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Article Title: Comparison of rosmarinic acid content in commercial tinctures produced from fresh and dried lemon balm (*Melissa officinalis*)

Year of publication: 2007

Citation: Sanchez-Medina, A. et al (2007) 'Comparison of rosmarinic acid content in commercial tinctures produced from fresh and dried lemon balm (*Melissa officinalis*).' *Journal of Pharmacy and Pharmaceutical Sciences* 10 (4) 455-463

Link to published version:

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DOI: (not stated)

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Comparison of rosmarinic acid content in commercial tinctures produced from fresh and dried lemon balm (*Melissa officinalis*)

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Received, June 26, 2007; Revised, September 28, 2007; Accepted, September 29, 2007; Published September 30, 2007.

ABSTRACT - Purpose. To measure the rosmarinic acid content of eight commercial tinctures derived from fresh (n= 5) and dried (n=3) *Melissa officinalis* herb. **Methods.** Rosmarinic acid and the internal standard (esculin) were purchased from Aldrich Chemical Co. The column used was a Luna C₁₈, 5 µm (150 x 4.6 mm I.D., Phenomenex) maintained at ambient room temperature. The HPLC system consisted of a Shimadzu SCL-6B controller, Shimadzu LC-6A pumps, Shimadzu SPD-6A UV single wavelength spectrophotometric detector set to 320 nm and Shimadzu SIL-6B autosampler. Gradient elution of the samples and standard were performed using ammonium formate (0.02 M; pH 6.25 at 27 °C; eluent A) and methanol (eluent B). The gradient elution initial conditions were 2% of eluent B with linear gradient to 60% at 30 min, followed by linear gradient to 90% of eluent B at 31 min, this proportion being maintained for 4 min. The column was then returned to the initial condition at 36 min and maintained until the end of the run at 43 min. The flow rate was 1 mL/min. The assay was validated for sensitivity, accuracy and reproducibility. **Results.** The content of rosmarinic acid in commercial tinctures was significantly higher in the tinctures made from dried plant material (2.96 – 22.18 mg/mL) compared to fresh plant tinctures (<= 0.92 mg/mL). **Conclusion.** These results have implications both for the manufacturers of commercial tinctures and also for herbal practitioners in the choice of tinctures for treating *Herpes simplex* infection.

INTRODUCTION

Tinctures, or hydroalcoholic solutions, of lemon balm herb (*Melissa officinalis* L.) are used in Western Herbal Medicine to treat cutaneous *Herpes simplex* infection as well as for carminative, diaphoretic and sedative actions

(1,2). However, herbal practitioners are faced with commercial tinctures from either fresh or dried *Melissa* leaf, that vary widely in the drug-to-extract ratio (ranging from 1:1 to 1:5) and alcohol concentration (25 to 45%), depending on the manufacturer (2). The phytochemistry of the essential oil, associated with the fresh leaf, is well known, albeit the total content of oil in the herbal drug is relatively low at 0.02-0.3% dry weight (3). Often, herbal practitioners consider the lemon scent, characteristic of fresh leaf, to be the main criterion of quality (4). The constituents responsible for this scent include geranial (citral A) and neral (citral B) (representing 45-90% of the oil) and *R*-(+)-citronellal (1-40%). It is the ratio of the content of geranial and neral (4:3), together with the presence of 6-methyl-5-heptene-2-one, that are considered to be the main criteria of the authenticity of the oil (4). Besides these essential oil constituents, a wide range of non-volatile bioactive compounds have also been reported. These include hydroxycinnamic acid derivatives (variously reported as 6-11%), primarily rosmarinic acid (2-7%), caffeic acid and chlorogenic acid (3-5) and flavonoids (0.5%) such as luteolin 3'-glucuronide (0.3%). The European Pharmacopoeia standard for dried *Melissa* leaf is not less than 4% hydroxycinnamic acid derivatives, expressed as rosmarinic acid derivatives, measured using spectrophotometry (3).

Melissa leaf has a long history of use as a topical antiviral by herbal practitioners and it is variously used directly on the skin, or as either a 1% tincture in cream, or dried aqueous extract in cream (1,2). The efficacy as a topical antiviral is supported by a couple of randomised, double-blind, placebo controlled clinical studies on *Herpes simplex* skin lesions.

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Such studies, primarily on *Herpes labialis*, have shown, significantly accelerated healing times ($p < 0.05$) compared with placebo (6-8). These trials involved treating lesions within 72 hours of the first symptoms, 2-4 times daily for 5-10 days, with a cream containing 1% of a lyophilised aqueous extract from dried Melissa leaf (70:1). One trial concluded that recurrence-free intervals were extended in comparison with topical virustatic preparations containing idoxuridine and tromantidine hydrochloride (9).

The antiviral activity of Melissa leaf in treating *Herpes simplex* infection has been reviewed elsewhere (10-13). Currently, antiviral research on *M. officinalis* has targeted the in-vitro activity against replication of the *Herpes simplex* virus type 1 and 2 (HSV-1 and HSV-2) (14, 15). Rosmarinic acid was also reported to inhibit the activities of both mutant and wild-type integrase of HIV-1 virus with IC_{50} values below 10 μ M (16, 17). However, regardless of the available scientific and clinical evidence for the role of dried Melissa leaf in a topical antiviral cream for treating *Herpes simplex* infection, there remains controversy amongst herbal practitioners as to whether fresh plant tinctures are more effective than those tinctures produced from dried plant material. The aim of this study was therefore to determine the rosmarinic acid content of commercial tinctures made from fresh and dried plant Melissa leaf.

MATERIALS AND METHODS

Chemicals

Standards of rosmarinic acid and the internal standard (IS, esculin) were purchased from Aldrich Chemical Co. (Dorset, UK). Ammonium formate (Riedel-de Haën, Seelze, Germany) was purchased from Fluka, UK. HPLC-grade methanol (ACROS Organics, Geel, Belgium) was purchased from BDH (Dorset, UK). Water was purified using a Milli-Q Organex System from Millipore (Molsheim, France). Ammonium formate solution (0.02 M; pH 6.25 at 27 °C) was prepared. NMR-grade D_2O for NMR experiments was supplied by Goss Scientific (Essex, UK).

Tinctures

Samples of either dried plant tinctures or specific (fresh plant) tinctures of *M. officinalis* L. were obtained from six companies commonly used by herbal practitioners in the UK. Test formulations from a particular manufacturer bore the same lot number and had an expiration date no earlier than

2 years from the date of purchase. The samples were randomly labelled A-H and their identity was not known until after the analyses were complete. For comparison, a fresh tincture was prepared (18) in our laboratory from fresh *M. officinalis* L. harvested from one of the author's (CJE) garden in Cheshunt, Hertfordshire on 10/05/06. The tincture was prepared within 2 h of harvest. Twenty grams of fresh *Melissa officinalis* herb (leaf and stem) were ground in a pestle and mortar for 1 min, after which 40 mL of EtOH:H₂O (45:55) was added and the resulting mixture triturated for a further 10 min. The green liquid was decanted off and filtered.

Sample preparation for HPLC

Eight commercial tinctures (Samples A-H) of lemon balm (*Melissa officinalis*) were used in this study. Comparison of rosmarinic acid content was standardised on the basis of milligrams of rosmarinic acid per mL of tincture. Thirty μ L of tincture was added to 970 μ L of methanol. Two hundred μ L of each solution were added to 200 μ L of esculin (100 μ g/mL in 0.02 M ammonium formate) solution. The resultant solutions were centrifuged at 13,000 min^{-1} for 5 min before injecting 20 μ L on the HPLC system. Samples were freshly prepared daily.

Preparation of NMR samples

Tincture A-H (1 mL) was placed in a 1.5 mL Eppendorf microfuge tube and the open tubes were placed in an Eppendorf Thermomixer at 35 °C for 18 h. Aliquots (100 μ L) of the resulting dark brown solutions were added to 500 μ L of D_2O in a 1.5 mL Eppendorf microfuge tube and the resultant liquid was centrifuged at 15,000 revolutions min^{-1} for 5 min. The supernatant liquid was pipetted into clean, dry 5 mm NMR tubes.

HPLC instrumentation

The quantification of samples and the calibration curves were performed using a high performance liquid chromatography system consisting of a Shimadzu SCL-6B controller, Shimadzu LC-6A pumps, Shimadzu SPD-6A UV single wavelength spectrophotometric detector and Shimadzu SIL-6B autosampler. The UV detector was set at 320 nm and data was acquired using Bruker Hystar software v2.1 (Bruker Biospin, Germany). The column used was a Luna C₁₈, 5 μ m (150 x 4.6 mm I.D., Phenomenex) maintained at ambient room temperature. Gradient elution of the samples and

standard were performed using ammonium formate 0.02 M at pH 6.25 (eluent A) and methanol (eluent B). The gradient elution was performed as follows: initial conditions were 2% of eluent B with linear gradient to 60% at 30 min, followed by linear gradient to 90% of eluent B at 31 min, this proportion being maintained for 4 min. The column was then returned to the initial condition at 36 min and maintained until the end of the run at 43 min. The flow rate was 1 mL/min. The sample injection volume was 20 μ L: two injections were performed for each sample and standard.

¹H-NMR Spectroscopy

All NMR spectra were acquired on a Bruker (Bruker GmbH, Rheinstetten, Germany) DRX 500 Spectrometer, operating at 500.13 MHz for the ¹H frequency. Spectra were the result of the summation of 128 free induction decays (FIDs), with data collected into 32K datapoints, and a sweep width of 14 ppm. Acquisition time was 2.34 s. The water signal was suppressed using a standard 1D-pulse sequence. Prior to Fourier transformation, an exponential line broadening equivalent to 0.3 Hz was applied to the FIDs. Spectra were referenced to the ethanol triplet centred at 1.05 ppm.

Quantitation of rosmarinic acid

Rosmarinic acid calibration curves (n=4) were obtained by preparing stock solutions (1000 μ g/mL) in methanol. Subsequent dilutions were made to give 200, 100, 50, 25, 12.5 and 6.25 μ g/mL. 200 μ L of each solution were added to 200 μ L of Esculin, IS (100 μ g/mL in 0.02 M ammonium formate) solution. After mixing, 20 μ L of each final concentration were injected in duplicate. Stock solutions and dilutions were prepared fresh daily. The linearity of the calibration curves was determined ($r^2 > 0.99$). The limit of detection (LOD) was calculated based on a signal-to-noise ratio (S/N: 3.3). The limit of quantitation (LOQ) was then calculated based on the signal-to-noise ratio (S/N: 10) using the lowest concentration in the calibration and the highest noise observed when injecting a blank (19). The intraday precision was calculated by comparison of the area obtained for 12 injections of Esculin solution (50 μ g/mL) in 0.02 M ammonium formate (pH 6.25) on two different days. For the interday precision, the ratio of the area of rosmarinic acid/area of esculin for all the

points in the calibration curve for the different days were summed and compared.

RESULTS

¹H NMR spectroscopy

The ¹H NMR spectra of the Melissa samples from the 8 different manufacturers are presented in Figure 1.

It can be seen that there is a wide range of concentrations across the samples. From the sample B spectrum, it was possible to discern several signals in the aromatic region (δ 6-8) that correspond theoretically to those of rosmarinic acid. The addition of rosmarinic acid standard then confirmed rosmarinic acid as a major constituent of some of the samples. Two distinctive signals for H7 and H8 are represented by two 1H doublets with $J = 16$ Hz at δ 6.1 and 7.4 respectively. H2 is represented by a 2 Hz doublet integrating for 1H at δ 7.0. The signals representing H5, H6, H2', H5' and H6' are overlapped in the region δ 6.6 to 6.9. A semi-quantitative ranking from high to low rosmarinic acid content was B > C > E, all were dried plant tinctures. Samples A, D, F, G and H, all from fresh plant tinctures, contained significantly less than the former. Because of the considerable overlap of NMR signals, further quantification of rosmarinic acid was carried out by HPLC-UV.

HPLC-UV determination of rosmarinic acid

The HPLC-UV_{320nm} chromatograms for samples A to H are illustrated in Figure 2. Retention times (t_R) were 15.5 min and 22.5 min for esculin and rosmarinic acid, respectively. The ranking of the samples in terms of rosmarinic acid concentration confirmed the semi-quantitative ranking already obtained by NMR spectroscopy. The quantification of the rosmarinic acid was acquired using peak area ratios of rosmarinic acid to internal standard. Esculin was chosen as internal standard as previous injections of the sample showed no major peaks in the samples at esculin t_R (15.5 min). The histogram in Figure 3 illustrates the data of the resulting concentrations of RA between the 8 commercial preparations. The amount of rosmarinic acid (mg/mL Mean \pm SD) present in batches was A (0.15 \pm 0.01); B (22.18 \pm 1.03); C (1.41 \pm 0.09); D (0.92 \pm 0.11); E (2.96 \pm 0.42); F (0.19 \pm 0.03); G (< LOD of 0.06); H (0.47 \pm 0.04). Linearity of the calibration curves was determined ($r^2 > 0.99$), confirming the linearity of the analytical method.

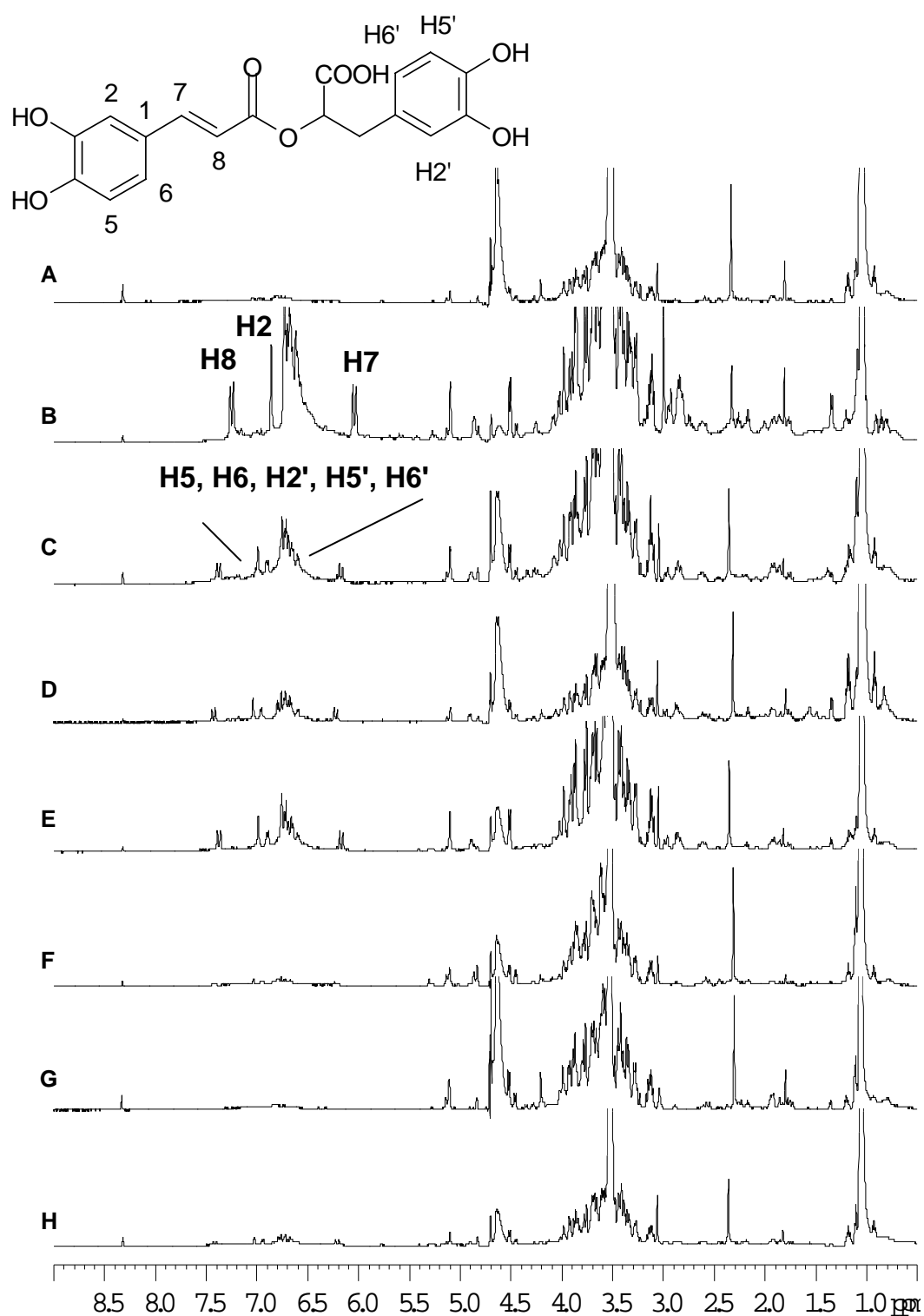


Figure 1. 500MHz ¹H NMR spectra of commercial tinctures (A-H) derived from fresh and dried *Melissa officinalis*.

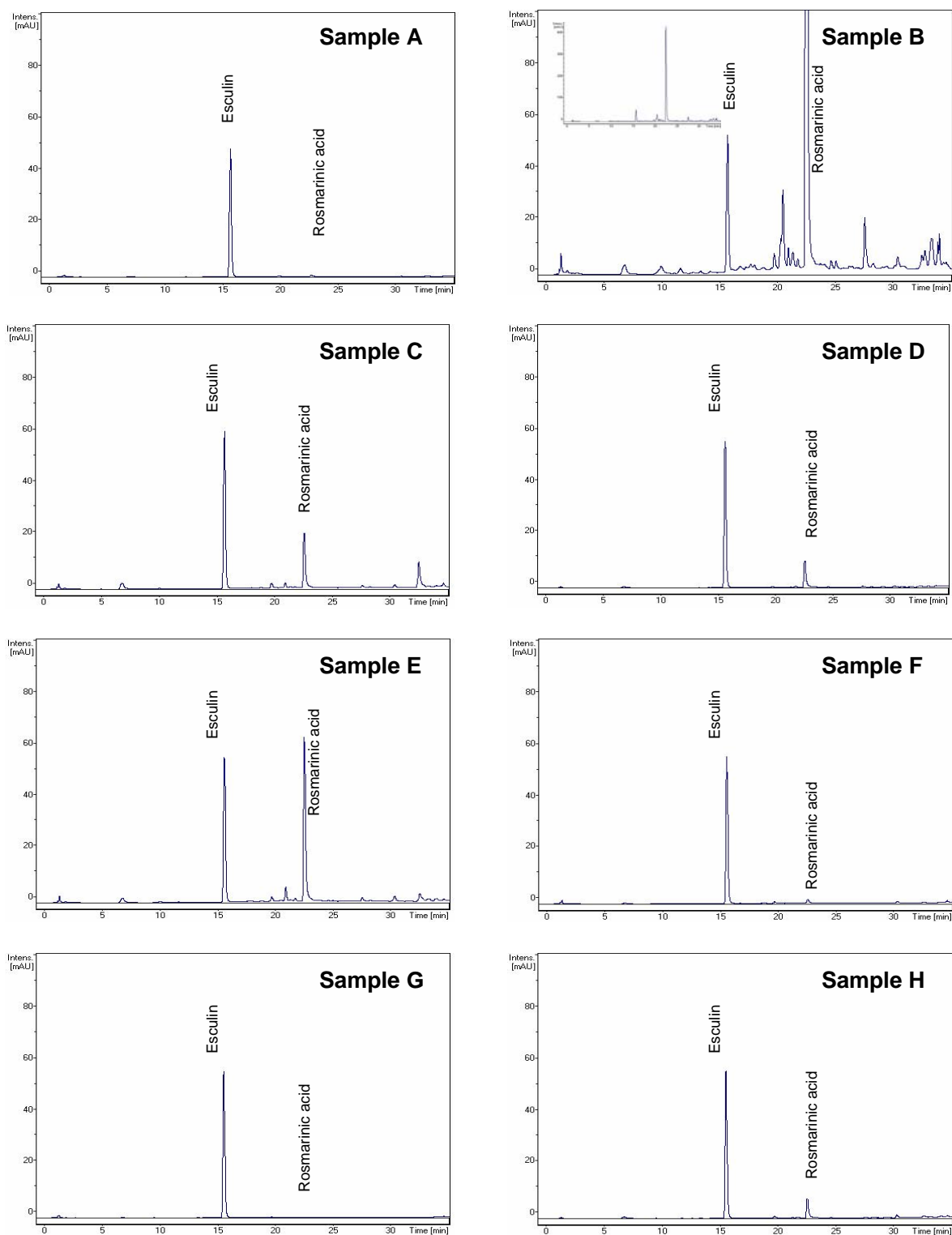


Figure 2. HPLC-UV_{320nm} chromatograms of commercial tinctures (A-H) produced from fresh and dried *Melissa officinalis*. Retention times of esculin and rosmarinic acid were observed at 15.5 and 22.5min respectively.

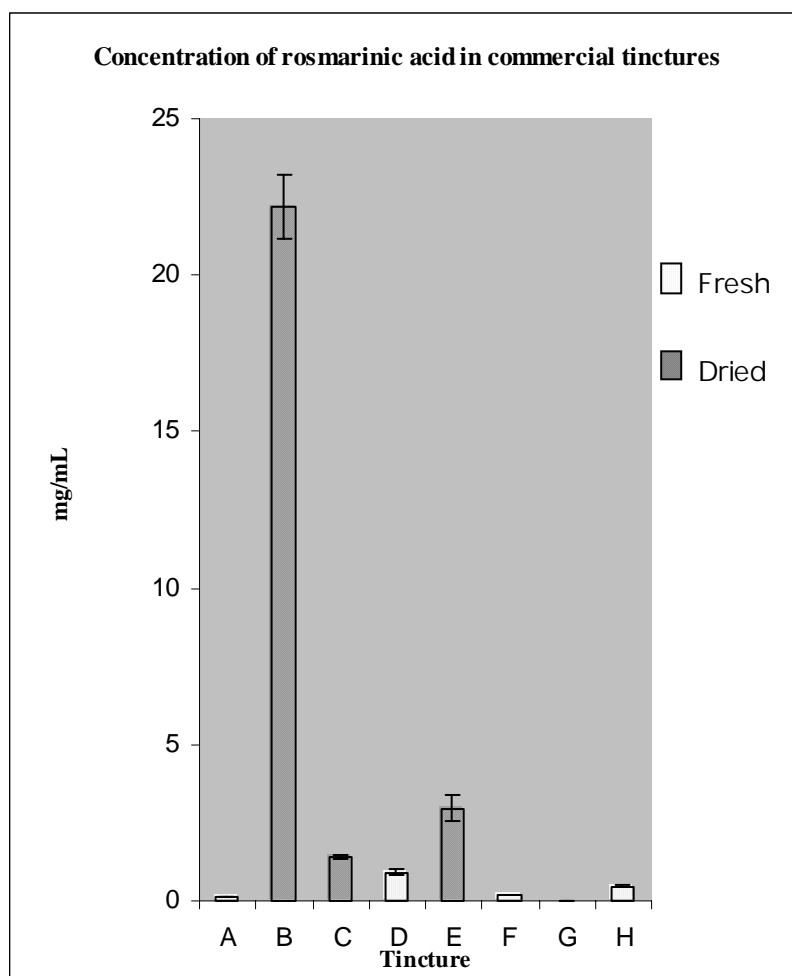


Figure 3. Rosmarinic acid content in commercial tinctures made from fresh and dried plant material.

The intraday precision was calculated by comparison of the area obtained for 12 injections of esculin solution (50 $\mu\text{g/mL}$) in 0.02 M ammonium formate (pH 6.25) on two separate days and the resulting coefficient of variation obtained (CV) was 1.98 and 2.28%. For the interday precision, the ratio of the area of rosmarinic acid/area of esculin for all the points in the calibration curve for the different days were added up and compared. The resulting CV obtained was 5.60%. The accuracy of the method was calculated after six injections of known concentration of rosmarinic acid (50 $\mu\text{g/mL}$) were carried out on three different days and were compared with the measured concentration ($50 \pm 3.20 \mu\text{g/mL}$) of rosmarinic acid. The mean of the determined values showed a variation of 1.9% with respect to the known values; this together with the low standard deviation observed supported the accuracy of the analytical method. CVs smaller than $\pm 15\%$ of the measured value

with respect to the known value reflect good accuracy of the method (19). The LOD (S/N: 3.3) and the LOQ (S/N:10) for rosmarinic acid using the developed method were 61 ng/mL and 185 ng/mL, respectively.

DISCUSSION

Due to relatively lax regulation of herbal products in the UK and abroad, commercial tincture products may contain significant differences in chemical composition. Concerning Melissa products, herbal practitioners in the UK are thus faced with a wide range of commercial tinctures, from either fresh or dried Melissa leaf, that vary widely in the drug-to-extract ratio (1:2 to 1:5) depending on the manufacturer (2). The concentration of alcohol used for Melissa tinctures typically ranges from 45% down to 25%. Taken together, this variability in drug-to-extract ratio and the range of alcohol concentrations may

partly explain the differential extraction of plant constituents, a common source of variability between commercial tinctures. Variability within and between batches can result from different methods of cultivation, extraction, seasonal factors, time of harvesting, storage conditions and natural intraspecific variation in *Melissa* leaf (4, 20). A further compounding factor is the manufacturing process: cold maceration or percolation being two preferred methods (2). Importantly, much of the official international pharmacopoeia data on *Melissa* leaf refers almost exclusively to dried plant. Although GMP regulations cover the manufacturing process they do not necessarily specify the quality of the starting plant material, nor whether fresh or dried is to be used. This state of regulation has therefore led to the wide range in quality of *Melissa* products that face herbal practitioners in the UK. However, the professional regulation of herbal practice is currently under review in the UK, and with the underlying principle of evidence-based practice informing primary healthcare, qualification of herbal practitioners arguably should involve an understanding of the pharmacological and pharmacodynamic basis of herbal medicine.

In general, fresh plant tinctures may contain lower amounts of bioactive principles due to a water content of typically 75 to 90%, resulting in a marked dilution effect (2). References to dried extracts are common in the British Herbal Pharmacopoeia, whereas fresh extracts are less frequently described (18). In the main, unless the bioactive components are volatile constituents, dried material has benefits in terms of storage and availability. Recent empirical research on greater celandine extracts indicated that fresh plant tinctures contain less total alkaloid content than dried counterparts (21). Despite this scientific evidence, many herbal practitioners still consider some fresh plant extracts such as greater celandine and *Galium aparine* to have superior activity.

Tincture products are popular with herbal practitioners because of availability, ease of dosage and for convenient storage. However, little research has been done on the chemical stability of tinctures due to the challenging multi-component nature of such analyses. Gafner and Bergeron (22) provide an excellent review of the environmental factors affecting chemical stability include pH, light and temperature. Fresh plant material is more susceptible to enzymatic degradation than is dried. The presence of air in

tincture products can lead to oxidation whilst the presence of metal ions mainly iron, copper and other transition metals, may cause hydrolysis and oxidation reactions with redox active molecules. Amber vials widely used to store tincture products contain significantly higher amounts of iron and titanium and may lead to leaching of metals leading to oxidation over a long storage period. Similarly, the presence of high levels of water has been shown in many studies to cause a more rapid decrease in important biomarker compounds. An important paper by Bilia and colleagues (23) reported a half life of only 3 months for the purported bioactive silymarin complex in alcoholic tincture products of Milkthistle, as measured by LC-DAD and LC-MS. This has serious implications for storage and labelling of tincture products.

The validation of LC-DAD methods for the analysis of important biomarkers in tincture products is reported mainly by academic groups as opposed to herbal manufacturers (22). This instrumentation and the associated methods are widely available, requiring little intellectual input and are highly automated. New EMEA guidelines highlight the use of chromatographic or spectroscopic patterns, the so-called metabolomics approach (24-26), as an important evolving technology. The use of NMR spectroscopy in metabolomics provides an opportunity of unrivalled potential to explore plant metabolism. This is reflected in the number of recent publications using NMR in a variety of applications as reviewed by Holmes *et al* 2006 (26). However, the level of expertise needed for the interpretation of NMR spectra is not widely available in the herbal industry, but sits more comfortably with the pharmaceutical industry (27). Here we illustrate the use of ^1H NMR spectroscopy as a rapid profiling tool for identifying rosmarinic acid as an important biomarker of antiviral activity in *Melissa* tincture products.

Traditionally, the choice of fresh or dried *Melissa officinalis* by herbalists has depended on the season. Differentiation of use between tinctures prepared from fresh or dried material has generally been based on personal preference rather than scientific evidence. Since there is currently no standardisation of methods for the preparation of phytomedicines, particularly tinctures that are administered as the ingredients of many herbalists' formulations, the choice of fresh or dried plant material has in the main been influenced by demand. The use of *Melissa*

officinalis as a topical antiviral is a relatively new application not mentioned in old herbals. Its use as such was confirmed by a series of comprehensive trials carried out in Germany (7) using a cream containing a dried extract from *Melissa officinalis*. The results of our study show that the use of fresh and dried plant material clearly affected the resultant constituent profile of the commercial tincture extracts, raising concern not only of potency but also of efficacy in terms of the compounds required to achieve a therapeutic effect. Due to their higher polyphenol content, i.e., rosmarinic acid, tinctures made from dried plant material are likely to be more effective as an antiviral than those made from the fresh plant, perhaps explaining, given their penchant for fresh plant tinctures, many herbalists' reported lack of success in treating herpes sores.

Due to a paucity of scientific research medical herbalists still, to a large degree, rely on anecdotal evidence in their prescribing of plant remedies. There is however, now a growing need to establish a scientific evidence base for the practice of herbal medicine to help herbalists to be more discriminatory in their choice of products. Additionally, in regard to quality, with currently no standardised methods of production or accepted measure in place for the manufacture of tinctures for use by herbalists in their prescribing there is no guarantee of potency or efficacy. There is thus an urgent need for a review by manufacturers of their production procedures of the herbs formulated as tinctures that are popularly used by herbal practitioners.

ACKNOWLEDGEMENTS

The authors would like to thank Mrs Jane Hawkes, King's College London for ¹H NMR experiments and Dr Geoff Webb, University of East London for valuable comments on the manuscript. The authors would also like to acknowledge a Promising Researcher Award from the University of East London to OC that partly-funded this study.

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