# Unlocking the full (medicinal) potential of *Artemisia annua*: A LC-MS and NMR investigation of the tea infusion

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**Abstract**

**Ethnopharmacological relevance:** A tea infusion prepared from *Artemisia annua* L. (Asteraceae) has been traditionally used to treat fevers and chills. From this plant the active compound, artemisinin, has been identified and is currently being prescibed as a first-line treatment for malaria. It is however claimed that the tea infusion contains numerous other compounds which improve the activity of artemisinin against malaria and also renders the tea infusion active against other diseases including cancer, HIV and diarrhea. Due to a lack of data on the chemical content of the tea infusion we embarked on a thorough chemical analysis of the tea infusion. Our main objectives were:

1. Identify the major chemical components in the tea infusion.
2. Test the stability of the tea infusion over a two week period.
3. Test the stability of the tea infusion prepared from tap water to simulate field conditions.

**Materials and methods:** Tea infusions were prepared using deionised water and tap water. The samples were subjected to LC-MS analysis to generate a chemical profile. The tea infusions were also subjected to semi-preparative fractionation followed by NMR analysis to confirm the identities of the major compounds based on their UV, MS and NMR spectral data.The stability of the tea infusions were studied under three different storage conditions and analysed at various time points over a two week period.

**Results:** Ten major compounds were identified based on their spectral properties of which two compounds, *cis*- and *trans* – melilotoside, are new for *Artemisia* spp. These compounds are known to be active against diarrhea causing pathogens. The tea infusions prepared from deionised water remained stable for two weeks while the tea infusion prepared from tap water showed a large degree of degradation after only 24 hours.

**Conclusion:** The tea infusion of *A. annua* is being used by thousands of people in developing nations to treat various ailments. It is therefore important to understand the chemistry of the tea infusion in order to develop improved methods of tea preparation and administration. In this study we took the first step by identifying the major compounds, however numerous minor compounds could be detected. This yielded valuable information as it identified compounds with anti-diarrheal activity and shows that the use of the tea infusion for the treatment of diarrhea can potentially be effective. It will be important to identify the minor compounds and to study the chemical interaction between the tea chemicals and common chemicals present in tap water. This interaction can lead to activity enhancement or a loss of activity. The variation of the chemical profile between different *A. annua* samples should also be investigated.

**Keywords:** *Artemisia annua;* tea infusion; malaria; HIV; cancer

## 1. Introduction

Many of today's drugs and drug leads are derived from traditional medicinal plants (Newman & Cragg, 2012). 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 (ART), during a large-scale screening for new antiplasmodial lead compounds. Today, ART and its derivatives are being used as a first-line treatment for uncomplicated malaria and once again highlights the importance of scientific research into medicinal plants.

However, herbalists claim that using the plant in the traditional way (tea infusion) will have a comparable, or even better efficacy against *Plasmodium falciparum* than the purified ART, and that this effect is mainly ascribed to synergism. Another claim is that *P. falciparum* cannot become resistant to ART if administered in its traditional way and that this effect is also due to synergism, as opposed to the use of pure ART and its derivatives which has lead to *P. falciparum* becoming resistant in Cambodia. It should be noted that the tea infusion has been used for centuries without resistance developing, while resistance against ART and derivatives has only occured now after the purified single compound has been used for a couple of decades (Amaratunga et al., 2012).

The plant material, and the tea infusion thereof, contains many different compounds including the active principle ART. We have recently shown that by using specific preparation methods for the tea infusion it is possible to extract up to 95% of the ART present in the plant material (Van der Kooy & Verpoorte, 2012). The co-extracted compounds in the tea infusion can have a synergistic effect on many different levels against *P. falciparum* for example: Stimulating the immune system, inhibiting secondary infections (thereby improving the immune response reaction), affect the physical properties of the infection site (e.g. anticoagulants), affecting non-lethal biochemical processes in *P. falciparum* (e.g. efflux channel inhibitors), having a direct activity against *P. falciparum* but at a different target site, improving the bioavailability of the active compound (e.g. ART) etc. All of these factors together (and there are more) are called synergism. To prove synergism is very difficult, due to its inherent complexity and the fact that we predominantly use *in vitro* bioassays. Scientists also tend to look mainly at what people used (e.g. *A. annua*) and not really at how they used it (as a tea infusion, food, smoked etc). The method of use can potentially add another layer of complexity to the problem.

During preparation of the tea infusion we used deionised water in the laboratory (acidic pH, low electrical conductivity) whilst people using the tea as a treatment use any locally available water (e.g. tap, borehole, river, rain water etc). The water chemistry will therefore be drastically different depending on the source of water. If we keep in mind the mechanism of action of ART is thought to occur via iron mediated activation (De Ridder 2008), it is quite possible that metal ions, other elements and salts in the water can have a profound influence on the chemistry of the tea infusion. Based on the possible chemical processes occurring during the preparation step, an inactive compound might become active or vica versa.

It is however also claimed the tea infusion is not only useful to treat uncomplicated malaria but also to treat HIV, diarrhea and even cancer. These assertions are directed by Non-goverment Organisations (NGO) who provide the plant material at a very low cost to many African and Asian communities. As these diseases are life-threatening and where especially children are vulnerable to malaria and diarrhea it is extremely important to investigate these claims, as many thousands of people lives are potentially affected. The main question that needs to be answered is: Are any of these claims true?

In order to find answers to this complex issue, our first step was to investigate the extraction efficiency of ART during the tea making process. We found that by boiling the leaf material for 2-3 min almost all the ART in the plant material was effectively extracted, and the ART content of the tea remained stable for at least 24 h (Van der Kooy & Verpoorte, 2011). This was followed by testing the tea infusion against HIV where we found it to be remarkably active and it appears that ART plays only a very minor role in the observed activity (Lubbe et al., 2012). We have also tested ART against 44 breast cancer cell lines and found it to be active against all cell lines tested with selectivity for the MDR cell lines (Liu et al., 2011).
In the last decade it was shown that ART is effective in treating different types of cancer (Efferth et al., 2002) and currently a consortium is studying ART and *A. annua* extracts and its potential synergistic effect (Ferreira et al., 2010). 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 scientific research has been conducted on the tea infusion. A literature survey revealed that the content of ART in the tea infusion and its *in vivo* pharmacokinetic properties was studied by Mueller et al., (2000, 2004) and Räth et al., (2004). These studies indicated the bioavailability of ART given in the form of a tea is significantly higher compared with the administration of pure ART. Weathers et al., (2011) also showed that mice given the dried leaf material had the same improved effect on the

bioavailability of ART as with the tea infusion (almost 50 times higher compared to pure ART). Other studies conducted on the chemistry of the tea infusion include Bilia et al., (2006) who quantified the ART and flavonoid content in a tea infusion but most of this work was performed on aqueous methanol extracts. Liu et al., (2010) 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 did however contain a large amount of flavonoids and other phenolics (Carvalho et al., 2011; Han et al., 2008). The potential for synergy between various compounds present in *A. annua* was also recently highlighted by Efferth et al., (2011) who have shown there are other bioactive compounds (anticancer and antitrypanosomal) present in the plant material other than ART.

We must 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. If we let it stand for a couple of hours the taste changes and it is usually discarded. This means that the chemistry has already started to change. This is the main reason why we decided to prepare the tea as prescribed and analyse it within a matter of minutes and, importantly, without any sample treatment (except filtering). Our main aim was therefore to directly analyse the tea infusion by LC-MS without any sample treatment and confirm the identity of the main components with NMR spectroscopy. Due to the low sensitivity and peak overlap 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 visible with both UV or MS spectroscopy, indicating the good dynamic range of NMR. The main objectives of this study were:

1. Study the stability of the tea infusions using distilled and tap water stored under different conditions.
2. Identify the main compounds in the tea infusion by LC-MS and NMR.
3. Conduct a literature survey on what is known on the biological activity and potential for synergism of the identified compounds.

## 2. MATERIALS AND METHODS

### 2.1. Chemicals and reagents

Chlorogenic acid and the 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) and the tap water (pH 7.9) was obtained from the tap in the laboratory.
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 (Germany) 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 (Van der Kooy and Verpoorte, 2011). In short, tea infusions were prepared by adding 10 mL of Millipore deionized boiling water to 90 mg of dried plant material. The mixture was allowed to boil for 3 min after which 1.5 mL was filtered into an HPLC vial for analysis. The plant material was not squeezed to remove residual water.

### 2.4. Stability test

In order to test the stability of the tea infusion over time and under different storage conditions, we analyzed fresh, one and two week old tea samples and compared their chemical profiles. Tea was made according to the abovementioned method and 1 mL was filtered into six HPLC vials. Three samples were freeze-dried while the other three samples were kept in liquid form. Two samples (one freeze-dried and one liquid) were stored at room temperature, another two in the fridge and finally two more in the freezer. Before analysis at the different time points the freeze-dried samples were dissolved in 1 mL of water. All samples were vortexed for 30 s just before analysis. One sample was also prepared with locally available tap water in duplicate. The fresh tea infusion was analysed immediately and after 24 hours.

### 2.5. LC-MS analysis

The LC-MS analysis was performed on an Agilent 1100 LC-MSD equipped with an auto-sampler, a binary pump system, a PDA and MSD single quadrupole detector. 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 ionization 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 $\mu$A for negative ionisation. For improved ionization 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.

### 2.6. Semi-purification of compounds

In order to aid the identification of the selected compounds, we fractionated the tea sample by semi-preparative HPLC. The column used to separate the compounds was a Phenomenex Luna $C_{18}(2)$ (250x10.00 mm, 5.0 $\mu$m) column. The mobile phase was the same as for LC-MS analysis but the flow-rate was increased to 2.0 mL/min and 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 every 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 infusions.

### 2.7. $^1$H NMR analysis

$^1$H-NMR was recorded at 25$^o$C 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 (PW) = 30$^o$ (11.3 µsec), and relaxation delay (RD) = 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. FIDs were Fourier transformed with LB = 0.3 Hz. The resulting spectra were manually phased and

baseline corrected, and calibrated to TSP 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-analysed in organic solvents (MeOD or $CDCl_3$) in order to compare the chemical shifts with existing literature (Table 2 gives the final solvent used for NMR analysis for each fraction).

## 3. RESULTS

### 3.1. Stability test

All samples prepared in deionized water, whatever its form (liquid or freeze-dried), its storage condition (room temperature, fridge or freezer) or its age (fresh tea, one week or two week old tea) showed the same chemical profile (Figure 1). This indicates that tea infusions prepared in a controlled environment appear to be stable for a two week period. The tea infusion prepared from the local tap water showed a large difference in the chemical profile after only one day. This sample was analysed immediately after preparation and after 1 day, upon which, a large degree of chemical degradation was observed (Figure 2).

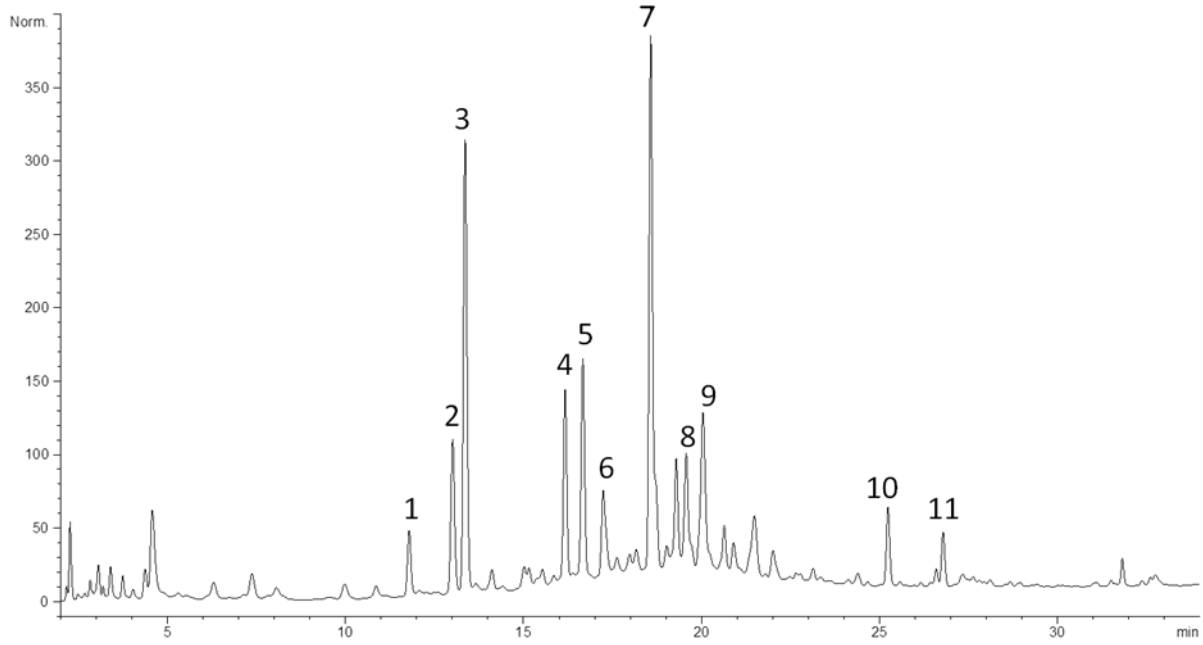



**Fig. 1: Chemical profile of *A. annua* tea at 254 nm. 1: scopolin; 2: *cis*-melilotoside; 3: chlorogenic acid; 4: 5-feruloylquinic acid; 5: *trans*-melilotoside; 6: scopoletin; 7: 3,5-dicaffeoylquinic acid; 8: rutin; 9: caffeoylferuloylquinic acid; 10: chrysosplenol D; 11: chrysosplenetin.**

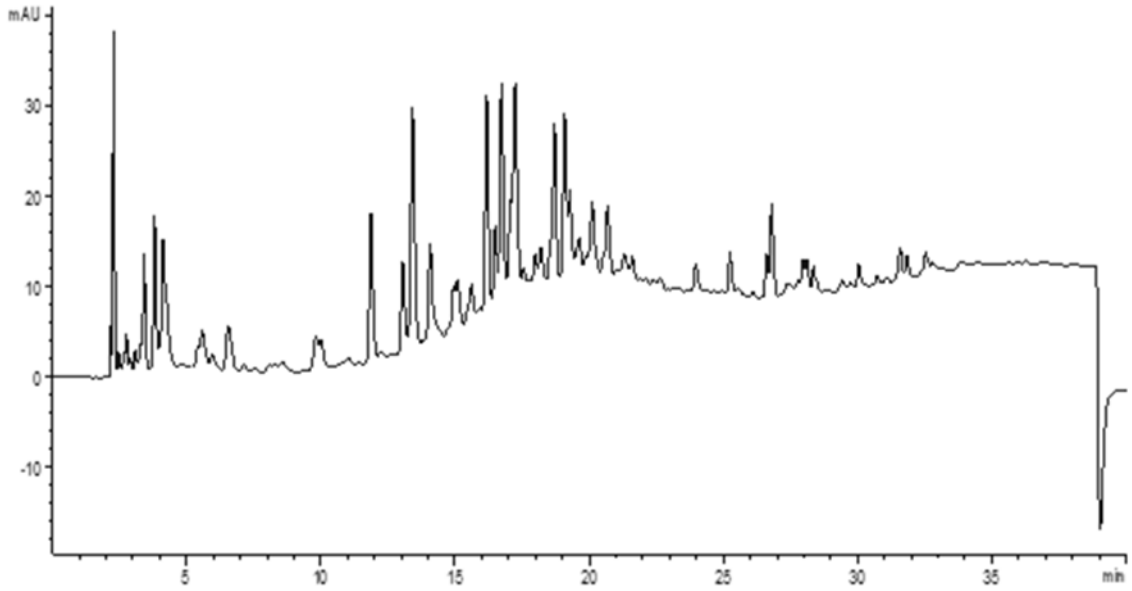



**Fig. 2: Chemical profile of the tea infusion prepared from tap water after 24 hours indicating the large amount of degradation.**

### 3.2. Compound identification

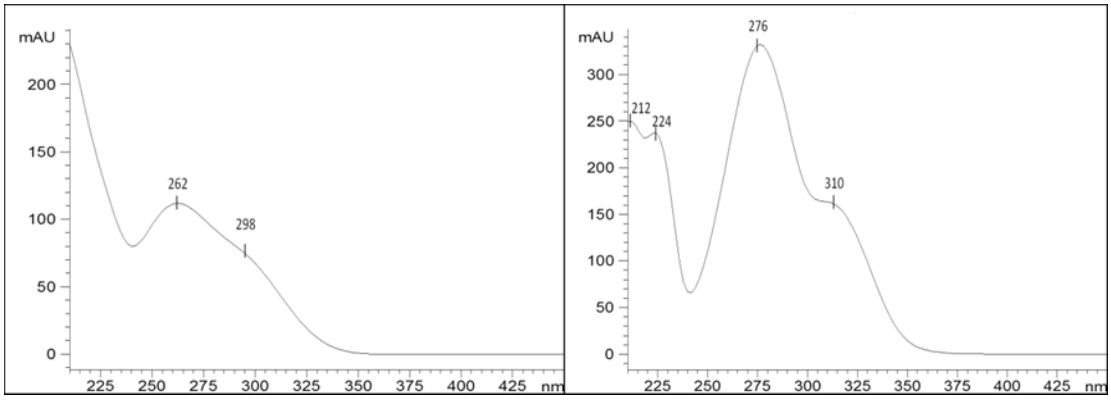
The choice of which components to regard as the main constituents was based on the UV absorbance at 210, 254, 330 nm and ease of ionization in the MS. Figure 1 shows the chemical profile of a typical *A. annua* tea infusion at 254 nm and Table 1 lists the identities of the main components identified in the tea based on their UV, mass and NMR spectral properties.

**Insert Table 1 here**

Peaks **1** and **6** were identified as Scopolin and Scopoletin respectively, based on their $^{1}$H NMR, MS and UV spectral data as compared to literature (Fliniaux et al., 1997; Han et al., 2008). The $^{1}$H NMR data of **1** matched to Fliniaux et al., (1997), 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 (Han et al., 2008). To add further evidence for the

identity of these compounds, a freshly prepared tea infusion was hydrolyzed by the addition of HCl. 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. According to our knowledge this is the first report that these compounds are identified in any *Artemisia* spp. Identification was based on UV properties (Figure 3) and mass spectral data as compared to literature (Yang et al., 2007). For both compounds, a quasimolecular ion of 344 and 325 were observed in the positive and negative ionization mode respectively. The mass of 344 corresponds to molecular ion with an addition of a water molecule. The fragments at *m/z* 165 and 163 observed in positive and negative mode respectively, correspond to the coumaric acid part of the molecule (Figure 4). The area of these peaks decreased upon hydrolysis, confirming the presence of a sugar moiety. $^{1}$H NMR data of the semi-purified fraction of **2** gave the distinctive ring signals at $\delta_H$ 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 $\delta_H$ 4.97 ppm (1H, d, J=7.4 Hz, H-1'). The typical *cis* configuration double bond signals could clearly be observed at $\delta_H$ 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**: $\delta_H$ 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 $\delta_H$ 6.56 (1H, d, J=16.2 Hz; H-8), 8.15 (1H, d, J=16.2 Hz, H-7). This data match perfectly with literature (Canuto et al., 2007; Yang et al., 2007).




**a)** **b)**

**Fig 3: UV spectrum of *cis*-melilotoside (a) and *trans*-melilotoside (b).**

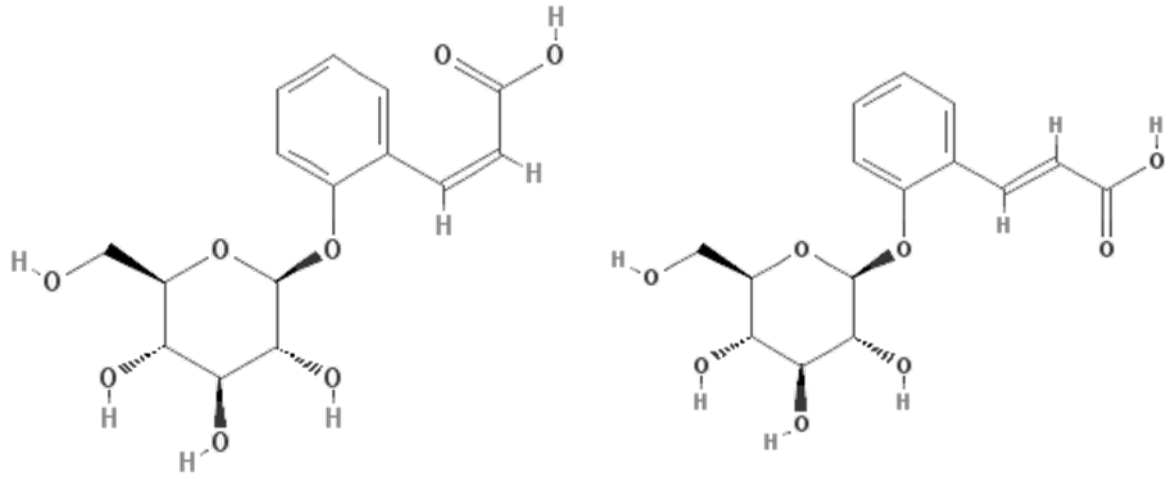


**Fig 4: Chemical structures of a) *cis*- and b) *trans* melilotoside**

Peaks **3**, **4**, **7** and **9** were identified as compounds belonging to the chlorogenic acids 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., (2007). To confirm the identity of these compounds, UV, mass and $^{1}$H NMR spectral properties were compared with literature (Han et al., 2008; Carbonara et al., 2012; Pauli et al., 1998).

Peaks **8**, **10** and **11** belongs to the flavonoid class of compounds and were identified as rutin ((*http://www.hmdb.ca/*) Mabry et al., 1970; Ibraheim et al., 2008), chrysosplenol D (Kraus and Roy, 2008) 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, (1998) but the $^{1}$H NMR data and UV properties did not match with those reported by Horie et al., (1989) and Ahmed et al., (1988). Peak **11** can therefore also possibly be Casticin or a chrysosplenetin isomer as reported by Horie et al., (1989); Asker et al., (2006) and Mesaik et al., (2009).

## 4. Discussion and conclusions

In order to conduct a phytochemical analysis of the tea infusion we decided to directly inject the samples without any treatment. This yielded a chemical profile of the tea infusion within 10 min of its preparation (Fig. 2). The chemical profile was also tested for stability and it was found that after 2 weeks no major changes occurred in the profile, when the tea is prepared with deionised water. The main components consist of a wide variety of phenolic compounds including chlorogenic acids (CGAs), feruloylquinic acids (FQAs), flavonols, coumarins and two new *0*-coumaric acid derivatives. Although only the main metabolites have been identified during this study at least 50 minor components could be detected. In the next section we will describe the stability and the biological activities of these compounds and their potential to interact synergistically.

### *Stability of the tea infusion*

Our previous analysis indicated that ART was stable for at least one day (Van der Kooy and Verpoorte 2012). During our current study we focussed only on the main compounds, other than ART, in the tea infusion and tested for stability over a two week period. We found that the tea infusion prepared from deionised water was remarkably stable. On the other hand, the tea infusion prepared from tap water displayed a large degree of chemical degradation after one day. This is a point of concern as most people do not have access to deionised water and will use any locally available water. Excluding the use of rain water, which closely resembles deionised water (low pH, low electrical conductivity), any other type of water source will potentially have a large influence on the chemistry of the tea infusion. This can however cause the tea infusion to become more active, less active and/or have the same activity for the treatment of a specific condition.

### *Coumarins*

No literature could be found that compounds **1** and **6** showed antiplasmodial activity. Compound **6** was tested by Gachet et al., (2011) where it was found to be inactive against *P. falciparum* at a concentration of 25 $\mu$M. Wu et al., (2001) tested both compounds for anti-HIV activity and found that they were inactive. Scopoline was tested by Cordero et al., (2004) against 4 different cancer cell lines but it did not show any appreciable activity. Scopoletin, on the other hand, showed good activity against certain types of cancer (Bhattacharyya et al., 2010, Arcos et al., 2006, Kim et al., 2005). Efferth et al., (2011) tested ART and scopoletin against various cancer cell lines and found that both showed good activity and importantly

that no cross resistance occured. This indicates that cell lines resistant against the one compound will not be resistant against the other and based on these *in vitro* results can therefore potentially be a useful combination therapy against certain types of cancer. The presence of scopoline in the tea infusion will lead to a slow release of scopoletin upon acid hydrolyses once consumed and it can therefore be seen as a pro-drug. No reports could be found on activity against diarrhea causing pathogens.

The coumarin class of compounds is well known for their anti-coagulation properties (eg warfarin). This physical effect on red blood cells might have an effect on the efficacy of ART against *Plasmodium*.

***CGAs***

No reports could be found on any antiplasmodial activity for these compounds. The known biological activities for compounds **3**, **4**, **7** and **9**, except for the well known antioxidant effects, including cancer preventative properties (Mechikova et al., 2010), antiproliferation effect on human ovarian cancer (Tai et al., 2010) and activity against breast cancer (Noratto et al., 2009), and other types of cancer (Iwai et al., 2004). This class of compounds is thought to exhibit good anti-HIV activity with patents covering their use (Harding et al 2011; Sun et al., 2008) as anti-HIV treatments. Cos et al. (2004) report that 3,5-dicaffeoylquinic acid does indeed show good activity against HIV integrase, although controversy remains around its potency and activity *in vivo*. Our latest finding based on the positive reports from African communities using the *A. annua* tea infusion for the treatment of HIV indicates that there is indeed strong anti-HIV activity in the *A. annua* tea infusion (Lubbe et al., 2012) and that this activity can therefore partly be ascribed to the chlorogenic acid class of compounds. One report identifies this class of compounds as showing activity against diarrhea causing pathogens (Tsou et al., 2000).

***Melilotosides***

This is 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 did however show potent acitivy against the diarrhea causing pathogens *Entamoeba histolytica* and *Giardia lamblia* ($IC_{50}$ = 12.5 and 16.8 μg/mL respectively) indicating the traditional use of *A. annua* for the treatment of diarrhea might be effective (Calzada et al., 2003).

***Flavonoids***

A large body of literature exists on the bioactivity of flavonoids. During this study two flavonoids (**8** and **10**) could be identified while the identity of compound **11** remains unclear due to contradictive literature reports. Ferreira et al., (2010) reviews the antiplasmodial and anticancer activity of flavonoids found in *A. annua*. Tao et al., (2007) reported on the strong antiHIV activity of the sodium-rutin-sulfate complex. This highlights the possibility that the salts and elements in tap water can 'activate' common compounds which might otherwise be inactive. Some reports indicated that **8** also exhibited some activity against diarrhea causing pathogens (Ho, et al., 1959; Gilani, et al., 2006). No reports on anti-HIV or antidiarrheal activity for **10** could be found.

## 5. Conclusions

In conclusion we report here on the stability and the identity of the major components in the *A. annua* tea infusion. These compounds show a diverse range of biological activities, including activity against certain types of cancer, HIV and with the identification of two new compounds (**1** and **5**) also against diarrhea. Although most of the reported biological activities were performed using *in vitro* analysis it remains to be seen how this can be translated into *in vivo* results. It is well known that most of the reported compounds have very low bioavailability and this will be our main focus of future research. The complex chemistry of the tea infusion will however be the most important part as this will explain if and how synergism occurs. Future work will also focus on the identification of minor compounds in the tea infusion, the variation of chemical makeup between various *A. annua* cultivars and the influence of metal ions and salts present in tap water on the chemical makeup. In the current study we took a first step to unlock the medicinal potential of this remarkable medicinal plant.

## 6. Acknowledgements

We thank Anamed for providing the plant material and Ros Priest and Suzannah Bourchier for proof reading the manuscript. No funding was received for this work.

## 7. Conflict of Interest

We declare no conflicts of interest.

## 8. REFERENCES


Ahmed, D., Choudhary, M.I., Turkoz, S., Sener, B., 1988. Chemical Constituents of *Buxus sempervirens*. Planta Medica. 54, 173–174.

Amaratunga, C., Sreng, S., Suon, S., Phelps, E.S., Stepniewska, K., Lim, P., Zhou, C., Mao, S., Anderson, J.M., Lindegardh, N., Jiang, H., Song, J., Su, X-Z., White, N.J., 2012. Artemisinin-resistant *Plasmodium falciparum* in Pursat province, western Cambodia: a parasite clearance rate study. The Lancet Infectious Diseases. 12, 851-858.

Arcos, M.L.B., Cremaschi, G., Werner, S., Coussio, J., Ferraro, G., Anesini, C., 2006. *Tilia cordata* Mill. extracts and Scopoletin (isolated compound): Differential Cell Growth Effects on Lymphocytes. Phytotherapy Research. 20, 34–40.

Asker, E., Akin, S., Hökelek, T., 2006. 5,3′-Dihydroxy-3,6,7,4′-tetra-methoxy-flavone. Acta Crystallographica Section E. 62, 4159–4161.

Bhattacharyya, S.S., Paul, S., Boujedaini, N., Khuda-Bukhsh, A.R., 2010. Anti-oncogenic potentials of a plant coumarin (7-hydroxy-6-methoxy coumarin) against 7, 12-dimethylbenz[a]anthracene-induced skin papilloma in mice: the possible role of several key signal proteins. Journal of Chinese Integrative Medicine. 8, 645-654.

Bilia, A. R., Melillo de Malgalhaes, P., Bergonzi, M. C., Vincieri, F. F.,2006. Simultaneous analysis of artemisinin and flavonoids of several extracts of *Artemisia annua* L. obtained from a commercial sample and a selected cultivar. Phytomedicine.13, 487-493.

Calzada, F., Velazquez, C., Cedillo-Rivera, R., Esquivel, B., 2003. Antiprotozoal activity of the constituents of *Teloxys graveolens*. Phytotherapy Research. 17, 731-732.

Canuto, K.M., Silveira, E.R., Bezerra, A.M.E., 2007. Estudo fitoquímico de espécimens cultivados de cumaru (*Amburana cearensis* AC Smith). Quím Nova, 33, 662–666.

Carbonara, T., Pascale, R., Argentieri, M.P., Papadia, P., Fanizzi, F.P., Villanova, L., Avato, P., 2012. Phytochemical analysis of a herbal tea from *Artemisia annua* L. Journal of Pharmaceutical and Biomedical Analysis. 62, 79–86.

Carvalho, I.S., Cavaco, T., Brodelius, M., 2011. Phenolic composition and antioxidant capacity of six *Artemisia* species. Industrial Crops and Products. 33, 382-388.

Cordero, C.P., Gomez-Gonzalez, S., Leo´n-Acosta, C.J., Morantes-Medina, S.J., Aristizabal., F.A. 2004. Cytotoxic activity of five compounds isolated from Colombian plants. Fitoterapia. 75, 225–227.

Cos, P., Maes, L., Vanden Berghe, D., Hermans, N., Pieters, L., Vlietinck, A., 2004. Plant Substances as Anti-HIV Agents Selected According to Their Putative Mechanism of Action. Journal of Natural Products. 67, 284-293.

De Ridder, S., Van der Kooy, F., Verpoorte, R., 2008. *Artemisia annua* as a self-reliant treatment for malaria in developing countries. Journal of Ethnopharmacology. 120, 302–314.

Efferth, T., Davey, M., Olbrich, A., Rücker, G., Gebhart, E., Davey, R., 2002. Activity of drugs from traditional Chinese medicine toward sensitive and MDR1- or MRP1-overexpressing multidrug-resistant human CCRF-CEM leukemia cells. Blood Cells, Molecules, and Disease. 28, 160–168.

Efferth, T., Herrmann, F., Tahrani, A., Wink, M., 2011. Cytotoxic activity of secondary metabolites derived from *Artemisia annua* L. towards cancer cells in comparison to its designated active constituent artemisinin. Phytomedicine. 18, 959-969.

Ferreira, J.F.S., Luthria, D.L., Sasaki, T., Heyerick, A., 2010. Flavonoids from *Artemisia annua* L. as Antioxidants and Their Potential Synergism with Artemisinin against Malaria and Cancer. Molecules. 15, 3135-3170.

Fliniaux, M. A., Gillet-Manceau, F., Marty, D., Macek, T., Monti, J.P., Jacquin-Dubreuil, A., 1997. Evaluation of the relation between the endogenous scopoletin and scopolin level of some solanaceous and papaver cell suspensions and their ability to bioconvert scopoletin to scopolin. Plant Science. 123, 205–210.

Ge, F., Ke, C., Tang, W., Yang, X., Tang, C., Qin, G., Xu, R., Li, T., Chen, X., Zuo, J., Ye, Y., 2007. Isolation of chlorogenic acids and their derivatives from *Stemona japonica* by

preparative HPLC and evaluation of their anti-AIV (H5N1) activity *in vitro.* Phytochemical Analysis. 18, 213–218.

Gilani, A.H., Khan A-U., Ghayur, M.N., Ali, S.F., Herzig, J.W. 2006. Antispasmodic effects of Rooibos tea (*Aspalathus linearis*) is mediated predominantly through K+ -channel activation. Basic & Clinical pharmacology & Toxicology. 99, 365-73.

Gachet, M.S., Kunert, O., Kaiser, M., Brun, R., Zehl, M., Keller, W., Munoz, R.A., Bauer, R., Schuehly, W., 2011. Antiparasitic Compounds from *Cupania cinerea* with Activities against *Plasmodium falciparum* and *Trypanosoma brucei rhodesiense.* Journal of Natural Products. 74, 559-566.

Han, J., Ye, M., Qiao, X., Xu, M., Wang, B., Guo, D-A., 2008. Characterization of phenolic compounds in the Chinese herbal drug *Artemisia annua* by liquid chromatography coupled to electrospray ionization mass spectrometry. Journal of Pharmaceutical and Biomedical Analysis. 47, 516–525.

Harding, N.M., Moodley, N., Meoni, P., Maharaj, V.J., Van Rooyen, S-W., Van der Merwe, M., Meyer, J.J.M., 2011. The use of an extract of helichrysum, for the treatment of HIV/AIDS. PCT Int. Appl. WO 2011001208 A1 20110106.

Ho, F-K., Chen, Y-P., 1959. On the flavonoid compounds and the antibacterial activities of *Crataeva religiosa*. Taiwan Yaoxue Zazhi. 10, 14-20.

Horie, T., Kawamura, Y., Yamada, T., 1989. Revised structure of a natural flavone from *Artemisia lanata.* Phytochemistry. 28, 2869–2871.

Ibraheim, Z.Z., Ahmed, A.S., Gouda,Y.G., 2008. Phytochemical and biological studies of *Adiantum capillus-veneris* L. Saudi Pharmaceutical Journal. 19, 65–74.

Iwai, K., Kishimoto, N., Kakino, Y., Mochida, K., Fujita, T., 2004. *In Vitro* Antioxidative Effects and Tyrosinase Inhibitory Activities of Seven Hydroxycinnamoyl Derivatives in Green Coffee Beans. Journal of Agricultural and Food chemistry. 52*,* 4893-4898.

Kim, E-K., Kwon, K-B., Shin, B-C., Seo, E-A., Lee, Y-R., Kim, J-S., Park, J-W., Park, B-H.,

Ryu, D-G., 2005. Scopoletin induces apoptosis in human promyeloleukemic cells, accompanied by activations of nuclear factor κB and caspase-3. Life Sciences. 77, 824-836.

Kraus, G., Roy, S., 2008. Direct Synthesis of Chrysosplenol D. Journal of Natural Products. 71, 1961–1962.

Liu, N. Q., Cao, M., Frédérich, M., Choi, Y. H., Verpoorte, R., Van der Kooy, F., 2010. Metabolomic investigation of the ethnopharmacological use of *Artemisia afra* with NMR spectroscopy and multivariate data analysis. Journal of Ethnopharmacology. 128, 230-235.

Liu, N. Q., Prager-van der Smissen, W., Ozturk, B., Smid, M., Van der Kooy, F., Foekens, J. A., Martens, J. W. M., & Umar, A. 2011. Molecular Portrait of Breast Cancer Cell Lines and Response to Artemisinin. European journal of Cancer. 47:152.

Lubbe, A., Seibert, I., Klimkait, T., & Van der Kooy, F. 2012. Ethnopharmacology in Overdrive: The remarkable anti-HIV activity of *Artemisia annua.* Journal of Ethnopharmacology. 141:854-859.

Mabry,T.J., Markham, K.R., Thomas, M.B., 1970. The systematic identification of flavonoids. Springer-Verlag.

Mesaik, M.A., Azizuddin., Murad, S., Khan, K.M., Tareen, R.B., Ahmed, A., Atta-ur-Rahman, Choudhary, M.I., 2009. Isolation and immunomodulatory properties of a flavonoid, casticin from *Vitex agnus-castus*. Phytotherapy Research. 23, 1516–1520.

Mechikova, G.Y., Kuzmich, A.S., Ponomarenko, L.P., Kalinovsky, A.I., Stepanova, T.A., Fedorov S.N., Stonik, V.A. 2010. Cancer-preventive Activities of Secondary Metabolites from Leaves of the Bilberry *Vaccinium smallii* A. Gray.. Phytotherapy Research. 24, 1730–1732.

Mueller, M.S., Karhagomba, I.B., Hirt, H.M., Wemakor, E., 2000. The potential of *Artemisia annua* L. as a locally produced remedy for malaria in the tropics: agricultural, chemical and clinical aspects. Journal of Ethnopharmacology. 73, 487-493.

Mueller, M.S., Runyambo, N., Wagner, I., Borrmann, S., Dietz, K., Heide, L., 2004.

Randomized controlled trial of a traditional preparation of *Artemisia annua* L. (Annual Wormwood) in the treatment of malaria. Transactions of the Royal Society of Tropical Medicine and Hygiene. 98, 318—321.

Newman, D.J., Cragg, G.M., 2012. Natural Products As Sources of New Drugs over the 30 Years from 1981 to 2010. Journal of Natural Products. 75, 311-335.

Noratto, G., Porter, W., Byrne, D., Cisneros-Zevallos, L., 2009. Identifying Peach and Plum Polyphenols with Chemopreventive Potential against Estrogen-Independent Breast Cancer Cells. Journal of Agricultural and Food chemistry. 57, 5219–5226.

Pauli, G.F., Poetsch, F., Nahrstedt, A., 1998. Structure assignment of natural quinic acid derivatives using proton nuclear magnetic resonance techniques. Phytochemical Analysis. 9, 177–185.

Räth, K., Taxis, K., Walz, G., Gleiter, C.H., Li, S-M., Heide, L. 2004. Pharmacokinetic study of Artemisinin after oral intake of a traditional preparation of *Artemisia annua* L. (Annual wormwood). American journal for Tropical Medicine and Hygiene. 70, 128–132.

Sy L-K., Brown, G.D., 1998. Three sesquiterpenes from *Artemisia annua.* Phytochemistry. 48, 1207–1211.

Sun, T., Wang, T., Chen, X., Lu, H., 2008. Traditional Chinese medicine composition for preventing AIDS and protecting liver. Faming Zhuanli Shenqing, CN 101185668 A 20080528

Tai, J., Cheung, S., Chan, E., Hasman D., 2010 Antiproliferation Effect of Commercially Brewed Coffees on Human Ovarian Cancer Cells *In Vitro*. Nutrition and Cancer. 62, 1044–1057.

Tao, J., Hu, Q., Yang, J., Li, R., Li, X., Lu, C., Chen, C., Wang, L., Shattock, R., Ben, K., 2007. *In vitro* anti-HIV and anti-HSV activity and safety of sodium rutin sulfate as a microbicide candidate. Antiviral Research. 75, 227-233.

Tsou, M. F., Hung, C.F., Lu, H.F., Wu, L.T., Chang, S.H., Chang, H.L., Chen, G.W., Chung, J.G., 2000. Effects of caffeic acid, chlorogenic acid and ferulic acid on growth and arylamine

N-acetyltransferase activity in *Shigella sonnei* (group D). Microbios. 101, 37-46.

Van der Kooy, F., Verpoorte, R., 2011. The Content of Artemisinin in the *Artemisia annua* Tea Infusion. Planta Medica. 77, 1754–1756.

Weathers, P.J., Arsenault, P.R., Covello, P.S., McMickle, A., Teoh, K.H., Reed, D.W., 2011. Artemisinin production in *Artemisia annua*: studies *in planta* and results of a novel delivery method for treating malaria and other neglected diseases. Phytochemical Reviews. 10, 173–183.

Wu, T-S., Tsang, Z-J., Wu, P-L., Lin, F-W., Li, C-Y., Teng, C-M., Lee, K-H. 2001. New constituents and antiplatelet aggregation and anti-HIV principles of *Artemisia capillaris.* Bioorganic & Medicinal Chemistry. 9, 77-83.

Yang, L., Nakamura, N., Hattori, M., Wang, Z., Bligh, S.W.A., Xu, L., 2007. High-performance liquid chromatography-diode array detection/electrospray ionization mass spectrometry for the simultaneous analysis of cis-, trans- and dihydro-2-glucosyloxycinnamic acid derivatives from *Dendrobium* medicinal plants. Rapid Communication in Mass Spectrometry. 21,1833–1840.