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# Ethnopharmacology in overdrive: The remarkable anti-HIV activity of *Artemisia annua*

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ABSTRACT

*Ethnopharmacological relevance: Artemisia annua* contains the well-known antimalarial compound artemisinin, which forms the backbone of the global malaria treatment regime. In African countries a tea infusion prepared from *Artemisia annua* has been used for the treatment of malaria only for the past 10–20 years. Several informal claims in Africa exist that the *Artemisia annua* tea infusions are also able to inhibit HIV. Since HIV is a relatively newly emerged disease, the claims, if substantiated, could provide a very good example of "ethnopharmacology in overdrive".

The objective of this study was to provide quantitative scientific evidence that the *Artemisia annua* tea infusion exhibits anti-HIV activity through *in vitro* studies. A second objective was to determine if artemisinin plays a direct or indirect (synergistic) role in any observed activity. This was done by the inclusion of a chemically closely related species, *Artemisia afra*, known not to contain any artemisinin in our studies.

*Materials and methods:* Validated cellular systems were used to test *Artemisia annua* tea samples for anti-HIV activity. Two independent tests with different formats (an infection format and a co-cultivation format) were used. Samples were also tested for cellular toxicity against the human cells used in the assays.

*Results:* The *Artemisia annua* tea infusion was found to be highly active with $IC_{50}$ values as low as 2.0 μg/mL. Moreover we found that artemisinin was inactive at 25 μg/mL and that a chemically related species *Artemisia afra* (not containing artemisinin) showed a similar level of activity. This indicates that the role of artemisinin, directly or indirectly (synergism), in the observed activity is rather limited. Additionally, no cellular toxicity was seen for the tea infusion at the highest concentrations tested.

*Conclusion:* This study provides the first *in vitro* evidence of anti-HIV activity of the *Artemisia annua* tea infusion. We also report for the first time on the anti-HIV activity of *Artemisia afra* although this was not an objective of this study. These results open the way to identify new active pharmaceutical ingredients in *Artemisia annua* and thereby potentially reduce the cost for the production of the important antimalarial compound artemisinin.



## 1. Introduction

In many parts of the world people rely on traditional medicines as sources for their primary health care needs (Farnsworth, 1985). Ethnopharmacology is the systematic study of the use of medicinal plants by specific cultural groups. Most societies have long histories of medicinal plant use, with knowledge of how to use plants being passed from generation to generation. A recently emerged, relatively "new" disease such as HIV/AIDS can therefore only have a limited ethnopharmacological background. The search for anti-HIV compounds is therefore usually approached by random screening or by investigating plant species known to contain potentially antiviral compounds or related structures (Vlietinck and Vanden Berghe, 1991; Cos et al., 2011).

In this paper we report on the remarkable *in vitro* anti-HIV activity of the Chinese medicinal herb *Artemisia annua* L. (Asteraceae). This plant is mainly used to treat malarial infections as it contains artemisinin (ART) an important antimalarial ingredient. This compound (and its derivatives) is now being used in combination with other antimalarials that possess a different mechanism of action in a drug regime called Artemisinin Combination Therapies. Although the traditional use of the plant in the form of an uncontrollable tea infusion to treat malaria is still widespread, it is strongly discouraged due to fears that a low content of ART may

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lead to the emergence of resistance (De Ridder et al., 2008). In order to address this aspect we recently demonstrated (Van der Kooy and Verpoorte, 2011) that by using the correct preparation methods the tea infusion can contain up to 95% of the ART present in the plant material.

*Artemisia annua* is native to Asia, but has been introduced in other parts of the world due to its antimalarial effectiveness. A survey about such use of the plant in Kenya and Uganda conducted from 2009 to 2011 revealed some interesting additional observations: more than half of the respondents had started using *Artemisia annua* for ailments other than malaria. Of these, about half had started to use *Artemisia annua* to treat HIV/AIDS (Willcox et al., 2011). In another survey of treatments prescribed by herbalists for people living with HIV/AIDS in Cameroon, *Artemisia annua* was one of the most frequently mentioned plants (Noumi, 2011). In both studies a tea was prepared from the plant, either alone or with other plant species. Informal feedback from partners of the NGO Anamed, which promotes the use of *Artemisia annua* as an antimalarial, also claims that the herb is often used by HIV patients (Willcox et al., 2011). If true, this will be an important case of how people in areas heavily affected by HIV infections explore plants available to them for possible medicines (Verpoorte et al., 2005). This information shows that people will relatively quickly adapt (by finding appropriate medicinal plants) to a changing environment (the emergence of a new disease) and is a good example of "ethnopharmacology in overdrive". The results of these surveys were the main reason for us to investigate the claimed *in vivo* anti-HIV activity of the traditional tea infusion in order to provide the first quantitative *in vitro* scientific evidence.

A literature survey revealed that various pharmacological activities of *Artemisia* species have recently been described by Bora (2011). Reported antiviral activities include the inhibition of HSV-1 and -2 by the essential oil of *Artemisia arborescens*, the inhibition of hepatitis B by a tablet containing *Artemisia capillaris*, and *in vitro* inhibition of HIV replication in H9 lymphatic cells by isolated compounds of *Artemisia capillaris*. The compounds responsible for inhibiting HIV replication were two flavonoids, arcapillin and isorhamnetin, and a coumarin aesculetin (Wu et al., 2001). A methanol extract of *Artemisia annua* was tested in a syncytium inhibition assay, which is based on the interaction between the HIV-1 envelope and the cellular membrane protein CD4 on T-lymphocytes (Chang, 2003). Some inhibition was seen at the concentration tested (15%), even though it was relatively low compared to some of the other 80 plants in the test series (Jung and Schinazi, 1994). Reports of other *Artemisia* species showing anti-HIV activity are limited to *Artemisia caruifolia* (Ma et al., 2001). Four compounds were isolated from a methanol extract of which *N*1,*N*5,*N*10-tri-*p*-coumaroylspermidine showed around 70% inhibition of HIV-1 protease at a concentration of 100 μg/mL. The three dicaffeoylquinic acid derivatives also isolated during this study did not show any appreciable activity against HIV-1 protease. Cos et al. (2008) did, however, report that 3,5-dicaffeoylquinic acid does indeed show good activity against HIV integrase although controversy remains around its potency and activity. In a metabolomic investigation of *Artemisia annua* and *Artemisia afra* coumaroylspermidine was not detected in either species tested (Liu et al., 2010a). No other reports could be found that this compound has been identified in *Artemisia annua* keeping in mind that *Artemisia annua* is probably one of the best studied *Artemisia* species. Moreover, the level of activity reported for this compound (around 100 μg/mL) is also far higher than the activity we report in this study for the tea infusion. No other reports could be found that any other extract of *Artemisia annua* significantly exhibits HIV expression.

A further literature survey revealed that the pure compound ART has been tested against various viruses. Efferth (2008) reported on the broad antiviral activity of ART and its semi-synthetic derivative artesunate. Artesunate showed inhibition of HIV at levels of 600 nM, but no reports on the activity of ART against HIV was given. Jung and Schinazi (1994) reported weak anti-HIV activity of ART, with $EC_{50}$ and $IC_{50}$ greater than 100 μM. Various patents exist that cover the broad biological activity including the antiviral activity of *Artemisia annua* in combination with other medicinal herbs (for example: Zhang, 2003; Chen, 2007; Xue, 2008a,b; Nagaura, 2009; Chen, 2010; Zhang and Zhang, 2010). Benedikt et al. (2005) have patented ART and some of its derivatives against various viruses. In the patent description the activity of ART was given to be insignificant against HIV-1 and -2.

In our current study we included *Artemisia afra* Jacq. ex Willd. (Asteraceae) as a control species, known not to contain artemisinin, although the further chemistry is similar (Van der Kooy et al., 2008; Liu et al., 2010a). This was done in order to determine if ART is responsible for the observed anti-HIV activity, or if a combination of ART and other components can explain the observed activity (synergism). We could not find any literature reports on any anti-HIV activity ascribed to *Artemisia afra* (Liu et al., 2009) except one report where HIV infected patients were given *Artemisia afra* together with standard HIV treatment in order to boost their immune systems (Mulholland and Drewes, 2004). For a further control plant we included the common tea and unrelated *Aspalathus linearis* (N.L. Burm.) R. Dahlgr. (Fabaceae) (common name: Rooibos tea) to act as a negative control in the bioassay.

In this study we prepared tea infusions of nine *Artemisia annua*, one *Artemisia afra*, and one Rooibos tea sample and tested their anti-HIV activity *in vitro*. We also determined the solid content of the different tea infusions in order to quantitatively determine the activity. The objective in this study was to provide quantitative scientific evidence that the *Artemisia annua* tea infusion exhibits anti-HIV activity.

## 2. Materials and methods

### 2.1. Plant material

*Artemisia annua* samples were obtained from the breeding programme of Anamed (Germany), collected in different years from different countries. Dr. Martin Hirt identified the plants as being *Artemisia annua*. Due to the fact that ART is known only to occur in appreciable quantities in this species, our quantitative analysis of ART in all the *Artemisia annua* samples acts as an additional positive identification of the plant material. Table 1 gives the harvest year and country of origin of all the samples. *Artemisia afra* plant material was purchased from a medicinal plant farm in December 2009 (Pharma Natura, Graskop, South Africa). This sample underwent in-depth NMR metabolomic analysis with authenticated *Artemisia afra* specimens and was shown to have the same chemical profile (Liu et al., 2010a). As an additional negative control we included the commonly available Rooibos tea (*Aspalathus linearis*), purchased from a local vendor (Vleuten, The Netherlands).

### 2.2. Artemisinin content

The ART content of all the samples (including *Artemisia afra*) was determined according to Liu et al. (2010b) (Table 1). In short, 50 mg of plant material was extracted with 10 mL of chloroform. The mixture was sonicated for 15 min and vortexed after which each sample was filtered, dried and reconstituted in a known volume of ethanol. The samples were transferred to HPLC vials and analysed on an Agilent 1200 series system equipped with a PL-ELS 2100 Ice detector

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**Table 1**
Country of cultivation, harvest period and plant parts used during this study. The ART content in this plant material was determined during a previous study (Liu et al., 2010b) and is compared to the current level of ART.

| Sample | Country of cultivation | Harvest period | Plant parts | ART content (%) 2009 | ART content (%) current study |
|---|---|---|---|---|---|
| *Artemisia annua* | | | | | |
| 1 | South Africa | 1999 | Leaves/flowers | 0.51 | 0.36 |
| 2 | South Africa | 2002 | Leaves/flowers | 0.33 | 0.30 |
| 3 | Tanzania | 2005 | Leaves | 0.59 | 0.49 |
| 4 | South Africa | 2006 | Leaves | 0.93 | 0.74 |
| 5 | Tanzania | 2006 | Leaves | 0.64 | 0.46 |
| 6 | Cameroon | 2007 | Leaves | 0.62 | 0.56 |
| 7 | Germany | 2007 | Leaves | 0.79 | 0.58 |
| 8 | Mozambique | 2007 | Leaves | 0.55 | 0.40 |
| 9 | Germany | 2009 | Leaves | 1.02 | 0.80 |
| *Artemisia afra* | | | | | |
| 10 | South Africa | >2008 | Leaves | n.d. | n.d. |

n.d.: not detected.

(Polymer Laboratories, Varian Inc.). For the accurate quantification a standard curve was prepared with pure ART (Sigma–Aldrich).

### 2.3. Tea preparation

Tea infusions were prepared as described earlier (Van der Kooy and Verpoorte, 2011). Ninety mg of plant material were carefully weighed off, and 10 mL of boiling distilled water was added to each sample. The samples were allowed to simmer for 3 min, after which 1.5 mL was filtered into HPLC vials (0.2 μm syringe filter) whilst it was still hot. The samples were sealed and sent for anti-HIV analysis.

### 2.4. Solid content

All samples were prepared in triplicate as described above and exactly 5 mL were transferred into falcon tubes (10 mL). The weight of the falcon tubes was determined beforehand. All samples were frozen at −80 °C and freeze dried after which it was weighed again. The solid content of all the tea samples are given in Table 2.

### 2.5. HIV bioassay

Various sample sets were prepared. The first set contained tea prepared from 1 sample in triplicate, the second set of samples was prepared from all plant samples in order to confirm the original result. Due to two samples being damaged on transport a third sample set was analysed consisting of the duplicate samples of the damaged samples.

The anti-HIV analysis was conducted using a validated cellular system by testing each sample at various dilutions in triplicates. Experimental results were used to determine the $IC_{50}$ values for each test sample. Two independent examinations were conducted and, importantly, no cytotoxicity was observed. The utilised format "iFIGS" (Infection format of "Fusion-induced gene stimulation") (Klimkait et al., 1998) represents an *in vitro* infection system: defined full length HIV-1 plasmids produce infectious virus after a transfection of DNA into human HeLa cells. During 48–60 h post transfection the cells release infectious HIV particles into the culture supernatant. This cell-free supernatant containing viral particles is then quantified and used as inoculum to infect reporter cells that contain a lacZ gene under the control of the HIV control region. Thereby, upon infection with HIV, the reporter gene will be induced in a quantifiable fashion, and the product, beta galactosidase, allows quantification of inhibitory effects of new chemical compounds or extracts. This system was infected in the presence of the sample (*Artemisia annua* tea) or Efavirenz (EFV) as a standard control drug. Efavirenz is a potent non-nucleosidic RT inhibitor currently in clinical use.

**Table 2**
The results are presented as the solid content for each sample and the dilution factor needed for each sample to reach the $IC_{50}$. Based on these two results the $IC_{50}$ was calculated as μg/mL. Not all samples were tested in both bioassays. The activity range of the positive control Efavirenz (EFV) used for each sample set is included and is expressed in nM. Pure ART was tested at 25 μg/mL.

| Sample | Solid content (mg/mL) | $IC_{50}$ dilution (iFIGS) | $IC_{50}$ (μg/mL) | $IC_{50}$ dilution (deCIPhR) | $IC_{50}$ (μg/mL) |
|---|---|---|---|---|---|
| **Sample set 1 (analysed May 2011)** | | | | | |
| 9 | 3.81 ± 0.07 | 33 | 115.6 | – | – |
| EFV | – | – | 21–32 nM | – | – |
| ART 25 μg/mL | – | n.a. | n.a. | – | – |
| **Sample set 2 (analysed June 2011)** | | | | | |
| 1 | 3.86 ± 0.25 | 1904 | 2.0 | 552 | 7.0 |
| 2 | 4.21 ± 0.47 | 909 | 4.6 | 247 | 17.0 |
| 3 | 3.04 ± 0.16 | 1147 | 2.7 | 343 | 8.9 |
| 4[a] | 3.48 ± 0.13 | n.t. | n.t. | – | – |
| 5 | 3.79 ± 0.28 | 1464 | 2.6 | 332 | 11.4 |
| 6 | 3.44 ± 0.15 | 1305 | 2.6 | 291 | 11.8 |
| 7 | 3.56 ± 0.13 | 795 | 4.5 | 177 | 20.1 |
| 8 | 3.22 ± 0.28 | 1280 | 2.5 | 329 | 9.8 |
| 9 | 3.81 ± 0.07 | 258 | 14.8 | 168 | 22.7 |
| 10[a] | 2.60 ± 0.36 | n.t. | n.t. | – | – |
| Rooibos | – | n.a. | – | – | – |
| EFV | – | – | 1–4 nM | – | 3–6 nM |
| **Sample set 3 (analysed August 2011)** | | | | | |
| 4 | 3.48 ± 0.13 | 60 | 58.0 | – | – |
| 10 | 2.60 ± 0.36 | 52 | 50.0 | – | – |
| EFV | – | – | 38–48 nM | – | – |

n.t.: not tested; n.a.: not active.
[a] Samples broken on transport. Tests performed on duplicate samples but at a later date.

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**Table 3**
Corrected $IC_{50}$ values for the iFIGS analysis. These values were obtained by adjusting all $IC_{50}$ values to the lowest (1 nM) and highest value (48 nM) obtained for the positive control EFV. This gives a better comparative analysis by minimising the effect of bioassay variability.

| Sample | $IC_{50}$ (μg/mL) | EFV nM | Corrected $IC_{50}$ Low (μg/mL) | Corrected $IC_{50}$ High (μg/mL) |
|---|---|---|---|---|
| **Sample set 1** | | | | |
| 9 | 115.6 | 25 | 4.6 | 221.9 |
| **Sample set 2** | | | | |
| 1 | 2.1 | 2 | 1.0 | 48.7 |
| 2 | 5.2 | 2 | 2.3 | 111.2 |
| 3 | 2.7 | 2 | 1.4 | 64.8 |
| 5 | 2.6 | 2 | 1.3 | 62.4 |
| 6 | 2.6 | 1 | 2.6 | 124.8 |
| 7 | 4.5 | 1 | 4.5 | 216.0 |
| 8 | 2.5 | 4 | 0.6 | 30.0 |
| 9 | 14.8 | 4 | 3.7 | 177.6 |
| **Sample set 3** | | | | |
| 4 | 58.0 | 48 | 1.2 | 58.0 |
| 10 | 50.0 | 48 | 1.0 | 50.0 |

For a second analysis the "deCIPhR" system ("dual-enhancement of Cell Infection to Phenotype Resistance") which is a co-cultivation procedure, in which the transfected cells are directly co-cultivated in the presence of drug with reporter cells. Both the infection (iFIGS) and co-cultivation (deCIPhr) format produced similar results, but the deCIPhR format permits direct and more rapid cell-to-cell spread of the virus and is therefore more stringent (more demanding on any inhibitor). As a consequence slightly higher drug concentrations are typically required for full inhibition.

### 2.6. Toxicity

All samples were tested for their cellular toxicity – Human cells that constitute the FIGS- and deCIPhR systems are also used for the determination of any unspecific inhibitory effects the extracts may have on the cellular viability. The cytotoxicity test is conducted precisely in the same way as the infection experiments, solely without applying any virus or viral DNA to the systems. This allows to quantitatively assess with appropriate DNA-staining agents any concentration-dependent impact the tea-extracts may exert on cellular viability.

## 3. Results and discussion

### 3.1. Artemisinin content

The content of ART in the plant material is presented in Table 1. The plant material used in this study was analysed for ART content in 2009 (Liu et al., 2010b) and during the current study. From Table 1 it can be calculated that ART degrades by 10–30% over a 2 year period if the material is stored dry and at room temperature (exposed to light and air). The results furthermore suggest that the ART content does not correlate well with the activity data presented in Table 2. No correlation can be drawn between the content of ART and the observed activity indicating that ART is probably not the main active compound in the tea infusion. Moreover, the pure ART standard was found to be inactive at 25 μg/mL. The maximum content of ART in the tea samples can be calculated as follow (for sample 1): 90 mg leaf material X 0.0036 (% ART)/1909 (dilution factor). The maximum content of ART for sample 1 is around 170 ng/mL (we assume a 100% ART extraction efficiency and no degradation). Furthermore, the most active sample (sample 1) had one of the lowest concentrations of ART whilst the sample with the highest content of ART had one of the lowest activities (sample 9). This indicates that ART does not appear to play any direct significant role in the observed activity.

### 3.2. Activity of Artemisia afra and synergism

Another possible explanation for the antiviral activity is synergism between ART and other compounds in the extract. With the inclusion of *Artemisia afra* (not containing ART) and the observed activity of this sample we can state that the possibility that synergism involving ART is rather limited, although it cannot be completely excluded. We have shown (Van der Kooy et al., 2008; Liu et al., 2010a) that these two species are chemotaxanomically closely related with the major exception that ART has not yet been detected in any *Artemisia afra* specimen. This does, however, not mean that the compound(s) responsible for the activity are the same in both species. With the inclusion of *Aspalathus linearis* we wanted to show that a well known tea (Rooibos) will not give a positive result and thereby act as a negative control. The tea prepared from this plant species did not show any activity, as was expected. Our intention was to have two non-ART-producing species as negative controls but to our surprise *Artemisia afra* showed a similar level of anti-HIV activity as *Artemisia annua*.

This is the first report of *Artemisia afra* possessing significant *in vitro* anti-HIV activity and adds credence to the reports of Mulholland and Drewes (2004) that patients given *Artemisia afra* in combination with standard HIV treatment reported improvement of symptoms compared to patients taking only standard HIV treatments. Keeping in mind the massive burden of HIV in southern Africa, can *Artemisia afra* become a flagship for Traditional African Medicines as asked by Liu et al. (2009). Here we provide scientific evidence that indeed this plant may have the potential to become a flagship for traditional African medicines although much more research will be needed.

### 3.3. Activity of Artemisia annua

The activity of the *Artemisia annua* tea infusions were found to be between 2.0 and 58.0 μg/mL. According to Cos et al. (2008) any pure natural product with an activity of below 25 μg/mL should be considered to have significant antiviral activity. If we look at the results of sample set 2, the $IC_{50}$ values for the tea infusions ranged between 2.0 and 14.8 μg/mL for the iFIGS bioassay format and 7.0–22.7 μg/mL for the more stringent deCIPhR bioassay format. The tea infusion therefore show potent activity in both bioassays used in this study. Based on this we can describe the tea infusion, consisting of many compounds, to be highly active. To place this into a better perspective we adjusted the $IC_{50}$ values in order to take into account the variability of the bioassay. This calculation is based on the reported $IC_{50}$ values for the positive control EFV (Table 2). During the first test (sample set 1) EFV had a relatively high inhibitory concentration of between 21–32 nM, whilst

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for sample set 2 and 3 it had inhibitory concentrations of between 1–4 nM and 38–48 nM respectively. This trend can also clearly be seen for the reported activities for the tea samples where sample set 2 had far lower $IC_{50}$ values than either of sample set 1 or 3 (Table 2). To have a better comparison between all the samples and to minimise the effect of variation in the bioassay, we adjusted all the $IC_{50}$ values to the lowest (1 nM) and highest (48 nM) reported value for the positive control. These corrected $IC_{50}$ values are presented in Table 3. This gives us a more accurate picture of the range of activity and the comparison between all the samples. The activity range for *Artemisia annua* can now be given as between 0.6 and 216.0 μg/mL and for *Artemisia afra* between 1.0 and 48.0 μg/mL. Sample 9 which was the first sample tested now also gives a far better comparison to the result obtained during its second analysis. Without the correction the $IC_{50}$ was found to be 115.6 μg/mL (EFV = 25 nM) and in the second test 14.8 μg/mL (EFV = 4 nM). With the $IC_{50}$ correction the lowest activity is now 4.6 and 3.7 μg/mL respectively (EFV = 1 nM).

The activities of all the samples appear to be relatively closely related indicating that the concentration of the active compound(s) in the samples is probably very similar keeping in mind the inherent variability of the sample preparation and the bioassay. This also indicates that the storage period (oldest sample ~10 years) and cultivation site does not seem to play a significant role in the presence/absence or quantity of these active compounds. No correlation can therefore be drawn between activity and site of cultivation or the age of the samples. In our current study our objective was not to identify the active components but to provide a quantitative measurement of the *in vitro* anti-HIV activity of *Artemisia annua*.

## 4. Conclusions

Although one has to be very critical about false positive findings (common compounds interfering with the bioassay) the use of this plant in the form of a tea infusion and the claim that it has *in vivo* efficacy against HIV give us confidence in the results of this study. We share the concern of the WHO that with the use of the tea infusion (low ART concentration) *Plasmodium falciparum* resistance may develop. Despite this voice, however, the uncontrolled use of the plant material in its traditional way will continue. This does create a potential problem. We aim at addressing this issue in two different ways. (1) We decided to publish our *in vitro* findings in order to create awareness of the full potential of *Artemisia annua*. At the moment there are shortages of ART on the world market leading to enormous price fluctuations. If a distinct anti-HIV compound(s) can be identified in this plant we may be able to produce a second lead compound or even a separate "active pharmaceutical ingredient" (API) from this plant. These API's may contribute to reducing the production cost of ART, if it can be co-produced with the use of existing facilities. Currently ART is extracted with apolar solvents (*e.g.* hexane) from which it is then purified. We have recently developed a polar (ethanol) extraction and purification protocol for ART and it is foreseen that these polar anti-HIV compound(s) will be co-extracted to some extent with the use of this protocol (Liu et al., 2011). If possible, this may lead to a stabilising economic effect on the ART world market. (2) It does not seem to be wise to principally ignore the long-standing traditional use of *Artemisia annua*. We rather suggest undertaking a thorough scientific investigation in order to fully understand what compounds are responsible for which activity. This could be followed by cultivating specific *Artemisia annua* cultivars containing the respective compounds at the exploitable levels. The identification of the key ingredients responsible for the observed activity will allow possibly managing, and more importantly, controlling their production in the plant. We have now embarked on a full metabolomic analysis with the aim to identify and quantify all key components in the tea infusion. Further careful examination as well as independent confirmation of the results presented in this paper will be essential before the discovery of new antiviral activities can lead to an expanded clinical exploitation from known and already industrially established plant-based preparations.

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