presence of ME. Selective 5-HT_6 antagonists may have potential as anxiolytics and anti-depressants (Wesolowska and Nikiforuk, 2007).

A previous study demonstrated an interaction between a *Zizyphus* seed extract (drug extract ratio of 7.3:1) and the serotonin receptor subtypes 5-HT_{1A} and 5-HT_2 with a concentration of 10 mg/ml (Liao et al., 1995). This study did not duplicate those results. However ZE in this study was tested at a concentrations 100 times lower than the previous study, which is closer to that expected in the human body following traditional use.

GABA is a neurotransmitter that modulates arousal and attention, anxiety, sleep and muscle tone. Sedation and relief from

anxiety are the effects of agents acting on GABAergic neurotransmission. Benzodiazepines are a class of drugs that act in this manner, reducing anxiety and inducing sleep through interactions with the $GABA_A$ receptor. ME was found to have a strong effect on the benzodiazepine binding site of the GABA receptor, working in the same direction as benzodiazepines. Previous studies have reported that honokiol, a chemical constituent of ME, interacts with the GABA receptor (Squires et al., 1999; Ai et al., 2001; Li and Bi, 2006). Magnolol, another constituent of ME, has been shown to be active in the oocyte assay used in this study (Kim et al., 2008). Honokiol and magnolol demonstrated activity in mice in the plus maze test with a dose of 0.2 mg/kg for 7 days. Honokiol exhibited activity at a dose similar to that of diazepam (a benzodiazepine), but did not exhibit the side effects known to be produced by this class of drugs (Kuribara et al., 1999a,b). Our studies did not duplicate a previous study that showed binding of a *Ziziphus* extract to the $GABA_A$ site (Liao et al., 1995). The reason may be that the previous study used a concentration 100 times higher than that used in these experiments.

The affinity of the *Magnolia* extract with the A_1 and GABA receptors is in line with earlier reports. The modest interaction of ME as an antagonist with 5-HT_6 has to the best of our knowledge not been previously reported. In addition, this may be the first report of interaction between the ZE and the 5-HT_{1B} receptor.

The activities in the receptor binding models are in agreement with the traditional uses of the extracts of *Magnolia* and *Ziziphus*. The study results may also provide some clues as to the activity of their proprietary combination (Seditol). Further investigations using in vivo models incorporating bioavailability and biotransformation are important to verify these findings.

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