European Journal of Medicinal Plants
4(1): 52-63, 2014

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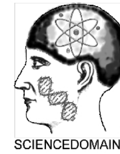

Identification of *cis-* and *trans-* Melilotoside within an *Artemisia annua* Tea Infusion

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Authors' contributions

Author JM managed the analyses and practical aspects of the study. Author FVK designed the study and wrote the manuscript. All authors read and approved the final manuscript.

Short Research Article

Received 13th August 2013
Accepted 6th September 2013
Published 11th October 2013

ABSTRACT

Aims: To identify the major chemical components contained within the *Artemisia annua* tea infusion, which has been traditionally used to treat fevers and chills.
Study Design: Currently, little chemical data exists on the tea infusion as previous work only focused on LC-MS analysis and although a valuable analytical tool it remains difficult to identify new compounds. Our study therefore employed LC-MS and NMR analysis in order to confirm the identities of the major compounds.
Place and Duration of Study: Natural Products Laboratory, Institute of Biology, Leiden University between Jan 2012 and December 2012.
Methodology: A thorough chemical analysis of the tea infusion was completed using both LC-MS and NMR analyses. Tea infusions were prepared using deionised water and were subjected to LC-MS analysis followed by semi-preparative fractionation and NMR analysis to confirm the identities of the major compounds.
Results: Eleven major compounds were identified including chlorogenic acids, feruloylquinic acids, flavonols, coumarins, of which two compounds, *cis-* and *trans-*melilotoside, are new for *Artemisia* spp.
Conclusion: The melilotosides are known to be active against diarrhoea causing pathogens and therefore might explain the traditional use of *A. annua* to treat diarrhoea. Future work will focus on the quantification of the melilotosides and the identification of

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other compounds (major and minor) in the tea infusion.

Keywords: Artemisia annua; tea infusion; malaria; melilotosides; HIV; cancer.

1. INTRODUCTION

Many of today's drugs and drug leads are derived from traditional medicinal plants [1]. One such example is *Artemisia annua* L. (Asteraceae) which has been used for centuries in China to treat fevers. In the 1970s Chinese scientists identified the active principle, artemisinin, during a large-scale screening for new antiplasmodial lead compounds. Today, artemisinin and its derivatives are being used as a first-line treatment for uncomplicated malaria and once again highlight the importance of scientific research into medicinal plants [2].

However, herbalists claim that using *A. annua* in its traditional way mainly in the form of a tea infusion will have a comparable or even better efficacy against *Plasmodium falciparum* than the purified artemisinin. The plant material, and the tea infusion thereof, contains many different compounds including the active principle artemisinin. The co-extracted compounds in the tea infusion can have a synergistic effect on many different levels against *P. falciparum*, although to prove synergism will be very difficult, due to its inherent complexity and the fact that we predominantly use *in vitro* bioassays. The synergistic action between compounds within *A. annua* was however recently demonstrated by Onimus et al. [3] who administered powderised *A. annua* leaves, known to contain a very low amount of artemisinin (>0.1%), to 25 patients. The treatment resulted in a 62% reduction in the number of parasites although the daily dose of artemisinin was only 0.5 mg. They concluded that the administration of powderised *A. annua* leaves was apparently more effective than the tea infusion but due to logistical reasons the tea infusion is still the preferred treatment option. It is however also claimed that the tea infusion is not only useful to treat uncomplicated malaria but also to treat HIV, diarrhoea and even cancer [4-7]. These assertions are directed by Non-governmental organisations that provide the plant material too many African and Asian communities. As these diseases are life-threatening and where especially children are vulnerable to malaria and diarrhoea it is extremely important to investigate these claims, as many thousands of people lives are potentially affected.

Investigating such a complex issue consisting of many scientific questions will be a time consuming endeavour. In order to find answers to this complex issue, we started with the investigation of the extraction efficiency of artemisinin during the tea making process. We found that by boiling the leaf material for 2-3 min almost all the artemisinin in the plant material was effectively extracted, and the artemisinin content of the tea remained stable for at least 24 h [8]. This was followed by testing the tea infusion against HIV (*in vitro*) where we found it to be remarkably active and it appears that artemisinin plays only a very minor role in the observed activity [4]. We have also tested artemisinin against 44 breast cancer cell lines and found it to be active against all cell lines tested with selectivity for the multi-drug resistant cell lines [6]. The synergistic effect of the *A. annua* tea infusion against *P. falciparum* was also investigated. Our results indicated that the *in vitro* antiplasmodial activity of the *A. annua* tea infusion could only be ascribed to artemisinin and therefore no synergism was present in our bioassay used [9]. This finding was however contradictory to the studies performed by Wright et al. [10] who found an 6-18 fold enhanced activity as compared to pure artemisinin.

With increasing reports on the various biological activities ascribed to the tea infusion and also the lack of any toxicity reported thus far, we find it surprising that very little research has been conducted on the tea infusion itself. At the heart of all of these claimed activities lies the chemistry of the tea infusion, which has been completely understudied. This lack of information and the presence of a large amount of contradictory results were reviewed by Van der Kooy and Sullivan [11] and the possible reasons and implications of this were reviewed by Van der Kooy [5]. Studies conducted on the chemistry of the tea infusion are limited to Bilia et al. [12] who quantified artemisinin and flavonoids but most of this work was performed on aqueous methanol extracts. Liu et al. [13] employed NMR metabolomics to compare an aqueous methanol extract of *A. annua* with *A. afra* for quality control purposes and also tested the tea infusion against *P. falciparum*. Polar extracts (mainly methanol) of the leaf material were shown to contain a large amount of flavonoids and other phenolics [14-15]. Carbonara et al. [16] performed a LC-MS analysis on a 24 h water extract of *A. annua* and reported a large amount of phenolic compounds to be present. However, no studies were conducted using both LC-MS and NMR in order to identify possible new compounds in the tea infusion.

In this study only compounds that gave adequate MS and NMR data were identified. Due to the low sensitivity of NMR spectroscopy the tea infusion was fractionated and concentrated in order to confirm the identities of the main compounds. These two analytical systems revealed both their strengths and weaknesses as the LC-MS was able to detect a large number of minor compounds which could not be detected with NMR. On the other hand many metabolites could be detected with NMR (e.g. sugars) which were not detectable with both UV and MS spectroscopy. We must also keep in mind that a tea infusion is prepared by adding boiling water to the plant material after which it is consumed within a matter of minutes. This is the main reason why we decided to prepare the tea as prescribed and analyzed it within a matter of minutes and, importantly, without any sample treatment (except filtering). Therefore, the main objective of this study was to identify the major chemical components in the tea infusion with MS and NMR analysis.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

LC-MS grade mobile phase were purchased from Sigma-Aldrich (Steinheim, Germany). Deionised water was obtained from a Millipore distilled water unit (pH 5.7). The deuterated solvents, D_2O, CD_3OD and $CDCl_3$ for NMR analysis were purchased from Andover (MA, USA).

2.2 Plant Material

Artemisia annua (Anamed A-3) plant material was obtained from the breeding program of ANAMED and identified by Dr Hans-Martin Hirt. The plants were grown in Ammerbuch (Germany), harvested in September 2010 and consisted of dried leaf material.

2.3 Sample Preparation

Artemisia annua tea was prepared as described earlier [8]. In short, tea infusions were prepared by adding 10 mL of deionised boiling water to 90 mg of A-3 plant material. The

mixture was allowed to boil for 2-3 min after which 1.5 mL was filtered (0.45 μm PTFE syringe filter) into an HPLC vial for analysis.

2.4 LC-MS Analysis

The LC-MS analysis was performed on an Agilent 1100 equipped with an auto-sampler, a binary pump system, a photo-diode array and MS single quadrupole detectors. The mobile phase consisted of water with 0.1% formic acid (FA) (solvent A) and methanol with 0.1% FA (solvent B). We used the following elution gradient: 0 min, 20% B; 5 min, 20% B; 30 min, 100% B; 35 min, 100% B; 36 min, 20% B, and 40 min 20% B. The flow-rate was 1.0 mL/min and UV detection was performed at 210, 254 and 330 nm.

The MS conditions were as follows: APCI positive and negative ionisation in the Scan mode (50-900 *m/z*). The fragmentor was set to 150V, drying gas flow 10 L/min, nebulizer pressure 50 psig, drying gas temperature 350°C, vaporizer temperature 500°C, capillary voltage 4000 V and the corona current 7 μA for positive and 25 μA for negative ionisation. For improved ionisation of peaks 8, 9 and 11 the fragmentor was set to 0 and the elution gradient was changed to: 0 min, 20% B; 5 min, 20% B; 40 min, 100% B; 45 min, 100% B; 46 min, 20% B; 50 min, 20% B.

2.5 Semi-purification of Compounds

In order to aid the identification of the selected compounds, we fractionated the tea sample (A-3) by semi-preparative HPLC. The column used to separate the compounds was a Phenomenex Luna C_{18}(2) (250x10.00 mm, 5.0 μm) column. The mobile phase was the same as for LC-MS analysis but the flow-rate was increased to 2.0 mL/min and the elution gradient was as follows: 0 min, 40% B; 2 min, 40% B; 35 min, 100% B; 40 min, 100% B; 41 min, 40% B; 45 min, 40% B. Fractions were collected for 1.5 min starting at 4 min after injection and ending at 40 min, yielding 24 fractions. Each fraction was re-injected into the LC-MS in order to obtain the UV and MS data of the main compounds in each fraction and match this data with the original chemical profile of the tea infusion.

2.6 ^{1}H-NMR Analysis

^{1}H-NMR was recorded at 25ºC on a 500 MHz Bruker DMX-500 spectrometer (Bruker, Karlsruhe, Germany) operating at a proton NMR frequency of 500.13 MHz. Each ^{1}H-NMR spectrum consisted of 128 scans requiring 10 min and 26 sec acquisition time with the following parameters: 0.16 Hz/point, pulse width = 30º (11.3 μsec), and relaxation delay = 1.5 sec. A pre-saturation sequence was used to suppress the residual H_2O signal with low power selective irradiation at the H_2O frequency during the recycle delay. Free induction decays were Fourier transformed with line broadening of 0.3 Hz. The resulting spectra were manually phased and baseline corrected, and calibrated to the internal standard trimethylsilyl propanoic acid at 0.0 ppm, using XWIN NMR (version 3.5, Bruker). All 24 fractions were dried under vacuum and re-dissolved in D_2O and based on the preliminary compound identification all fractions were re-analyzed in organic solvents (MeOD or $CDCl_3$) in order to compare the chemical shifts with existing literature.

3. RESULTS

3.1 Compound Identification

The choice of which components to regard as the main constituents was based on the UV absorbance at 210, 254, 330 nm and ease of ionisation in the MS. Fig. 1 shows the chemical profile of a typical *A. annua* tea infusion at 254 nm and Table 1 lists the identities of the main components identified in the tea based on their MS and NMR spectral properties. Due to artemisinin not containing a chromophore it does not appear in the chromatogram. That artemisinin is present in the tea infusion in appreciably quantities has been shown by a number of authors as reviewed by Van der Kooy and Sullivan [11].

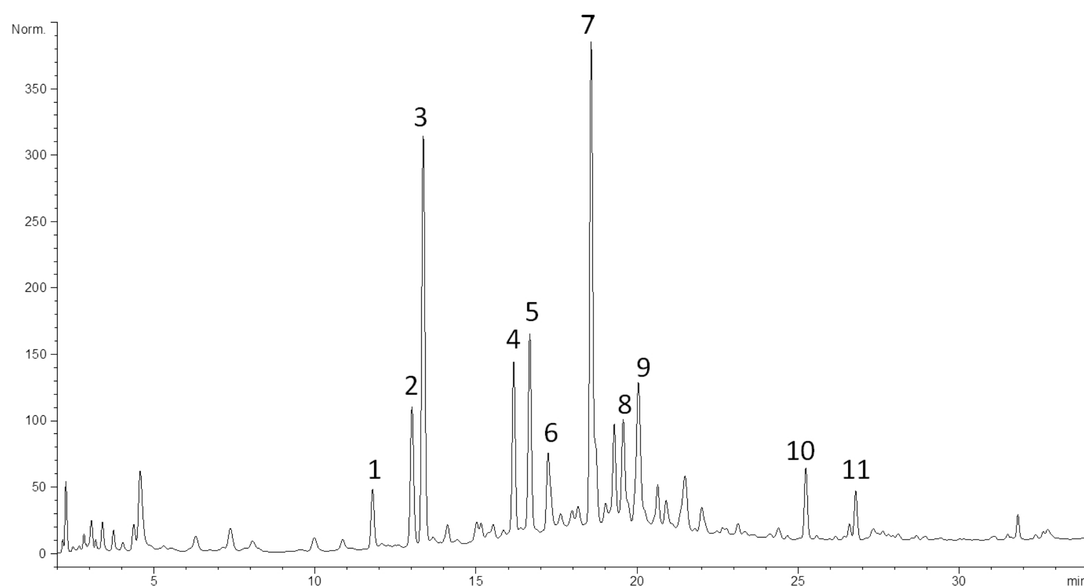

Fig. 1. Chemical profile of the *A. annua* tea infusion at 254 nm. 1: scopolin; 2: cis-melilotoside; 3: chlorogenic acid; 4: 5-feruloylquinic acid; 5: trans-melilotoside; 6: scopoletin; 7: 3,5-dicaffeoylquinic acid; 8: rutin; 9: caffeoylferuloylquinic acid; 10: chrysosplenol D; 11: chrysosplenetin

Table 1. Identification of the main phenolic compounds in the *A. annua* tea infusion based on their MS (+ and -) and ^{1}H NMR data

n°	RT (min)	MS (+)	MS (-)	MW	^{1}H NMR					Proposed compounds	References
					δ (ppm)	Integ.	Pattern	J (Hz)	H		
1 (D_2O)	11.79	355; 193	399; 191; 176	354	7.93	1	d	9.5	4	scopolin	[17]
					7.22	1	s	-	5		
					7.18	1	s	-	8		
					6.39	1	d	9.5	3		
					5.24	1	d	7.3	1'		
					3.92	3	s	-	OMe		
2 (MeOD)	13.01	327; 165; 147	325; 163; 119	326	7.57	1	d	7.8	6	*cis*-melilotoside	[18,19]
					7.35	1	d	12.5	7		
					7.32	1	t	7.8	4		
					7.22	1	d	7.8	3		
					7.00	1	t	7.8	5		
					5.99	1	d	12.5	8		
					4.97	1	d	7.4	1'		
3 (MeOD)	13.36	355; 195; 163	353; 191	354	7.55	1	d	16.0	7'	chlorogenic acid (5-caffeoylquinic acid)	[15,16,20,21]
					7.04	1	d	2.0	2'		
					6.94	1	dd	8.2, 2.0	6'		
					6.77	1	d	8.2	5'		
					6.25	1	d	16.0	8'		
					5.33	1	m	-	5		
4 (MeOD)	16.17	369; 209; 177; 151	367; 191	368	7.62	1	d	15.9	7'	5-feruloylquinic acid	[15,16,20,21]
					7.19	1	d	1.8	2'		
					7.08	1	dd	8.2, 1.8	6'		
					6.80	1	d	8.2	5'		
					6.35	1	d	15.9	8'		
					5.33	1	m	-	5		
					3.89	3	s	-	OMe		

Table 1 continues…………

5 (MeOD)	16.67		327; 165;	325; 163;	326	8.15	1	d	16.2	7	*trans*-melilotoside	[18,19]
			147	119		7.66	1	d	7.9	6		
						7.41	1	t	7.9	4		
						7.29	1	d	7.9	3		
						7.09	1	t	7.9	5		
						6.56	1	d	16.2	8		
						5.02	1	d	7.8	1'		
6 (MeOD)	17.24		193	191; 176	192	7.89	1	d	9.4	4	scopoletin	[15]
						7.15	1	s	-	5		
						6.81	1	s	-	8		
						6.24	1	d	9.4	3		
						3.94	3	s	-	OMe		
7 (MeOD)	18.57		517; 499;	515; 497;	516	7.61	1	d	15.9	7'	3,5-dicaffeoylquinic acid	[15,16,20,21]
			355; 337;	353		7.57	1	d	15.9	7''		
			195; 163			7.06	1	d	1.9	2'		
						7.05	1	d	1.9	2''		
						6.97	1	dd	8.1, 2.0	6'		
						6.96	1	dd	8.1, 2.0	6''		
						6.77	1	d	8.1	5'		
						6.77	1	d	8.1	5''		
						6.34	1	d	16.0	8'		
						6.26	1	d	16.0	8''		
						5.42	1	m	-	3		
						5.37	1	m	-	5		
8 (MeOD)	19.57		611; 495;	609; 493;	610	7.73	1	dd	2.1, 8.5	6'	rutin	[22,23]
			333; 303	477		7.58	1	d	2.1	2'		
						6.87	1	d	8.5	5'		
						6.41	1	d	2.1	8		
						6.20	1	d	2.1	6		
						5.11	1	d	7.7	1''		
						4.52	1	d	1.3	1'''		
						1.12	3	d	6.2	Me		

Table 1 continues……….

9 (MeOD)	20.04	513; 499;	529; 515;	530	7.61	1	d	16.0	7'	3,5-caffeoylferuloylquinic	[15,16,20,21]
		355;	367; 353		7.51	1	d	16.0	7''	acid	
		209; 177;			7.10	1	d	1.6	2'		
		163			7.09	1	d	1.9	2''		
					7.00	1	dd	9.7, 1.9	6'		
					6.96	1	dd	8.0, 2.0	6''		
					6.81	1	d	8.2	5'		
					6.77	1	d	8.0	5''		
					6.35	1	d	15.9	8'		
					6.34	1	d	15.9	8''		
					5.42	1	m	-	3		
					5.38	1	m	-	5		
					3.87	3	s	-	OMe		
10 ($CDCl_3$)	25.24	361; 303	359; 301	360	12.50	1	s	-	OH-5	chrysosplenol D	[24]
					7.77	1	d	2.0	2'		
					7.57	1	dd	8.4, 2.0	6'		
					7.00	1	d	8.4	5'		
					6.50	1	s	-	8		
					3.95	3	s	-	OMe		
					3.91	3	s	-	OMe		
					3.82	3	s	-	OMe		
11 ($CDCl_3$)	26.79	375; 221	373	374	12.60	1	s	-	OH-5	chrysosplenetin	[25]
					7.70	1	d	1.9	2'		
					7.66	1	dd	8.4, 2.0	6'		
					7.04	1	d	8.5	5'		
					6.50	1	s	-	8		
					3.98	3	s	-	OMe		
					3.96	3	s	-	OMe		
					3.92	3	s	-	OMe		
					3.86	3	s	-	OMe		

Peaks 1 and 6 were identified as scopolin and scopoletin respectively, based on their ^1H-NMR, MS and UV spectral data as compared to literature [15,17]. The ^1H-NMR data of 1 matched to Fliniaux et al. [17], for scopolin but all signals were shifted by + 0.2 ppm, due to a difference in the pH of D_2O used in the analysis. The mass spectrum of 1, revealed a quasimolecular ion of 355 in positive mode with the major fragments of 193 and 191 in positive and negative mode respectively, indicating the loss of one glucose molecule. The NMR data of peak 6 revealed this compound to be a coumarin and the MS (quasimolecular ion of 193 and 191 in the positive and negative mode respectively) and UV data confirmed it to be scopoletin [15]. To add further evidence for the identity of these compounds, a freshly prepared tea infusion was hydrolysed by the addition of HCl (0.1 mL 1N HCl added to 1mL of tea and allowed to react at 80 ºC for 1 hour). Upon hydrolysis the peak area of 1 decreased while the area of 6 increased indicating a correlation between 1 and 6.

Peaks 2 and 5 were identified as *cis*-melilotoside and *trans*-melilotoside respectively (Fig. 2). According to our knowledge this is the first report that these compounds are identified in any *Artemisia* spp. Identification was based on UV properties and mass spectral data as compared to literature [18]. For both compounds, a quasimolecular ion of 327 and 325 were observed in the positive and negative ionisation mode respectively. The fragments at m/z 165 and 163 observed in positive and negative mode, respectively, correspond to the coumaric acid part of the molecule. The area of these peaks decreased upon hydrolysis, confirming the presence of a sugar moiety. ^1H-NMR data of the semi-purified fraction of 2 gave the distinctive ring signals at δ_H 7.00 (1H, t, J=7.8 Hz, H-5), 7.22 (1H, d, J=7.8 Hz, H-3), 7.32 (1H, t, J=7.8 Hz, H-4), 7.57 (1H, d, J=7.8 Hz, H-6) and the H-1' of the glucose moiety at δ_H 4.97 ppm (1H, d, J=7.4 Hz, H-1'). The typical *cis* configuration double bond signals could clearly be observed at δ_H 5.99 (1H, d, J=12.5 Hz; H-8), 7.35 (1H, d, J=12.5 Hz, H-7). A similar pattern was observed for 5: δ_H 7.09 (1H, t, J=7.9 Hz, H-5), 7.29 (1H, d, J=7.9 Hz, H-3), 7.41 (1H, t, J=7.9 Hz, H-4), 7.66 (1H, d, J=7.9 Hz, H-6), 5.02 (1H, d, J=7.8 Hz, H-1') but with the distinctive *trans* double bond configuration at δ_H 6.56 (1H, d, J=16.2 Hz; H-8), 8.15 (1H, d, J=16.2 Hz, H-7) [18,19].

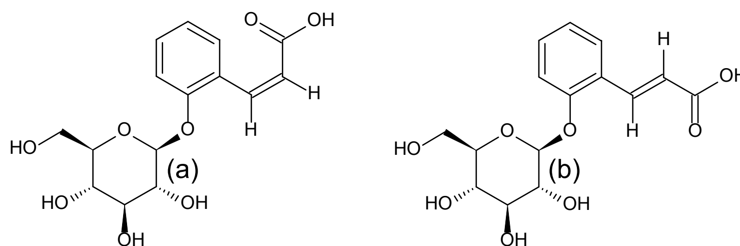

Fig. 2. a) *cis* – and b) *trans* – Melilotoside

Peaks 3, 4, 7 and 9 were identified as compounds belonging to the chlorogenic acid family, named respectively chlorogenic acid (5-caffeoylquinic acid), 5-feruloylquinic acid, 3,5-dicaffeoylquinic acid, caffeoylferuloylquinic acid. Peak 3 was positively identified based on the NMR, MS and UV spectral data and was confirmed by the analysis of a pure reference standard of chlorogenic acid. The position of the bond between caffeoyl or feruloyl moiety and quinic acid were defined based on Ge et al. [20]. To confirm the identity of these compounds, UV, MS and ^1H-NMR spectral properties were compared with literature [15,16,21].

Peaks 8, 10 and 11 belong to the flavonoid class of compounds and were identified as rutin [22,23], chrysosplenol D [24] and chrysosplenetin respectively. For peak 11 the reported literature data were not very clear. Indeed, peak 11 was identified as chrysosplenetin by Sy and Brown, [25] but the ^{1}H-NMR data and UV properties did not match those reported by Horie et al. [26] and Ahmed et al. [27]. Peak 11 can therefore also possibly be casticin or a chrysosplenetin isomer as reported by Horie et al. [27]; Asker et al. [28] and Mesaik et al. [29].

4. DISCUSSION AND CONCLUSIONS

The main objective of this study was the identification of the main compounds in the tea infusion. In order to achieve this we decided to directly inject the sample without any treatment, yielding a chemical profile of the tea infusion within 10 min of its preparation (Fig. 2). All major compounds that gave adequate spectral data were identified. The main components consist of a wide variety of phenolic compounds including chlorogenic acids, feruloylquinic acids, flavonols, coumarins and two new o-coumaric acid derivatives. Although only the main metabolites have been identified during this study, at least 50 minor components could be detected. Some major compounds (e.g. the peak between compound 7 and 8) did not give adequate spectral data and also remains unidentified. This is however the first report on the identification of *cis*- and *trans*- melilotoside in *A. annua* and any other *Artemisia* spp. No reports on any activity against *P. falciparum*, cancer or HIV could be found for these compounds. These relatively rare compounds were shown to exhibit good activity against the diarrhoea causing pathogens *Entamoeba histolytica* and *Giardia lamblia* (IC50 = 12.5 and 16.8 µg/mL respectively) indicating that the traditional use of *A. annua* for the treatment of diarrhoea might be effective [30]. It is however well known that most phenolic compounds suffer from having a low *in vivo* bioavailability, but this aspect does however not apply to the melilotosides and their activity against *E. histolytica* and *G. lamblia*. These parasites occur in the lumen of the digestive tract and they should be inhibited here without the melilotosides having to be absorbed into the bloodstream. In conclusion, we report here on the identity of the major components in the *A. annua* tea infusion and by combining LC-MS and NMR analysis we were able to identify two new compounds for *Artemisia* spp. With this study we have taken only the first small step in unlocking the full medicinal potential of *A. annua.* Future work will focus on the quantification of the melilotosides in different *A. annua* samples and the identification of other compounds (major and minor) in the tea infusion.

ACKNOWLEDGEMENTS

We thank Anamed for providing the plant material and Ros Priest and Suzannah Bourchier for proof reading the manuscript. We also thank CompleMed, University of western Sydney for providing the facilities to complete this work and Shaun Sullivan for converting the manuscript into the journals format.No funding was received for this work.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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