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Bioassay-guided Fractionation of Lemon Balm (*Melissa officinalis* L.) using an *In Vitro* Measure of GABA Transaminase Activity

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A novel pharmacological mechanism of action for the anxiolytic botanical *Melissa officinalis* L. (lemon balm) is reported. The methanol extract was identified as a potent *in vitro* inhibitor of rat brain GABA transaminase (GABA-T), an enzyme target in the therapy of anxiety, epilepsy and related neurological disorders. Bioassay-guided fractionation led to the identification and isolation of rosmarinic acid (RA) and the triterpenoids, ursolic acid (UA) and oleanolic acid (OA) as active principles. Phytochemical characterization of the crude extract determined RA as the major compound responsible for activity (40% inhibition at 100 μg/mL) since it represented approximately 1.5% of the dry mass of the leaves. Synergistic effects may also play a role.

Keywords: lemon balm; GABA transaminase; rosmarinic acid; ursolic acid; oleanolic acid.

INTRODUCTION

Lemon balm (*Melissa officinalis* L., Lamiaceae), also known as common balm or sweet balm, is a perennial lemon-scented herb in the mint family native to the Mediterranean and to Southern Europe. It was introduced to North America and now grows commonly in gardens and at roadsides (Small, 1997). *M. officinalis* was used in traditional medicine dating back as far back as ancient Greek and Roman times for treating disorders of the nervous system and melancholy (Grieve, 1998). Today, herbalists in Lebanon recommend the leaves to treat migraines and stomach ailments, strengthen the heart and improve memory (Salah and Jäger, 2005). Also, in Danish folk medicine, lemon balm is used to treat sleeplessness caused by heartbreak and sadness (Jäger *et al.*, 2006). Recently, *M. officinalis* has also shown anxiolytic, mood and memory-enhancing effects (Ballard *et al.*, 2002; Akhondzadeh *et al.*, 2003; Kennedy *et al.*, 2002, 2003, 2004, 2006), as well as sedative (Soulimani *et al.*, 1991), antioxidant, antimicrobial and antitumor actions (Dragland *et al.*, 2003; de Sousa *et al.*, 2004; Mimica-Dukic *et al.*, 2004). For a review of the scientific literature available to date on *M. officinalis*, please refer to Ulbricht *et al.* (2005).

Anxiety and related neurological disorders often result from low levels of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) (Brambilla *et al.*, 2003). While $GABA_A$ receptor binding is relatively well studied in anxiolytic botanicals, one of the other mechanisms to increase GABA levels in the brain, and potentially to control anxiety, is to inhibit the enzyme GABA transaminase (GABA-T) (Ashton and Young, 2003). A survey of ten anxiolytic botanicals was reported recently and an extract of *M. officinalis* was found to be the best inhibitor of *in vitro* GABA-T activity from rat brain (Awad *et al.*, 2007). However, the phytochemical constituent(s) responsible for GABA-T inhibition is currently unknown. *M. officinalis* contains a variety of compounds: primarily polyphenolics, flavonoids and terpenes (Mrlianová *et al.*, 2002; Patora *et al.*, 2003; Žiaková *et al.*, 2003). The principal objective of this study was to isolate and identify the active component(s) of *M. officinalis* via bioassay-guided fractionation using the *in vitro* enzyme assay for GABA-T inhibition. The successful isolation of three active phytochemicals and evidence for a novel mechanism of action is presented.

MATERIALS AND METHODS

Plant material and extraction. Certified organic dried aerial parts of lemon balm (*Melissa officinalis* L., lot # L9123CO, University of Ottawa Herbarium # 19806) was obtained from Blessed Herbs (Oakham, MA, USA). Briefly, 100 g was extracted sequentially with 1 L of solvent (1:10 m/v) for 24–48 h each, in the following order: hexane, ethyl acetate, methanol and water. This yielded four crude extracts of varying polarity. Each extract was evaporated to dryness, freeze-dried, sealed and stored in the dark at 4 °C. They were reconstituted in solvent and passed through a 0.2 μm PTFE Chromspec filter for the enzyme assays. The final solvent concentration was diluted to less than 1% (in wells) for all trials.

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***In vitro* GABA-T activity using rat brain.** The GABA-T bioassay was performed according to Awad *et al.* (2007). The use of animals for the *in vitro* assays followed a standard protocol approved by the University of Ottawa Ethics Committee and the Canadian Council of Animal Care. The crude extracts were tested in triplicate at final concentrations in the range 0–4 mg/mL (in wells), to determine the IC_{50} concentration. The relative activity of each concentration was compared with the solvent control, which was set to 100%. Fractions collected from the *M. officinalis* methanol extract were tested in triplicate at a final concentration of 10 µg/mL (in wells).

Bioassay-guided fractionation of *M. officinalis* methanol extract. For column chromatography, 5 g of the dried methanol extract of *M. officinalis* was mixed evenly with 5 g of polyamide 6 'Baker TLC' reagent for placement on the column head, and separated by column chromatography using a column bed of 4 cm × 40 cm. Elution was achieved using solvent gradients of 200 mL aliquots of water (100–0%): methanol (0–100%) and methanol (100–0%): acetone (0–100%) in 10% increments. A total of 86 (50 mL) aliquots were collected, evaporated and run on silica gel thin layer chromatography (TLC) plates using a solvent system of 25% methanol: 75% ethyl acetate. Aliquots with similar TLC profiles were combined to yield eight fractions in total, labeled A–H. Fractions were re-dissolved in 95% EtOH and tested for *in vitro* GABA-T inhibition as described above.

Fraction E was active and selected for further purification. An attempt was made to purify this fraction using reverse-phase HPLC, but this method was unsuitable due to the polar nature of this fraction. It was therefore subjected to size exclusion chromatography using lipophilic Sephadex (1 cm × 60 cm column; LH-20 beads) with an isocratic gradient of 100% methanol. Sixteen (5 mL) aliquots were collected and pooled according to similar TLC profiles to yield seven fractions, labeled E1–E7. Fraction E4 was subjected to a second Sephadex column using a 100% methanol gradient, and 30 additional aliquots (1 mL) were collected, labeled E4-1–E4-30. Fraction E4-27 contained a single spot on TLC, and was tested for activity, found to be active and identified as described below.

The combined fraction of F and G was also selected for further purification and was subjected to a polyamide column using a solvent gradient of ~300 mL aliquots of methanol (100–0%): acetone (0–100%) in 10% increments. A total of 342 (10 mL) aliquots were collected, and pooled according to similar TLC profiles to yield five major fractions, labeled FG1 to FG5. Fraction FG4 had a single spot on TLC and was subsequently tested for activity and identified as described below. Fraction FG2 contained three spots on the TLC and so an attempt to purify these compounds was made. Since there was very little material to work with, the purification was conducted using the analytical HPLC system. A method was developed to separate the compounds on a Phenomenex Gemini column (250 mm × 4.6 mm) using a gradient of 30–100% MeCN in 15 min. Aliquots of fraction FG2 were made and 100 µL were injected onto the column several times and the major peaks were collected and pooled. Two subfractions FG2-1 and FG2-2 were collected and re-run on the HPLC to verify purity. It was estimated that there was less than 1 mg of each subfraction collected, which was insufficient to accurately confirm their identity.

Compound identification. Isolated compounds were identified by nuclear magnetic resonance (NMR) spectroscopy using deuterated methanol (CD_3OD) on a Bruker AMX-300 NMR spectrometer. Mass spectra were obtained for ursolic and oleanolic acids from LC/MS using an APCI mass spectrometer (Agilent Technologies Inc., Palo Alto, CA). Absorption spectra of identified compounds were compared with those of known standards using HPLC.

Phytochemical characterization. For verification of plant identity, the crude extract of *M. officinalis* was characterized using HPLC DAD and LC/MS. Standards, rosmarinic acid and caffeic acid, were purchased from Sigma Chemical Company (St Louis, MO). Analysis of phenolic acids was performed using a YMC ODS-AM 100 mm × 2 mm column, on a Hewlett Packard Series 1100 LC system (Agilent Technologies, Mississauga, ON.) with Chem Station for LC 3D software (Rev. A.09.01). Briefly, the solvents were A: acetonitrile (MeCN) + 0.05% trifluoroacetic acid (TFA) and B: HPLC grade water + 0.05% TFA. The gradient was as follows: 5–20% A in 7 min, 20–50% C in 3 min, 50–90% in 5 min, hold 90% C for 2 min, return to 5% C in 2 min, equilibrate for 5 min; flow rate at 0.4 mL/min; oven temperature 50 °C. Samples (1 µL) were injected and detected at either 210 or 326 nm.

GABA-T inhibition of identified compounds. Rosmarinic acid and ursolic acid were the two primary compounds identified through bioassay-guided isolation. Commercial standards of each were tested in triplicate at 1, 10 and 100 µg/mL (final concentration in wells). Oleanolic acid was also identified in fraction E4-27 and it was tested at 10 µg/mL. Since this fraction contained a mixture of the two triterpenoids, ursolic and oleanolic acids were tested in combination in a 1:1 ratio (5 µg/mL UA: 5 µg/mL OA) to determine if GABA-T activity was affected when both compounds were present.

Statistical analysis. Linear regression using Probit software (Hubert and Carter, 1990) was performed to determine the IC_{50} values of the primary extracts (±95% CI).

RESULTS

Bioassay-guided fractionation

Bioassay-guided fractionation of the dried leaves of *M. officinalis* was performed to determine the active constituent(s) responsible for GABA-T inhibition previously observed in the crude extract (Awad *et al.*, 2007). After sequential extraction with solvents of different polarity, each extract was tested for activity (Table 1). The methanol extract had the greatest inhibition against GABA-T and showed a classic dose response (Fig. 1). Inhibition decreased in the following order: methanol extract, water extract, ethyl acetate extract and hexane extract (not inhibitory). Therefore, the polar fractions were more active than the non-polar fractions in this assay.

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Table 1. Extract yield (g) and 50% inhibitory concentration (IC_{50}; mg/mL) and 95% confidence interval (CI) of the four primary extracts obtained from sequential extraction of *M. officinalis* dry leaves

Extract	Yield (g)	IC_{50} (95% CI)
Hexane	2.1	NI
Ethyl acetate	2.0	2.55 (2.28, 2.82)
Methanol	8.9	0.55 (0.53, 0.57)
Water	10.6	0.82 (0.79, 0.85)

NI, non inhibitory within the tested range, IC_{50} > 4 mg/mL.

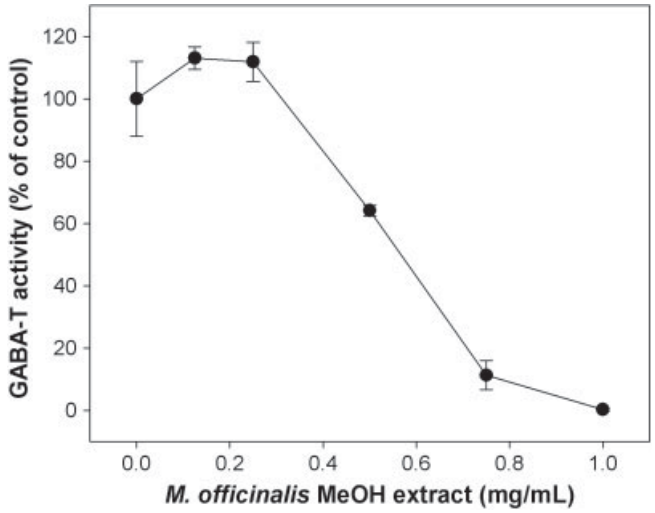

Figure 1. Dose response curve for the effect of the methanol fraction of *M. officinalis* on *in vitro* GABA transaminase (GABA-T) activity expressed as percent of control (100%). Values are mean ± SE (n = 3) for each concentration tested.

The methanol extract was chosen for further phytochemical characterization. The structures of the identified phytochemicals are illustrated in Fig. 2. Following the first polyamide column, three of eight fractions collected had greater than 20% GABA-T inhibition at 10 µg/mL. The results outlined in Fig. 3A show that fraction F was the most active (47.5% ± 4.6% inhibition), followed by fractions E (29.9% ± 13.9% inhibition) and A (23.5% ± 10.2% inhibition). Fractions F and G had similar compound profiles by TLC and were combined as one sample, labeled as fraction FG.

Further purification led to the identification of two triterpenoids, ursolic acid (UA) and oleanolic acid (OA) in fraction E4-27. These compounds are isomers of each other and are difficult to separate because they have an identical molecular weight and differ only on the location of one methyl (CH_3) group (Fig. 2). Since there was inadequate material no attempt was made to separate the individual compounds. However, NMR analysis revealed that they were present in a 3:1 UA:OA ratio in the sample. Fraction E4-27 had moderate activity and inhibited GABA-T by 22.5% ± 5.2% (Fig. 3B) at 10 µg/mL.

Fraction FG was purified to yield two active fractions; FG2 and FG4. Fraction FG4 was identified to be rosmarinic acid (RA) (Fig. 2) and NMR indicated that this sample was approximately 95% pure. It also showed moderate activity by inhibiting GABA-T by approximately 27.3% ± 7.4% (Fig. 3B) at 10 µg/mL. Interestingly, fraction FG2 was more active and had 36.2% ± 7.9% GABA-T inhibition (Fig. 3B). It underwent several steps of purification and ultimately yielded two fractions which were pure according to the analytical HPLC. There was less than 1 mg of each and that amount was insufficient for bioactivity and NMR analyses.

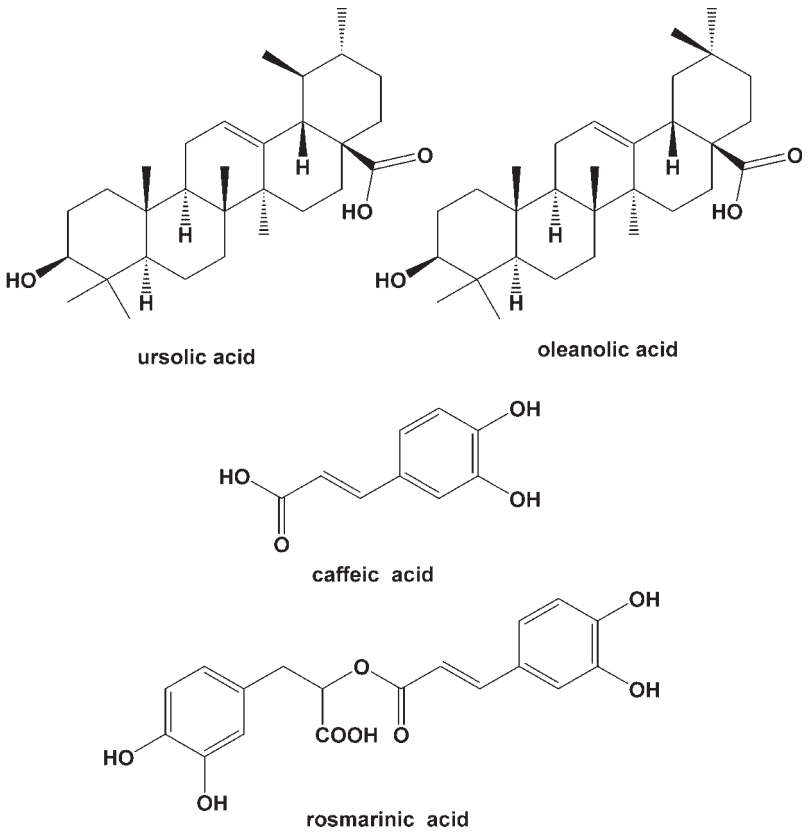

Figure 2. Chemical structures of identified compounds from the *M. officinalis* methanol extract.

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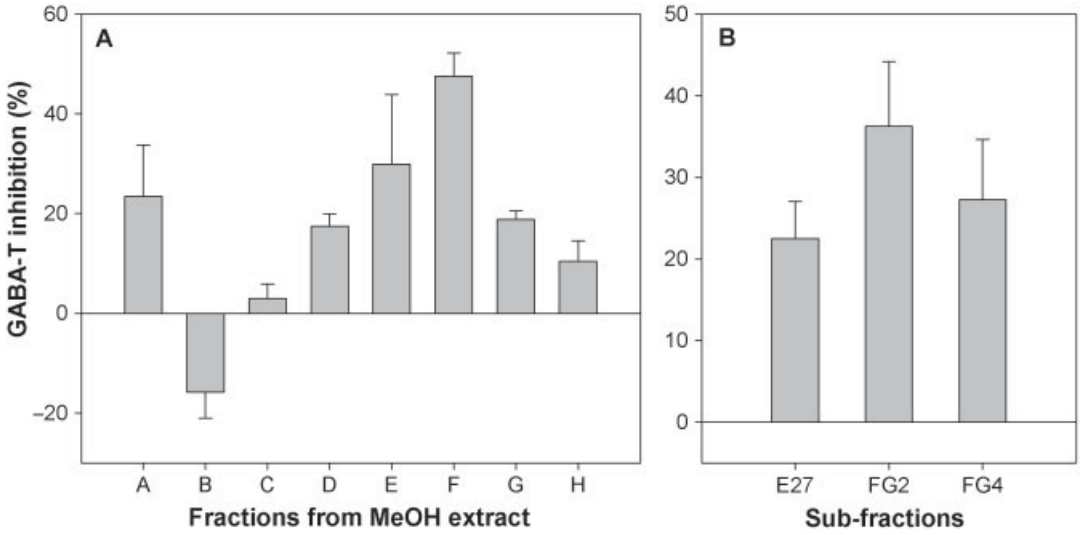

Figure 3. Percent GABA-T inhibition of selected fractions (10 μg/mL) collected from the separation of *M. officinalis* extracts. (A) Fractions A–H collected from the methanol extract and (B) fractions E4-27, FG2 and FG4 collected from active fractions E and FG. Values are mean ± SE (n = 3).

Table 2. Percent GABA-T inhibition by the methanol extract of *M. officinalis* and the commercial standards tested at various concentrations

Sample	% GABA-T inhibition ± SE	Final concentration (in well) μg/mL	Final concentration (in well) μM
Methanol extract	50[a]	550	–
Rosmarinic acid	–	93.5	259.5
Rosmarinic acid standard	40.4 ± 3.2	100	277.5
	4.6 ± 2.4	10	27.7
	4.6 ± 2.2	1	2.7
Ursolic acid standard[b]	19.9 ± 10.6	100	219.0
	13.6 ± 9.7	10	21.9
	5.0 ± 3.6	1	2.2
Oleanolic acid standard[b]	20.2 ± 11.1	10	21.9
UA:OA combined	21.9 ± 11.6	5 UA:5 OA	11 UA:11 OA

Rosmarinic acid (RA) was quantified by HPLC and the final concentration (in well) was calculated, which represents the amount RA present in the extract at 550 μg/mL; all values are mean ± SE (n = 3).

[a] 50% inhibition based on the IC_{50} value of the MeOH extract (0.55 mg/mL).

[b] Ursolic and oleanolic acids were not quantified in the extract since the LC/MS method available is not capable of separating these two compounds; therefore an accurate measure was not possible.

GABA-T inhibition by identified compounds

The activities of the commercial standards were tested and the results are outlined in Table 2. The rosmarinic acid (RA) standard showed 40% inhibition at the highest dose tested (100 μg/mL). Interestingly, this concentration was similar to the amount determined in the methanol extract. Fifty percent inhibition (IC_{50}) occurred at 0.55 mg/mL (Table 1). Based on HPLC analysis, the RA concentration was calculated to be 94 μg/mL at that dose; and represents approximately 15.1 mg RA/g dry material, or 1.5%. This result confirms that the activity of the *M. officinalis* methanol extract is primarily due to the high levels of RA. Fraction FG4 contained 95% RA, which means that at the tested dose of 10 μg/mL there would be at most 9.5 μg/mL of RA in the well. However, this fraction showed more activity (27% inhibition) compared with pure RA at 10 μg/mL (5% inhibition). The HPLC profile indicates that there is an additional peak in fraction FG4 (Fig. 4) at approximately 10.2 min, which may contribute to the activity of that fraction.

Since RA is a dimer of caffeic acid, it is also possible that RA may have been chemically modified during the fractionation process. Caffeic acid (CA) was identified and quantified in the crude methanol extract. There was approximately 0.18 mg CA/g dry material, or 0.02%. The IC_{50} dose of 0.55 mg/mL of the methanol extract was therefore calculated to have approximately 11.2 μg/mL (62 μM) of CA present. This small amount of CA may have contributed to the overall activity of the extract. Caffeic acid or other cinnamic acid derivatives may have greater inhibition compared with RA, although they are not present in high amounts and are therefore likely to be minor contributors. However, it is also possible that these compounds interact together either in a synergistic or additive way to increase the overall activity of the extract. Further studies are required to verify this.

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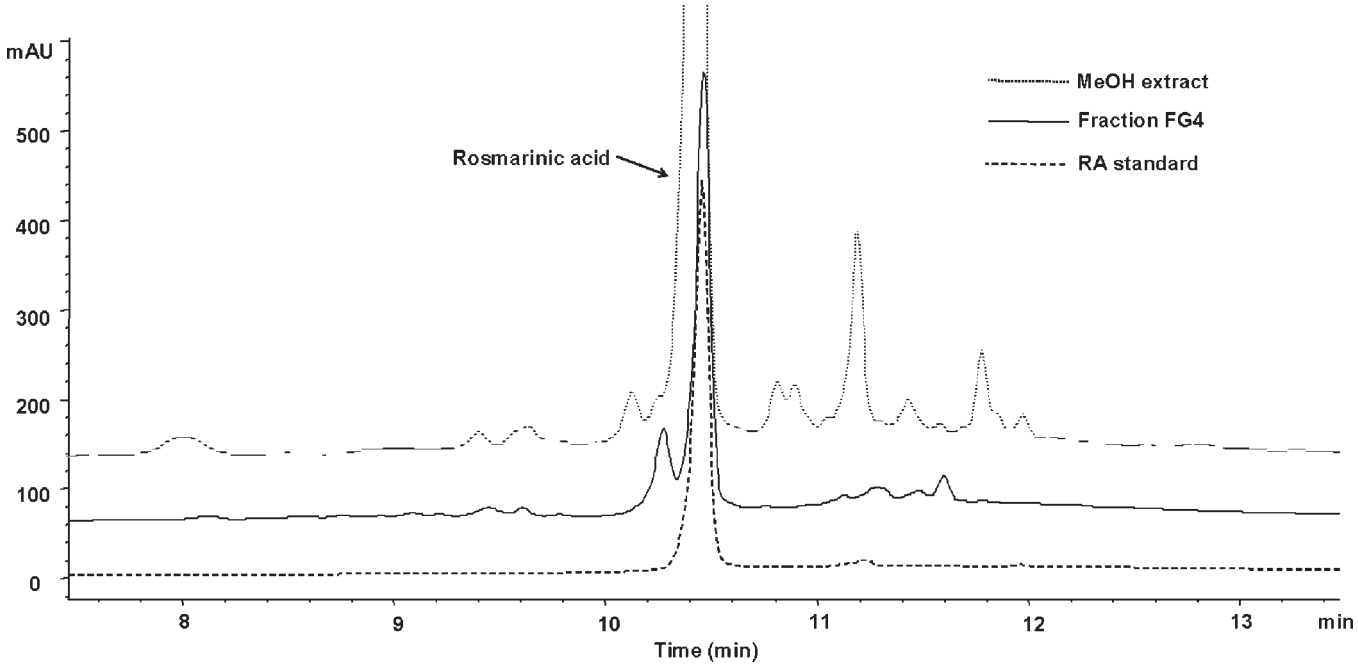

Figure 4. Overlay of HPLC chromatographs comparing the *M. officinalis* methanol (MeOH) extract to fraction FG4 (containing 95% rosmarinic acid) and the rosmarinic acid (RA) standard at 210 nm. Note the baselines have been shifted upwards to highlight the differences between samples.

Ursolic acid (UA) made up 75% of Fraction E4-27, which inhibited GABA-T by 22.5% at 10 μg/mL. The UA standard was tested at 10 μg/mL and it only showed low activity (13.6% inhibition). On the other hand, oleanolic acid (OA) which constituted 25% of the fraction had greater activity (20% inhibition) at the same dose. When an equal amount of UA and OA were combined in a 1:1 ratio (5 μg/mL each) and tested, they inhibited GABA-T by about 22%, which was very similar to the activity of the isolated fraction. Since the activity of UA and OA combined was not greater than the activity of either UA or OA alone, additive and (or) synergistic effects between these two compounds tested at this dose is unlikely. When UA was tested at the higher dose of 100 μg/mL it inhibited GABA-T activity by 20%, and is therefore half as active as RA. It is clear from the above results that each of the compounds isolated from the *M. officinalis* methanol extract inhibit *in vitro* GABA-T activity to varying degrees. Since RA is present in such a high quantity in the extract, it is likely to be the major compound responsible for activity. However, the triterpenoids UA and OA also contribute; and other compounds in the extract may be active inhibitors of GABA-T.

DISCUSSION

The successful isolation of three phytochemicals from *M. officinalis* is reported for the first time with a novel mechanism of action to inhibit the major GABA metabolic enzyme GABA-T. Bioassay-guided fractionation of the methanol extract led to the identification of rosmarinic, ursolic and oleanolic acids as active principles. Rosmarinic acid was only active at a high concentration, but HPLC analysis revealed that it represented approximately 1.5% of the dry mass of the leaves, which is considered to be high for a secondary metabolite.

The anxiolytic and mood altering effects of *M. officinalis* have been documented in numerous studies; however, the mechanism(s) of action are unclear. Until now, only a few studies have investigated the CNS pharmacology of *M. officinalis.* Earlier work by Soulimani *et al.* (1991) demonstrated sedative actions of lemon balm in mice, and the activity was attributed to the hydroalcohol extract rather than the essential oils. Cholinergic effects have been noted in alcohol extracts which had varying affinities to nicotinic and muscarinic receptors from human brain (Wake *et al.*, 2000; Kennedy *et al.*, 2003). Since some of these effects appeared to be non-specific it was suggested that another mechanism could be involved. Later, Salah and Jager (2005) and Jager *et al.* (2006) showed moderate affinity of an ethanol extract of *M. officinalis* to the benzodiazepine (BZD) site of the $GABA_A$ receptor; and no activity was seen in a serotonin (5-HT) transporter assay. The present study reports that *M. officinalis* also inhibits GABA-T and the major constituent for this activity is rosmarinic acid since it is present in very high quantities.

Rosmarinic acid has been reported in a wide variety of plant families and species, most notably the aromatic herb rosemary (*Rosmarinus officinalis* L.) (Petersen and Simmonds, 2003). Rosmarinic acid is well known for its antioxidant and antimicrobial properties (al-Sereiti *et al.*, 1999; Moreno *et al.*, 2006; Mencherini *et al.*, 2007). It also possesses anxiolytic activity at low doses in mice, however, it does show some neurotoxic effects at higher doses (Pereira *et al.*, 2005). Interestingly, two studies by Takeda *et al.* (2002a, 2002b) demonstrated that rosmarinic acid and caffeic acid had antidepressant actions in the forced swimming test and the conditioned fear stress paradigm in mice. They speculated that rosmarinic acid affected the monoamine system, a principle target in depression, but it did not inhibit monoamine oxidase A or B, or the monoamine transporters. Since rosmarinic acid showed moderate activity in the present study, it is possible that the CNS-depressant

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effects observed *in vivo* are in part due to inhibition of GABA-T as this would be expected to increase GABA levels *in vivo*.

Other compounds that may also play a role in the activity of *M. officinalis* are cinnamic acid derivatives including caffeic acid, as well as the triterpenoids ursolic acid and oleanolic acid. Ursolic acid and oleanolic acid have also been reported in a large variety of plant species worldwide, and have a wide range of pharmacological properties. They are particularly known for their hepatoprotective, antiinflammatory, antioxidant, antitumor, antiviral and antimicrobial effects (Liu, 1995; Novotný *et al.*, 2001; Abe *et al.*, 2002). Interestingly, numerous CNS-related activities have been reported for ursolic acid. Chung *et al.* (2001) demonstrated that ursolic acid isolated from oregano (*Origanum majorana* L.) inhibited the enzyme acetylcholinesterase (AChE), a primary target in Alzheimer's disease. In addition, ursolic acid was identified from the active fraction of *Mallotus peltatus* (Geist) Muell Arg. (Euphorbiaceae) leaves, which had CNS-depressant actions on behaviour and sleep (Chattopadhyay *et al.*, 2003). Shih and colleagues (2004) found that ursolic acid was capable of protecting hippocampal neurons from kainate-induced excitotoxicity in rats. Furthermore, a recent study showed that ursolic acid from the catnip species *Nepeta sibthorpii* Bentham (Lamiaceae) had sedative and anticonvulsant activity in mice (Taviano *et al.*, 2007). The authors suggested that the activity of ursolic acid may be mediated through the GABAergic system since it increased the latency time of seizures induced by pentylenetetrazole (PTZ), a $GABA_A$ receptor antagonist. To complement these findings, work done in our laboratory by Mullally *et al.* (2007, unpublished data) found that ursolic acid exhibited moderate affinity to the BZD site of the $GABA_A$ receptor. Based on the results obtained in this study, it is possible that the moderate GABA-T inhibition observed *in vitro* may also contribute to the sedative and anticonvulsive effects observed *in vivo*. This strengthens the argument that the anxiolytic mode of action of ursolic acid, or other related triterpenoids, involves effects on GABAergic systems.

In conclusion, this study demonstrates that lemon balm is a GABA-T inhibitor *in vitro*, and provides evidence for a novel mechanism of action for rosmarinic, ursolic and oleanolic acids. *In vivo* studies are needed to confirm that GABA-T inhibitory actions of the *M. officinalis* extracts and pure compounds identified in this *in vitro* analysis translate to whole animals. Certainly, other active principles may also be present in the extract and future isolation and activity studies are warranted.

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