In vitro inhibition of *Plasmodium falciparum* early and late stage gametocyte viability by extracts from eight traditionally used South African plant species

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Abstract

Ethnopharmacological relevance: Extracts of plant species, used traditionally to treat malaria, have been extensively investigated for their activity against *Plasmodium* intraerythrocytic asexual parasites in search of new antimalarial drugs. However, less effort has been directed towards examining their efficacy in blocking transmission. Here, we report the results of the *in vitro* screening of extracts from eight selected plant species used traditionally to treat malaria in South Africa for activity against *P. falciparum* NF54 early and late stage gametocytes. The species used were *Khaya anthotheca, Trichilia emetica, Turraea floribunda, Leonotis leonurus, Leonotis leonurus* ex Hort, *Olea europaea* subsp. Africana, *Catha edulis* and *Artemisia afra*.

Aim of the Study: To investigate the activities of extracts from plant species traditionally used for malaria treatment against *P. falciparum* gametocytes.

Material and Methods: Air-dried and ground plant leaves were extracted using acetone. Primary two point *in vitro* phenotypic screens against both early and late stage gametocytes were done at 10 and 20 µg/ml followed by full IC_{50} determination of the most active extracts. Inhibition of gametocyte viability *in vitro* was assessed using the parasite lactate dehydrogenase (pLDH) assay.

Results: Of the eight crude acetone extracts from plant species screened *in vitro*, four had good activity with over 50-70% inhibition of early and late stage gametocytes' viability at 10 and 20 µg/ml, respectively. *Artemisia afra* (Asteraceae), *Trichilia emetica* (Meliaceae) and *Turraea floribunda* (Meliaceae) were additionally highly active against both gametocyte stages with IC_{50} values of less than 10 µg/ml while *Leonotis leonurus* ex Hort (Lamiaceae) was moderately active ($IC_{50}<20$ µg/ml). The activity of these three highly active plant species was significantly more pronounced on late stage gametocytes compared to early stages.

Conclusion: This study shows the potential transmission blocking activity of extracts from selected South African medicinal plants and substantiates their traditional use in malaria control that broadly encompasses prevention, treatment and transmission blocking. Further studies are needed to isolate and identify the active principles from the crude extracts of *A. afra*, *T. emetica* and *T. floribunda*, as well as to examine their efficacy towards blocking parasite transmission to mosquitoes.

Graphical Abstract
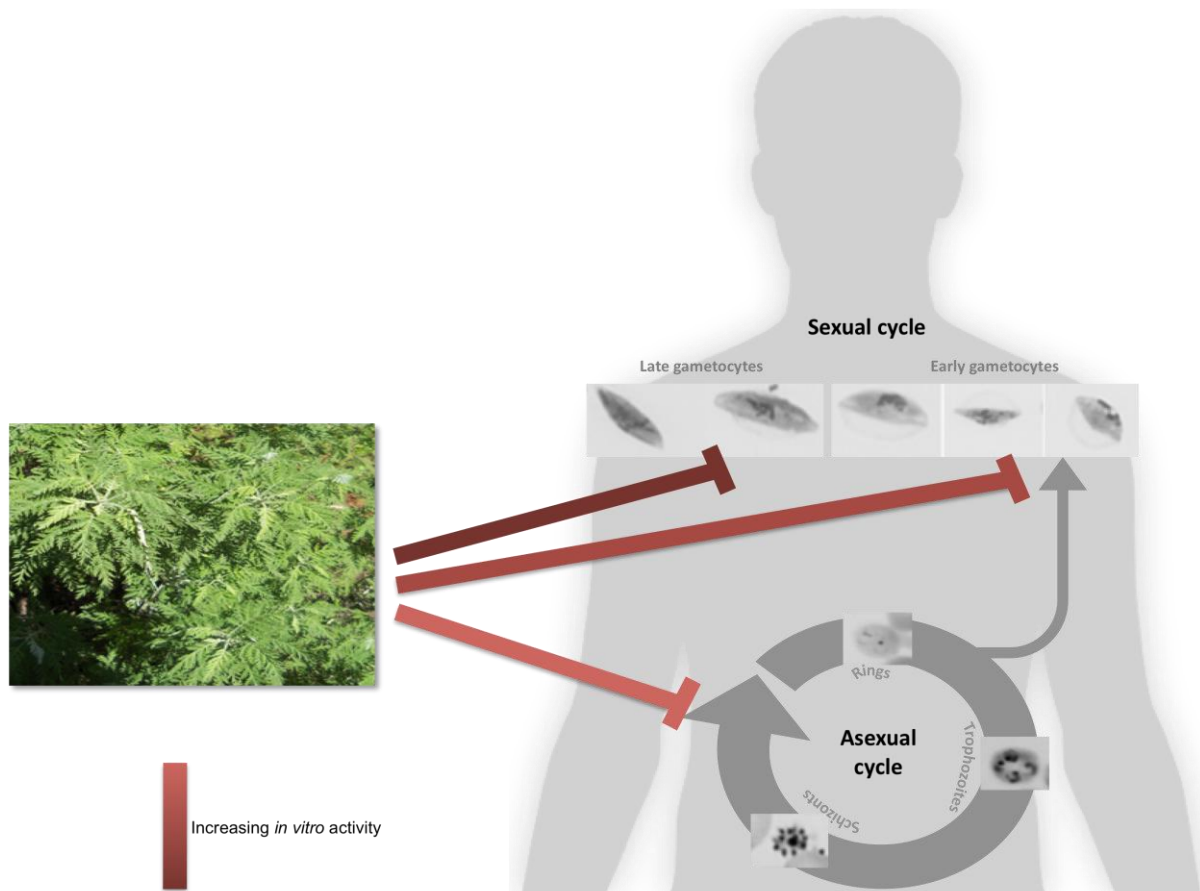
1.   Introduction

For the past decades, the management of malaria has primarily relied on vector control and chemotherapeutic drugs such as chloroquine and the artemisinin-based combination therapies (ACT's). While the benefits of adopting these measures have been evident (Bhatt *et al.*, 2015), unfortunately so too has been the emergence of insecticide resistant mosquitoes (Benelli, 2015; Dai *et al.*, 2015; Edi *et al.*, 2012; Ranson *et al.*, 2011) and drug resistant strains of *Plasmodium falciparum* parasites (Ashley *et al.*, 2014; Dondorp *et al.*, 2009; Tun *et al.*, 2015). There is thus a dire need to advance the efficacy of these approaches with novel interventions, drugs and insecticides in the fight against this disease. In the current era of renewed calls for malaria elimination and eradication, there is a need to devise new strategies to complement current interventions (Roberts and Enserink, 2007). One such identified strategy is to block transmission from the human host to the mosquito vector (Alonso *et al.*, 2011).

Human-to-mosquito transmission blocking entails targeting the sexual stages of the parasite, the gametocytes. This is the only stage of *Plasmodium* that can infect a female *Anopheles* mosquito for the sexual development of the parasite into sporozoites that can in turn infect humans and thereby perpetuate the lifecycle (Baker, 2010). Inhibiting gametocyte development would significantly reduce the number of infective mosquitoes and thus the number of newly infected patients. A single low dose primaquine (0.25 mg/kg) remains the only gametocytocidal drug recommended by the World Health Organisation against *P. falciparum* gametocytes (White *et al.*, 2014). However, its use is restricted due to adverse effects on patients with glucose-6-phosphate dehydrogenase deficiency (Baird and Hoffman, 2004), a genetic disorder that is prevalent among populations in malaria endemic areas (Nkhoma *et al.*, 2009). While some antimalarial drugs are active against the early stages of *P. falciparum* gametocytes (Abay, 2013; Butcher, 1997; Lucantoni *et al.*, 2013; Mackerras and Ercole, 1949; Price *et al.*, 1996; White *et al.*, 2014), they are not effective at clinically relevant concentrations on the late stage, transmittable gametocytes. There is therefore an urgent need to find new safe and efficacious transmission blocking drugs that can target late stage gametocytes.

Plant-derived natural products have played a fundamental role in the control of malaria. They have been a vital source of some of the mainstay drugs in malaria treatment such as the alkaloid, quinine and sesquiterpene lactone, artemisinin (Wells, 2011). These drugs were identified following intensive screens of hundreds of plant extracts for activity against the intraerythrocytic, asexual parasites of *Plasmodium* (Tu, 2011; Zhang, 2011). By contrast, less effort has been channeled towards examining extracts of plants for their activity against the sexual stages of the parasite. Currently, only three plant species have been directly screened for their malaria transmission blocking capacity: *Azadirachta indica* (Meliaceae) (Dhar *et al.*, 1998; Jones *et al.*, 1994; Lucantoni *et al.*, 2010; Udeinya *et al.*, 2006, 2008; Yerbanga *et al.*, 2014), *Vernonia amygdalina* (Asteraceae) (Abay *et al.*, 2015, 2013) and *Guiera senegalensis* (Combretaceae) (Yerbanga *et al.*, 2014). *A. indica* has been comprehensively studied and shown to have good gametocytocidal activity *in vitro* (Dhar *et al.*, 1998; Jones *et al.*, 1994; Udeinya *et al.*, 2008, 2006), *in vivo* (Lucantoni *et al.*, 2010) and *ex vivo* (Yerbanga *et al.*, 2014). The active components are a diverse range of limonoid compounds (Jones *et al.*, 1994; Lucantoni *et al.*, 2010; Yerbanga *et al.*, 2014). *V. amygdalina* gametocytocidal activity was demonstrated *in vivo* with the active principles identified as the sesquiterpene lactones vernadalol and vernolide (Abay *et al.*, 2015, 2013). *G. senegalensis* did not have any transmission blocking properties (Yerbanga *et al.*, 2014).

Even given the complexities involved in plant-based screening for antimalarial activity, the paucity of information of plant species with potency against early and late stage gametocytes encourages studies to identify malaria transmission blocking capabilities of plant extracts. In the current study, we explored the anti-gametocyte properties of eight plant species traditionally used to treat malaria in South Africa. This was investigated by using a stage-specific *in vitro* phenotypic screen of crude extracts of plants for activity against early and late stage *P. falciparum* NF54 gametocytes.

2.   Materials and Methods

2.1 Plant selection, collection and crude acetone extract preparation

The subset of eight plant species investigated in this study was selected as follows: Plants were first and foremost chosen for being used traditionally for either malaria or fever treatment as documented in ethnobotanical studies (Alam *et al.*, 2012; Clarkson *et al.*, 2004; Van Wyk, 2008; Table 1). From the ethnobotanical set, plants were selected based on two parallel criteria: 1) previous reports of either good or moderate activity against the intraerythrocytic asexual parasites of *P. falciparum* parasites (IC$_{50}$ ≤10 µg/ml − good; 20≥IC$_{50}$>10 − moderate) and 2) evidence of the presence of compounds (at plant family level) that are structurally similar to those known to be gametocytocidal either *in vitro*, *in vivo* or *ex vivo* (Abay *et al.*, 2015; Adjalley *et al.*, 2011; D'Alessandro *et al.*, 2013; Duffy and Avery, 2013; Jones *et al.*, 1994; Lucantoni *et al.*, 2013, 2010; Saenz *et al.*, 2013; Sun *et al.*, 2013; Yerbanga *et al.*, 2014). Using these two parallel selection strategies, the ethnobotanical set was narrowed down to a hundred plant species from which eight were collected and screened in the current study.

Leaves of the eight plant species were collected from the Manie van der Schijff Botanical Garden at the University of Pretoria, Hatfield campus in July 2014. Plants were identified by a curator and voucher specimens were made and deposited at the H.G.W.J. Schweickerdt Herbarium of the University of Pretoria (Table 1) and confirmed on www.theplantlist.org. Collected plant material was air dried, ground to fine powder and extracted in acetone as described elsewhere (Dzoyem and Eloff, 2015). Phytochemical profiling of dry crude extracts was performed using thin layer chromatography (TLC) (Kotze and Eloff, 2002).

2.2 In vitro cultivation of asexual and sexual stage P. falciparum parasites

The chloroquine sensitive and high gametocyte yielding *P. falciparum* NF54 strain was established in human erythrocytes (O^{+}, suspended at 5% haematocrit), cultured asexually and synchronised at least two times on ring stage parasites.

Gametocytogenesis and gametocyte culturing was performed as described by Reader *et al.* (2015). Contaminating asexual cultures were removed by N-acetyl glucosamine (NAG) (50 mM) treatment for 96 hours (Reader *et al.*, 2015). Gametocyte development from stages I to V was monitored daily by microscopic examination of Giemsa-stained smears of cultures. A suspension of uninfected erythrocytes was co-cultured along with infected cell cultures

under similar conditions and used as background attributable to uninfected erythrocytes for the gametocyte assays.

2.3 In vitro assessment of gametocyte viability

The ability of extracts from plants to inhibit *P. falciparum* early and late stage gametocyte viability was assessed *in vitro* using the parasite lactate dehydrogenase (pLDH) assay. Modified protocols of the method of Makler and Hinrichs (1993) were used to set up the assays (D'Alessandro *et al.*, 2015, 2013) and for examining gametocyte viability spectrophotometrically (Reader *et al.*, 2015).

Dried crude acetone extracts were dissolved in DMSO as stock solutions and diluted in culture medium prior to their use in assays (highest concentration of DMSO to which parasites were exposed to was 0.4% (v/v) which is non-toxic to gametocytes (D'Alessandro *et al.*, 2015, 2013; Duffy and Avery, 2013; Lucantoni *et al.*, 2013). These extracts were transferred into 96 well sterile plates and seeded with an equal volume (100 µl/well) of gametocyte suspension (0.8-3% gametocytaemia) to achieve a final haematocrit of 1% (D'Alessandro *et al.*, 2013). Dual primary point screens (using 10 and 20 µg/ml plant extract) were followed by the determination of the IC_{50} values (concentration of extract required to inhibit gametocyte viability by 50%) using a range of starting concentrations of the most active plant extracts (final concentration range of 0.156-40 µg/ml). Methylene blue (10 µM) served as the positive drug control.

Plates were incubated at 37ºC for 72 hours, followed by the replacement of spent medium with extract-free culture medium (75% medium change) (D'Alessandro *et al.*, 2015, 2013). Plates were then incubated for a further 72 hrs before assessing viability by measuring pLDH activity (Reader *et al.*, 2015). All assays were done in technical triplicates with three independent biological repeats each. Non-linear regression analysis (using GraphPad Prism, version 5.0) was used to determine IC_{50} values.

2.4 In vitro antiplasmodial activity against intraerythrocytic asexual parasites

The activity was determined as described by Clarkson *et al.* (2003). Dual point screens were set up at 10 and 20 µg/ml. Chloroquine (0.5 µM) was used as positive drug control.

3. Results

3.1 Plant selection, collection and extraction

Eight plant species representing five different families and seven genera were collected for the study. The three plant species investigated namely, *L. leonurus*, *L. leonurus* ex Hort and *Olea europaea* (Oleaceae) were selected based on previously reported activity against the intraerythrocytic asexual parasites of *P. falciparum* (Table 1) (Clarkson *et al.*, 2004). *Khaya anthotheca* (Meliaceae) was selected as the Meliaceae family, has been shown to produce limonoids that are potent against gametocytes (Jones *et al.*, 1994; Lucantoni *et al.*, 2010; Yerbanga *et al.*, 2014). The remainder of plant species was selected based on both activity and natural compound production (Table 1). Different extraction yields (grams acetone plant extract/grams dry plant starting material) were obtained, with *K. anthotheca* producing the highest yield (11%) while *T. emetica* had the lowest (~3%; Table 1). Major compound classes identified through TLC phytochemical profiling included phenols, terpenoids and stilbenes. *A. afra* was the only species observed to have compounds that fluoresce under UV light (Fig. 1S).

Table 1. Plant species selected and evaluated for potential inhibition of asexual or sexual stage *P. falciparum* parasites.

Plant specie (Family)	Common names	Selection criteria	Extraction Yield (%)	Voucher No.
Khaya anthotheca (Welw) C.DC. (Meliaceae)	Rooimahonie (Afrikaans), red mahogany (English)	c	11.05	PRU 121 391
Trichilia emetica Vahl subsp. Emetica (Meliaceae)	Umkhulu (Zulu), bosveldrooiessenhout (Afrikaans)	a, c	3.25	PRU 121 390
Turraea floribunda Hochst. (Meliaceae)	Umadlozane (Zulu), umhlatholana (Xhosa), kanferfoelieboom (Afrikaans)	a, c	7.12	PRU 121 387
Leonotis leonurus (L.) R. Br. (Lamiaceae)	Wilde dagga (Afrikaans), lion's ear (English)	a	5.85	PRU 121 393
Leonotis leonurus ex Hort (yellow) (Lamiaceae)	Wilde dagga (Afrikaans)	a	3.85	PRU 121 394
Olea europaea subsp. Africana (Oleaceae)	Wild olive (English), Olienhout (Afrikaans)	b	7.08	PRU 121 388
Catha edulis (Vahl) Endl (Celastraceae)	Umhlwazi (Zulu), igqwaka (Xhosa), khat (Afrikaans)	a	3.33	PRU 121 392
Artemisia afra Jacq. ex Wild. (Asteraceae)	African wormwood (English), Wilde-als (Afrikaans), Umhlonyane (Zulu)	a, c	6.38	PRU 121 389

a – good activity against asexual parasites ($IC_{50} \leq 10$ µg/ml); b – moderately active against asexual parasites ($20 \geq IC_{50} > 10$); c – chemical class type production. All plant species names have been checked and confirmed as acceptable on www.theplantlist.org.

3.2.1 Gametocyte viability (Dual point screens)

The effects of crude acetone extracts of eight different plants on early and late stage gametocyte viability were assessed *in vitro* using a pLDH assay (Fig. 1). Gametocyte cultures used for both early and late stage assays contained >85% of each respective stage being investigated (Fig. 1A), validating their use in the stage-specific evaluation of gametocytocidal activity analysis. Furthermore, quality evaluation of assay performance revealed that for both stage-specific assays, a signal-to-background (S/B) ratio of >3 was obtained; as well as acceptable signal-to-noise ratios (S/N >20) (Fig. 1B). Assay reproducibility was high as indicated by Z'-factors being consistently >0.8.
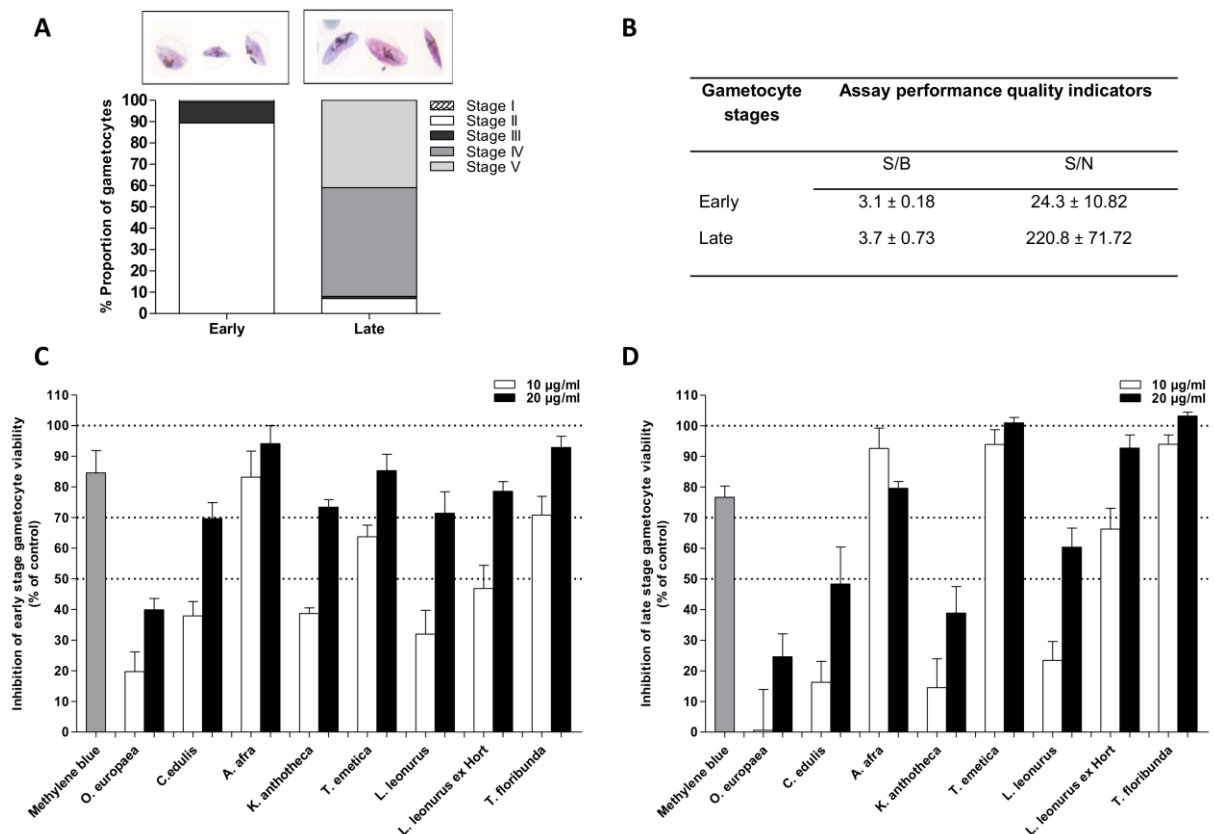
Figure 1. *In vitro* **inhibition of early and late stage *P. falciparum* NF54 gametocyte viability by crude acetone extract of plants.** (A) Stage-specific distribution of gametocyte populations in cultures used for dual point screens. (B) Assay quality indicator parameters. Dual point primary screens of eight different plant extracts at 10 and 20 μg/ml on (C) early and (D) late stage gametocytes (0.8-3% gametocytaemia), evaluated with a 72+72 hr pLDH assay. Data have been normalised against untreated gametocyte controls. Positive control drug used was methylene blue (10 μM). Results are the mean ±SEM of three independent biological repeats each done in triplicate.

Dual point evaluation (10 and 20 µg/ml) of crude extracts of the eight plant species on the *in vitro* viability of early stage gametocytes indicated dose dependent inhibition by the crude plant extracts (Fig. 1C). This reduction ranged between 20% to 94% with *O. europaea* and *A. afra* extracts having the lowest and highest activities, respectively. While seven plant extracts produced >70% inhibition of early stage gametocyte *in vitro* viability at 20 µg/ml, only three plant extracts (*A. afra*, *T. emetica* and *T. floribunda*) showed >50% inhibition at 10 µg/ml (Fig. 1C).

Results for late stage gametocyte screens mirrored those of early stages with a few exceptions. The plant extracts showed concentration dependent pattern of inhibition ranging from 99.4% to 0% (Fig. 1D). Extract from *O. europaea* was the least active while *A. afra*, *T. emetica*, *T. floribunda* and *L. leonurus* ex Hort extracts were the most potent. *K. anthotheca*, *O. europaea* and *C. edulis* extracts had a lower activity against the late stage gametocytes in comparison to the early stages. By contrast, *A. afra*, *T. emetica*, *T. floribunda* and *L. leonurus* ex Hort extracts demonstrated increased activity. The range of inhibition of gametocyte viability *in vitro* for these four species of plants was from 47-94% to 66-100% against the early and late stage gametocytes, respectively (Fig. 1C and D). Only four plant extracts (*A. afra*, *T emetica*, *T. floribunda* and *L. leonurus* ex Hort) displayed over 50% and 70% reduction on viability at either 10 or 20 µg/ml, respectively, against the late stage gametocytes (Fig. 1D). From the dual point assays, *A. afra*, *T. emetica*, *T. floribunda* and *L. leonurus* ex Hort extracts were selected for full dose-response and IC_{50} determination.

3.2.2 Gametocyte viability (IC_{50} determination)

Three of the four plant species investigated in the study exhibited IC_{50} values of less than 10 µg/ml against both early and late stage gametocytes of *P. falciparum* (Fig. 2). Methylene blue as positive drug control had *in vitro* gametocyte viability inhibition values of 88.2±1.1% and 87.4±2.7% against early and late stages, respectively. The assay performance for dose-response evaluation remained consistent with S/B and S/N ratios of 3-4 and 53-111, respectively.

Of the four selected plants species, *A. afra* was the most potent with IC_{50} values of 5.7±0.3 and 3.2±0.2 µg/ml against early and late stage gametocytes, respectively. This was closely followed by *T. emetica* (7.6±0.9 µg/ml – early stages; 3.8±0.5 µg/ml – late stages) and *T. floribunda* (9.2±0.2 µg/ml – early stages; 4.6±0.7 µg/ml – late stages) while *L. leonurus* ex Hort showed moderate activity (12.8±1.4 µg/ml – early stages; 13.9±2.6 µg/ml –

late stages). Interestingly, three of the four plant extracts tested *(A. afra, T. emetica* and *T. floribunda)* exhibited significantly higher (n = 3, P <0.05, unpaired Student's *t*-test) inhibition of late stage gametocyte viability in comparison to early stage gametocyte viability, as indicated by an approximately two-fold lower IC_{50} value (Fig. 2).
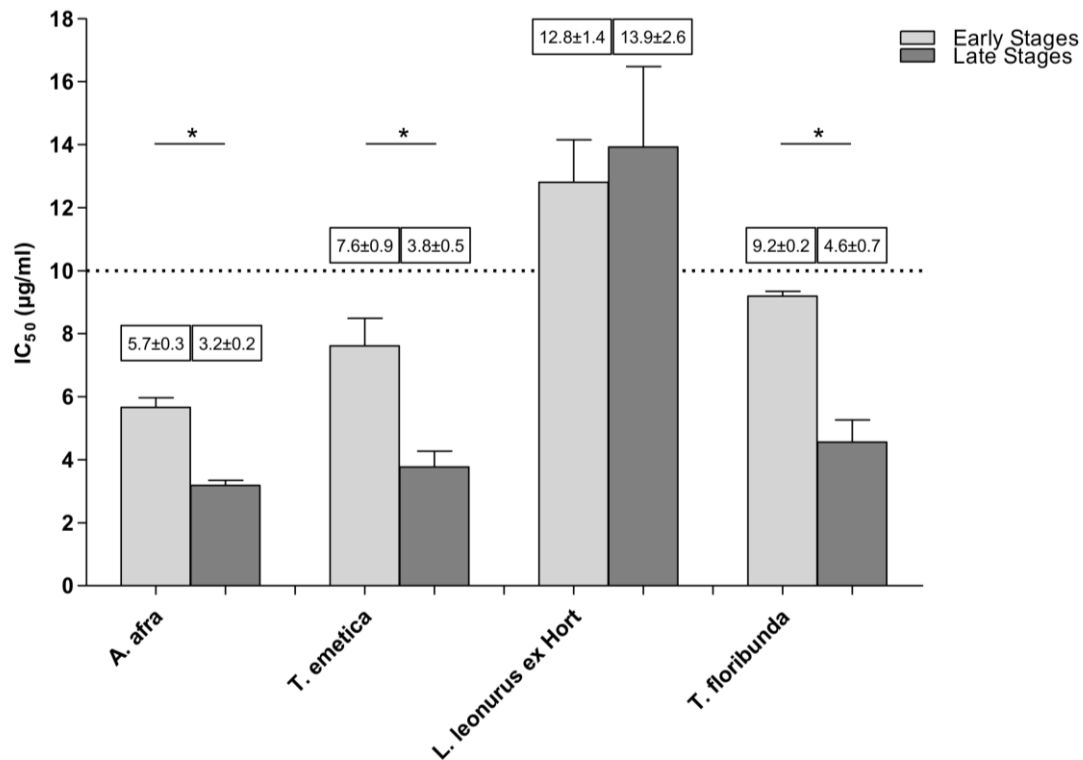

Figure 2. Dose-response evaluation of selected plant extracts against early and late stage *P. falciparum* NF54 gametocytes. IC_{50} values (in µg/ml) for plant extracts on early and late stage gametocytes (0.8-3% gametocytaemia), determined using the 72+72 hr pLDH assay. Positive drug control was methylene blue. Data are the mean ±SEM of three independent biological repeats each done in triplicate. Statistically significant differences between the IC_{50} values obtained against early compared to late stage gametocytes are indicated (* P < 0.05) as determined by Student's *t*-test.

3.2.2 Activity of plant extracts on gametocytes in comparison to asexual parasites

Plant extracts were additionally evaluated for their ability to block the proliferation of intraerythrocytic asexual *P. falciparum* NF54 parasites, compared to their inhibition of viability of both early and late stage gametocytes (Fig. 3). With varying degrees of potency, the plant extracts displayed polypharmacology by inhibiting both asexual parasites and early stage gametocytes (Fig. 3A). *O. europaea* extracts was poorly active against both gametocyte stages as well as asexual parasites (Fig. 3A). By contrast, *T. floribunda*, *A. afra*, *T. emetica*, *L. leonurus* ex Hort, *L. leonurus* and *C. edulis* extracts were all highly active against both asexual parasites and early stage gametocytes (≥70% inhibition of viability at 20 µg/ml). *K.*

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anthotheca extracts was highly active on the early stage gametocytes while being moderately effective against asexual parasites (between 50-70% inhibition of viability at 20 µg/ml).

Comparison of the inhibitory activity of plant extracts between late stage gametocytes and asexual parasites showed similarities to that of early gametocyte stages. *A. afra*, *T. emetica*, *T. floribunda* and *L. leonurus* ex Hort extracts were the most active against both late stage gametocytes as well as asexual parasites, exhibiting over 80% inhibition in both instances (Fig. 3B).
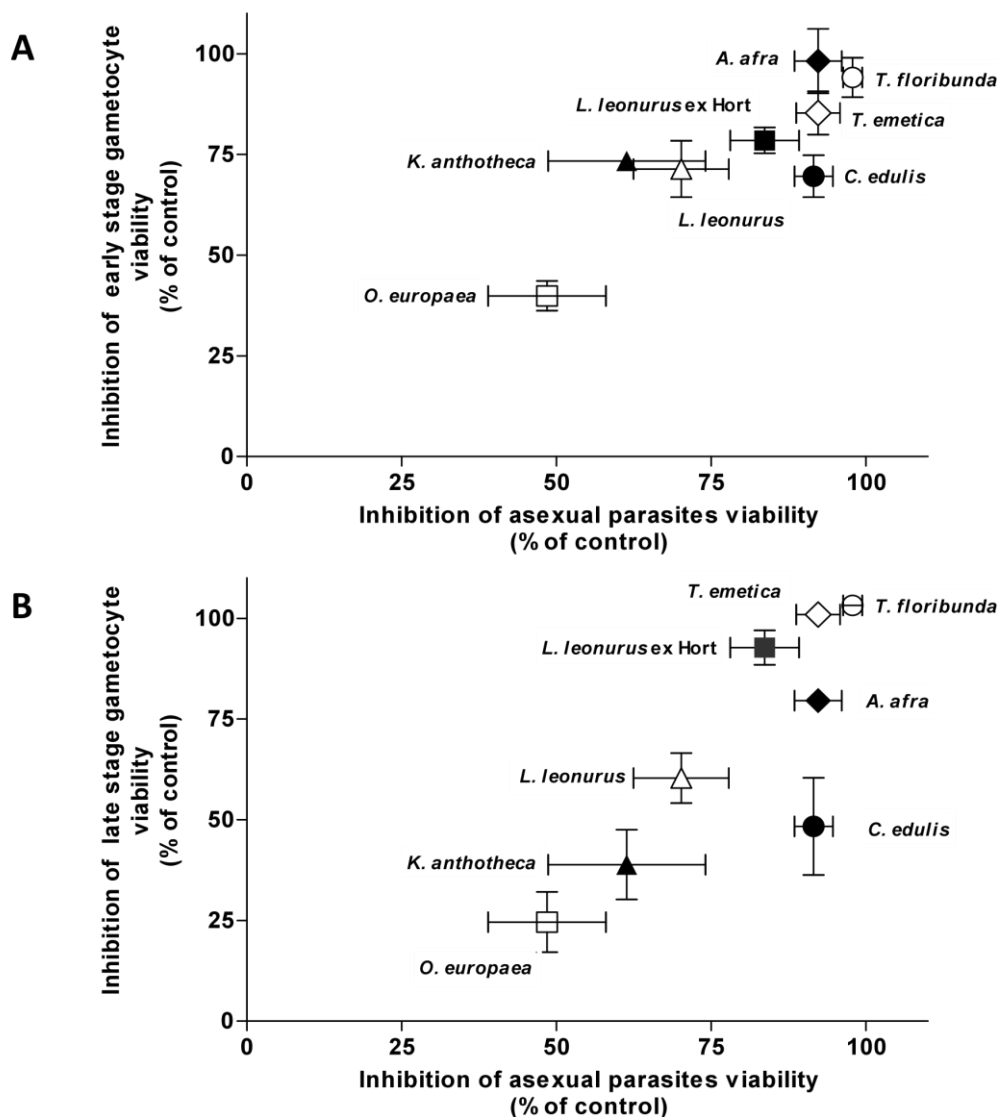
Figure 3. Polypharmacology of crude acetone plant extracts on the *in vitro* viability of both *P. falciparum* NF54 gametocytes and intraerythrocytic asexual parasites. Plant extracts were screened at 20 µg/ml using the pLDH assay for both gametocytes and asexual parasites. Inhibition of asexual parasite proliferation was compared to inhibition of either early (A) or late (B) gametocyte viability. Data are expressed as % viability of untreated controls and are the mean ±SEM of three independent experiments each done in triplicate. For asexual parasite assays, chloroquine (0.5 µM) was used as positive control inhibiting 89.2±5.3% parasite viability.

4.   Discussion

At the time malaria patients seek medication, there would already be late stage IV and V gametocytes circulating in their system (Eziefula *et al.*, 2014). Late stage gametocytes particularly make a scientifically credible stage to block transmission as they are the only form of *P. falciparum* capable of infecting a female *Anopheles* mosquito (Sinden *et al.*, 2012a). However, the currently available set of chemotherapeutics is largely only capable of impairing development of early stage gametocytes. The goal of discovering a single drug targeting all malaria stages (Burrows *et al.*, 2013) motivated the evaluation of the ability of plant extracts to inhibit both early and late stage gametocytes *in vitro*.

There are a number of plants that have been identified and documented in ethnobotanical studies for treating malaria. However, for bio-prospecting purposes, there is need for rational based criteria for prioritising plants to be screened for activity against gametocytes, similar to the approach used for screening against intraerythrocytic asexual parasites (Clarkson *et al.*, 2004). This has the advantage of narrowing down the number of plant species for a study in a systemic manner with a higher probability of getting hits. However, the disadvantage is that other species with gametocytocidal activity may then not be screened. Due to the vast pool of knowledge available on activity of plant extracts against asexual parasites, a criterion was set to screen those with good to moderate activity on asexuals against gametocytes. This approach is also being used in screening currently available malaria treatment drugs against gametocytes (Sinden *et al.*, 2012a). The second selection criterion was based on chemical classes of natural compounds produced by different species of plant families known to have gametocytocidal activity. The importance of extraction method, temperature and solvent is critical with significant consequences on activity as classically illustrated on the role they played in the discovery of artemisinin (Tu, 2011; Zhang, 2011). In our investigation we used a non-destructive shaking method at room temperature (Dzoyem and Eloff, 2015). We selected acetone as the solvent because of its ability to extract many compounds of different polarities from plants, its non-toxic nature and easy removal hence avoiding interference with subsequent assays (Eloff, 1998).

In this study, extracts from two species of the Meliaceae and one of the Asteraceae plant families had good activity against both early and late stage gametocytes. *T. emetica* and *T. floribunda* (from the Meliaceae family) both had IC$_{50}$ values below 10 µg/ml with greater potency towards late stages, which are generally regarded to be the least susceptible to drug treatment (White, 2008). *A. indica*, a species of the Meliaceae family, has been shown to be equipotent (IC$_{50}$ = 0.001 µg/ml) against both early and late gametocyte stages of *P. falciparum* (Udeinya *et al.*, 2006).

The limonoid, azadirachtin, was identified as the active principle in *A. indica* (Jones *et al.*, 1994). Its mode of action was deciphered to be through an interruption of mitotic spindle formation, a process of great significance in microgametogenesis (Billker *et al.,* 2002). Other limonoids such as gedunin, azadirone and azadiradione from *A. indica* have also been suggested to be gametocytocidal (Yerbanga *et al.*, 2014). The two Meliaceae species used in this study, *T. emetica* and *T. floribunda*, are known to produce limonoids, a common feature of this plant family (Roy and Saraf, 2006). This natural product chemical class may be responsible for the gametocytocidal properties of the crude acetone extracts of these plants observed in this study. Both these species were also highly active against intraerythrocytic asexual parasites, a common characteristic of *A. indica* (Udeinya *et al.*, 2006). However, *K. anthotheca*, also a member of the Meliaceae family and a known producer of limonoids (Roy and Saraf, 2006) was shown in this study to have a markedly variable efficacy on multiple stages of the malaria parasite. It has been suggested in other studies that limonoids could act as a scaffold upon which new multiple stage active antimalarial drugs could be designed (Yerbanga *et al.*, 2014). Further enthuse for working on this natural class of compounds is evident from the fact that it also significantly compromises the fitness of the mosquito vector (Dembo *et al.*, 2015).

A. afra (Asteraceae) is a widely used medicinal plant species in South Africa for the treatment of a number of different ailments (Philander, 2011; Van Wyk, 2011, 2008). While it is highly active against the asexual parasites (Clarkson *et al.*, 2004) there has been no report of its potency on *P. falciparum* NF54 gametocyte stages. However, *A. annua* (Asteraceae), that produces a sesquiterpene lactone, artemisinin, has gametocytocidal properties *in vitro* (Duffy and Avery, 2013; Lucantoni *et al.*, 2013). The genus *Artemisia* is well known for the production of sesquiterpene lactones (Al-khathlan *et al.*, 1992) and *A. afra* is no exception (Al-khathlan *et al.*, 1992; Jakupovic *et al.*, 1988) but there is no evidence for the presence of artemisinin in the latter specie (Van der Kooy *et al.*, 2008; Liu *et al.*, 2009). To our

knowledge none of the sesquiterpene lactones (or any other natural compounds from *A. afra*) were screened for activity against either early or late stage gametocytes of *Plasmodium*. *V. amygdalina* (Asteraceae) has been shown to be potent against *P. berghei* gametocytes *in vivo* with two sesquiterpene lactones having been identified as the active compounds (Abay *et al.*, 2015, 2013). It also has good activity against asexual parasites with an IC$_{50}$ value of 8.72 μg/ml against *P. falciparum* 3D7 strain (Zofou *et al.*, 2011). These sesquiterpene lactones may be responsible for the observed inhibition of gametocyte viability by the crude acetone extract of *A. afra*.

L. leonurus ex Hort (yellow) is a garden cultivar of the wild type *L. leonurus* (L.) R.Br. There is currently no documented report on the gametocytocidal activity of either of the two species or any family member. In this study, *L. leonurus* ex Hort (yellow) had the most pronounced activity of the two against both gametocytes and intraerythrocytic asexual parasites. Just as was the case with *T. emetica*, *T. floribunda* and *A. afra*, *L. leonurus* ex Hort extracts was equally highly potent against both gametocytes and asexual parasites. All plant extracts that were highly active against gametocytes (particularly late stages) were equally active against asexual parasites. This pattern of activity may suggest that selecting plants for screening against gametocytes based on their known activity against asexual parasites may be of merit. However, it must be noted that this observation has been made from a rather small data set and may not necessarily apply to all plant species. It is also worth taking into consideration that *A. afra*, *T. emetica* and *T. floribunda* were also selected based on the classes of compound they are known to produce in addition to inhibition of asexual parasites. Hence, this criterion may also be worth using in selecting other plant species to study.

A drawback of the pLDH assay is its inability to interrogate viability of male and female gametocytes separately in a culture containing both sexes. The implication is that extracts, which may be active against one specific sex, (in which case no complete inhibition is observed) may be flagged as false negatives (Sinden *et al.*, 2012b). Hence extracts from *C. edulis*, *L. leonurus* and *K. anthotheca* that have been used in this study may still be considered as potential transmission blocking agents.

5.   Conclusion

While extensive studies have been carried out in screening extracts of plants against *Plasmodium* intraerythrocytic asexual parasites, comparatively less screenings have been undertaken against gametocyte stages. The present study has given an insight into the gametocytocidal properties of traditional South African medicinal plant species extracts. To the best of our knowledge, this is the first report on the *in vitro* inhibition of gametocyte viability by the crude acetone extracts used in this study. Based on the findings of this study and those from the limited literature available, there is merit in channeling more effort towards screening extracts of plants in the transmission blocking agenda. The next step is to isolate and identify the active natural products especially from those plant species, namely *A. afra, T. emetica* and *T. floribunda*, shown here to inhibit both the asexual and late gametocyte stages. Subsequent studies would include the *in vivo* "gold standard" membrane-feeding assay, which will indeed provide additional data on the transmission blocking properties of these plant extracts.

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Supplementary material
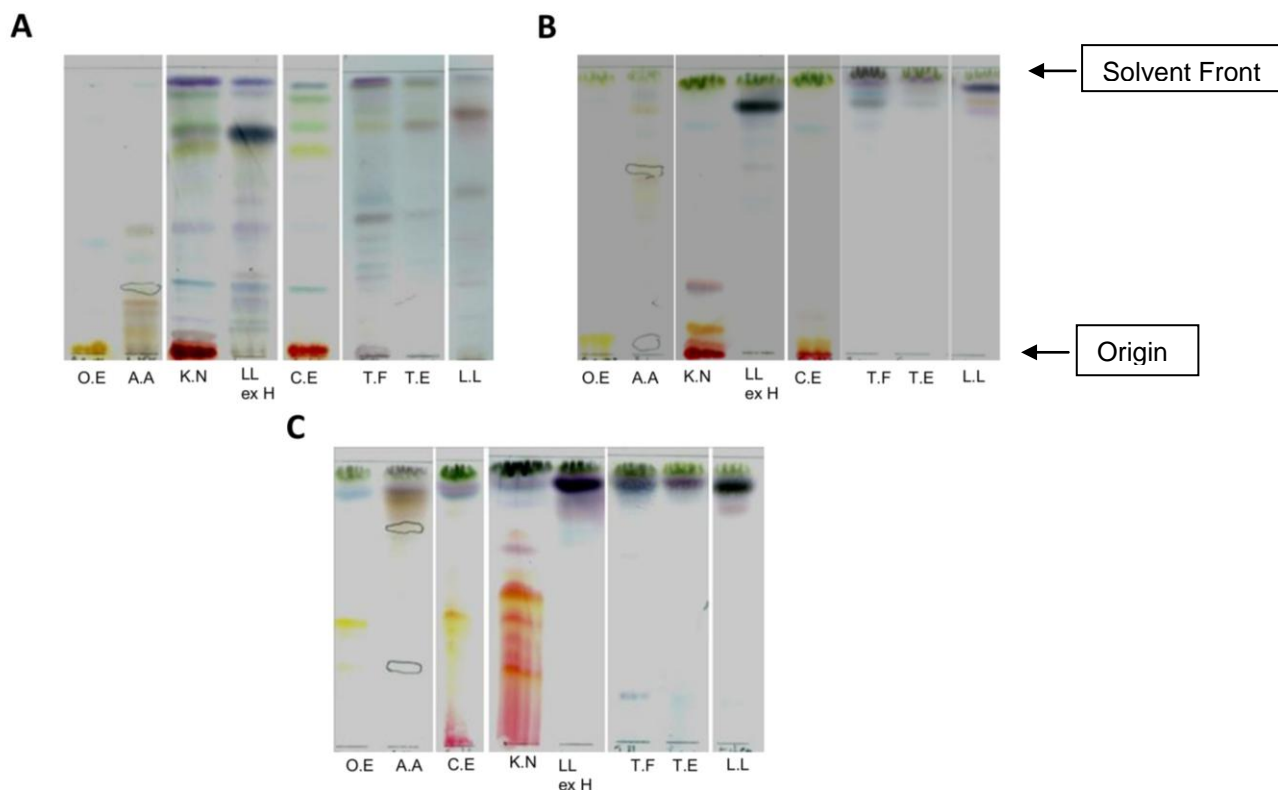
Figure 1S. Thin-layer chromatograms of crude acetone extracts from plants. Three different mobile solvent systems were used for developing the chromatograms. (A) benzene, ethanol and ammonium hydroxide solution (90:10:1), (B) chloroform, ethyl acetate and formic acid solution (50:40:10) and (C) ethyl acetate, methanol and water solution (40:6.5:5). Compounds were visualised with vanillin-sulphuric acid (Kotze and Eloff, 2002). Major compound classes identified include include phenols (yellow), terpenoids (purple or bluish purple) and stilbenes (bright red). *A. afra* was the only species observed to have compounds that fluoresce under UV light (circled) (O.E - *Olea europaea*; A.A - *Artemisia afra*; K.N - *Khaya anthotheca*; L.L ex *H - Leonotis leonurus* ex Hort; C.E - *Catha edulis*; T.F - *Turraea floribunda*; T.E - *Trichilia emetica*; L.L - *Leonotis leonurus*)