Identification of eugenol as the major determinant of $GABA_A$-receptor activation by aqueous *Syzygium aromaticum* L. (clove buds) extract

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1. Introduction

γ-Amino butyric acid (GABA) is a major inhibitory neurotransmitter in the brain, which binds as a ligand to the heteropentameric $GABA_A$ receptors. After binding, the receptors, which are members of the Cys-loop superfamily of ligand-gated ion channels (Collingridge, Olsen, Peters, & Spedding, 2009; Connolly & Wafford, 2004), open an intrinsic pore permeable for chloride ions, resulting in hyperpolarization or shunt inhibition. This inhibitory activity is the reason for $GABA_A$ receptors to be a target of sedative, hypnotic, and anxiolytic drugs (Sigel & Steinmann, 2012). In humans, $GABA_A$ receptors consist of eight subunit families with 19 different subunits in total (α1–α6, β1–β3, γ1–γ3, ρ1–ρ3, δ, ε, θ, π) (Olsen & Sieghart, 2008; Simon, Wakimoto, Fujita, Lalande, & Barnard, 2004). The pentameric subunit arrangement determines the pharmacological properties of the $GABA_A$ receptors (Karim et al., 2013; Olsen & Sieghart, 2008; Sieghart & Sperk, 2002). In molecular-pharmacological studies, α- and β-subunits are often assembled to heteromeric $GABA_A$ receptors to decrease the number of transfected subunits leading to an almost homogenous receptor population (Krasowski & Harrison, 2000). All α-subunits generally affect the efficacy of GABA in receptors consisting of binary α- and β- subunits (Karim et al., 2013), whereas many allosteric modulators, including various anesthetics, address the β2-subunit (Reynolds et al., 2003).

$GABA_A$-receptor activity is enhanced by several foods and food components that are known for their sedative, anxiolytic, or calming effects (Aoshima & Hamamoto, 1999; Aoshima et al., 2001; Campbell, Chebib, & Johnston, 2004; Hossain, Aoshima, Koda, & Kiso, 2002; Hossain, Hamamoto, Aoshima, & Hara, 2002; Kessler, Villmann, Sahin-Nadeem, Pischetsrieder, & Buettner, 2012; Kessler et al., 2014; Sahin, Eulenburg, Kreis, Villmann, & Pischetsrieder, 2016; Zaugg et al., 2010, 2011). Recently, we reported that the aqueous food extracts of *Sideritis* species, green tea, sage, lavender, lemon balm, chamomile, and hops potentiated the response to GABA of the $GABA_A$ receptor composed of human

Abbreviations: γ-Amino butyric acid, GABA; (ultra) high-performance liquid chromatography, (U)HPLC; gas chromatography, GC; two-electrode voltage clamp, TEVC; diode array detector, DAD; mass spectrometry, MS; standard error of the mean, SEM.

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α1β2-subunits (Sahin et al., 2016). Kessler et al. also described the $GABA_A$ receptor-modulating activity of volatile *Sideritis* extracts (Kessler et al., 2012) and identified some terpenoid substances from this extracts that are responsible for $GABA_A$-receptor modulation (Kessler et al., 2014). Zaugg et al. reported that ethyl acetate extracts of black pepper (*Piper nigrum* L.) fruits potentiate $GABA_A$-receptor activity (Zaugg et al., 2010). The traditional Asian medicine uses black pepper because of its antiepileptic, anxiolytic, sedative and sleep-inducing health benefits (Ahmad et al., 2012). Furthermore, piperine was identified as the extract component that is predominantly responsible for these effects (Zaugg et al., 2010). It has been shown before that piperine and its derivatives are potent antiepileptics (Pei, 1983).

Potentiation of GABAergic synaptic transmission is used as a well-established target for the treatment of insomnia, anxiety disorders, and epilepsy (Möhler, 2006; Roth & Draguhn, 2012). More recently, the $GABA_A$ receptor was also suggested as an emerging drug target for pain treatment (Li & Zhang, 2012). The spice *Syzygium aromaticum* L. (clove buds) has been reported to exert topical anesthetic and analgesic effects *in vivo* and is, therefore, used in dentistry (Alqareer, Alyahya, & Andersson, 2006; Hosseini, Kamkar-Asl, & Rakhshandeh, 2011). Although the *in vivo* activity of clove has been demonstrated, its mechanism of action in the nervous system is not fully understood. The aim of the present study was, therefore, to investigate if $GABA_A$ receptors may be a target for modulation by clove that could explain the anesthetic and analgesic effects of clove and clove components. Hence, in the first step the $GABA_A$ receptor-modulatory effect of aqueous clove extract was studied using the previously developed and validated three-step test system (Sahin et al., 2016) and then compared to the effects of aqueous black pepper extract. In the next step, we identified the active extract components by activity profiling based on high-performance liquid chromatography (HPLC) and gas chromatography (GC).

2. Material and methods

2.1. Chemicals

Unless noted otherwise, chemicals were supplied by Sigma-Aldrich (Taufkirchen, Germany), including GABA ≥ 99%, glycine ≥ 99%, and ortho-phthaldialdehyde. Eugenol (99%) was obtained from Alfa Aesar (Karlsruhe, Germany) and acetyleugenol from Carl Roth (Karlsruhe, Germany). All solvents for HPLC or UHPLC analyses were purchased from Fisher Scientific (Schwerte, Germany).

2.2. Plant material and preparation of extracts

Clove buds (*Syzygium aromaticum* L., from Hallesche Essig- und Senffabrik, Bad Dürrenberg, Germany) and powdered black pepper (*Piper nigrum* L., from Ostmann Gewürze, Dissen, Germany) were purchased in a local supermarket. Clove buds were coarsely ground before extraction, whereas black pepper was used directly. Aliquots of 2.5 g of the test substances were mixed with 100 mL of boiling water each followed by 15 min of stirring at room temperature. After filtration through Whatman filter paper, the filtrates were frozen and lyophilized. The lyophilized samples were solved in water (1 mg/mL) before experiments.

2.3. Three-step test system to analyze specific $GABA_A$ receptor modulation by the food extracts

The experiments to test for the specific modulation of $GABA_A$ receptors by food extracts were set up as described before (Sahin et al., 2016). Briefly, ionotropic α1β2-$GABA_A$- and glycine receptors were expressed in oocytes of *Xenopus laevis* after cRNA injection. Test solutions were prepared from pure GABA (or glycine, respectively) in buffer, adding the test extracts in various concentrations. Then the maximal current responses of ionotropic $GABA_A$ receptors during perfusion by the test solutions were measured in the transfected oocytes by two-electrode voltage clamp (TEVC). In TEVC, the absolute GABA-induced current amplitude in $GABA_A$ receptor-expressing *Xenopus laevis* oocytes does not only depend on the quantity and quality of the injected cRNA, but also on the oocyte charge and time of expression and, thus, varies among different experiments. Therefore, all readouts for the test compounds were normalized to the signal of 1 μM GABA obtained in the same oocyte, which was set to 100%. Additionally, an increase of the GABA signal during one test run was observed (Sahin et al., 2016). Therefore, the mean of the GABA signals that were administered directly before and after the test compound was used for normalization.

Any endogenous GABA content was quantified in the spice extracts after derivatization with ortho-phthaldialdehyde by HPLC–fluorescence light detection. To determine whether the test substances specifically addressed $GABA_A$ receptors, analyses were repeated accordingly with glycine test solutions/glycine receptors.

2.4. Semi-preparative reversed-phase liquid chromatographic fractionation

Aqueous clove extract was fractionated by reverse phase HPLC. The applied Jasco (Groß-Umstadt, Germany) HPLC unit was equipped with autosampler (AS-2057), degasser (DG 980-50), two pumps (PU-2087) and UV/Vis detector (UV-2077). For HPLC analysis, 500 μL of the clove extract was injected to a VP 250/10 NUCLEODUR 100-5 C_{18} ec column (Macherey-Nagel, Düren, Germany) and eluted with water (mobile phase A) and methanol (mobile phase B). Analysis started from 80% A /20% B with B increasing to 95% at 0–25 min. At 25–30 min, solvent B was kept at 95% followed by linear gradient change to 20% B at 30–35 min; at 35–40 min, solvent B was kept at 20%. The flow speed was 3 mL/min, and detection was performed at 254 nm. In total, 13 peak-based fractions were collected by a fraction collector (CHF 122SB, Advantec Toyo Seisakusho Kaisha, Kashiwa, Japan). The solvent was removed with a vacuum evaporator (Heidolph Instruments, Schwabach, Germany). Afterwards, the fractions were lyophilized and dissolved in MilliQ-water (1 mg/mL).

2.5. UHPLC–DAD

The aqueous solutions of the clove fractions were analyzed by a Dionex Ultimate 3000 RS liquid chromatography system consisting of a pump with degasser, autosampler, column compartment, and diode array detector (DAD). Ten microliters of each fraction was injected to a YMC-Ultra HT Pro C18 column (2 μm particle size; 2 × 50 mm, YMC, Kyoto, Japan) at 30 °C using a gradient composed of water (solvent A) and acetonitrile (solvent B). The gradient started 5 min before data acquisition. The elution profile between −5 and 0 min was kept at 5% B followed by linear gradient change to 95% B at 0–10 min; at 10–11 min, B was kept at 95%; at 11–14 min B was reduced to 5%; at 14–15 min, B was kept at 5%. The flow rate was set to 0.3 mL/min. Signals were detected by DAD allowing for the collection of full UV spectra. The ingredients were identified in the clove extract and its fractions by comparing the retention times and DAD spectra of the unknown peaks with those of the standard compounds. For quantification of eugenol in the extracts, 0.5 mg of eugenol was solved in 1 mL of MilliQ-water and used as stock solution. The calibration curve was established from the mean peak areas of triplicate UHPLC–DAD analyses of 12 concentrations of the eugenol-standard (0.5, 0.75, 1, 2.5, 5,

7.5, 10, 20, 25, 35, 45, and 50 μg/mL). The eugenol concentration in clove extract and its fractions was calculated using the equation of the external calibration curve. The coefficient of determination (R^2) of the calibration curve was 0.9996, the method's limit of detection 0.53 μg/mL and limit of quantification 1.6 μg/mL. To determine the eugenol concentration in the clove extract and its fractions, clove extract was injected into the UHPLC system in a concentration of 0.25 mg/mL and the fractions in a concentration of 1 mg/mL. The eugenol concentration of the samples, which were used for electrophysiological experiments in a concentration of 5 μg/mL, were calculated from these results.

2.6. Gas chromatography–mass spectrometry analysis

Aqueous clove extract (100 μL) was mixed on a Thermomixer Comfort (Eppendorf AG, Hamburg, Germany) with 200 μL of dichloromethane for 10 min at room temperature at a shaking speed of 14000 rpm ($1.97 \times 10^3 g$). After separation, the dichloromethane phase was dried over anhydrous sodium sulfate and transferred directly into vials for GC analysis.

The GC analysis was carried out on an Agilent 6890 series gas chromatograph connected to a Hewlett Packard 5973 mass spectrometer. The analytical capillary column used was DB-FFAP (J&W Scientific, Fisons Instruments, Mainz, Germany) in the dimension 30 m × 0.25 mm × 0.25 μm. The GC temperature was kept at 40 °C for 7 min, increased to 240 °C at a rate of 8 °C/min and held for 15 min. Helium was used as carrier gas at constant flow of 1 mL/min. Sample aliquots of 2 μL were injected by MPS2 autosampler (Gerstel, Duisburg, Germany). Mass spectrometry (MS) transfer line and ion source temperature were set to 240 °C and 200 °C, respectively. MS detection mode was electron ionization (EI) with a current of 70 eV (full scan, m/z 30–300). For the identification of substances, mass spectra and retention indices of all detected compounds in clove extract were compared with spectra of the injected reference compounds and with spectra in NIST mass spectral library (NIST MS Search 2.0).

2.7. Data analysis

The electrophysiological data were expressed as means ± standard error of the mean (SEM) fitting concentration–response data for agonist-evoked currents with the Hill equation by Origin 9.1 (OriginLab, Northampton, MA, USA). HPLC analysis results were expressed as mean ± SD. Statistical data analysis was done by GraphPad Prism 6 (GraphPad Software, La Jolla, USA) using repeated measures two-way ANOVA for the evaluation of $GABA_A$-receptor- and glycine-receptor response elicited by food extracts. Following ANOVA, the Holm-Sidak multiple comparisons test was performed with significance levels *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

3. Results and discussion

3.1. Specific allosteric modulation of human α1β2-$GABA_A$ receptor by aqueous extracts of clove buds and black pepper

Aqueous extracts of clove and black pepper were screened by an established three-step test system (Sahin et al., 2016) to determine specific $GABA_A$-receptor modulators. In the first step, the effects of aqueous spice extracts on the response of the human $GABA_A$-α1β2 heteropentameric receptors expressed in *Xenopus laevis* oocytes were tested by TEVC analysis. For this purpose, the test extracts were administered together with 1 μM GABA, which corresponded to the $EC_{5\text{-}15}$ range of GABA on this receptor (Sahin et al., 2016). In this range, small modulatory effects are recorded most sensitively (Hossain et al., 2002; Hossain et al., 2002). Fig. 1 illustrates the potentiation of the $GABA_A$-receptor responses elicited by the spice extracts that were applied at various concentrations (0.5–5 μg/mL). In both cases, a concentration-dependent increase of the maximum current was observed. The largest current amplitudes were recorded for clove applied in a concentration of 5 μg/mL (426 ± 23%; Fig. 1A/B; the potentation of $GABA_A$-receptor responses is always given as mean ± SEM). Using the same concentration, black pepper caused an increase by 274 ± 33%. The differences between the GABAergic activities of both spices are mainly caused by their molecular composition. However, it cannot be excluded that the form of the spice applied for extraction may also influence the test results. The use of powder instead of coarsely ground material may improve the extraction efficiency of active components. On the other hand, oxidation processes may occur during storage of the powder leading to the formation or degradation of active components. Zaugg et al. reported that the ethyl acetate extract of black pepper in a concentration of 100 μg/mL augmented the GABAergic responses by 169.1 ± 2.4% (Zaugg et al., 2010). These results indicate that the aqueous extract of black pepper may be even more active than the ethyl acetate extract. However, it is difficult to compare the latter study with the present work, because different test parameters were used, such as the $GABA_A$-subunit composition.

The present findings show that the aqueous extracts of clove and black pepper elicited strongly augmented GABA-evoked responses at the $GABA_A$ receptor of our test system (Sahin et al., 2016). Previously, this three-step test system identified aqueous extracts of hop cones and *Sideritis* as the most potent specific $GABA_A$-receptor modulators. At the lowest concentration of 0.5 μg/mL, the substances potentiated α1β2-$GABA_A$-receptor activity by 182 ± 27% (hop cones) and 173 ± 18% (*Sideritis sipylea)*, respectively. At the highest test concentration (5 μg/mL), hop cone- and *Sideritis stricta* extracts caused an increase of GABA-evoked currents of 348 ± 26% and 364 ± 66%, respectively (Sahin et al., 2016).

To exclude that the observed effects resulted from endogenous GABA in the spice extracts, their GABA concentrations were measured by HPLC in the second step. Only very low GABA-concentrations were determined in the analyzed black pepper extract corresponding to a concentration of 31 nM in the most concentrated test sample, whereas no GABA was detected in the clove extract (Table 1). The potency of GABA at the $GABA_A$ receptor is dependent on the subunit composition. Whereas, for example, α4/δ-containing $GABA_A$ receptors are activated by mid-nanomolar concentrations of GABA, the α1β2-containing receptor investigated in the current study does not react to GABA in this concentration range (Karim et al., 2013; Sahin et al., 2016). Therefore, it is very unlikely that the augmentation of the GABA response during co-application of GABA with clove- or black pepper extracts was induced by the additional endogenous GABA content of the extracts. Consistently, the current induced by the spice extracts in the absence of GABA, which may be caused by residual GABA or other agonists in the extract, was very low reaching only 6–15% of the response to 1 μM GABA (Table 1). These findings indicate that the observed positive modulatory effects of the analyzed food extracts on $GABA_A$ receptors were indeed caused by allosteric modulators.

Finally, to examine if the observed effects are specific to $GABA_A$ receptors, modulation of the activity of the human glycine receptor-α1 by the spice extracts was investigated in the third step. The glycine receptor, which is another inhibitory receptor of the same superfamily of ligand-gated ion channels, shows high homology to the $GABA_A$ receptor (Collingridge et al., 2009). None of the tested spice extracts potentiated the glycine-receptor function in the presence of glycine (Table 2). In contrast, even a low, but significant inhibition of the receptor response was observed.

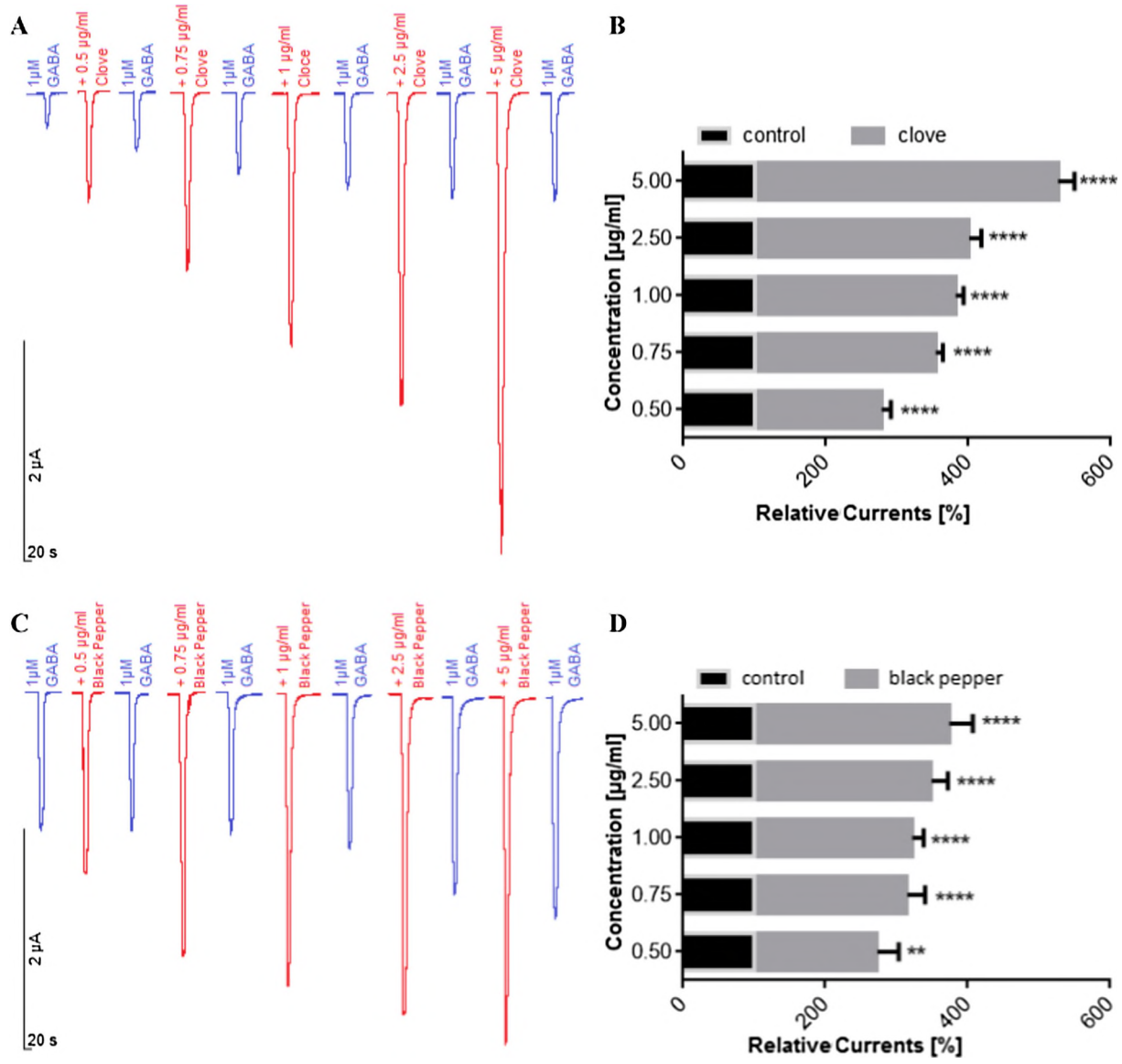

Fig. 1. Modulation of human α1β2-GABA$_A$ receptors expressed in *Xenopus laevis* oocytes by clove (A-B) and black pepper (C-D). Potentiation of GABA-induced current by various extract concentrations (0.5–5 µg/mL), control responses to 1 µM GABA as indicated (A, C). B and D depict mean current amplitudes (relative currents in %) compared to the response to pure GABA (100%) ± standard error of the mean (SEM) from at least three independent batches of oocytes. Because a slight time-dependent increase of the current was observed in all experiments, the average of the GABA current before and after the application of the extracts was set as 100%; $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

Table 1
Endogenous GABA contents of test food extracts and current amplitudes of α1β2-GABA$_A$ receptor-responses to the extracts without GABA addition.

Food extract	GABA content [µg/mL][a]	GABA$_A$ receptor response I_{rel} [%][b]	n[c]
Black pepper	0.633 ± 0.07	15 ± 2	6
Clove	nd[d]	6 ± 2	4

[a] Endogenous GABA contents of food extracts (1 mg/mL) determined by reverse phase HPLC fluorescence detection. Values are the mean ± SD from triplicate analysis;
[b] Current amplitudes of α1β2-GABA$_A$ receptor- responses to the food extracts (concentrations 7.5 µg/mL) without GABA addition. Mean current amplitudes (I_{rel}) are shown compared to the response to 1 µM pure GABA (100%) ± SEM, measured in at least two independent batches of oocytes.
[c] Number of *Xenopus laevis* oocyte recordings;
[d] nd: below level of detection.

Table 2
Modulatory effects of test food extracts on glycinergic responses of the human glycine receptor α1.

Food extract	Glycine- receptor response I_{rel} [%][a]	n[b]	Significance value[c]
Black pepper	74 ± 7	5	*
Clove	64 ± 18	4	**

[a] Modulatory effects of test food extracts at 5 µg/mL on the maximum current (I_{max}) response of glycine-hα1 receptor elicited by 30 µM glycine (30 µM glycine without addition = 100%). Mean current amplitudes compared to control ± SEM from at least two independent batches of oocytes;
[b] Number of *Xenopus laevis* oocyte recordings;
[c] Statistical significance of differences compared with pure glycine by using two-way ANOVA with Holm-Sidak multiple comparisons test is indicated as $^{*}p < 0.05$ and $^{**}p < 0.01$.

Thus, it can be concluded that the measured potentiation effects of black pepper and clove on the $GABA_A$ receptor are indeed receptor-specific.

3.2. Identification of the GABAergic components of aqueous extracts of clove buds by activity-guided fractionation

Because aqueous clove extract showed the highest activity to potentiate GABA-induced responses of the $GABA_A$ receptor in our study, the components responsible for its modulating activity were determined by HPLC-based activity profiling. This approach had identified piperine as the main GABAergic active compound of the ethyl acetate extract of black pepper before (Zaugg et al., 2010). The clove extract was separated by semi-preparative HPLC and 13 peak-based fractions were collected (Fig. 2A). Then, the $GABA_A$ receptor-modulating activity profiles of the clove fractions were measured in a concentration of 5 μg/mL analogously to the extracts. Here, fraction 11 corresponding to the peak that eluted at a retention time of 23.7 min (Fig. 2A) evoked the highest positive modulatory effect on the $GABA_A$ receptor with a potentiation of about 780% compared to sole application of GABA (Fig. 2B). Therefore, the composition of fraction 11 was further investigated by UHPLC–DAD: One main peak was recorded at a retention time of 6.9 min (Fig. 3A). The UV-spectrum of this peak suggested the presence of eugenol or a eugenol derivative. Therefore, an authentic eugenol reference was analyzed in the same way, revealing identical retention time and UV spectrum as the unknown compound. Additionally, the presence of eugenol was confirmed in the same way in the non-fractionated aqueous clove extract (Fig. 3B). Additionally, GC–MS analysis was performed to verify the identity of

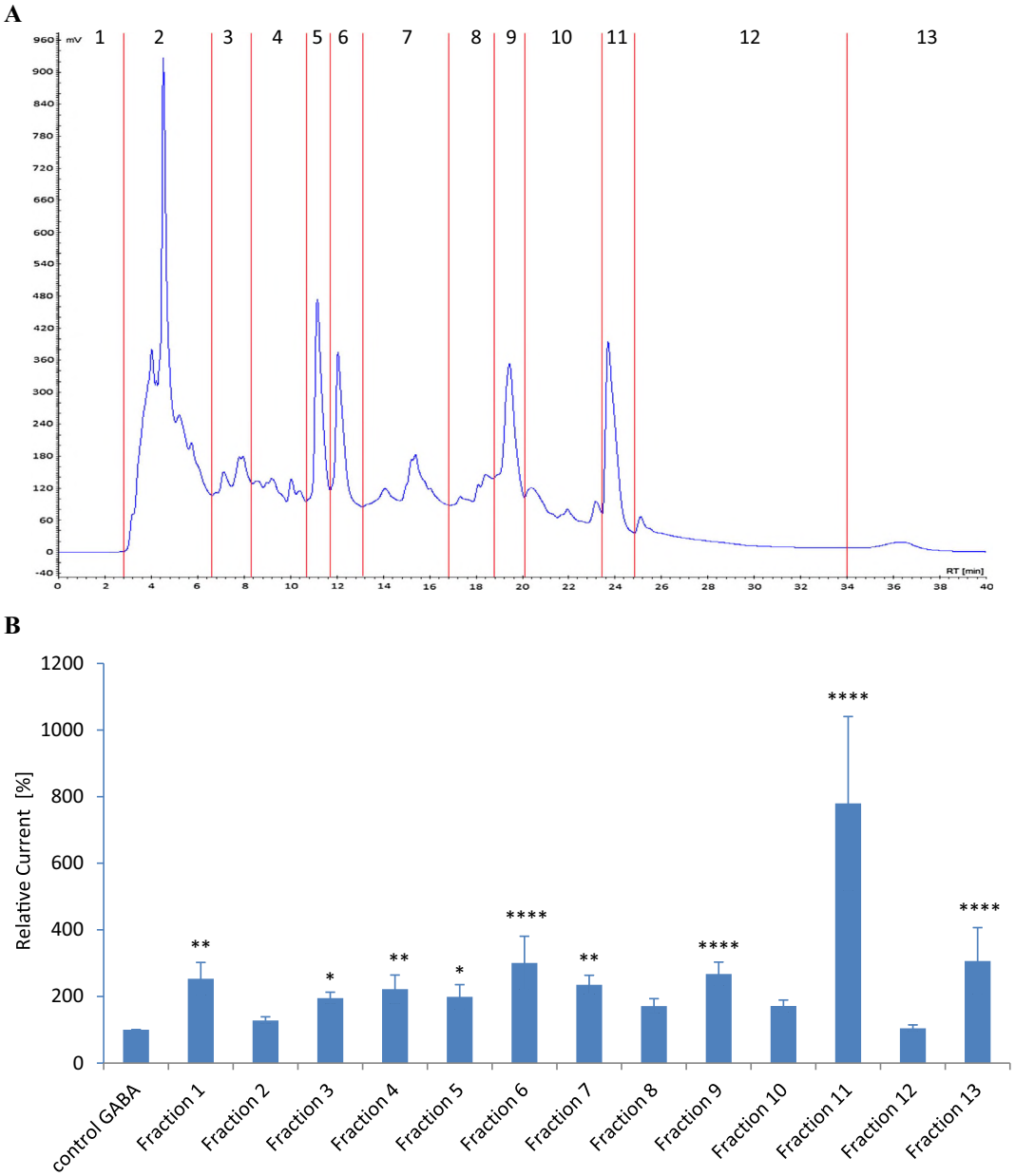

Fig. 2. Chromatogram of a semi-preparative separation of aqueous clove extract and the corresponding activity profile of the fractions. (A) Semi-preparative HPLC chromatogram of 20 mg/mL clove extract recorded at 254 nm. The numbers (1–13) indicate fraction numbers. The collection period of each fraction is shown by vertical lines. (B) Modulatory effects of the fractions (5 μg/mL) on the relative current responses of α1β2-$GABA_A$ receptors elicited by 1 μM GABA. Application of 1 μM pure GABA was set to 100%. Mean current amplitudes compared to control ± SEM from 4–10 oocytes; *p < 0.05 **p < 0.01, ***p < 0.001, and ****p < 0.0001.

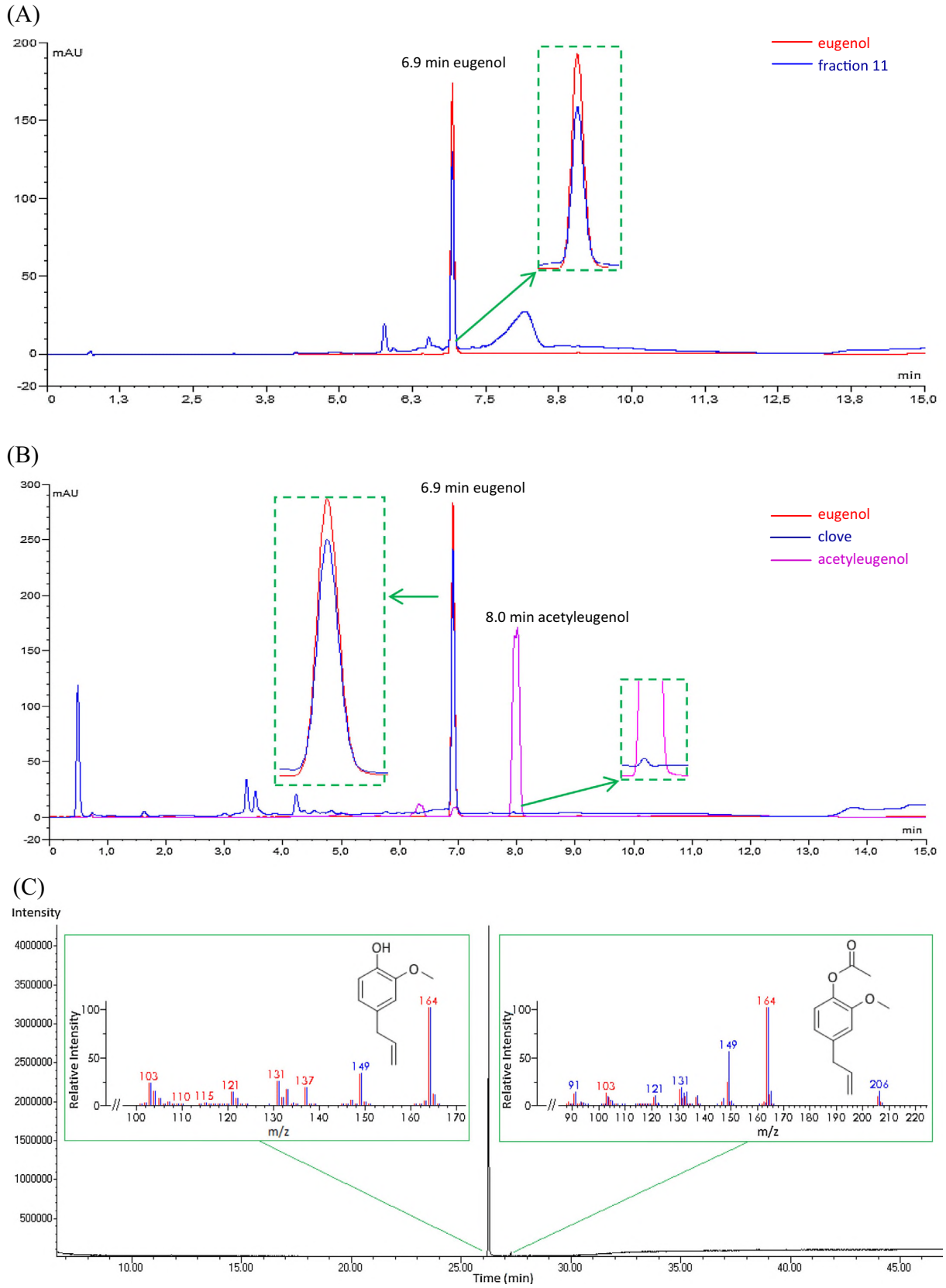

Fig. 3. (A) UHPLC–DAD chromatograms of fraction 11 of the clove extract (blue line) and eugenol standard (red line) recorded at 282 nm with an expanded view of the spectra in the inset. (B) UHPLC–DAD chromatograms of clove extract (blue line), eugenol (red line), and acetyleugenol standard (pink line) at 282 nm. The insets demonstrate expanded views of the eugenol and acetyleugenol signals. (C) GC–MS total ion chromatogram of clove extract, eugenol (*m*/*z* 164), and acetyleugenol (*m*/*z* 206). The insets on the picture provide expanded views of the eugenol and acetyleugenol mass spectra (red lines: present clove extract, blue lines: spectra given by NIST Mass spectral library).

eugenol in the clove samples. Analysis yielded the same MS spectrum as the authentic reference (Fig. 3C), thus unequivocally confirming the identity of eugenol as a main component in fraction 11 and its presence in the clove extract. Despite these results, however, it cannot be excluded that other components, which do not absorb UV light, might be responsible for the high activity of fraction 11. Therefore, the contribution of eugenol to the overall $GABA_A$ receptor-modulating activity of the clove extracts was determined. For this purpose, eugenol was quantified in the whole clove extract by UHPLC–DAD revealing a content of 0.2 μg/μg.

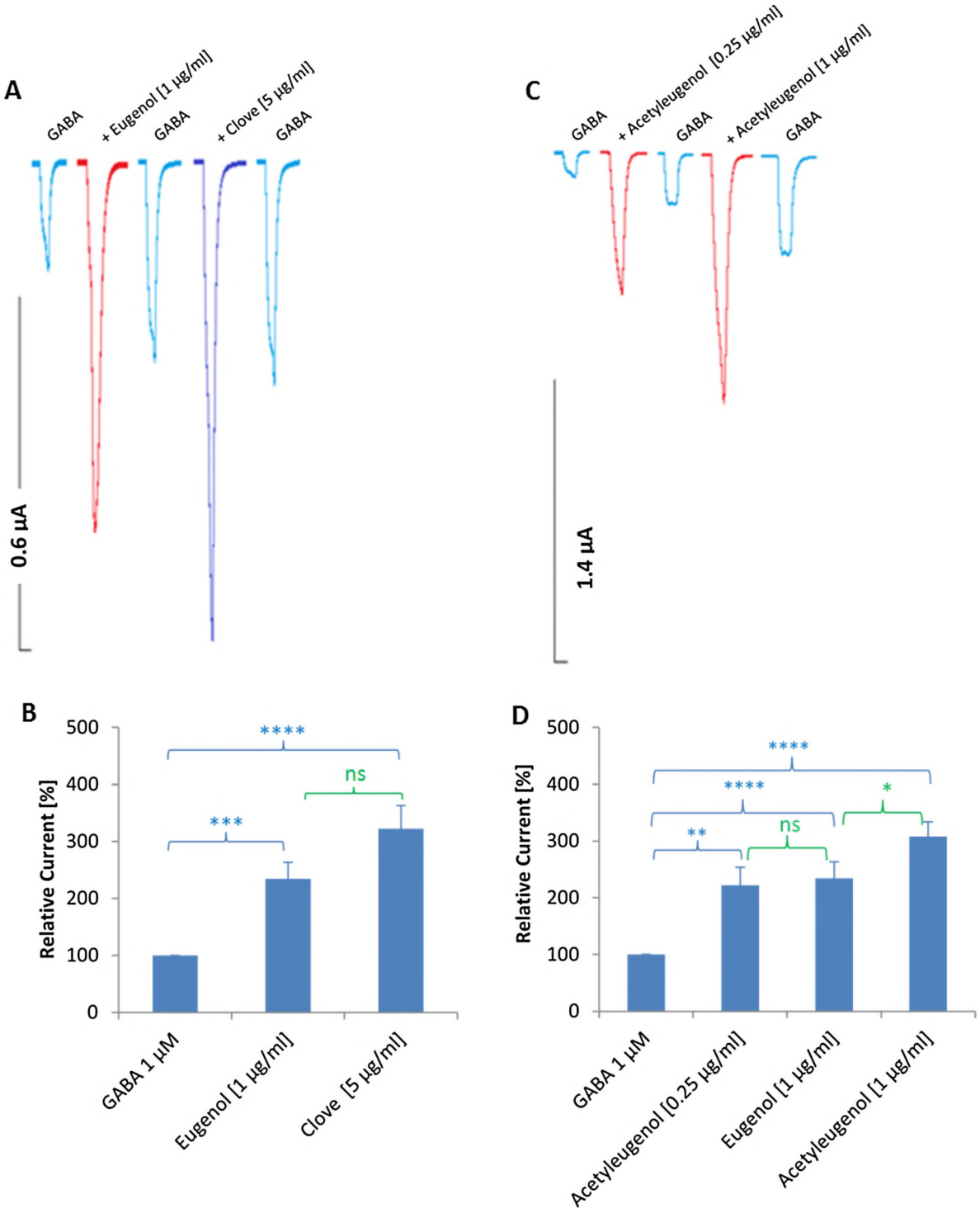

Fig. 4. Potentiation of human α1β2-$GABA_A$ receptors expressed in *Xenopus* oocytes by eugenol, acetyleugenol, and clove extract. (A) Current responses of the modulators eugenol (1 μg/mL) and clove extract (5 μg/mL). (B) Mean current amplitudes compared to sole application of GABA (100%) ± SEM from 5–10 oocytes of two to four independently transfected batches. (C) Representative current traces following sole application of GABA or GABA in combination with acetyleugenol at concentrations of 0.25 and 1 μg/mL, and clove extract at 5 μg/mL. (D) Potentiating effects of acetyleugenol on $GABA_A$ receptor. The difference between the effects of 0.25 and 1 μg/mL acetyleugenol was not significant. Application of 1 μM pure GABA was set to 100%. Because of a slight time-dependent increase of the current in all experiments, the average of the GABA current before and after application of the extracts was used for mean subtraction. Mean current amplitudes compared to control ± SEM from 7–10 oocytes; $^{*}p < 0.05$ $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

Eugenol was then tested separately in the same concentration as present in the clove extract, which was analyzed in parallel by measuring the response of α1β2-containing $GABA_A$ receptors during co-application of 1 μM GABA (Fig. 4A). All samples significantly enhanced the GABA-gated currents compared to the sole application of GABA (Fig. 4B). Clove extract at the concentration of 5 μg/mL produced a potentiation of up to 322 ± 41%, while 1 μg/mL eugenol induced an enhancement of agonist response up to 234 ± 29% (Fig. 4B). The differences between both samples, however, were not significant ($p = 0.35$). Thus, it can be concluded that eugenol is the major determinant of the $GABA_A$ receptor-modulating activity of the clove extract. However, minor contribution of other components cannot be excluded.

3.3. GABAergic activity of acetyleugenol

Besides eugenol, acetyleugenol was detected in the aqueous clove extracts by UHPLC–DAD and GC–MS analysis (Fig. 3B/C), but in concentrations below the limit of quantification. To determine possible $GABA_A$ receptor-modulating activity, acetyleugenol was tested in a concentration of 1 μg/mL by TEVC analysis (Fig. 4C). Acetyleugenol evoked an augmentation of 308 ± 26%, which was significantly higher ($p < 0.05$) compared to the signal enhancement by the identical concentration of eugenol (234 ± 29%) (Fig. 4D).

Although acetyleugenol proved to be a very potent modulator of $GABA_A$ receptors, its contribution to the activity of the aqueous clove extract was presumably relatively small, because the concentration of acetyleugenol in the aqueous extracts was very low. In contrast to the present aqueous extract, it was reported that clove bud oil is composed of up to 21% acetyleugenol and up to 85% eugenol (Mittal, Gupta, Parashar, Mehra, & Khatri, 2014; Razafimamonjison et al., 2014). To estimate if acetyleugenol may have a relevant effect on the $GABA_A$ receptor in clove bud oil, the effects of eugenol and acetyleugenol were tested in the same ratio as reported in the oil. Therefore, the response of 0.25 μg/mL acetyleugenol was compared to the response of 1 μg/mL eugenol (Fig. 4D). At the aforementioned concentrations, acetyleugenol potentiated $GABA_A$-receptor activity by up to 222 ± 32% evoking only a slightly lower signal ($p = 0.93$, nonsignificant) compared to 1 μg/mL eugenol (Fig. 4D). Therefore, it can be assumed that, in contrast to aqueous clove extracts, acetyleugenol and eugenol may have similar $GABA_A$ receptor-modulating activities in clove bud oil. Due to its lower water solubility, however, acetyleugenol is not recovered in the aqueous extract.

Additionally, β-caryophyllene had been identified in clove bud essential oil before (Mittal et al., 2014; Razafimamonjison et al., 2014). Because high concentrations of β-caryophyllene have been reported to exert some $GABA_A$ receptor-modulating activity (Kessler et al., 2014), its presence in the aqueous clove extracts was analyzed by GC–MS. However, β-caryophyllene was not detected in the aqueous clove samples indicating that this compound is not sufficiently water-soluble (data not shown).

Eugenol belongs to the phenylpropanoid group (Chaieb et al., 2007) and is, in contrast to acetyleugenol and caryophyllene, slightly soluble in water (Rahimi, Ashnagar, & Hamideh, 2012).

It was shown previously that eugenol potentiates the response of $GABA_A$ receptors expressed in *Xenopus* oocytes injected with rat whole-brain mRNA (Aoshima & Hamamoto, 1999) or with bovine brain cRNAs of $GABA_A$ receptors composed of α1- and β1-subunits (Aoshima et al., 2001). Similarly, eugenol was shown to increase the GABA-mediated Cl^- influx in primary cultures of cortical neurons in a dose-dependent manner (Reiner et al., 2013). In the present study, we demonstrated that eugenol increased the GABA-evoked chloride currents resulting from potentiated activity of $GABA_A$ receptors, which were composed of human α1- and β2-subunits expressed in an oocyte system. Under these conditions, eugenol proved to be the main determinant of the $GABA_A$ receptor-modulating activity of an aqueous clove extract.

To our knowledge, the modulatory potential of acetyleugenol on $GABA_A$-receptor activity has not been described before. Ding et al. identified methyleugenol, which is an ortho-alkylated derivative of eugenol, as a novel agonist of $GABA_A$ receptors. They reported that methyleugenol enhanced the GABA-evoked currents in hippocampal neurons at a concentration of 30 μM (~5 μg/mL), and also potentiated the GABA-gated responses and thereby $GABA_A$-receptor activity in HEK-293T cells expressing α1β2γ2- or α5β2γ2 $GABA_A$-receptor subunits with an EC_{50} of 290 μM (~52 μg/mL) and 198 μM (~35 μg/mL), respectively (Ding et al., 2014). In the present study, it was observed that eugenol and its derivative, acetyleugenol, significantly increased the α1β2-containing $GABA_A$-receptor activity at very low concentrations (0.1–1 μg/mL). These findings show that the GABAergic modulatory effect of eugenol and its derivatives (methyl- and acetyleugenol) can be related to structural similarities.

Obviously, the phenolic structure is an important determinant interacting with the GABAergic system. For example, thymol or its isomer carvacrol also enhanced the function of $GABA_A$ receptors consisting of human subunits α1β1γ2, α6β3γ2, and α1β3γ2 (thymol) (Priestley, Williamson, Wafford, & Sattelle, 2003) or via native $GABA_A$ receptor in primary cultures of cortical neurons (thymol, carvacrol) (Garcia, Bujons, Vale, & Sunol, 2006; Reiner et al., 2013). In addition to allosteric modulatory effects on $GABA_A$ receptors, thymol directly activates the $GABA_A$ receptor (Garcia et al., 2006; Mohammadi et al., 2001; Priestley et al., 2003). The anesthetic propofol, an ortho-alkylated phenolic compound similar to thymol, also directly activated the $GABA_A$ receptor (Mohammadi et al., 2001; Priestley et al., 2003), and this agonist effect may account for its sedative-hypnotic actions (Mohammadi et al., 2001). Despite their high structural similarity, eugenol and carvacrol did not evoke chloride influx through $GABA_A$ receptors in the absence of GABA (Reiner et al., 2013). In the present study, we also observed that eugenol in clove extracts did not induce chloride currents by an agonistic mechanism.

Data on the bioavailability of eugenol across the blood brain barrier are scarce. However, in several *in vivo* studies, orally administered eugenol exerted direct effects in the brain indicating a passage through the blood brain barrier, which is consistent with its hydrophobic structure (Irie et al., 2004; Said & Rabo, 2017). Since acetyleugenol is even more hydrophobic than eugenol, at least a similar transition into the brain can be expected. In animal studies performed on mice and rats, eugenol suppressed epileptiform activity and exerted antidepressant effects, which may be related to GABA signaling (Müller, Page, Speckmann, & Gorji, 2006). Furthermore, eugenol can be used in dental clinics for its anesthetic and analgesic effects. It was shown that eugenol showed topical anesthetic effects elicited by a significant attenuation of pain in humans using the homemade clove gel (Alqareer et al., 2006). Moreover, animal studies demonstrated the anesthetic effect of eugenol in male Sprague–Dawley rats (Guenetta, Beaudry, Marier, & Vachon, 2006) and in *Xenopus laevis* frogs (Goulet, Hélie, & Vachon, 2010). Thus, it can be hypothesized that the GABAergic effects observed in the present study may also be of relevance *in vivo*. On the other hand, substituted alkenylbenzenes, mainly methyleugenol, can act as genotoxic carcinogen (Groh et al., 2016) raising the question if eugenol intake may also have adverse effects. However, possible negative effects of eugenol consumption have been revised by the Joint FAO/WHO Expert Committee on Food Additives concluding that the available data do not support a carcinogenic potential of eugenol setting an acceptable daily intake of 0–2.5 mg/kg of body weight (JECFA., 1982).

4. Conclusion

The spice *Syzygium aromaticum* L. (clove buds) exerts anesthetic and analgesic effects.

Since GABA signaling is involved in the pathophysiology of pain (Li & Zhang, 2012), the present results strongly suggest that the analgesic effects of clove and eugenol may be at least partially mediated by the potentiation of $GABA_A$-receptor activity. Further studies are now required to determine if $GABA_A$-receptor signaling is involved in the analgesic activity of clove *in vivo*. Furthermore, it remains to be elucidated if $GABA_A$-receptor modulation by clove and eugenol may also result in sedative and sleep-enhancing effects.

Acknowledgement

We thank Prof. Christoph Korbmacher from the Institute of Cellular and Molecular Physiology, FAU, for providing *Xenopus laevis* oocytes and Christine Meissner for proofreading the manuscript. Funding by the Turkish Ministry of National Education for Sümeyye Sahin is gratefully acknowledged.

Conflict of interest

The authors declare no conflict of interest. Funders did not influence the research.

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