Original Research Article

^{1}H NMR-based metabolomics of antimalarial plant species traditionally used by Vha-Venda people in Limpopo Province, South Africa and isolation of antiplasmodial compounds

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Abstract

Ethnopharmacological relevance: The Vha-Venda people living in rural areas of Limpopo Province of South Africa regularly use traditional plant-based medicines to treat malaria. In our earlier publication, twenty indigenous plant species used to treat malaria or its symptoms by Vha-Venda people were evaluated for antiplasmodial activity. The main objective of the current study was to assess the robustness of NMR-based metabolomics in discriminating classes of secondary compounds that are responsible for the observed antimalarial activity and the isolation of antiplasmodial compounds.

Materials and methods: Twenty dichloromethane extracts were reconstituted in $CDCl_3$, subjected to ^1H NMR-based metabolomic analysis on a Varian 600 MHz spectrometer and the acquired ^1H NMR spectra were then evaluated collectively using multivariate data analysis (MDA). Principal Component Analysis (PCA) and Orthogonal Projections to Latent Structures–Discriminant Analysis (OPLS-DA) were used to 'globally' discern antiplasmodial profiles. A contribution plot was then generated from the OPLS-DA scoring plot in an attempt to determine the classes of compounds that are responsible for the observed grouping. Further phytochemical analyses were conducted on the lipophilic extracts of *Tabernaemontana elegans* and *Vangueria infausta* subsp. *infausta*. These best candidates were fractionated, purified and their isolated compounds identified based on conventional chromatographic and spectroscopic techniques.

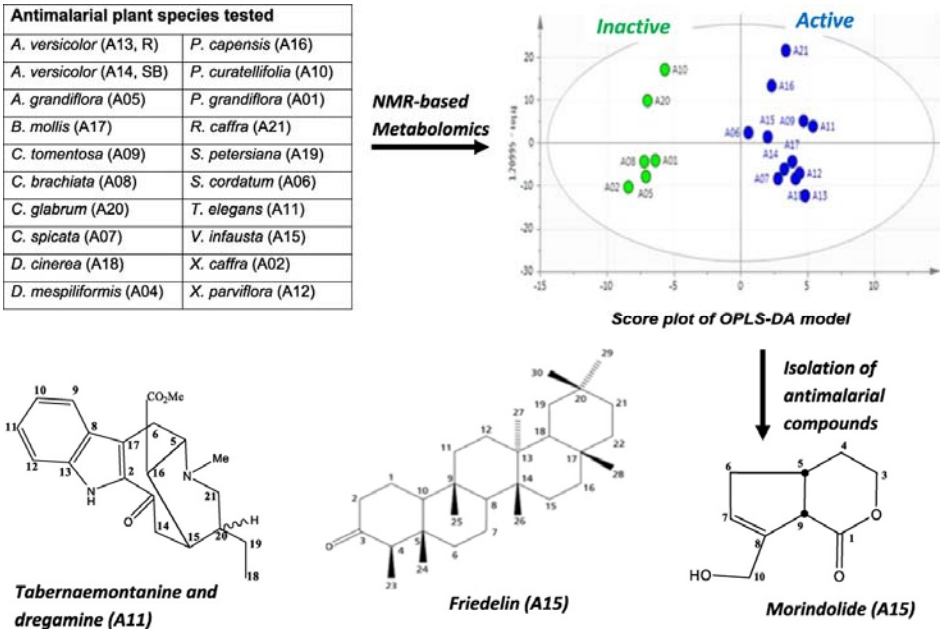
Results: The PCA did not separate the acquired profiles according to the detected antiplasmodial bioactivity. Application of a supervised OPLS-DA on the ^1H NMR profiles resulted in a discrimination pattern that could be correlated to the observed antimalarial bioactivity. A contribution plot generated from the OPLS-DA scoring plot illustrated the classes of compounds responsible for the observed grouping. Prominent peaks were observed in the aromatic, sugar-based/N-containing and aliphatic spectral regions of the contribution plot. Two known indole alkaloids were isolated from *T*. *elegans*, and identified as tabernaemontanine (IC_{50} = 12.0±0.8 µM) and dregamine (IC_{50} = 62.0±2.4 µM). Friedelin (IC_{50} = 7.20±0.5 µM) and morindolide (IC_{50} = 107.1±0.6 µM) were isolated from *V*. *infausta* subsp. *infausta*. This is the first report of the rare iridoid lactone, morindolide's antimalarial activity. While these two

compounds have been previously identified, this is the first account of their occurrence in the genus *Vangueria*.

Conclusion: The study illustrated the potential of NMR-based metabolomics in discriminating classes of compounds that may be attributed to antiplasmodial activity. Additionally, the study demonstrated the potential of discovering novel antiplasmodial scaffolds from medicinal plants and the rationale for the bioprospecting antimalarial plant species used by Vha-Venda people.

Keywords: NMR- based metabolomics, *Tabernaemontana elegans*, *Vangueria infausta* subsp. *infausta*, Antiplasmodial activity, Dregamine, Tabernaemontanine, Friedelin, Morindolide, South Africa

Graphical abstract



1. Introduction

While substantial progress has been achieved in reducing the adverse impact tropical diseases in recent years, malaria remains the main cause of mortality in endemic countries (Camponovo et al., 2017). The World Health Organization (WHO) estimates that between the years 2010 and 2015 the global malaria occurrences declined by 21% while malaria-specific mortality rates decreased by 29% (Cibulskis et al. 2016; WHO, 2017). These accomplishments have been driven by several factors including increased funding, effective vector control schemes, strengthening of endemic health systems, efficient treatment regimens as well as sustained surveillance to monitor progress (White et al., 2014; WHO, 2017). Chemotherapy has continued to be part of the indispensible tools used at all phases of the elimination of malaria in some regions. Although artemisinin-based combination therapies (ACTs) are currently the most effective treatments available for malaria, the emergence of drug resistant strains in south-east Asia and other recently identified areas, is a cause for concern (Dondorp et al., 2017). In view of the emergent vectorial and plasmodial resistance to the currently available tools, as well as the limitations experienced with the development of malaria vaccine, novel approaches are needed in order to overcome the challenges encountered in malaria eradication.

The malaria research agenda for global eradication has identified key priority research areas that need to be addressed in an effort to eliminate the disease. One such area is the optimization of ACTs and other available antimalarial drugs in an attempt to maximize their functional lifespan (The MalERA Group, 2011; Duparc et al., 2012; O'Neill et al., 2017). The advantage of this approach is that the safety and pharmacokinetic profiles of the modified compounds are well established, however,

cross-resistance remains a threat (Held et al., 2014). Another area of major focus is the continuation of research and development of new antimalarial drugs with novel mechanisms of action. Natural plant products and their derivatives have been the major basis for the current malaria therapy and its chemoprevention. Most plant species have not been adequately exploited for their potential as sources of new bioactive antiplasmodial scaffolds. It is now apparent that selection, collection, extraction and biological evaluation are no longer barriers to the flow of plant samples through the discovery pipeline (Brusotti et al., 2014). The major obstacles to drug discovery are the bioactivity-directed fractionation, isolation and structure elucidation of the biologically active principles. These steps are labour intensive and the isolation of relatively few compounds and their subsequent screening represent the culmination of most investigations on medicinal plants.

Metabolomics is one of the approaches that are currently being explored in an endeavour to increase the efficiency through which novel compounds can be discovered from natural products (Shyur and Yang, 2008; Kim, 2010; Schripsema, 2010; Heyman and Meyer, 2012; Sheridan et al., 2012; Leonti and Casu, 2013; Quansaha and Karikari, 2016). One major focus of most metabolomic investigations relating to medicinal plants has been the quality control of plant samples that are used for herbal preparations. Several studies have shown the potential of NMR-based metabolomics in chemical fingerprinting of herbal preparations thereby implying a global overview of all the plant constituents contained in such preparations (Schaneberg et al., 2003; Van der Kooy et al., 2008; Heyman and Meyer, 2012; Mncwangi et al., 2014). Other studies have demonstrated the ability of NMR spectroscopy coupled with multivariate analysis in predicting the bioactivity of the assessed plant extracts (Bailey et al., 2004; Heyman et al., 2015). In particular,

Heyman et al., (2015) showed that metabolomics could be used to discriminate bioactive fractions and to further identify compounds responsible for predicted activity in complex matrices without bioassay-guided fractionation and purification steps. Very few studies have investigated the potential of NMR-based metabolomics in bioprospecting plant species belonging to different families for novel chemotherapeutic agents. In our previous study, we demonstrated the antiplasmodial activity of medicinal plants used by Vha-Venda people against malaria (Bapela et al., 2014). Consequently, the main objective of this study was to assess the robustness of NMR-based metabolomics in discriminating classes of secondary compounds that are responsible for antiplasmodial activity in antimalarial plants used by Vha-Venda people and to isolate bioactive compounds.

2. Materials and methods

2.1. ^{1}H NMR spectroscopy of plant extracts

A detailed description on the traditional uses, plant collection, plant extraction and *in vitro* screening was previously published (Bapela et al., 2014). Based on the apparent lack of antiplasmodial activity by the investigated polar plant extracts, the metabolomic analysis was conducted on the dichloromethane plant extracts only, using 1D ^{1}H NMR spectroscopy (Table 1). NMR spectra of the DCM extracts were acquired on a Varian 600 MHz spectrometer (CSIR, Pretoria, South Africa) operating at a proton NMR frequency of 600.13 MHz. Each crude DCM extract was reconstituted in deuterated chloroform ($CDCl_3$) at a concentration of 15 mg/ml, with 0.1 % tetramethylsilane (TMS) as the internal frequency lock (0.00 ppm). The dissolved plant extracts (600 µl) were transferred into 5 mm NMR tubes and analysed on the spectrometer. Each spectrum was acquired with 64 scans per

sample, a spectral width of 14 ppm, and the temperature was kept constant at 25 °C (Heyman et al., 2015). Prior to statistical analyses, all ^{1}H NMR spectra were referenced to TMS, manually phased and baseline corrected (Whittaker smoother).

2.2. Multivariate data analysis (MDA)

^{1}H NMR spectra were reduced to ASCII files using MestReNova 8.1.1 (Mestrelab Research). Normalisation was done by scaling the spectral intensities to 0.1% of TMS and the region of 0.00 – 14.00 ppm was then integrated into bins of 0.04 ppm in width, resulting in 350 variables. The ASCII files generated were then imported into Microsoft Excel 2010 for secondary variable labelling after which the files were imported into SIMCA-P 13.0.0 (Umetrics, Umeå, Sweden). The acquired data was scaled (Pareto scaling) prior to Principal Component Analysis (PCA) and Orthogonal Projections to Latent Structures–Discriminant Analysis (OPLS-DA) algorithms. Pareto scaling is commonly used to reduce the influence of intense peaks so that large and small peaks are treated with equal emphasis in the MDA (Hendriks et al., 2011). A PCA model was constructed with the aim of detecting natural clustering patterns and for the identification of outliers. Further application of OPLS-DA analysis established a model that could be used to differentiate selective (antiplasmodial and not toxic) (SI ≥ 10) from non-selective (SI < 10) antiplasmodial classes in the dataset analysed. A contribution plot was then derived from the OPLS-DA scores plot in order to determine the spectral domains that are associated with the antiplasmodial metabolites of the analysed plant extracts.

2.3. Extraction and isolation of compounds

2.3.1. *Tabernaemontana elegans* (Stapf.) (Apocynaceae)

In our previous study, the dichloromethane extract recovered from the bark of *T. elegans* when extracted in dichloromethane (DCM): 50% methanol (MeOH) (1:1) showed a high selective antiplasmodial activity (IC_{50} = 0.331 µg/ml; SI = 14), which prompted further phytochemical investigation of the extract in an attempt to identify the compounds responsible for the observed antiplasmodial activity (Bapela et al., 2014). Additionally, given the documented bioactivity of indole alkaloids from the Apocynaceae family and the fitness of the NMR spectrum of *T. elegans* to the active profile shown by the metabolomics, alkaloids were targeted for isolation (Van Beek et al., 1984; Delorenzi et al., 2001, Mansoor et al., 2009; Girardot et al., 2012). Ground stem bark (1.0 kg) of *T. elegans* (120337) was extracted in DCM: 50% MeOH (1:1), separated into polar and non-polar fractions and dried under vacuum (Choi et al., 2004). The recovered DCM (37 g) extract was partitioned between DCM and 1% aqueous HCl (pH = 2) yielding separate DCM and aqueous layers. The acidic layer was collected at pH 3, basified to pH 9 with a solution of 10% NaOH and then extracted in DCM, resulting in a two-phase mixture, the neutral and the basic components. The basic layer was collected and concentrated *in vacuo* to yield a dry crude alkaloidal fraction (3.64 g). The neutral and the basic fractions were evaluated for antiplasmodial activity.

The crude alkaloidal fraction was then subjected to column chromatography (CC) on silica gel, with hexane:ethyl acetate: methanol as an eluent mixture starting at 7:3:0.1 with increasing polarity up to 6.75:3:1.6 (approx. 2000 ml of each solvent mixture). Eluates were combined into 11 fractions (I – XI) based on qualitative thin layer chromatography (TLC) analysis. Compounds ***1*** (42 mg) and **2** (106 mg) were

respectively recovered from fractions III and VI through recrystallization using the solvents MeOH and $CHCl_3$. ^1H NMR and ^{13}C NMR spectra of the isolated compounds were recorded in $CDCl_3$ on a Varian 200 MHz spectrometer. The configuration of the isolated compounds was based on comparison of the acquired spectroscopic data with published ^1H NMR and ^{13}C NMR spectral data of identified compounds.

2.3.2. *Vangueria infausta* Burch. subsp. *infausta* (Rubiaceae)

Dichloromethane root extract of *V. infausta* subsp. *infausta* (120338) was selected as a candidate for further chromatographic analysis as it showed significant antiplasmodial activity (IC_{50} = 1.84 µg/ml, SI = 25) and displayed a better correlation pattern with the antiplasmodial activity profile from the metabolomics experiment. Ground roots (3.0 kg) of *V. infausta* subsp. *infausta* were extracted in dichloromethane DCM: 50% MeOH (1:1) and the recovered DCM extract (3.0 g) was subjected to silica column chromatography (CC) eluting with hexane: ethyl acetate (EtOAc) (98:2 – 9:1), DCM: MeOH (98:2 – 96:4) and EtOAc: MeOH: H_2O (94:4:2 – 60:25:15) to yield 16 fractions (I – XVI). *In vitro* antiplasmodial screening was then conducted on the fractionated extracts. Bioactive fractions were further subjected to column chromatography in an effort to isolate antiplasmodial compounds. Fraction III was chromatographed on silica eluting with hexane: ethyl acetate (9:1) with increasing polarity to yield compound ***3*** (7 mg) through recrystallization. Compound ***4*** (4 mg) was isolated from fraction VIII through rechromatography of partially purified fractions. ^1H NMR and ^{13}C NMR spectra of the isolated compounds were recorded in $CDCl_3$ or CD_3OD on a Bruker 400 MHz spectrometer. The configuration of the isolated compounds was based on comparison of the acquired spectroscopic data with published ^1H NMR and ^{13}C NMR spectral data of identified compounds.

2.4. *In vitro* antiplasmodial assay

The acquired fractions and one compound (morindolide) were subjected to *in vitro* screening against *Plasmodium falciparum* (NF54) strain using a [^{3}H]hypoxanthine incorporation assay (Desjardins et al., 1979; Matile and Pink, 1990). The test organism was the chloroquine sensitive NF54 strain and the standard drug was chloroquine (Sigma C6628) (Ponnudurai et al., 1981). All tests were performed in 96-well microtiter plates, conducted in duplicates and repeated twice. Each fraction/compound was dissolved in 10% DMSO to afford a stock solution with a concentration of 10 mg/ml. Dissolved analytes were added to parasite cultures and then incubated in RPMI 1640 medium without hypoxanthine. The medium was supplemented with HEPES (5.94 g/l), $NaHCO_3$ (2.1 g/l), neomycin (100 U/ml), Albumax® (5 g/l) and washed human red cells A^+ at 2.5% haematocrit (0.3% parasitaemia). Serial drug dilutions of eleven 3-fold dilution steps covering a range from 100 to 0.002 µg/ml were prepared. Plates were incubated in a humidified atmosphere at 37 °C, 4% CO_2, 3% O_2 and 93% N_2. After 48 h, 50 µl of [^{3}H]hypoxanthine was added to each well.

The plates were then incubated for a further 24 h under the same conditions and then harvested with a Betaplate™ cell harvester (Wallac). Red blood cells were transferred onto a glass fibre filters and washed with distilled water. The dried filters were inserted into a plastic foil with 10 ml of scintillation fluid and counted in a Betaplate™ liquid scintillation counter (Wallac). IC_{50} values were calculated from sigmoidal inhibition curves by linear regression using Microsoft Excel (2010) (Huber and Koella, 1993). Microsoft Excel (2010) was also used to determine the mean values of the tested samples and the standard deviation of the isolated compound. Each IC_{50} value obtained represents means of two independent assays that were conducted in duplicates.

2.5. Cytotoxicity assay

Column chromatography fractions and morindolide were subjected to an antiproliferative bioassay in an attempt to determine their potential lethality or safe therapeutic application against mammalian cells. Assays were performed in 96-well microtiter plates, each well containing 100 µl of RPMI 1640 medium supplemented with 1% L-glutamine (200 mM), 10% fetal bovine serum and 4000 rat skeletal myoblast L6 cell line (Ahmed et al., 1994). Podophyllotoxin was used as positive control (Ahmed et al., 1994). Serial drug dilutions of eleven 3-fold dilution steps covering a range from 100 to 0.002 µg/ml were prepared. After 70 hours of incubation the plates were inspected under an inverted microscope to assure growth of the controls and sterile conditions. Alamar Blue (10 µl) was then added to each well and the plates were incubated for another 2 hours.

The plates were then read with a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation) using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. The IC_{50} values were calculated by linear regression from the sigmoidal dose inhibition curves using SoftmaxPro software (Molecular Devices Cooperation) (Huber and Koella, 1993). Microsoft Excel (2010) was also used to determine the mean values of the tested samples and the standard deviation of the isolated compound. Each IC_{50} value is a mean of two independent assays that were conducted in duplicates.

3. Results and discussion

As part of our continued search for novel antimalarial plant products (Prozesky et al., 2001; Tetyana et al., 2002; Adelekan et al., 2008), twenty South African indigenous plant species used to treat malaria and/or malarial symptoms by Vha-Venda people, were previously evaluated for their antiplasmodial activity

(Bapela et al., 2014). Of the extracts that were assessed, ten plant extracts exhibited significant *in vitro* antiplasmodial activity (IC_{50} ≤ 5 µg/ml), with non-polar extracts displaying more activity than polar extracts (Table 1). Consequently, a metabolomic analysis was conducted only on the dichloromethane plant extracts using ^{1}H NMR spectroscopy. The acquired ^{1}H NMR spectra were then subjected to Principal Component Analysis (PCA) in an attempt to investigate whether the descriptors are capable of separating the training set according to the observed antiplasmodial activity. Two significant outliers were identified based on the application of Distance to Model X (DMod[X]), Hotelling's T2Range Plots (T2Range) as well as visual observations of the PCA scores plot (Figure 1). Outlier points *A04* (*Diospyros mespiliformis*) and *A19* (*Senna petersiana*) were investigated and then excluded from the dataset. The resulting PCA scores plot did not show any clustering pattern that may be correlated to the observed antiplasmodial activity (Figure 1). The observed lack of an apparent PCA clustering pattern could be attributed to the biochemical variation inherent in the analysed crude extracts. The plant samples were harvested from sixteen different plant families which are characterized by diverse classes of secondary metabolites. Therefore, the observed antiplasmodial activity of the analysed plant extracts could be due to many chemically unrelated secondary compounds. It could also mean that most of the variability between the samples groups is due to inconsistencies in factors other than the biochemical diversity. The effects of factors such as geographical characteristics, climatic conditions, developmental stages and the plant parts harvested could be best controlled by increasing the biological replications in these types of investigations, which may be extremely costly.

Owing to the lack of an inherent clustering by the PCA algorithm, a supervised OPLS-DA was then applied to the ^{1}H NMR data in an attempt to enhance the poor clustering obtained. The OPLS-DA scatter plot showed a clear discrimination between the selective (antiplasmodial and not toxic) and non-selective classes by principal component (PC) 1 (t[1]) while PC 2 (t[2]) indicated the variation of secondary metabolites within class (Figure 2). The observed low modelled variation along PC 1 (0.058) may be attributable to the distinctive bioactive functional groups being present in the bioactive plant species, which could mean that their antiplasmodial activity resulted from different classes of compounds. The robustness of a model generated by the OPLS-DA algorithm is most commonly interpreted by means of R^2 and Q^2 values calculated by the supervised correlation with the secondary variable. The value of the cross-validated variance (R^2) indicates the amount of data explained by the model and gives a general overview of the fitness of the model whereas the variance (Q^2) value is a measure of the predictability of the model (Eriksson et al., 2006). The OPLS-DA model generated in this study was successful in discriminating selective from non-selective sample classes (R^2 = 0.95). However, the low value (Q^2 =0.09) calculated for the OPLS-DA model derived from the dataset showed that the model generated is not robust enough to predict the antiplasmodial activity of unknown samples (Bailey et al., 2004). Models that explain the data well and are not overfitted usually have R^2 and Q^2 values of ~0.5 and the observed disparate values between R^2 and Q^2 (R^2 >> Q^2) indicate that model is overfitting in supervised analyses (Hendriks et al., 2011). This observation is often encountered as a result of the nature of the datasets in MDA where the number of observations is significantly different from the total number of variables, as was the case in this study.

Ideally it is aimed to further interpret the OPLS-DA scoring plot in terms of the original variables used in the analysis, which in this study are represented by chemical shifts (ppm). To find out precisely which spectral regions of the ^{1}H NMR spectra are responsible for the detected separation between selective and non-selective plant extracts, a contribution plot was derived from the OPLS-DA scoring plot (Figure 3). The chemical shifts of interest could then be correlated to classes of secondary metabolites found within the plant kingdom. Antiplasmodial regions were tentatively identified as aliphatic/terpenoids (0.4 – 1.44 ppm), sugar-based/N-containing (3.63 - 4.00 ppm) and aromatic/ethylenic (6.60 – 7.40 ppm) compounds and prominent bars representing non-active regions were identified as vinylic (5.41 – 5.60 ppm), (acetylenic 1.80 – 3.10 ppm) as well as ketonic (1.80 - 2.5 ppm) compounds. However, the signals of the specified regions could not be precisely identified due to the complexity of the metabolite profile. Compounds in the aliphatic region appear to have played a significant role in this discrimination. Although the major bars are significantly correlated to the observed antiplasmodial activity, the importance of the minor bars should not be overlooked as some secondary metabolites are restricted to certain plant families or genera and also accumulate in relatively trace amounts. Weaker signals possessing a discriminatory power could provide novel antiplasmodial scaffolds and should therefore be investigated in further characterization studies.

Visual inspection of two spectra of the most active plant extracts, *Tabernaemontana elegans* and *Vangueria infausta* subsp. *infausta*, illustrated qualitative correlation to the global active profile (Figure 4). The spectrum of *T. elegans* showed some prominent peaks in the aliphatic/terpenoid and the aromatic/ethylenic regions whereas major peaks of *V. infausta* subsp. *infausta*

corresponded to aliphatic/terpenoid as well as sugar-based/N-containing regions of the active profile. Indole alkaloids and terpenoids compounds have been previously isolated from *T*. *elegans*, which could explain the observation (Van Beek et al., 1984). Phytochemical analysis of *V. infausta* subsp. *infausta* indicated the presence of terpenoids and glycosides, which fit the profile (Abeer, 2011).

The acid-base extraction of the dichloromethane extract of *T. elegans* yielded two fractions, which were then subjected to antiplasmodial screening. Alkaloidal fraction (IC_{50} = 0.06 µg/ml, SI = 47.8) demonstrated significant antiplasmodial activity when compared to the neutral fraction (IC_{50} = 6.05 µg/ml, SI = 4.1). The observed bioactivity is attributable to the indole alkaloids, which are concentrated in the basic fraction. In an attempt to identify these indole alkaloids and to determine their respective antiplasmodial activities, the basic fraction was then subjected to column chromatography. Further fractionation and purification of the alkaloidal fraction led to the isolation of two compounds, dregamine and tabernaemontanine (Figure 5). The ^{1}H NMR spectra of the isolated compounds shared many spectral features and displayed a biogenetic correlation to indole alkaloids. Structural elucidation and configuration of the isolated compounds were based on the acquired spectroscopic data and facilitated by comparison with published ^{1}H and ^{13}C NMR data of the compounds (Ahond et al., 1976; Van der Heijden et al., 1986). The two corynanthean indole alkaloids are epimers differing only at one stereogenic centre (position 20), where the hydrogen atom is in the α position on tabernaemontanine and in the β position for dregamine (Figure 5). There is no published data on the antiplasmodial activity of indole alkaloids isolated from *T. elegans*. Nevertheless, studies conducted by Girardot et al., (2012) on indole alkaloids isolated from *Muntafara sessilifolia* demonstrated significant antiplasmodial activities by dregamine

(62.0±2.4 μM) and tabernaemontanine (12.0±0.8 μM) when tested against the chloroquine-resistant strain of *P. falciparum* (FcB1). Tabernaemontanine exhibited a relatively higher selectivity index (SI = 8.4) compared to its isomer and may therefore be a potential antiplasmodial drug candidate. The antiplasmodial activity of dregamine (SI = 3.1) could be due to the compound exhibiting higher cytotoxicity at lower concentrations. The stereochemistry at C-20 seems to possess an effect on the antiplasmodial activity of these two acyl indole alkaloids.

Fractionation of the dichloromethane extract of V. *infausta* subsp. *infausta* yielded 16 fractions (I – XVI), which were subsequently subjected to *in vitro* antiplasmodial screening against chloroquine sensitive NF54 strain of *P. falciparum*. Fractions III, VII, VIII, IX, X, XI and XII demonstrated high antiplasmodial activity (IC_{50} ≤ 5 μg/ml) (Appendix A). However, their respective IC_{50} values were not significantly different from that of the crude extract, thus highlighting the complexity of the fractions. Friedelin (**3**) and morindolide (**4**) were isolated from fractions III and VIII respectively. Friedelin, a pentacyclic, triterpenoid is very abundant in nature and has been previously isolated from several plant species (Lenta, et al., 2007; Mann et. al., 2011; Sousa et al., 2012) however; this is the first report on its presence in the genus *Vangueria* (Figure 6). Most triterpenoids frequently occur together, are not restricted to any specific plant family and have exhibited good antiplasmodial activities in the micromolar ranges (Lenta et al., 2007; Ngouamegne et al., 2008). Friedelin has demonstrated good antiplasmodial activity (IC_{50} = 7.70 μM) against *P. falciparum* strain K1 and an (IC_{50} of 7.20±0.5 μM when tested against chloroquine-resistant strain (W2) of *P. falciparum* (Lenta, et al., 2007 Ngouamegne et al., 2008) (Table 2). Antiplasmodial efficacy of the related fraction (III) was found to be 3.94 μg/ml, which may be mostly attributed to friedelin (Appendix A). Morindolide is a rare

iridoid lactone with an undocumented antimalarial activity. Although the compound has been previously isolated from the Rubiaceae family (Yoshikawa et al., 1995), this is the first account of its presence in the genus *Vangueria* as well as its documented antiplasmodial activity (Figure 7). In this study, morindolide demonstrated moderate antimalarial activity (IC_{50} = 107.1±0.6 µM, SI = 3.32) and has shown to be toxic to the mammalian cells tested (Table 2). Iridoid glucosides and related aglycones have showed a wide range of pharmacological activities including significant antiplasmodial activity (Tchimene et al., 2013; He et al., 2014). Additional studies on morindolide may include its derivatization, which could possibly ameliorate its toxicity while optimising its relatively low antiplasmodial activity.

Conclusions

The study illustrated the potential of NMR-based metabolomics in discriminating classes of compounds that may be attributed to antiplasmodial activity. Although the model generated lacked sufficient power to predict the antimalarial efficacy of unknown samples, it yielded a global active profile for the plants investigated. Further studies should be carried out on a larger sample size and must also include a biological replication in a blind, new dataset for cross validation of the model. The current study also demonstrated the potential of discovering novel antiplasmodial scaffolds from medicinal plants and further validated the ethnomedicinal use of the investigated plant species by Vha-Venda people on the *in vitro* level. More efforts should be directed at the identification all the metabolites responsible for the separation of samples.

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Appendix A: Supplementary information

Supplementary data associated with this article can be found in the online version at

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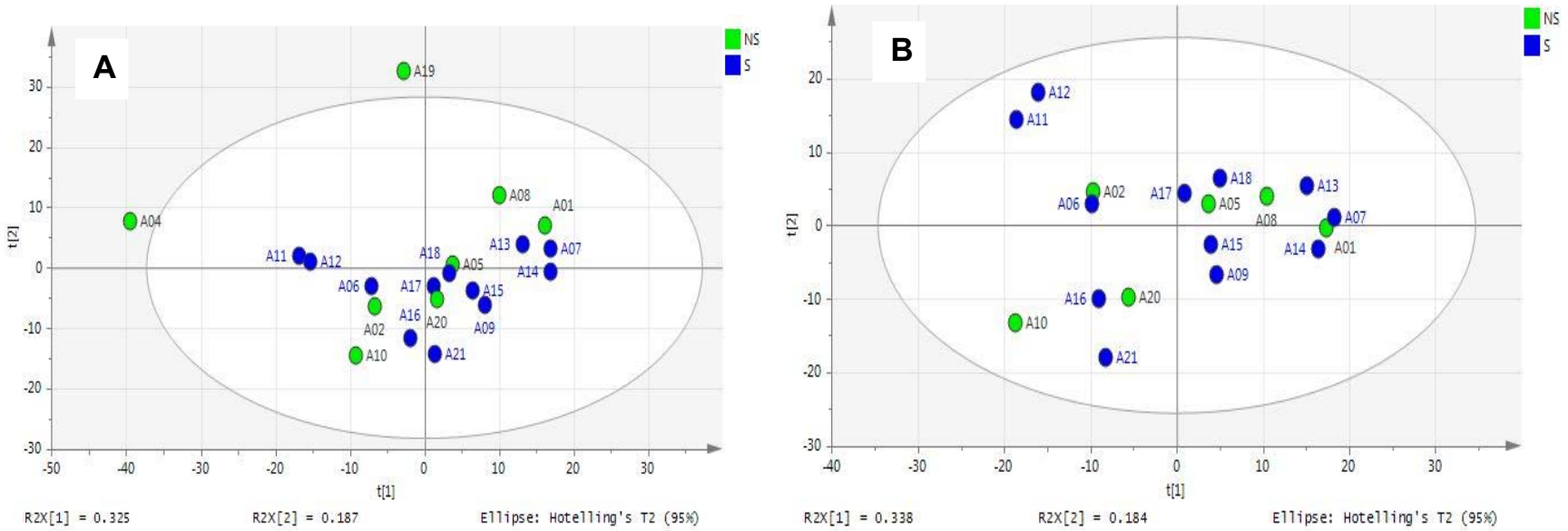


Figure 1. Principal component analysis (PCA) score plots of dichloromethane extracts, based on the selective indices of the extracts. Score plot A shows outliers A04 and A19, while score plot B illustrates the grouping after the removal of the outliers.

Groupings: S- selective and NS – not selective

Observations: N = 18, Variables: K = 352 (X = 350, Y = 2)

Plant codes: *A. versicolor* (A13, root), *A. versicolor* (A14,stem bark) *A. grandiflora* (A05), *B. mollis* (A17), *C. tomentosa* (A09), *C. brachiata* (A08), *C. glabrum* (A20), *C. spicata* (A07), *D. cinerea* (A18), *D. mespiliformis* (A04), *P. capensis* (A16), *P. curatellifolia* (A10), *P. grandiflora* (A01), *R. caffra* (A21), *S. petersiana* (A19), *S. cordatum* (A06), *T. elegans* (A11), *V. infausta* (A15), *X. caffra* (A02), *X. parviflora* (A12)

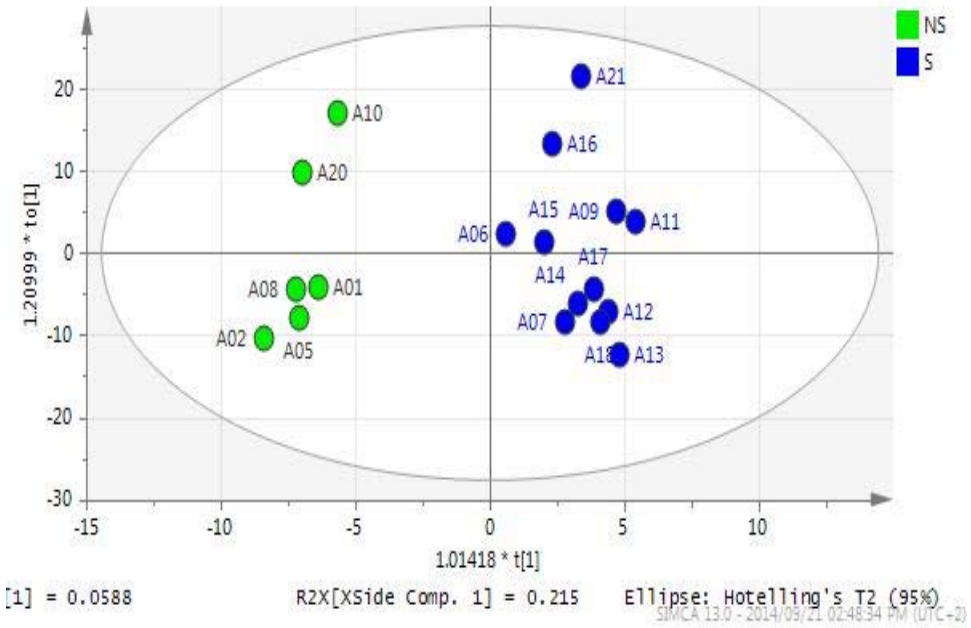


Figure 2. Scores plot of OPLS-DA model based on the selective index of non-polar extracts classified into two groups: S- selective and NS – not selective (Observations: *N* = 18, Variables: *K* = 352 (*X* = 350, *Y* = 2))

Plant codes: *A. versicolor* (A13, root)*, A. versicolor* (A14,stem bark) *A. grandiflora* (A05), *B. mollis* (A17), *C. tomentosa* (A09), *C. brachiata* (A08), *C. glabrum* (A20), *C. spicata* (A07), *D. cinerea* (A18), *P. capensis* (A16), *P. curatellifolia* (A10), *P. grandiflora* (A01), *R. caffra* (A21), *S. cordatum* (A06), *T. elegans* (A11), *V. infausta* (A15), *X. caffra* (A02), *X. parviflora* (A12)

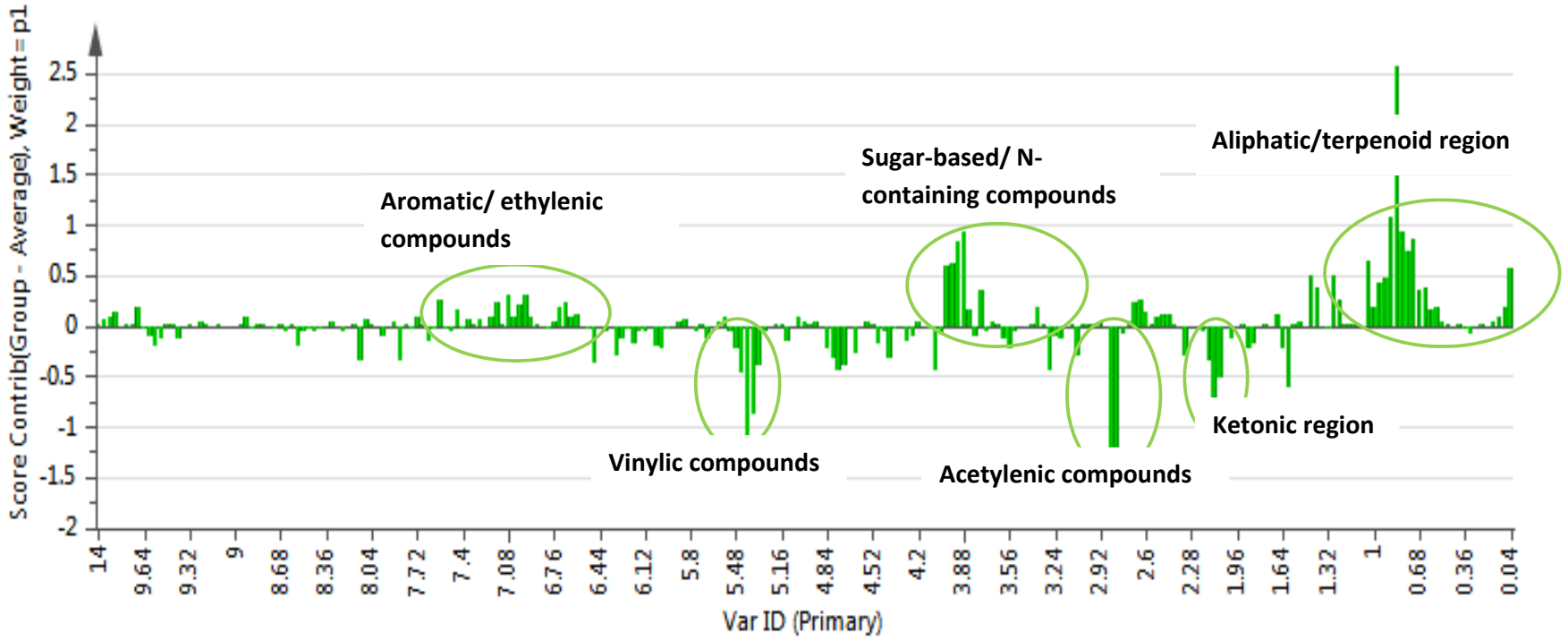


Figure 3. Contribution plot of non-polar plant extracts showing the comparison between selective and non-selective clusters.
(Selective – bars projecting upwards and Non-selective – bars projecting downwards)

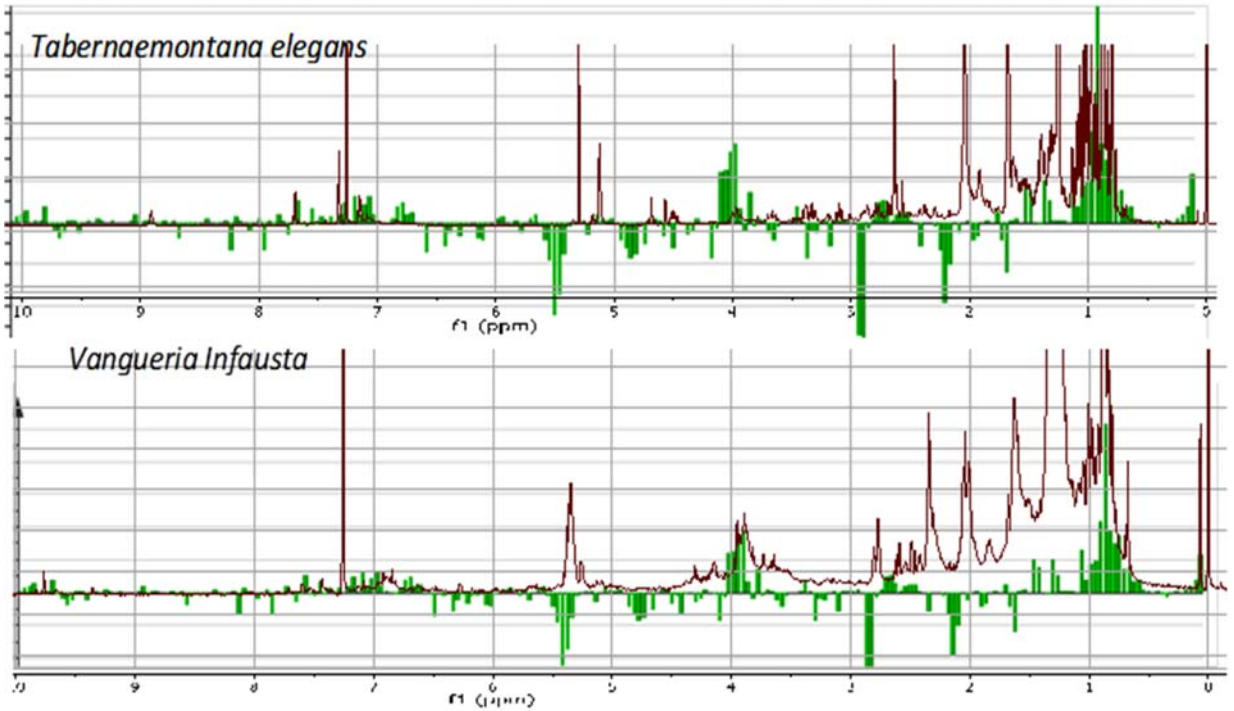
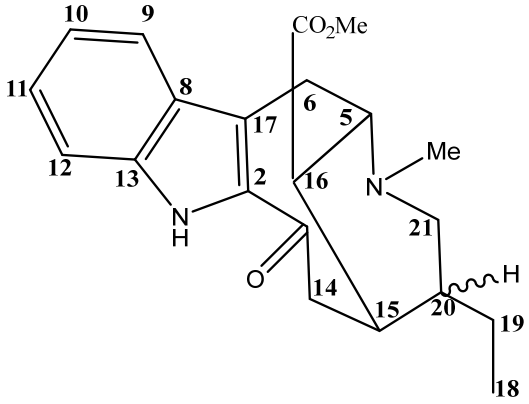


Figure 4. ^{1}H NMR spectra of the DCM extracts of *T. elegans* and *V. infausta* when compared to the active profile of the contribution plot



1. 20β-H *2*. 20α-H

Figure 5. Molecular structures of dregamine (***1***) and tabernaemontanine (***2***) isolated from *Tabernaemontana elegans* (Ahond et. al., 1976; Van der Heijden et. al., 1986)

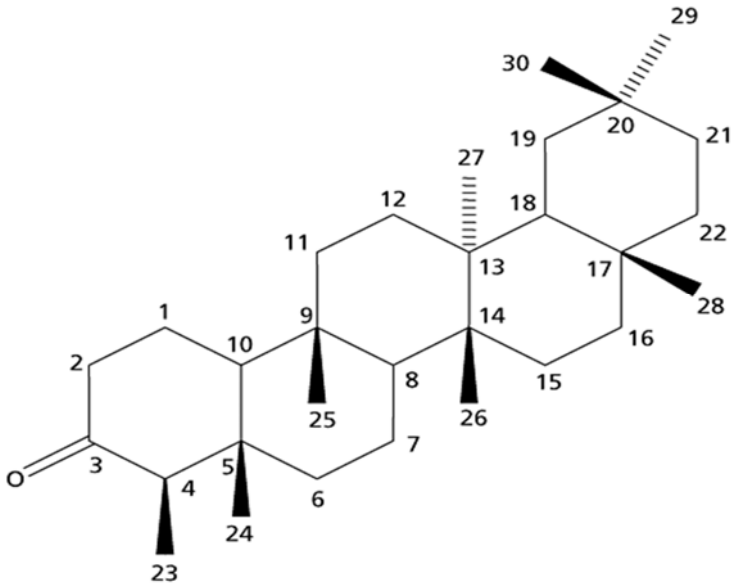


Figure 6. Molecular structure of friedelin (Lenta, et al., 2007; Mann et. al., 2011)

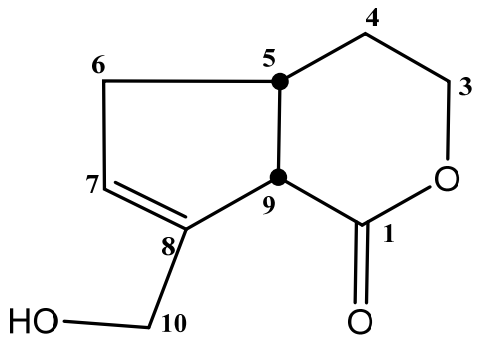


Figure 7. Molecular structure of morindolide lactone (Yoshikawa et al., 1995)

Table 1. *In vitro* screening of antimalarial plant species traditionally used by Vha-Venda people. Each IC_{50} value represents a mean of two independent assays conducted in duplicates.

Plant species*, family, voucher number	**Collected plant part**	**Extraction solvent**	**[a]Antiplasmodial activity (IC_{50}) µg/ml**	**[b]Cytotoxicity (IC_{50}) µg/ml**	**Selectivity Index (SI)**
Albizia versicolor Welw. ex Oliv. (Fabaceae)	Roots	DCM	8.69	55.6	6
120322		50% MeOH	>50	70.1	ND
A. versicolor	Stem bark	DCM	7.08	72.1	10
120322		50% MeOH	27.3	52.3	2
Anthocleista grandiflora Gilg. (Loganiaceae)	Twigs	DCM	2.12	55.1	26
120323		50% MeOH	23.8	42.0	2
Bridelia mollis Hutch. (Phyllanthaceae)	Roots	DCM	3.06	51.4	17
120324		50% MeOH	28.5	49.6	2
Capparis tomentosa Lam. (Capparidaceae)	Roots	DCM	2.19	40.8	19
120325		50% MeOH	29.2	70.4	2
Clematis brachiata Thunb. (Ranunculaceae)	Roots	DCM	5.36	42.6	8
120326		50% MeOH	>50	72.3	ND
Clerodendrum glabrum E. Mey. (Verbenaceae)	Leaves	DCM	8.89	62.2	7
120327		50% MeOH	>50	72.7	ND
Cussonia spicata Thunb. (Araliaceae)	Root bark	DCM	3.25	47.8	15
120328		50% MeOH	>50	69.1	ND
Dichrostachys cinerea Wight et Arn. (Fabaceae)	Roots	DCM	2.10	51.6	25
120329		50% MeOH	>50	65.3	ND
Diospyros mespiliformis Hochst. ex A.DC. (Ebenaceae)	Roots	DCM	4.40	24.3	6
120330		50% MeOH	28.4	60.4	2
Pappea capensis Eckl. & Zeyh. (Sapindaceae)	Twigs	DCM	5.47	54.0	10
120331		50% MeOH	24.8	55.2	2

Parinari curatellifolia Planch. Ex Benth. (Rosaceae)	Stem bark	DCM	6.99	57.6	8
120332		50% MeOH	16.9	55.4	3
Pyrenacantha grandiflora Baill. (Icacinaceae)	Roots	DCM	5.82	0.52	0
120333		50% MeOH	>50	10.5	ND
Rauvolfia caffra Sond. (Apocynaceae)	Stem bark	DCM	2.13	26.9	13
120334		50% MeOH	10.8	57.2	5
Senna petersiana (Bolle) Lock. (Fabaceae)	Leaves	DCM	22.5	59.3	3
120335		50% MeOH	22.1	66.8	3
Syzygium cordatum Hochst. (Myrtaceae)	Leaves	DCM	6.15	65.7	11
120336		50% MeOH	10.4	53.8	5
Tabernaemontana elegans Stapf. (Apocynaceae)	Stem bark	DCM	0.33	4.68	14
120337		50% MeOH	0.83	38.2	46
Vangueria infausta Burch. subsp. *Infausta* (Rubiaceae)	Roots	DCM	1.84	45.7	25
120338		50% MeOH	>50	71.5	ND
Ximenia americana Linn. (Olacaceae)	Roots	DCM:50% MeOH	3.01	8.68	3
120339					
Ximenia caffra Sond. (Olacaceae)	Leaves	DCM	>50	74.1	ND
120340		50% MeOH	28.2	69.1	2
Xylopia parviflora (A.Rich.) Benth. **Oliv.** (Annonaceae)	Roots	DCM	2.19	51.5	24
120341		50% MeOH	14.2	78.3	6
Chloroquine			0.003		
Podophyllotoxin				0.007	

* The plant species were collected based on their ethnomedicinal uses published in literature. (Watt and Breyer-Brandwijk, 1962; Arnold and Gulumian, 1984; Mabogo, 1990; Bandeira et al., 2001)

[a]Chloroquine resistant *Plasmodium falciparum* NF54 strain

[b]Rat skeletal muscle L-6 cell line

Table 2. *In vitro* antiplasmodial activity of extracts, fractions and isolated compounds.

Tabernaemontana elegans			***Vangueria infausta* subsp. *infausta***		
	[a]Antiplasmodial activity (IC_{50}) µg/ml	[b]Cytotoxicity (IC_{50}) µg/ml		[a]Antiplasmodial activity (IC_{50}) µg/ml	[b]Cytotoxicity (IC_{50}) µg/ml
DCM extract	0.331	4.68	DCM extract	1.84	45.7
Neutral fraction	6.05	25.1	Fraction III	3.94	ND
			Friedelin	[d]7.20±0.5 µM	ND
				[e]7.70 µM	ND
Basic fraction	0.06	2.87	Fraction VIII	2.18	ND
[c]Dregamine	62.0±2.4 µM	195.8±8.6 µM	Morindolide	107.1±0.6 µM	366.1±0.8 µM
[c]Tabernamontanine	12.0±0.8 µM	100.7±9.1 µM			
Chloroquine	0.002	ND	Chloroquine	0.002	ND
Podophyllotoxin	ND	0.005	Podophyllotoxin	ND	0.005

[a]Chloroquine resistant *Plasmodium falciparum* NF54 strain

[b]Rat skeletal muscle L-6 cell line

[c]Chloroquine resistant strain FcB1 *of P. falciparum* (Girardot et al., 2012)

[d]Friedelin was tested against the W2 strain of *P. falciparum* (Ngouamegne, et al., 2008)

[e]Friedelin was tested against *P. falciparum* strain K1 (Lenta, et al., 2007)