Advances in Biological Chemistry, 2014, 4, 148-159
Published Online April 2014 in SciRes. http://www.scirp.org/journal/abc
http://dx.doi.org/10.4236/abc.2014.42019

$GABA_A$ Receptor Modulation by Compounds Isolated from *Salvia triloba* L.

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Received 20 February 2014; revised 21 March 2014; accepted 28 March 2014

Abstract

***Salvia triloba*, traditionally known as Greek sage, has been used to enhance memory, as a sedative and to treat headaches. Pharmacological evaluation of purified extracts and isolated compounds of *S. triloba* were carried out on functional assays using two-electrode voltage clamp methods on recombinant GABA receptors expressed in *Xenopus laevis* oocytes. Bio-assay guided fractionation led to seven compounds being isolated from *S. triloba*: ursolic acid, carnosol, oleanolic acid, salvigenin, rosmanol, cirsimaritin and hispidulin. The purified extracts of *S. triloba* inhibited 54% of the current produced by 300 µM GABA at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors. Ursolic acid, carnosol, oleanolic acid and rosmanol also acted as negative allosteric modulators. The flavonoids salvigenin, cirsimaritin and hispidulin acted as positive modulators when applied in the presence of low concentrations of GABA but in the presence of high concentrations of GABA acted as negative modulators, demonstrating a biphasic action. These results are consistent with the concept that *Salvia triloba* may have cognition enhancing properties. In most cases these activities are likely to be occurring via different modulatory sites on $GABA_A$ receptor complexes. It may be that the combination of these activities permits cognition enhancement whilst offering protection from convulsant activity.**

Keywords

$GABA_A$ Receptors; *Salvia triloba*; Flavonoids; Sedative; Anxiolytics; Chromatography; Allosteric Modulation

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How to cite this paper: Abdelhalim, A., *et al.* (2014) $GABA_A$ Receptor Modulation by Compounds Isolated from *Salvia triloba* L. *Advances in Biological Chemistry*, **4**, 148-159. http://dx.doi.org/10.4236/abc.2014.42019

1. Introduction

Salvia triloba L. (Lamiaceae), traditionally known as Greek sage, is also known by several other names including *Salvia fruticosa*, *Salvia libanotica*, *Salvia lobryana*, and *Salvia cypria* due to a taxonomic confusion over the years. It is a perennial Mediterranean herb and has been used to enhance memory [1], as a sedative [2] and to treat headaches [3]. Many phytochemicals are known to interact with receptors for the major inhibitory neurotransmitter GABA [4]. Such interaction may underlie some of the pharmacological effects of *S. triloba*. The constituents of an ethanolic extract of *S. triloba* have been shown to have moderate affinity to $GABA_A$ benzodiazepine receptor sites [5]. Another study demonstrated that compounds isolated from this plant increased the hypnotic effect of hexobarbital in the rat [6] [7], suggesting a benzodiazepine like effect.

Ionotropic GABA receptors are ligand gated chloride channels that mediate much of the fast inhibitory neurotransmission in the brain [8]. $GABA_A$ receptors are antagonised by the convulsant alkaloid bicuculline [9] and modulated by drugs such as benzodiazepines and barbiturates as well by a range of phytochemicals including flavonoids [4] [10]. The less numerous homomeric $GABA_C$ receptors are insensitive to bicuculline and most of the chemicals that modulate $GABA_A$ receptors. The receptor complex is a pentamer composed of a combination of five subunits of these subunits (α1-6, β1-3, γ1-3, δ, ε, π, θ). However, most $GABA_A$ receptors appear to be formed by 2α, 2β subunits in combination with one subunits form another class. The most common subunit combination in the human brain was found to be $\alpha_1\beta_2\gamma_2$ [8] [11].

We have studied the effects of extracts and isolated compounds from *S. triloba* on functional assays using two-electrode voltage clamp methods on recombinant $GABA_A$ receptors containing $\alpha_1\beta_2\gamma_{2L}$ subunits expressed in *Xenopus laevis* oocytes. Using bio-assay guided fractionation we found seven phytochemicals that modulated these receptors.

2. Materials and Methods

2.1. Plant Materials

Dried *Salvia triloba* plants were purchased from herbal markets located in Amman. The plants were identified by Prof. Dawud AL-Eisawi (Department of Biological Sciences, Faculty of Science, University of Jordan). Voucher specimens were deposited at the herbarium of the University of Jordan.

2.2. Chemicals, Materials and Instrumentation

All chemicals used were purchased from Aldrich Chemical Co. Ltd (St Louis, MO, USA) unless otherwise stated and were of highest commercially available purity. Solvents were distilled by standard techniques prior to use. Silica gel for column chromatography (CC) was performed on silica gel (Merck silica gel 60H, particle size 5 - 40 μm) and Sephadex LH-20 gel. Routine thin layer chromatography (TLC) was performed on Merck aluminium backed plates, pre-coated with silica (0.2 mm, 60F254).

The UV-Spectra were recorded on Hitachi U-2000 double beam UV/Vis Spectrophotometer. Mass spectra were carried out on a ThermoFinnigan (Waltham, MA, USA) PolarisQ Ion Trap system using a direct exposure probe. Nuclear magnetic resonance ^{1}H NMR and ^{13}C NMR spectra were recorded on 400 MHz Varian Gemini spectrometer (Palo Alto CA, USA) in DMSO-d_6 (Sigma-Aldrich, USA) with tetramethylsilane as internal standard. Melting points were determined using a Stuart (Stone, Staffordshire, UK) SMP10 melting point apparatus.

2.3. Preparation of the Extracts and Solvent Fractionation

The dried material (5 kg) was ground into fine powder and defatted by extraction with petroleum ether at room temperature for 7 days. After filtration, the remaining materials were then extracted extensively with ethanol for (3 times, 10 days each) at room temperature. The resulting ethanol extract was partitioned between $CHCl_3$ and H_2O (1:1 v/v). The dried chloroform extract was further partitioned between *n*-hexane and 10% aqueous methanol. The dried methanolic extract was then extracted with ethyl acetate to give the final extract.

2.4. Isolation of Constituents

The final extract (43 g) was chromatographed on a silica gel column chromatography (Φ28 × 8 cm) eluted with

a gradient of MeOH/$CHCl_3$ of increasing polarity resulting in six fractions (SI-SVI). Each fraction was further purified by a combination of column chromatography and preparative thin layer chromatography using suitable solvent systems.

The addition of ethyl acetate to the methanolic extracts of *S. triloba* yielded an impure solid that was then washed with methanol for further purification and afforded ursolic acid (3-hydroxy-urs-12-en-28-oic acid). Adding chloroform to fraction SI lead to the precipitation of impure solid which was then recrystallize by methanol to give carnosol as a white crystals. The TLC of the mother liquor of fraction SI showed a major UV active spot resulting in precipitation of an impure solid which give upon further purification by chloroform salvigenin (5-hydroxy-6,7,4'-trimethoxyflavone). Fraction SII was subjected to column chromatography (Φ31 × 4 cm) and was eluted with hexane/$CHCl_3$ mixtures of increasing polarity until pure chloroform was used providing three subfractions (SII-1- SII-3). Fraction SII-2 afforded a pure white solid that was then identified as oleanolic acid (3-hydroxy-olean-12-en-28-oic acid). The addition of methanol to fraction SIV yielded an impure solid that produced two major UV active spots on TLC with R_f values of 0.4 and 0.3 (2% MeOH/$CHCl_3$ as the solvent system). This solid was separated by column chromatography on a Sephadex LH-20 gel (Φ21 × 4 cm) to yield rosmanol (R_f = 0.4) and cirsimaritin (R_f = 0.3) (5,4'-dihydroxy-6,7-dimethoxyflavone). An impure solid precipitated upon treating fraction SV with methanol. Further washing with hot acetonitrile of this precipitate gave hispidulin (5,7,4'-trihydroxy-6-methoxyflavone) as pure yellow solid.

2.5. Physical and Spectroscopic Data of the Isolated Compounds

Ursolic acid (1): light white powder, m.p. 209˚C - 220˚C; ESMS *m/z* (%): 479.4 (100), 457 (5), 439 (15), 425 (44), 413 (12). ^{1}H-NMR (DMSO-d6) δ ppm: 5.11 (H, t, J = 3.4 Hz, H-12), 2.99 (H, m, H-3), 2.08 (H, d, J = 11.6 Hz, H-18), 1.02 (3H, s, Me-27), 0.89 (3H, s, Me-23), 0.87 (3H, s, Me-26) 0.84 (H, d, J = 6 Hz, H-30), 0.79 (H, d, J = 6 Hz, H-29), 0.73 (3H, s, Me-24), 0.65 (3H, s, Me-25); ^{13}C-NMR (DMSO-d6) δ ppm: 178.7 (C-28), 138.6 (C-13), 125.0 (C-12), 77.3 (C-3), 55.2 (C-5), 52.8 (C-18), 47.5 (C-9), 47.3 (C-17), 42.1 (C-14), 39.5 (C-8), 38.9 (C-4), 38.9 (C-19), 38.8 (C-20), 38.7 (C-1), 37.0 (C-10), 36.8 (C-22), 33.2 (C-7), 30.6 (C-21), 28.7 (C-15), 28.0 (C-23), 27.4 (C-2), 24.3 (C-16), 23.7 (C-27), 23.3 (C-11), 21.5 (C-30), 18.4 (C-6), 17.5 (C-29), 17.4 (C-26), 16.5 (C-24), 15.7 (C-25).

Carnosol (2): white crystals, m.p. 209˚C - 220˚C; UV λ_{max} (MeOH) nm: 209 and 285; ESMS *m/z* (%): 683.4 (100), 331.0 (19); ^{1}H-NMR (DMSO-d_6) δ ppm: 6.68 (H, s, H-14), 5.44 (1H, dd, *J* = 3.8, 1.4 Hz, H-7), 3.21 (1H, sept, *J* = 6.8, H-15), 2.64 (1H, dd, *J* = 14, 1.6 Hz, H-1α), 2.43 (1H, ddd, *J* = 14, 4.6 Hz, H-1β), 2.01 (1H, ddd, H-6α), 1.6 (1H, m, H-6β), 1.59 (1H, dd, *J* = 10.6, 5.4 Hz, H-5), 1.50 (1H, m, H-2β), 1.42 (1H, dd, *J* = 13, 1.4 Hz, H-3α), 1.21 (1H, ddd, *J* = 13.4, 3.2 Hz, H-3β), 1.10 (6H, d, *J* = 6.8, H-17, H-16), 0.79 (3H, s, H-18), 0.77 (3H, s, H-19); ^{13}C-NMR (DMSO-d_6) δ ppm: 175.9 (C-20), 143.7 (C-11), 143.5 (C-12), 134.7 (C-13), 132.0 (C-8), 122.3 (C-9), 111.7 (C-14), 77.4 (C-7), 48.3 (C-10), 45.4 (C-5), 41.0 (C-3), 34.6 (C-4), 31.8 (C-18), 29.7 (C-6), 29.2 (C-1), 26.6 (C-15), 23.2 (C-17), 23.1 (C-16), 19.8 (C-19), 19.0 (C-2).

Oleanolic Acid (3): light white powder; ESMS *m/z* (%): 456 (100), 431 (15), 391 (8); ^{1}H-NMR (DMSO-d_6) δ ppm: 5.14 (H, t, H-12), 2.98 (H, t, H-3), 2.78 (H, dd, *J* = 6.8, 4 Hz, H-18), 1.07 (3H, s, Me-27), 0.87 (3H, s, Me-25), 0.85 (6H, s, Me-30, 24), 0.70 (H, s, H-29), 0.66 (3H, s, Me-26), 0.65 (3H, s, Me-23); ^{13}C-NMR (DMSO-d_6) δ ppm: 179.0 (C-28), 144.3 (C-13), 122.0 (C-12), 77.3 (C-3), 55.2 (C-5), 47.5 (C-9), 46.1 (C-19), 45.9 (C-17), 41.8 (C-14), 41.3 (C-18), 38.8 (C-8), 38.5 (C-1), 37.0 (C-10), 33.8 (C-21), 33.3 (C-29), 32.9 (C-22), 32.5 (C-7), 30.8 (C-20), 28.7 (C-23), 27.6 (C-15), 27.4 (C-2), 26.0 (C-27), 23.8 (C-30), 23.3 (C-16), 23.1 (C-11), 18.5 (C-6), 17.3 (C-26), 16.5 (C-24), 15.5 (C-25).

Salvigenin (4): light yellow powder; UV λ_{max} (MeOH) nm: 332 (Band I), 277 (Band II); +NaOMe, 327, (Band I), 278 (Band II); +$AlCl_3$, 349 (Band I), 300 (Band II); +HCl, 349 (Band I), 299 (Band II); ESMS *m/z* (%): 350.9 (100), 328.9 (4); ^{1}H-NMR (DMSO-d_6) δ ppm: 12.86 (1H, s, 5-OH), 8.06 (2H, d, *J* = 8.8 Hz, H-2', H-6'), 7.11 (2H, d, *J* = 8.8 Hz, H-3', H-5'), 6.95 (1H, s, H-8), 6.93 (1H, s, H-3), 3.92 (3H, s, 7-OMe), 3.85 (3H, s, 4'-OMe), 3.72 (3H, s, 6-OMe); ^{13}C-NMR (DMSO-d_6) δ ppm: 182.7 (C-4), 164.1 (C-2), 162.9 (C-4'), 159.2 (C-7), 153.1 (C-5), 152.5 (C-9), 132.4 (C-6), 128.8 (C-2', C-6'), 123.2 (C-1'), 115.1 (C-3', C-5'), 105.6 (C-10), 103.8 (C-3), 92.1 (C-8), 60.5 (6-OMe), 56.9 (7-OMe), 50.1 (4'-OMe).

Rosmanol (5): light white powder; ESMS *m/z* (%): 314.9 $[M-CH3]^+$ (100), 369.0 (93), 301.0 (28), 347 (8); ^{1}H-NMR (DMSO-d_6) δ ppm: 6.68 (H, s, H-14), 5.08 (1H, d, *J* = 4.4 Hz, H-7), 4.13 (1H, t, *J* = 4 Hz, H-6), 3.21 (1H, sept, *J* = 1.6 Hz, H-15), 2.59 (1H, d, *J* = 14.4 Hz, H-1α), 2.57 (1H, m, H-5), 2.53 (1H, ddd, *J* = 13.8, 4.4 Hz,

H-1β), 1.60 (1H, m, H-2α), 1.19 (1H, d, J = 4 Hz, H-3α), 1.49 (1H, m, H-2β), 1.21 (1H, ddd, J = 13.4, 3 Hz, H-3β), 1.25 (3H, d, J = 1.6 Hz, H-17), 1.11 (3H, d, J = 1.6 Hz, H-16), 0.94 (3H, s, H-18), 0.79 (3H, s, H-19); ^{13}C-NMR (DMSO-d_6) δ ppm: 175.2 (C-20), 143.9 (C-11), 143.4 (C-12), 134.6 (C-13), 128.8 (C-8), 122.2 (C-9), 115.2 (C-14), 80.0 (C-6), 67.7 (C-7), 55.0 (C-5), 47.9 (C-10), 41.1 (C-3), 34.3 (C-4), 32.3 (C-18), 29.2 (C-1), 26.7 (C-15), 23.3 (C-17), 23.0 (C-16), 21.3 (C-19), 18.9 (C-2).

Cirsimaritin (6): light yellow crystals, m.p. 267˚C - 269˚C; UV λ_{max} (MeOH) nm: 333 (Band I), 275 (Band II); +NaOMe, 387, (Band I), 272 (Band II); +$AlCl_3$, 353 (Band I), 299 (Band II); +HCl, 353 (Band I), 299 (Band II); ESMS *m/z* (%): 336.8 (100), 314.9 (43), 300.9 (4); ^{1}H-NMR (DMSO-d_6) δ ppm: 12.91 (1H, s, 5-OH), 7.94 (2H, d, J = 8.8 Hz, H-2', H-6'), 6.91 (2H, d, J = 8.8 Hz, H-3', H-5'), 6.91 (1H, s, H-8), 6.82 (1H, s, H-3), 3.90 (3H, s, 7-OMe), 3.71 (3H, s, 6-OMe); ^{13}C-NMR (DMSO-d_6) δ ppm: 182.7(C-4), 164.5 (C-2), 161.9 (C-4'), 159.0 (C-7), 153.1 (C-5), 152.5 (C-9), 132.3 (C-6), 129.0 (C-2', C-6'), 121.4 (C-1'), 115.4 (C-3', C-5'), 105.5 (C-10), 103.1 (C-3), 92.0 (C-8), 60.5 (6-OMe), 56.9 (7-OMe).

Hispidulin (7): light yellow powder, m.p. 290˚C - 292˚C; UV λ_{max} (MeOH) nm: 334 (Band I), 274 (Band II); +NaOMe, 392, (Band I), 276 (Band II); +$AlCl_3$, 353 (Band I), 300 (Band II); +HCl, 351 (Band I), 299 (Band II); ESMS *m/z* (%): 322.9 (100), 300.9 (98), 271.0 (13), 238.9 (8), 197.7 (7); ^{1}H-NMR (DMSO-d_6) δ ppm: 13.06 (1H, s, 5-OH), 7.90 (2H, d, J = 8.8 Hz, H-2', H-6'), 6.90 (2H, d, J = 8.8 Hz, H-3', H-5'), 6.76 (1H, s, H-3), 6.57 (1H, s, H-8), 3.73 (3H, s, 6-OMe); ^{13}C-NMR (DMSO-d_6) δ ppm: 182.6 (C-4), 164.2 (C-2), 161.6 (C-4'), 157.7 (C-7), 153.2 (C-5), 152.8 (C-9), 131.8 (C-6), 128.9 (C-2', C-6'), 121.7 (C-1'), 116.4 (C-3', C-5'), 104.5 (C-10), 102.8 (C-3), 94.7 (C-8), 60.4 (6-OMe).

2.6. Pharmacological Analysis

Pharmacological evaluation of the final extract and the isolated compounds from *Salvia triloba* were carried out on functional assays using two-electrode voltage clamp methods on recombinant GABA receptors expressed in *Xenopus laevis* oocytes using the methods described previously [12]. Bio-assay guided fractionation led to seven compounds being isolated from *S. triloba*; these are: ursolic acid, carnosol, oleanolic acid, salvigenin, rosmanol, cirsimaritin and hispidulin.

3. Results

The final extract of *Salvia triloba* tested at 200 mg/mL did not show any effect at uninjected oocytes. The extract of *S. triloba* activated the receptor by 53.2% ± 0.2% when applied alone at $\alpha_1\beta_2\gamma_{2L}$ GABA receptors and inhibited currents due to 300 µM GABA by 54.1% ± 0.2%.

3.1. Ursolic Acid

Ursolic acid it did not show any effect at sham injected oocytes or at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors when administered alone. However, At 300 µM ursolic acid inhibited currents due to 100 µM GABA by 29.0% ± 0.4% (**Figure 1**, **Table 1**). GABA dose response curves were carried out both with and without ursolic acid (**Figure 1**) at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors. Ursolic acid (100 µM) shifted the GABA dose response curves at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors to the right, increasing the mean GABA EC_{50} from 123.3 to 210.4 µM (95% CI: 29.4 to 1508), with a Hill slope of 0.48 ± 0.16 (compared to 0.73 ± 0.07 in the case of GABA alone). The effect of ursolic acid was greater at higher concentrations of GABA, with no effect at concentrations below 30 µM GABA.

3.2. Oleanolic Acid

Oleanolic acid (**Figure 2**) did not have any effect on sham-injected oocytes (n = 3, data not shown). Also, oleanolic acid demonstrated no activity at $\alpha_1\beta_2\gamma_{2L}$ GABA receptors when administered alone but inhibited currents due to 3000 µM GABA by 31.0% ± 1.6% (**Table 1**).

3.3. Carnosol

Carnosol (**Figure 3**) did not have any effect at sham-injected oocytes (n = 3, data not shown) or at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors when administered alone, but inhibited currents due to 100 µM GABA with an IC_{50} of 80.11 µM (95% CI: 69.6 to 92.3) and a Hill coefficient of 6.0 ± 1.5 (**Figure 3(a)**). GABA dose response curves were

Table 1. Percentage inhibition and enhancement values for the isolated compounds.

Compound	% Inhibition[a]	% Enhancement[b]
Ursolic acid	29.0	-
Carnosol	60.2	-
Oleanolic acid	31.0	-
Salvigenin	40.2	89.9
Rosmanol	26.7	99.7
Cirsimaritin	23.0	89.9
Hispidulin	81.7	52.2

[a]Percentage inhibition of maximal GABA response by 100 µM compound, [b]Percentage enhancement of GABA EC_5response by 100 µM compound.

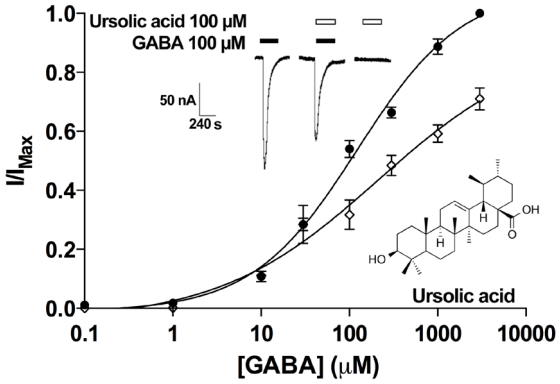

Figure 1. Dose response curves of GABA (●) and GABA in the presence of 100 µM ursolic acid (◊) at $\alpha_1\beta_2\gamma 2L$ $GABA_A$ receptors expressed in Xenopus oocytes. Data are the mean ± SEM (n = 3 - 6 oocytes). Data are the mean ± SEM (n = 3 - 6 oocytes). Inset: representative current traces from individual oocytes for GABA (100 µM) alone and in the presence of 100 µM ursolic acid at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors expressed in Xenopus oocytes.

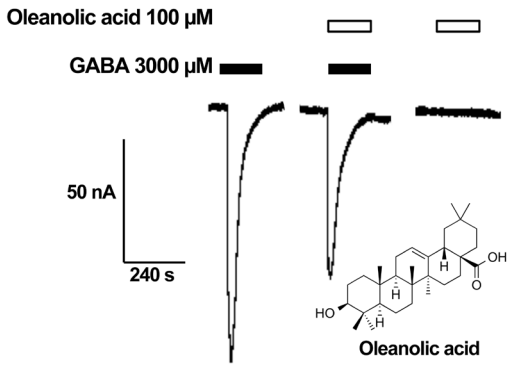

Figure 2. Representative current traces from individual oocytes for GABA (3000 µM) alone and in the presence of 100 µM oleanolic acid at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors expressed in *Xenopus* oocytes.

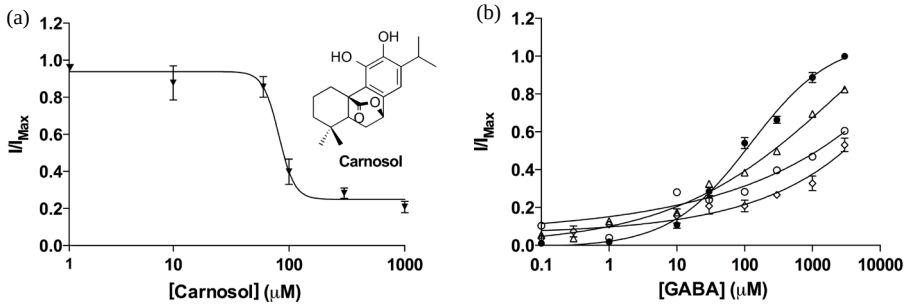

Figure 3. (a) Effect of carnosol in the presence of GABA (100μM) at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors expressed in *Xenopus* oocytes. (b) Dose response curves of GABA (●) and GABA in the presence of 100 (Δ), 300 (○) and 1000 μM (◊) carnosol at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors expressed in *Xenopus* oocytes. All data are the mean ± SEM (n = 3 - 6 oocytes).

in the presence and absence of carnosol (100, 300 and 1000 μM) at $\alpha_1\beta_2\gamma_{2L}$ GABA receptors. Carnosol had no significant effect on EC_{50} of GABA. However, 100, 300 and 1000 μM of carnosol inhibited currents due to 3000 μM GABA by 17.5% ± 4.0%, 40.7% ± 3.3% and 58.5% ± 3.5%, respectively (**Figure 3(b)**). Carnosol appears to have a slight positive modulation of GABA at lower does of GABA.

3.4. Salvigenin

Salvigenin (**Figure 4**) did not have any effect on sham-injected oocytes and had no activity at $\alpha_1\beta_2\gamma_{2L}$ or $\alpha_1\beta_2$ $GABA_A$ receptors when administered alone. Salvigenin exhibited a biphasic effect at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors. At high concentrations of GABA (100 μM), salvigenin (100 μM) inhibited currents due to GABA by 40.2% ± 0.7% and this action was unaffected by the addition of 10 μM flumazenil, while at low concentrations of GABA (10 μM) salvigenin positively modulated the current by 89.9% ± 1.5% (**Table 1**). This enhancement was reduced to 21.8% ± 0.2% by flumazenil (10 μM). At $\alpha_1\beta_2$ $GABA_A$ receptors (data not shown), no potentiation of current due to low doses of GABA was observed, however salvigenin reduced the current due to 10 μM GABA by 49.2% ± 0.3% at 100 μM.

3.5. Rosmanol

Rosmanol (**Figure 5**) did not have any effect on sham-injected oocytes (n = 3, data not shown) and had no activity at $\alpha_1\beta_2\gamma_{2L}$ or $\alpha_1\beta_2$ $GABA_A$ receptors when administered alone. Rosmanol appears to have two modes of action at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors. At high concentrations of GABA (3000 μM), rosmanol (100 μM) inhibited currents due to GABA by 26.7% ± 2.1% and this inhibition was not affected by the addition of flumazenil (10 μM), while at low concentrations of GABA (10 μM) rosmanol positively modulated the current by 99.7% ± 0.5% (**Table 1**) and this enhancement was reduced to 50.8% ± 0.3% by flumazenil (10 μM). At $\alpha_1\beta_2$ $GABA_A$ receptors, no enhancement of the GABA response was observed, however, rosmanol inhibited currents due to 10 μM GABA by 29.2% ± 0.2% at 100 μM (data not shown), similar to its effect at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors. Rosmanol (100 μM) decreased the maximal GABA response by approximately 50% and shifted the GABA EC_{50} and decreased the EC_{50} of GABA from 123.3 (95% CI: 91.5 to 166.2) to 19.51 μM (95% CI: 9.5 to 40.1), with a Hill slope of 0.88 ± 0.15 (**Figure 5**).

3.6. Cirsimaritin

Cirsimaritin (**Figure 6**) exhibited a biphasic effect at $\alpha_1\beta_2\gamma_{2L}$ receptors, inhibiting currents due to 100 μM GABA by 23.0% ± 0.5% at 100 μM and positively modulating currents due to 10 μM GABA by 89.9% ± 1.5%. The inhibitory effect of cirsimaritin at high concentrations of GABA showed further inhibition in the presence of 10 μM flumazenil, however, this is most likely due to receptor desensitization. Flumazenil had no effect on the enhancement of the GABA response at low concentrations of GABA. Cirsimaritin was inactive at $\alpha_1\beta_2$ $GABA_A$ receptors (data not shown). At $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors cirsimaritin (60 μM) shifted the GABA dose response curve to the right at low GABA concentrations (<60 μM) and to the left at higher GABA concentrations.

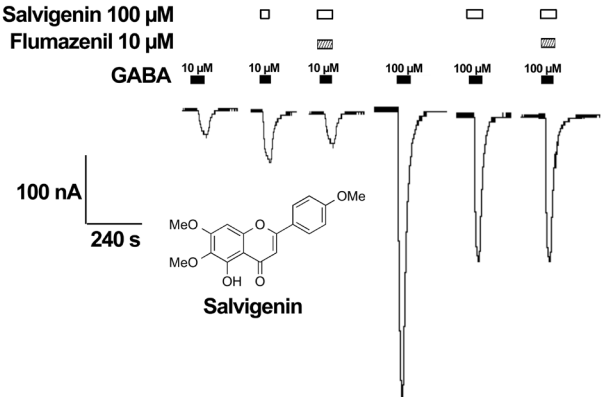

Figure 4. Representative current traces from individual oocytes showing that the effect of a low dose of GABA (10 µM) is potentiated by 100 µM salvigenin and that this potentiation is inhibited by 10 µM flumazenil; and that a high dose of GABA (100 µM) is inhibited in the presence of 100 µM salvigenin and that this inhibition in is unaffected by 10 µM flumazenil at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors.

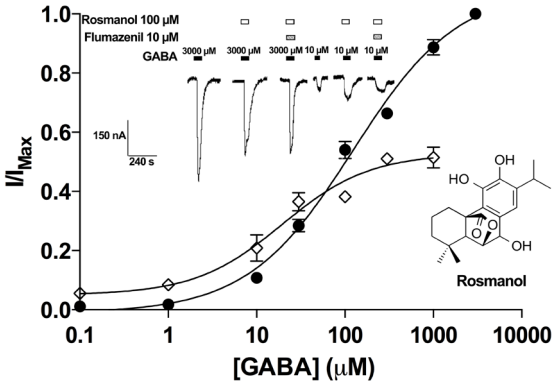

Figure 5. Dose response curves of GABA (●) and GABA in the presence of 100 µM (◊) rosmanol at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors expressed in *Xenopus* oocytes. Data are the mean ± SEM (n = 3 - 6 oocytes). Inset: Representative current traces from individual oocytes showing that the effect of GABA (3000 µM) is inhibited by 100 µM rosmanol and this inhibition is unaffected by 10 µM flumazenil; and thatthe effect ofGABA (10 µM) is enhanced by 100 µM rosmanol and that this enhancement is inhibited by 10 µM flumazenil at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors.

3.7. Hispidulin

Hispidulin (**Figure 7**) produced no effect on sham-injected oocytes (n = 3, data not shown) or at $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_1\beta_2GABA_A$ receptors when administered alone but inhibited currents due to 100 µM GABA with an IC_{50} of 81.7 µM (95% CI: 45.24 to 147.8) and a Hill coefficient of 1.6 ± 0.64. At $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptorshispidulin (100 µM) shifted the GABA dose response curve to the right at GABA concentrations less than 300 µM and to the left at higher GABA concentrations. This resulted in a change to the GABA EC_{50} from 123 µM (95% CI: 91.5 to 166.2) to 60 µM (95% CI: 19.5 to 185.5). Hispidulin acts as a positive modulator when applied at low concentrations of GABA but at high concentrations it acts as a negative modulator. This resulted in a change to the GABA EC_{50} from 123 µM (95% CI: 91.5 to 166.2) to 60 µM (95% CI: 19.5 to 185.5). Hispidulin acts as a

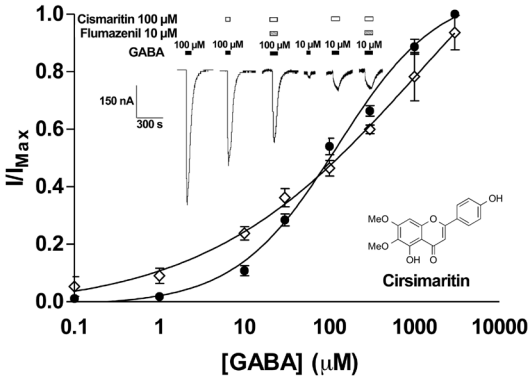

Figure 6. Dose response curves of GABA (●) and GABA in the presence of 60 µM (◊) cirsimaritin at human $\alpha 1\beta 2\gamma 2$L $GABA_A$ receptors expressed in *Xenopus* oocytes. Data are the mean ± SEM (n = 3 - 6 oocytes). Inset: Representative current traces from individual oocytes expressing $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors showing that the effect of GABA (100 µM) is inhibited by 100 µM cirsimaritin and that this inhibition is unaffected by 10 µM flumazenil; the effect of GABA 10 µM is enhanced by the presence of 100 µM cirsimaritin and this enhancement is unaffected by 10 µM flumazenil.

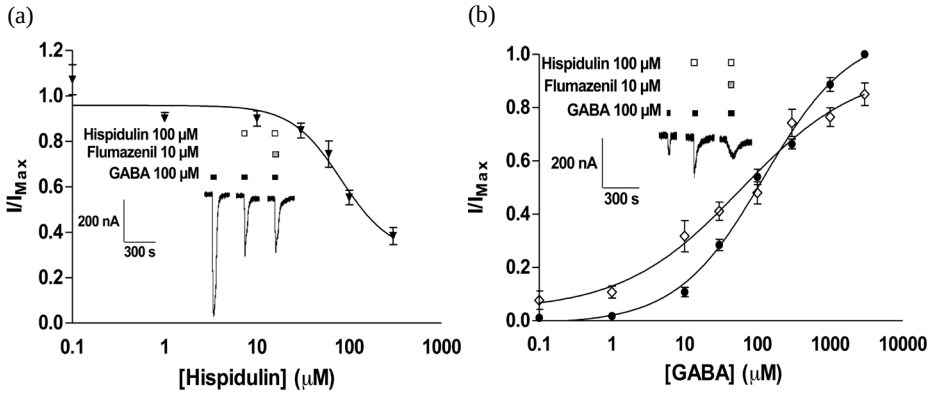

Figure 7. (a) Effect of hispidulin in the presence of GABA (100 µM) at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors expressed in *Xenopus* oocytes. Data are the mean ± SEM (n = 3 - 6 oocytes) Inset: Representative current traces from individual oocytes showing that the effect of a high dose of GABA (100 µM) is inhibited by 100 µM hispidulin and that this inhibition of the GABA response is unaffected by 10 µM flumazenil (b) Dose response curves of GABA (●) and GABA in the presence of 100 µM (◊) hispidulin at human $\alpha_1\beta_2\gamma_{2L}GABA_A$ receptors expressed in *Xenopus* oocytes. Data are the mean ± SEM (n = 3 - 6 oocytes) Inset: Representative current traces from individual oocytes showing that the effect of GABA 10 µM is enhanced by the presence of 100 µM hispidulin and this enhancement is inhibitedby 10 µM flumazenil at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors.

positive modulator when applied at low concentrations of GABA but at high concentrations it acts as a negative modulator. The effect of hispidulin on low concentrations of GABA was blocked by 10 µM flumazenil. However, the effect of hispidulin on high concentrations of GABA was not affected by 10 µM flumazenil. At $\alpha_1\beta_2$ $GABA_A$ receptors, hispidulin inhibited currents due to 100 µM GABA by 52.2% ± 0.5% at 100 µM (**Table 1**). The maximum concentration of hispidulin applied was 300 µM due to the limits of its solubility.

4. Discussion

Ursolic acid is a pentacyclic triterpenoid that exists widely in many medicinal plants used in traditional medicine, and it appears that ursolic acid and carnosol are partly responsible for the antitumorigenic activity of rosemary [13]. Although ursolic acid has been reported to inhibit GABA transaminase (GABA-T) [14], to date, no studies have investigated the action of ursolic acid at $GABA_A$ receptors. In the current study ursolic acid inhibited currents due to 100 µM GABA with an IC_{50} of 98.7 µM and shifted the GABA dose response curve at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors to the right increasing the GABA EC_{50}approximately two-fold to 210.4 µM. Interestingly, this result is in contrast to the *in vivo* results reported in a recent study by Taviano *et al.* in which ursolic acid showed significant CNS depressant properties in mice when administrated orally. In this study ursolic acid produced a potentiation of pentobarbital-induced sleeping time and a protective action against pentylenetetrazol (PTZ) induced convulsion [15]. As GABA is an inhibitory neurotransmitter, inhibition of the GABA response would be expected to have an overall excitatory response, and in fact most $GABA_A$ antagonists are convulsants. This suggests that ursolic acid is exerting its CNS depressant actions either through a different mechanism or a different $GABA_A$ receptor sub-type.

Carnosol is primarily responsible for the high antioxidant activity of *Rosmarinus officinalis* [13] [16] [17]. Carnosol significantly increased tyrosine hydroxylase activity suggesting that it may be a potential for the treatment for Parkinson's disease (PD) [18]. In a study by Rutherford and colleagues, carnosol inhibited the binding of *t*-butylphosphorothionate (TBPS) to the chloride channel of the GABA/benzodiazepine receptor but it had no effect on the binding of muscimol, diazepam or flunitrazepam, suggesting that the site of action of carnosol to be directly on the chloride channel [19].

The current study investigates the effects of carnosol at human recombinant $\alpha_1\beta_2\gamma_{2L}$ receptors expressed in *Xenopus laevis* oocytes. Carnosol had no activity at $\alpha_1\beta_2\gamma_{2L}$ GABA receptors when administered alone but inhibited currents due to 100 µM GABA with an IC_{50} of 80.11 µM. Carnosol was found to non-competitively block GABA, being more effective at high dose of GABA. These features are characteristic of a channel blocker.

Oleanolic acid is an isomer of ursolic acid, differing only on the location of one methyl group. In a study by Ha *et al.*, oleanolic acid was tested for its ability to modulate binding to $GABA_A$ benzodiazepine receptors *in vitro*; in this study oleanolic acid did not affect the binding of [^{3}H] Ro15-1788 or [^{3}H] flunitrazepam in the presence of GABA [20]. In another study oleanolic acid was found to inhibit GABA transaminase (GABA-T) by 20% at 10 µM/mL [14]. In the current study oleanolic acid inhibited currents due to 3000 µM GABA by 30% most probably by acting as a channel blocker with weaker activity than carnosol.

To date, no studies have investigated the action of salvigenin at $GABA_A$ receptors. In the current study salvigenin inhibited currents due to 100 µM GABA by 40% at 100 µM and positively modulated currents due to 10 µM GABA by 90%. This compound appears to have two modes of action, with positive modulation occurring via the high-affinity flumazenil benzodiazepine site and the inhibitory action occurring via a site that is independent of the presence of the γ-subunit.

Rosmanol is one of the compounds that gives sage free radical scavenging activity [21] [22]. 7-Methoxyrosmanol and galdosol are two derivatives of rosmanol that have been shown to competitively inhibit^{3}H-flumazenil bindingto the benzodiazepine receptor with IC_{50} values of 7.2 and 0.8 µM, respectively [23]. In the current studyrosmanol at 100 µM inhibited currents due to 3000 µM GABA by 25%. GABA dose response curves were carried out both without and with rosmanol (100 µM) at $\alpha_1\beta_2\gamma_{2L}$ GABA receptors shifting the GABA dose response curves to the right with a GABA EC_{50} 19.51 µM. Rosmanol has a biphasic mode of action, positively modulating the effect of GABA at low concentrations of GABA and inhibiting the response to high concentrations of GABA. This suggests that rosmanol may have two sites of action on the GABA receptor complex.

The two phases of rosmanol at $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors are thought to be mediated via two distinct mechanisms. The positive modulation of the GABA response by rosmanol at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors was sensitive to antagonism by flumazenil only at low concentrations of GABA indicating the involvement the "high affinity" benzodiazepine binding site which requires the γ subunit. The inhibitory second phase action of rosmanol is not affected by flumazenil and is observed at receptor combinations both with and without a γ subunit, indicating that the presence of the γ subunit is not a requirement. Therefore the inhibitory phase is not mediated via the high-affinity flumazenil sensitive benzodiazepine site. This inhibitory phase may occur via the "low-affinity" benzodiazepine binding site, which is known to be present on $\alpha\beta$ combinations or at a novel site independent of benzodiazepine activity.

Competitive inhibition of ^{3}H-flumazenil binding was detected for cirsimaritin when tested for its affinity to the benzodiazepine receptor in a membrane preparation from human frontal cortex with an IC_{50} of 350 μM [23]. Cirsimaritin inhibited the binding of [methyl-^{3}H] diazepam to rat brain benzodiazepine receptors with an IC_{50} of 23 μM and induced a small increase in [^{35}S]TBPS binding [24]. In the current study, the positive modulation by cirsimaritin of low concentrations of GABA at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors was found to be insensitive to antagonism by flumazenil indicating that the high affinity benzodiazepine binding site is not involved which suggests the involvement of the "low-affinity" binding site, or an alternative novel biding site. At high concentrations of GABA, the inhibitory effect of cirsimaritin appeared to be slightly increased by the presence of flumazenil, however this is most likely due to desensitization of the GABA receptors. Interestingly, although cirsimaritin was insensitive to flumazenil, it showed no activity at $\alpha_1\beta_2$ $GABA_A$ receptors, indicating that the presence of the γ subunit is essential for activity, suggesting that cirsimaritin may modulate the response to GABA via a novel binding site.

The fact that cirsimaritin displaces benzodiazepine binding, yet is flumazenil insensitive in functional assays is not without precedent. For example 6-methylflavone has been reported to competitively displace [^{3}H]-Ro 15-1788 binding in assays on rat brain membranes *in vitro* and human recombinant $GABA_A$/BZD receptors expressed in Sf-9 insect cells [25]. However, 6-methylflavone has been shown to be a flumazenil insensitive positive modulator at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors expressed in *Xenopus* oocytes [26]. Similarly, amentoflavone has been shown to be on of the most potent non-nitrogen containing ligands for the benzodiazepine site in binding assays with K_i = 6 nM [27]. However, at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors expressed in *Xenopus* oocytes, amentoflavone has been shown to be a flumazenil insensitive negative modulator [28] [29].

Hispidulinwas tested for its affinity to the benzodiazepine receptor and was found to competitively inhibit the binding of ^{3}H-flumazenil with an IC_{50}'s of 1.3 μM [23] and 8 μM [30]. Synthetic hispidulin was investigated at recombinant $GABA_A$ receptors expressed in *Xenopus laevis* oocytes and found to act as a positive allosteric modulator at $GABA_A$ receptor subtypes ($\alpha_{1\text{-}3,5,6}\beta_2\gamma_2$) being more potent at $\alpha_{1,2,5}\beta_2\gamma_2$ subtypes than at $\alpha_{3,6}\beta_2\gamma_2$ [31]. In the same study, hispidulin was shown also to have an anticonvulsant action in seizure prone mongolian gerbils and to cross the blood brain barrier. Unlike diazepam, hispidulin was found to act as a positive modulator at $\alpha_6\beta_2\gamma_{2L}$ $GABA_A$ receptors at which 10 μM of hispidulin enhanced the action of GABA at these receptors by 65%, with the enhanced response being reduced to 37% by 1 μM flumazenil [31].

Hispidulin also appears to have a biphasic mode of action at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors: acting as a positive modulator when applied with low concentrations of GABA but at high concentrations of GABA it acts as a negative modulator. The two phases of hispidulin at $\alpha_1\beta_2\gamma_2$ $GABA_A$ appear to be mediated via two distinct mechanisms. The positive modulation of low concentrations of GABA by hispidulin at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors was sensitive to antagonism by flumazenil indicating the involvement the "high affinity" benzodiazepine binding site that requires the γ subunit. The second phase or inhibitory action of hispidulin is not affected by flumazenil and is observed at receptor combinations both with and without a γ subunit, indicating that it is not acting at the high-affinity benzodiazepine binding site.

5. Conclusions

Although previous studies have investigated some of the chemical constituents of sage at the $GABA_A$/benzodiazepine complex and cholinergic receptors, few studies have investigated the constituents of *Salvia* at $GABA_A$ receptors using a functional electrophysiological assay.

$GABA_A$ receptors are known to be implicated in memory and cognition with enhancers of GABA function such as benzodiazepines having a well-documented detrimental effect on cognitive function. However, many GABA inhibitors or antagonists are not sub-type selective, acting at GABA receptors throughout the central nervous system and usually have the significant activity as convulsants, making them unsuitable for use as cognitive enhancers. In this study, the majority of compounds demonstrated some inhibitory activity at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors, which supports the concept that this herb may have cognition enhancing properties. Interestingly, many of the compounds demonstrated a biphasic activity at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors, enhancing the activity of GABA at lower concentrations of GABA and showing inhibition at higher GABA concentrations. In most cases these activities are likely to be occurring via different modulatory sites on the $GABA_A$ receptor complex. It may be that the combination of these activities permits cognition enhancement whilst offering protection from convulsant activity.

The action of salvigenin, rosmanol, and hispidulin at low concentrations of GABA were found to be flumazenil sensitive, these compounds also demonstrated flumazenil insensitive actions at high concentrations of GABA. Particularly interesting is the action of cirsimaritin, which was found to be flumazenil insensitive, but requires the presence of the γ-subunit, suggesting that cirsimaritin may act at a novel modulatory site on the $GABA_A$ receptor complex. Further studies to determine the properties of this site may provide another target for therapeutic modulation of $GABA_A$ function.

Conflict of Interest

The authors have no conflict of interest.

Acknowledgements

A. A. gratefully acknowledges the financial support of the Government of Australia for an Endeavour International Postgraduate Research Scholarship (EIPRS).

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