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Metabolomic investigation of the ethnopharmacological use of *Artemisia afra* with NMR spectroscopy and multivariate data analysis

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ABSTRACT

Ethnopharmacological relevance: Artemisia afra has been used as an infusion to treat malaria throughout the southern parts of Africa, in much the same way as the antimalarial plant *Artemisia annua* in China. The antiplasmodial activity of purified components from an apolar fraction of *Artemisia afra* has been shown in the past. No data on the efficacy of the tea infusion prepared from *Artemisia afra* are however available.

Objective: To investigate the antiplasmodial activity of various extracts of *Artemisia afra* including an ethnopharmacological prepared sample. To identify polar metabolites in *Artemisia afra* and *Artemisia annua* and by using multivariate data analysis investigate the metabolic differences between these species.

Materials and methods: The antiplasmodial activity of *Artemisia afra* and *Artemisia annua* extracts were tested for activity against *Plasmodiam falciparum* 3D7 (chloroquine-sensitive strain) with chloroquine, quinine and artemisinin as positive controls. Hydrophilic metabolites in *Artemisia afra* and *Artemisia annua* were identified directly from the crude extracts through 1D- and 2D-NMR spectra. The NMR spectra were also used to differentiate between the two species using principal component analysis (PCA) for quality control purposes.

Results: The apolar fractions of both *Artemisia afra* and *Artemisia annua* showed activity against *P. falciparum* while activity was only found in the tea infusion of *Artemisia annua*. Metabolomic studies using 1D- and 2D-NMR spectroscopy identified 24 semi-polar components in *Artemisia afra* including three new phenylpropanoids for this species: caffeic acid, chlorogenic acid and 3,5-dicaffeoyl quinic acid. PCA analysis conducted on the samples yielded good separation between the polar extracts of *Artemisia afra* and *Artemisia annua*.

Conclusion: These findings shows that there are no *in vitro* activity in the tea infusion of *Artemisia afra* and lists the identified metabolites causing the metabolic differences between *Artemisia afra* and *Artemisia annua* for quality control purposes.

1. Introduction

Malaria is a vector-borne epidemic caused by the protozoan *Plasmodium* parasite and infects approximately 515 million people annually, of which about one million cases are fatal (WHO, 2008). The majority of the fatalities occur in children under the age of 5 years in sub-Saharan African countries (Snow et al., 2005). There are four species of *Plasmodium* parasites involved in causing malaria, including *P. falciparum*, *P. malariae*, *P. ovale* and *P. vivax* (Spinazzola et al., 2007), among which *P. falciparum* is responsible for 80% of all the malarial infections and 90% of all lethal malarial cases (Trampuz et al., 2003). The endemic area of *P. falciparum* is mainly in tropical Africa, while *P. vivax* is the predominant species in other areas such as Asia and Latin America (Steffen et al., 2003). *Artemisia annua* L. (Asteraceae), known as Chinese wormwood (qing hao), is a well-known antimalarial herb, from which the antiplasmodial compound, artemisinin, is derived. Claims do exist that artemisinin occurs in some other *Artemisia* species especially in the African *Artemisia afra* Jacq. (Asteraceae), also known as the African wormwood. *Artemisia afra* is a widely used herb for the treatment of various ailments in the southern parts of Africa. In traditional African medicine, the infusion of this plant has been employed for treatment of malaria by drawing a quarter cup of fresh leaves in a

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cup of boiling water for 10 min (Roberts, 1990). As an alternative to *Artemisia annua*, there are also herbal companies selling *Artemisia afra* or mixtures containing *Artemisia afra* as medicine for the treatment of malaria. "Anti-Malaria Artemisia" (Nordman Superior Food Supplements: http://www.nordman.co.za/) and "X-Malaria" (Herbal Africa: http://herbalafrica.co.za/Products/Xmalaria.htm), are two such companies, among which Nordman Superior Food Supplements claimed that artemisinin is responsible for the antiplasmodial effects of their product. Van der Kooy et al. (2008) failed to find any trace of artemisinin in their products and developed a quality control protocol with the use of NMR and PCA analysis based on the apolar fractions of these two species. Although Kraft et al. (2003) proved that flavones and sesquiterpene lactones isolated from *Artemisia afra* posses *in vitro* antiplasmodial activity to some extent, none of the compounds had the same efficacy compared to artemisinin. Hydrophilic extracts of *Artemisia afra* were also reported to be inactive against *P. falciparum* (Kraft et al., 2003). This provided *in vitro* evidence that this herb might be ineffective in treating *P. falciparum*. The phytochemical analysis of *Artemisia afra* has been reported with most of the research focusing on the non-polar compounds and essential oils including non-volatile sesquiterpenes and triterpenes. The polar components in the plant have not been studied extensively to date. The reported metabolites occurring in *Artemisia afra* was recently reviewed by Liu et al. (2009).

Metabolomic techniques are fast becoming a hotspot of global biological research. With the development of nuclear magnetic resonance spectroscopic (NMR) techniques and multivariate data analysis, metabolomics is increasingly playing an important role in every aspect of the biomedical and phytochemical research fields, including biomarker screening (Connor et al., 2004; Viant et al., 2003), quality control (Van der Kooy et al., 2008), activity and toxicity prediction (Bailey et al., 2004), clinical chemistry (Bamforth et al., 1999), chemotaxonomy (Alcantara et al., 2007) and environmental metabolism (Bundy et al., 2001; Jahangir et al., 2008a). Modern high resolution NMR spectrometers and various two-dimensional (2D) NMR techniques enable phytochemists to directly elucidate the chemical structures of some secondary metabolites in plant extracts without purification, thereby reducing the costs and time involved.

During this study nine different *Artemisia annua* and seven different *Artemisia afra* samples (including commercial samples) were collected to investigate the differences in their metabolic profiles and to identify polar components in *Artemisia afra*. Both species are currently being used to treat malaria by consuming a tea preparation. This practice has been studied for *Artemisia annua* and it was found that while the symptoms of malaria decreases, or disappear altogether, the recrudescence rate (reappearance of the disease) was too high (Mueller et al., 2000; Rath et al., 2004). We therefore tested the antiplasmodial activity of tea samples prepared from both species and in addition we also prepared polar and apolar extracts and determined the *in vitro* antiplasmodial activity.

2. Experimental

2.1. Sample preparation

2.1.1. Samples for metabolomic analysis

Nine samples of *Artemisia annua* (obtained from Anamed, Winnenden, Germany) and seven samples of *Artemisia afra* (collected in South Africa) were prepared for metabolomic investigation. Table 1 gives the relevant information for all the samples. All the *Artemisia afra* samples, excluding the two commercial products, were collected from the botanical garden of the University of Pretoria, South Africa at different times of the year. The *Artemisia afra* leaves, aerial parts and roots were separately ground into fine powders and allowed to air dry for 1 week. Of each sample 100 mg were extracted with 1.5 ml of water, 1.5 ml of methanol and 3 ml of chloroform by first vortexing for 10 s followed by sonication of 30 min. Two milliliters of the water–methanol fraction and the chloroform fraction were separately removed and dried completely under nitrogen gas. For the chloroform samples, 1 ml of CH_3OH-d_4 was added and for the water–methanol samples a mixture (1:1) of CH_3OH–d_4 and KH_2PO_4 buffer in H_2O–d_2 (pH 6.0) containing TMSP (0.05%) were added. This was followed by 10 min sonication and centrifuging for

Table 1
The nine *Artemisia annua* and seven *Artemisia afra* samples and related information.

Sample	Cultivated areas	Plant parts	Storage conditions	Harvest period
Artemisia annua				
1	South Africa	Leaves/flowers	Not controlled	1999
2	South Africa	Leaves/flowers	Not controlled	2002
3	Tanzania	Leaves	Not controlled	2005
4	South Africa	Leaves	Controlled	2006
5	Tanzania	Leaves	Controlled	2006
6	Cameroon	Leaves	Controlled	2007
7	Germany	Leaves	Controlled	2007
8	Mozambique	Leaves	Controlled	2007
9	–	Leaves	–	–
Artemisia afra				
1	Nordman[a]	Aerial parts	–	–
2	South Africa	Leaves	–	2008.06
3	South Africa	Leaves	–	2008.10
4	South Africa	Leaves	–	2008.11
5	Wilde Als[b]	Aerial parts	–	–
6	South Africa	Leaves	–	2008.12
7	South Africa	Roots	–	2008.12

[a] *Note*: Commercial capsules from Nordman Company in South Africa.
[b] Commercial herbs from Pharma Natura medicinal herb farm.

Table 2
Antiplasmodial activity of *Artemisia afra* and *Artemisia annua* samples.

Sample	Storage age of samples (month)	IC_{50}[a] (μg/ml)
Chloroform extracts[b]		
Afra 1	Unknown	8.55 ± 2.47
Afra 2	7.5	8.85 ± 5.59
Afra 3	3.5	8.75 ± 2.76
Afra 4	2.5	8.40 ± 0.84
Afra 5	Unknown	11.05 ± 4.03
Afra 6	1.5	12.35 ± 4.31
Afra 7	1.5	NA[c]
Annua 1	>12	0.067 ± 0.001
Annua 2	>12	0.050 ± 0.003
Annua 4	>12	0.065 ± 0.030
Water–methanol extracts[d]		
Afra 1	Unknown	NA
Afra 2	7.5	NA
Afra 3	3.5	NA
Afra 4	2.5	NA
Afra 5	Unknown	NA
Afra 6	1.5	NA
Afra 7	1.5	NA
Annua 1	>12	NA
Annua 2	>12	NA
Annua 4	>12	NA
Boiling water extracts[e]		
Afra 2	7.5	NA
Afra 5	Unknown	NA
Afra 6	1.5	NA
Afra 7	1.5	NA
Annua 4	>12	0.264 ± 0.062

[a] *Note*: Half maximal inhibitory concentration.
[b] Samples dissolved in dimethyl sulfoxide (DMSO) for bioassay.
[c] No activity was found at the highest concentration tested (20.00 μg/ml).
[d] Samples dissolved in aqueous alcohol for bioassay.
[e] Samples dissolved in water for bioassay.

Table 3
^{1}H-NMR characteristic of identified polar metabolites.

Compound name	Chemical shifts (ppm) and coupling constants (Hz)	*Artemisia annua*	*Artemisia afra*
Acetic acid	δ 1.93 (s)	+	+
Adenine	δ 8.21 (s), δ 8.19 (s)	+	+
Alanine	δ 1.49 (H-3, d, J = 7.0 Hz), δ 3.78 (H-2, q, J = 7.0 Hz)	+	+
Aspartic acid	δ 2.82 (H-3, dd, J = 8.0, 17.5 Hz), δ 2.96 (H-3′, dd, J = 3.6, 17.5 Hz), δ 3.96 (H-2, dd, J = 3.6, 8.0 Hz)	+	+
Caffeic acid	δ 7.57 (H-7, d, J = 16.1 Hz), δ 7.15 (H-2, d, J = 2.1 Hz), δ 7.07 (H-6, dd, J = 8.4, 2.1 Hz), δ 6.89 (H-5, d, J = 8.4 Hz), δ 6.31 (H-8, d, J = 16.1 Hz)	+	+
Chlorogenic acid	δ 7.64 (H-7′, d, J = 16.1 Hz), δ 7.15 (H-2′, d, J = 2.1 Hz), δ 7.07 (H-6′, dd, J = 8.4, 2.1 Hz), δ 6.89 (H-5′, d, J = 8.4 Hz), δ 6.37 (H-8′, d, J = 16.1 Hz), δ 5.34 (H-3, td, J = 10.0, 4.8 Hz), δ 4.22 (H-5,), δ 3.81 (H-4, dd, J = 10.0, 3.2 Hz), δ 2.15 (H-2β, H-6β, m), δ 2.03 (H-2α, H-6α, m)	+	+
Choline	δ 3.22 (s)	+	+
Citric acid	δ 2.71 (H-β, d, J = 17.6 Hz), δ 2.54 (H-β′, d, J = 17.6 Hz)	+	+
3,5-Dicaffeoyl quinic acid	δ 7.65 (H-7′, d, J = 16.1 Hz), δ 7.62 (H-7″, d, J = 16.1 Hz), δ 7.19 (H-2′, d, J = 2.1 Hz), δ 7.15 (H-2″, d, J = 2.1 Hz), δ 7.09 (H-6′, dd, J = 8.4, 2.1 Hz), δ 7.07 (H-6″, dd, J = 8.4, 2.1 Hz), δ 6.89 (H-5′, d, J = 8.4 Hz), δ 6.83 (H-5″, d, J = 8.4 Hz), δ 6.48 (H-8′, d, J = 16.1 Hz), δ 6.39 (H-8″, d, J = 16.1 Hz), δ 5.49 (H-3, td, J = 10.0, 4.8 Hz), δ 5.43 (d, J = 2.2 Hz), δ 4.03 (dd, 5.2, 2.8 Hz), δ 2.35 (H-2β, H-6β, m), δ 2.10 (H-2α, H-6α, m)	+	+
1-*O*-Ethyl-β-D-glucoside	δ 1.19 (H = 2′, t, J = 7.0 Hz)	+	+
Formic acid	δ 8.48 (s)	+	+
Fumaric acid	δ 6.54 (s)	+	+
α-Glucose	δ 5.20 (H-1, d, J = 3.8 Hz)	+	+
β-Glucose	δ 4.59 (H-1, d, J = 7.9 Hz)	+	+
Glutamic acid	δ 2.35 (H-4, m), δ 2.08 (H-3, m)	+	+
p-Hydroxy benzoic acid	δ 7.92 (H-3, H-5, d, J = 8.8 Hz), δ 6.94 (H-2, H-6, d, J = 8.8 Hz)	–	+
Leucine or isoleucine	δ 0.97 (d, J = 6.8 Hz), δ 0.99 (d, J = 6.8 Hz)	+	–
Malic acid[a]	δ 4.28 (dd, J = 10.0, 3.2 Hz), δ 2.69 (dd, J = 16.0, 3.5 Hz), δ 2.42 (dd, J = 15.0. 7.0 Hz)	+	+
Phosphatidylcholine	δ 3.28 (s)	+	+
Proline	δ 4.08 (H-2, dd, J = 8.6, 6.4 Hz), δ 3.41 (H-5, m), δ 2.34 (H-3, m), δ 2.01 (H-4, m)	+	+
Quercetin analogue or luteolin analogue	δ 7.62 (H-6′, dd, J = 8.6, 1.8 Hz), δ 6.98 (H-5′, d, J = 8.6 Hz), δ 6.50 (H-8, d, J = 1.8 Hz), δ 6.30 (H-6, d, J = 1.8 Hz)	–	+
Rhamnose in flavonoid	δ 1.10 (rhamnoside, H-6, d, J = 6.4 Hz)	+	+
Succinic acid	δ 2.45 (s)	+	+
Sucrose	δ 5.42 (H-1, d, J = 3.8 Hz), δ 4.18 (H-1, d, J = 8.5 Hz)	+	+
Threonine	δ 1.33 (H-5, d, J = 6.6 Hz)	+	+
Valine	δ 1.01 (H-4, d, J = 8 Hz), δ 1.06 (H-5, d, J = 8 Hz), δ 2.28 (H-3, m)	+	+

[a] Chemical shift changeable with pH and concentration.

15 min at 13,000 rpm. The supernatants of all the samples (800 ml) were transferred into NMR tubes for ^{1}H-NMR analysis.

2.1.2. Samples for antiplasmodial analysis

All seven *Artemisia afra* samples and three samples of *Artemisia annua* (samples no. 1, 2 and 4) were prepared for antiplasmodial activity testing. Of each sample 100 mg were extracted with a mixture of water (5 ml), methanol (5 ml) and chloroform (10 ml) by first vortexing for 10 s, followed by 30 min of sonication. The extracts were filtered and the polar and apolar fractions were dried completely under nitrogen gas. For the ethnopharmacological study, 100 mg of four *Artemisia afra* samples (samples no. 2, 5, 6 and 7) and one *Artemisia annua* (sample no. 4) were allowed to simmer in boiling water for 10 min. After filtration the samples were dried in a freeze drier (*Artemisia afra* sample no. 1 is sold in capsules which should be swallowed and was therefore excluded). All the dried extracts were weighed and sent to University of Liège (Belgium) for antiplasmodial activity testing.

2.2. Antiplasmodial bioassay

The parasites were maintained in continuous *in vitro* culture as previously described (Trager and Jenssen, 1976). *P. falciparum* 3D7 (chloroquine-sensitive strain) was cultivated under an atmosphere of 5% CO_2, 5% O_2, and 90% N_2. The host cells were O+ or A+ human red blood cells. The culture medium was composed of RPMI-1640 containing HEPES (25 mM), L-glutamine, $NaHCO_3$, and supplemented by glucose (2 g/l), gentamicin (0.1%) and heat inactivated human O+ or A+ plasma (10%). The antiplasmodial activity was assessed by measurement of the parasite lactate dehydrogenase (pLDH) activity (Makler et al., 1993), using chloroquine, quinine and artemisinin as positive controls and the medium as negative control. Each sample was dissolved in DMSO (1 mg/50 ml) and 10 μl were transferred in a small flask containing 1 ml of medium. In a 96-well microplate, references, samples and medium were dispensed in duplicate in a series of fourfold dilutions. A volume of 225 μl of an infected erythrocyte suspension (parasitemia 2%, hematocrit 1%) was added to each well, giving a total volume of 250 μl per well, and the plates were incubated at 37 °C for 48 h. After mixing the content of each well, an aliquot of 20 μl was transferred in the corresponding well of a new plate, added to 100 μl of lysis buffer (3 ml Triton X-100, 1 g lithium lactate, 200 mg 3-acetylpyridine adenine dinucleotide (APAD), 10 mg saponin 100, adjusted to 100 ml with Tris buffer pH 8.0). The plate was then incubated for 15 min at room temperature (RT). 20 ml of a solution 1:1 of nitroblue tetrazolium (2 mg/ml) and phenazine ethosulfate (0.2 mg/ml) were added to each well and the plate was re-incubated for 30 min at RT in the dark. Acetic acid (100 μl) was added to each well to stop the reaction and the absorbance was measured at 590 nm.

2.3. Multivariate data analysis and structure elucidation

2.3.1. NMR analysis and data bucketing

^{1}H-NMR, 2D J-resolved and ^{1}H–^{1}H-correlated spectroscopy (COSY) spectra of *Artemisia afra* and *Artemisia annua* samples were recorded at 25 °C on a 500 MHz Bruker DMX-500 spectrometer (Bruker, Karlsruhe, Germany) operating at a proton frequency of 500.13 MHz. The heteronuclear multiple bonds coherence (HMBC) was performed on a 600-MHz Bruker DMX-600 spectrometer (Bruker) operating at a proton frequency of 600.13 MHz.

^{1}H-NMR spectra were acquired with 128 scans, requiring 10 min and 26 s acquisition time. The following parameters were used during the acquisition: 0.16 Hz/point, pulse width (PW) = 30° (11.3 μs), and relaxation delay (RD) = 1.5 s. A presaturation sequence was used to suppress the residual water signal. FIDs were Fourier transformed with LB = 0.3 Hz. 2D J-resolved NMR spectra were acquired using 8 scans per 128 increments for F1 and 8 k for F2 using spectral widths of 5000 Hz in F2 (chemical shift axis) and 66 Hz in F1 (spin–spin coupling constant axis). A 1.5 s relaxation delay was performed, leading to a total acquisition time of 56 min. Datasets were zero-filled to 512 points in F1 and both dimensions were multiplied by sine-bell functions (SSB = 0) prior to double complex FT. J-resolved spectra tilted by 45° was symmetrized about F1 and then calibrated by XWIN NMR (version 3.5, Bruker). The COSY spectra were acquired with 1.5 s relaxation delay, 5000 Hz spectral width in both dimensions. The window function for COSY spectra was sine-bell (SSB = 0). The HMBC spectra were obtained with 2.0 s relaxation delay, 6361 Hz spectral width in F2 and 30,183 Hz in F1. Qsine (SSB = 2.0) was used for the window function of the HMBC. The optimized coupling constant for HMBC was 145 Hz.

2.3.2. Multivariate data analysis

The ^{1}H-NMR spectra were automatically reduced to ASCII files using AMIX (v. 3.7, Bruker Biospin). Spectral intensities were scaled to the total intensity and reduced to integrated regions of equal width (δ 0.04) corresponding to the region of δ 0.30–δ 10.00. The regions of δ 3.28–δ 3.34 and δ 4.70–δ 5.00 were excluded from the analysis due to the residual signal of methanol and water. PCA analysis was performed by SIMCA-P software (ver 11.0, Umetrics, Umeå, Sweden). PCA involves a mathematical procedure that transforms a number of possibly correlated variables into a smaller number of uncorrelated variables called principal components. The first principal component accounts for as much of the variability in the data as possible, and each succeeding component accounts for as much of the remaining variability as possible. With the use of this procedure a large number of complex data sets can be reduced to less complex dimensionless datasets which are easier to interpret.

3. Results and discussion

3.1. Antiplasmodial activity

The chloroform samples showed to be effective against *P. falciparum*, exhibiting a half maximal inhibitory concentration (IC_{50}) (n = 2) ranging between 8.55 μg/ml and 12.35 μg/ml for the *Artemisia afra* samples and between 0.050 μg/ml and 0.067 μg/ml for the *Artemisia annua* samples (Table 2). On average *Artemisia annua* showed more than a hundred-times higher antiplasmodial activity compared with *Artemisia afra* which can be explained by the presence of artemisinin in the *Artemisia annua* samples. The polar samples (aqueous methanol) of both species showed no activity at the highest concentration tested. This is an interesting result and shows that very little of the artemisinin in the *Artemisia annua* samples were extracted into the polar solvent. The extraction technique used during the preparation of the samples caused artemisinin to dissolve completely in the chloroform phase and not enter the polar phase. The tea infusion of *Artemisia annua* did show some activity as was expected (Mueller et al., 2000). For the tea infusion of the *Artemisia afra* samples no activity was found at the highest concentration tested. This indicates that the traditional approach to prepare this herb might not be effective for the treatment of malaria.

3.2. ^{1}H-NMR and identification of metabolites in Artemisia annua and Artemisia afra

All the *Artemisia annua* and *Artemisia afra* samples were subjected to metabolomic analysis by ^{1}H-NMR and diverse 2D NMR techniques. The ^{1}H-NMR signals of all the common metabolites such as amino/organic acids, carbohydrates and phenolic compounds were assigned with the use of a ^{1}H-NMR library containing over 500 metabolites. All the metabolites were identified with ^{1}H-NMR and the presence of these compounds was further confirmed with the aid of 2D NMR including J-resolved, ^{1}H–^{1}H COSY, HSQC and HMBC. Most of the metabolites identified were reported in previous publications from our group (Abdel-Farid et al., 2007; Jahangir et al., 2008b; Liang et al., 2006; Verpoorte et al., 2007; Widarto et al., 2006). Table 3 summarizes the chemical shifts and coupling constants of all the identified metabolites present in the two species.

The ^{1}H-NMR spectrum can be divided into three different regions consisting of the aromatic region (δ 10.00–δ 6.00), sugar region (δ 6.00–δ 3.00), and organic and amino acid region (δ 3.00–δ 0.00). In the organic and amino acid region, the signals observed included aspartic acid (δ 2.82), citric acid (δ 2.71), malic acid (δ 2.69), succinic acid (δ 2.45), glutamic acid (δ 2.35), proline (δ 2.01), acetic acid (δ 1.93), alanine (δ 1.48), threonine (δ 1.33), valine (δ 1.01) and leucine or isoleucine (δ 0.99). Other typical signals were also present in this region such as 1-*O*-ethyl-β-D-glucoside (δ 1.19) and rhamnose (δ 1.10). Sucrose (δ 5.42), α-glucose (δ 5.20), β-glucose (δ 4.59) and choline (δ 3.22) were identified in the sugar region. In the aromatic region, the most intensive signals were identified as phenylpropanoids and further confirmed by J-resolved and COSY spectra. The presence of a conjugated system between alkenyl group and carboxyl or ester group gives contribution to typical *trans*-coupling of phenylpropanoids with a coupling constant of 16 Hz between H-7 or H-7′ (ca. δ 7.6) and H-8 or H-8′ (ca. δ 6.4). Three major phenylpropanoids were identified as 3,5-dicaffeoyl quinic acid (δ 7.65, δ 7.62, δ 6.48 and δ 6.39, J = 16.1 Hz), chlorogenic acid (δ 7.64 and δ 6.37, J = 16.1 Hz) and caffeic acid (δ 7.57 and δ 6.31, J = 16.1 Hz). The presence of these compounds was further confirmed by J-resolved (Fig. 1A) and COSY (Fig. 1B) analysis and the spectra compared to our database. The double triplets at δ 5.49 and δ 5.34 were also evidence of the presence of these compounds. Other components identified included formic acid (δ 8.48), adenine (δ 8.21), p-hydroxy benzoic acid (δ 7.92), quercetin or luteolin analogue (δ 7.62) and fumaric acid (δ 6.54).

3.3. PCA of the ^{1}H-NMR metabolite differences between Artemisia annua and Artemisia afra

PCA was performed on the water–methanol fractions of all the samples (excluding the root sample of *Artemisia afra*). The scatter plot clearly showed discrimination between *Artemisia afra* and *Artemisia annua* by principal component (PC) 1, while PC 2 mainly indicates the variation of metabolites within the species (Fig. 2A). The loading plot of PC 1 (Fig. 2B) indicates the signals of the metabolites which are responsible for the observed separation. The signals in the phenolic region (δ 7.64–δ 7.56, δ 7.20–δ 7.04, δ 6.88–δ 6.84, δ 6.48 and δ 6.40–δ 6.36) generally had a positive effect in PC 1, mainly caused by four phenylpropanoids (caffeic acid, chlorogenic acid, dicaffeoyl quinic acid and ferulic acid). These signals

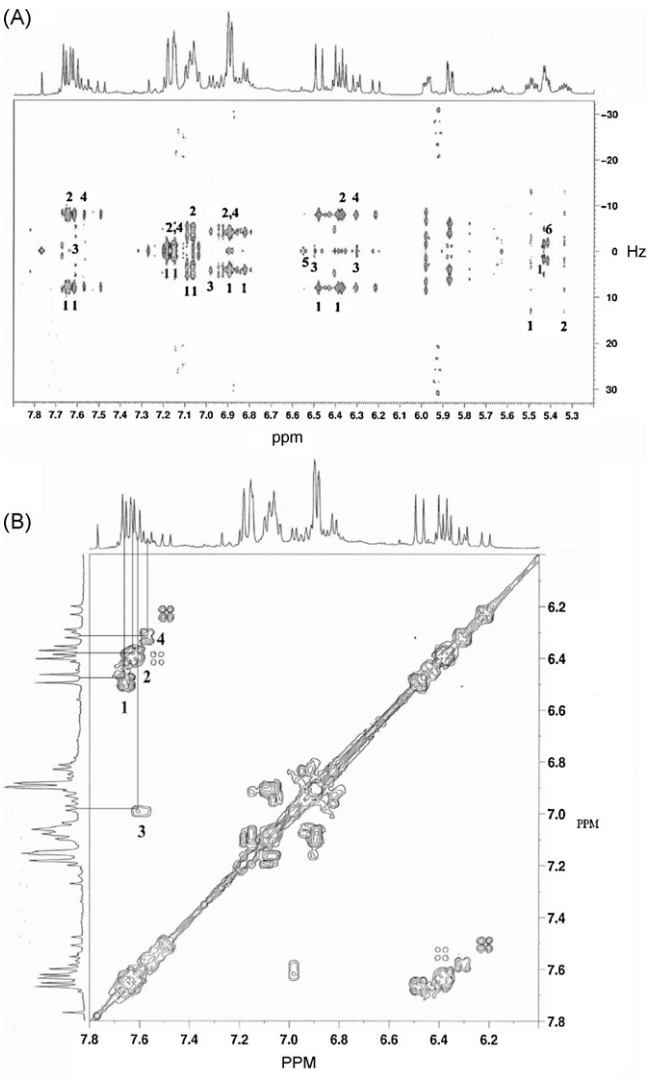

Fig. 1. 1. 3,5-dicaffeoyl quinic acid, 2. chlorogenic acid, 3. quercetin analogue or luteolin analogue, 4. caffeic acid, 5. fumaric acid, 6. sucrose; (A) The x-axis is expressing the chemical shift in ppm and the y-axis the coupling constant in Hz. The typical coupling of phenylpropanoids (H-7 or H-7′ and H-8 or H-8′) is *trans*-coupling of the double bond contributing to a coupling constant of around 16.0 Hz. Ortho- and meta-coupling (ca. 8.5 Hz and 2.0 Hz) from the B ring of flavonoids are also a typical pattern in the aromatic region; (B) Both x and y-axis are expressing the chemical shift in ppm. Typical coupling between H-7 or H-7′ and H-8 or H-8′ in phenylpropanoids and between H-5′ and H-6′ in flavonoids.

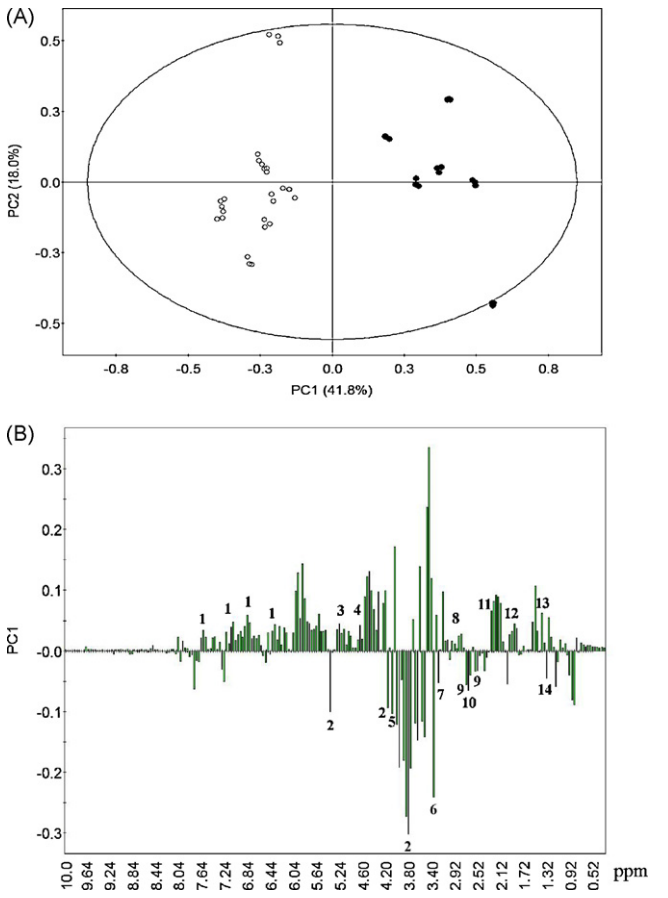

Fig. 2. Score (PC 1 vs. PC 2, A) and loading (PC 1, B) plot of PCA results obtained from ^{1}H-NMR spectra of *A. afra* and *A. annua* samples; (A) A. afra (●) and A. annua (○); (B) 1. phenylpropanoids, 2. sucrose, 3. α-glucose, 4. β-glucose, 5. proline, 6. phosphatidylcholine, 7. choline, 8. aspartic acid, 9. citric acid, 10. malic acid, 11. valine, 12. acetic acid, 13. alanine, 14. threonine.

demonstrate the related components are higher in *Artemisia afra* than in *Artemisia annua*. The signals of α-glucose (δ 5.20) and β-glucose (δ 4.60) also gave positive contribution to PC 1, as well as the signals from some amino acids and organic acid such as aspartic acid (δ 2.96–δ 2.92) and δ 2.84–δ 2.80), valine (δ 2.28), acetic acid (δ 1.92) and alanine (δ 1.48). Some unidentified signals were also found to largely contribute to the discrimination between the two species. These signals at δ 3.48–δ 3.44, δ 6.00–δ 5.96 and δ 5.88–δ 5.84, and especially the signals in the region δ 6.00–δ 5.96 (ddd, *J* = 10.0, 5.0, 2.0 Hz) and δ 5.88–δ 5.84 (dd, *J* = 10.0, 2.0 Hz) were determined to be from the same compound through COSY analysis. This unidentified compound might be a unique biomarker for *Artemisia afra* as these signals are completely absent in the *Artemisia annua* spectrum.

In comparison, the signals with negative values indicate that related components are present in higher amounts in *Artemisia annua* compared to *Artemisia afra*, including sucrose (δ 5.40), fructose moiety of sucrose (δ 4.16), proline (δ 4.08), phosphatidylcholine (δ 3.28), choline (δ 3.20), citric acid (δ 2.72, δ 2.56–δ 2.52), malic acid (δ 2.68) and threonine (δ 1.32). The signals from sucrose and phosphatidylcholine account for large weight of the holistic discrimination.

4. Conclusions

Antiplasmodial activity was shown to occur in the non-polar fraction of *Artemisia afra* while the traditional used tea infusion did not show any activity at a concentration of 20 μg/ml. Phenolic compounds present in this species were reported to exhibit various activities, including activity against degenerative diseases such as cancer and cardiovascular diseases (Magalhaes et al., 2009). This group of compounds are known to chelate metals, inhibiting lipoxygenase and to scavenge free radicals (Martinez-Valverde et al., 2000), all of which are closely related to degenerative diseases. *Artemisia afra* is also used to treat diabetes (Thring and Weitz, 2006), and there is also reports available on the cardiovascular effects of this species (Guantai and Addae-Mensah, 1999). Several publications report on the components already identified in *Artemisia annua* and *Artemisia afra*. These reports mainly focus on the apolar compounds present in the essential oils such as monoterpenes, sesquiterpenes, and triterpenes, among which the sesquiterpene lactones were thoroughly investigated due to presence of artemisinin in *A annua*. Flavonoids form the major part of the reported polar metabolites in these two species (Bhakuni et al., 2001; Liu et al., 2009). No previous publications reported on phenylpropanoids present in these two species. Three different

phenylpropanoids, caffeic acid, chlorogenic acid and 3,5-dicaffeoyl quinic acid, were identified in both species, which may partially explain the antidiabetic application of *Artemisia afra* infusion in traditional African medicine (Erasto et al., 2005; Mahop and Mayet, 2007). It has been reported that chlorogenic acid may influence glucose metabolism and insulin sensitivity and therefore decrease the risk for Type 2 diabetes (Paynter et al., 2006).

In this research, more than 25 polar compounds in *Artemisia afra* were elucidated and assigned through proton NMR and 2D-NMR spectra of crude extracts, including some phenylpropanoids which may be related to above activities. This data is valuable for further phytochemical and activity research of this species. Metabolomic differentiation between the polar fractions of *Artemisia afra* and *Artemisia annua* was also re-investigated in this research as previous work was conducted only on the apolar fractions. Phenylpropanoids are generally present in higher amounts in *Artemisia afra* compared to *Artemisia annua*, while sucrose and phosphatidylcholine were found to be higher in *Artemisia annua*.

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