Accepted Manuscript

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PII: S0378-8741(08)00358-9
Reference: JEP 5150

To appear in: Journal of Ethnopharmacology

Received date: 3-6-2008
Revised date: 2-7-2008
Accepted date: 8-7-2008


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Phytochemistry and *in vitro* pharmacological activities of South African *Vitex* (Verbenaceae) species

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Received 5 June 2008; accepted in revised form 8 July 2008

Abstract

The *in vitro* antimicrobial, anti-oxidant, anti-inflammatory, antimalarial activities and toxicity profile of the crude extracts of five South African *Vitex* species (Verbenaceae); *V. obovata* ssp. *obovata*, *V. obovata* ssp. *wilmsii*, *V. pooara*, *V. rehmannii* and *V. zeyheri* were investigated. The antimicrobial activity was assessed using the minimum inhibitory
concentration assay on *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Enterococcus faecalis*, *Salmonella typhimurium* and *Cryptococcus neoformans*. All the species mostly showed good inhibition against Gram-positive bacteria (0.02 - 8.00 mg/ml) and lower activity against the Gram-negative bacteria and the yeast (0.50 - 8.00 mg/ml). Through bioactivity-guided fractionation, the fraction responsible for the antimicrobial activity of *V. rehmannii* was purified to give a labdane diterpene as an inseparable epimeric mixture of 12S,16S/R-dihydroxy-ent-labda-7,13-dien-15,16-olide. Circimaritin was also isolated and identified from *V. rehmannii*. This is the first report on the presence of both these compounds in *Vitex* species. The anti-oxidant activity was evaluated using the 2,2-diphenyl-1-picrylhydrazyl assay and all the species, apart from *V. zeyheri*, exhibited scavenging activity (IC$_{50}$: 22.14 ± 1.74 to 33.06 ± 1.68 µg/ml). To assess the anti-inflammatory activity of the extracts the 5-lipoxygenase assay was used, but none of the species exerted any activity at 100 µg/ml. The antimalarial activity of the extracts and the labdane diterpene isolated from *V. rehmannii* was also investigated on the chloroquine-resistant Gambian FCR-3 strain of *Plasmodium falciparum* using the tritiated hypoxanthine incorporation assay. All the extracts and the labdane diterpene exhibited good activity, with the labdane diterpene being the most active (IC$_{50}$: 2.39 ± 0.64 µg/ml). The toxicity profile of the extracts and the labdane diterpene was evaluated on human kidney epithelial cells using the tetrazolium cellular viability assay and all the test extracts were shown to be highly toxic, displaying safety index values ranging from 0.53 to 2.59.

**Keywords:** 12S,16S/R-Dihydroxy-ent-labda-7,13-dien-15,16-olide; Anti-inflammatory; Antimalarial activity; Antimicrobial; Anti-oxidant; Toxicity; *Vitex* spp.
Vitex has been reported to be used in traditional medicine to treat a wide range of ailments, such as depression, venereal diseases, malaria, asthma, allergy, wounds, skin diseases, snake bite and body pains (Neuwinger, 2000). In South Africa, an infusion of the leaves of *V. rehmannii* is administered by Zulu’s as an enema for stomachache, while that of *V. obovata* ssp. *wilmsii* is administered as an enema for body pains (Watt and Breyer-Brandwijk, 1962). Previous pharmacological studies carried out on various Vitex spp have illustrated its safety and efficacy to treat different diseases (You et al., 1998; Samy et al., 1998; Gupta et al., 1999; Hernandez et al., 1999; Taiwo et al., 1999; Berger et al., 2000; Hossain et al., 2001; Alam and Gomes, 2003; Dharmasiri et al., 2003), which has made *Vitex* a popular subject in phytochemical and ethnomedicinal research.

In our previous paper (Nyiligira et al., 2004), we reported on the chemical composition and *in vitro* antimicrobial and anti-inflammatory activity of the essential oils of South African *Vitex* spp. As part of our continuous pharmacological investigation on South African *Vitex* spp, we herein report the *in vitro* antimicrobial, anti-oxidant, anti-inflammatory, anti-malarial activities as well as the toxicity profile of solvent extracts from the above-mentioned five taxa. The pharmacological assays were selected on the basis of their anti-infective traditional...
use. Free radicals are often associated with various conditions such as inflammation and hence the anti-oxidant activity was included in the pharmacological assays. To our knowledge, this is the first report on the phytochemical and pharmacological investigation of the non-volatile compounds from South African *Vitex* spp.

2. Material and methods

2.1. Collection of plant material

Fresh plant material was collected from natural populations in South Africa and the taxonomy confirmed by the South African National Biodiversity Institute, Pretoria. Voucher specimens have been maintained in the Department of Pharmacy and Pharmacology, University of the Witwatersrand and duplicate copies have been deposited at the South African National Biodiversity Institute, Pretoria.

2.2. Preparation of extracts

Fresh aerial parts were dried in an oven at 30 °C for 12 h. The pulverized aerial parts (1 kg) were macerated overnight in acetone or methanol (2 L) at room temperature, and then filtered before the extraction solvent was evaporated at 35 °C. The acetone and methanol extracts were concentrated to dryness under reduced pressure and kept at 4 °C prior to analysis.

2.3. In vitro bioassays
2.3.1. Antimicrobial activity

The antimicrobial activity was performed on acetone extracts using the minimum inhibitory concentration (MIC) assay (Eloff, 1998). Three Gram-positive bacteria, Staphylococcus aureus (ATCC 6538), Bacillus cereus (ATCC 11778) and Enterococcus faecalis (ATCC 29212), two Gram-negative bacteria, Escherichia coli (ATCC 11775) and Salmonella typhimurium (ATCC 14028) and one yeast, Cryptococcus neoformans (ATCC 90112) were used. Two commercial antimicrobials, ciprofloxacin and amphotericin B, were used as controls for the bacteria and yeast, respectively.

2.3.2. Anti-oxidant activity

The anti-oxidant activity of both acetone and methanol extracts were assessed using a modified quantitative 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (Shimada et al., 1992). Vitamin C was used as a control.

2.3.3. Anti-inflammatory activity

The anti-inflammatory activity of acetone extracts was carried out using the 5-lipoxygenase assay (5-LO) (Baylac and Racine, 2003). Nordihydroguaiaretic acid (NDGA) was used as a control.

2.3.4. Antimalarial activity

The antimalarial activity of the acetone extracts was assessed on the chloroquine-resistant Gambian FCR-3 strain of Plasmodium falciparum using the tritiated hypoxanthine incorporation assay (Desjardins et al., 1979; Van Zyl and Viljoen, 2002). Chloroquine and quinine were used as controls.
2.3.5. Toxicity

The toxicity profile of the acetone extracts was assessed on Graham cells (transformed human kidney epithelium cells) using the tetrazolium (MTT) cellular viability assay (Mosmann, 1983; Van Zyl and Viljoen, 2002). Chloroquine and quinine were used as controls. The safety index values of the test compounds and controls were calculated with reference to the antimalarial activity.

2.4. Bioactivity-guided fractionation and isolation

The isolation of active compound(s) was based on bioactivity-guided fractionation and was performed on *V. rehmannii* using the bio-autographic assay (Meyer and Dilika, 1996). *Staphylococcus aureus* (ATCC 6538) was used as the test pathogen. The acetone extract (5 g) was subjected to silica gel 60 column chromatography with the eluent gradient of CHCl₃-CH₂OH (9.8:0.2 to 6:4) to afford fractions 1-210. Fractions 141-186 (218 mg) were rechromatographed on silica gel 60 to give fractions 211-376. After one night at 4 °C, fractions 263-277 afforded 5 mg of yellow crystals (compound 1). The crude acetone extract (20 g) was subjected to column chromatography on silica gel 60 and sequentially eluted with *n*-hexane-CH₂Cl₂ (9:1), CH₂Cl₂-CH₂OH (6:1) and methanol to give fractions 1-4. Fraction 2 (13.6 g) was rechromatographed over the same stationary phase using sequentially *n*-hexane-ethylacetate (6:4) and CH₂Cl₂-CH₂OH (6:1) to afford fractions 5-179. Fractions 19-89 (8 g) were subjected to silica gel column chromatography and eluted with CHCl₃-CH₂OH (9:0.2) to give fractions 180-320. Fractions 244-294 (2.9 g) was chromatographed on Sephadex LH-20 column with CHCl₃-CH₂OH (9:0.2) to afford compound 2 (white amorphous powder: 600 mg).
2.5. *High performance liquid chromatography analysis*

To determine if 1 and 2 were also present in the other investigated species, the acetone extracts of the five taxa were subjected to high performance liquid chromatography (HPLC)-mass spectrometry analysis using a Waters 2690 HPLC system (Phenomenex Aqua C18 column, 250 mm x 2.1 mm at 80 °C) equipped with a 996 photodiode array detector (PDA) and Thermabeam mass selective detector (TMD). The Thermabeam produced classical electron impact spectra which were compared against the commercial NIST® (version 2) MS spectra library. The samples were dissolved in methanol and injection (10 µl) was done under the following conditions: mobile phase flow rate: 0.2 ml/min, nebuliser gas flow: 30 l/h, nebuliser temperature: 80 °C, expansion region: 90 °C, and source of temperature: 225 °C. The initial mobile phase was 10% acetonitrile in 10 mM aqueous formic acid and the solvent ratio was changed through a linear gradient to 90% acetonitrile, 10% 10 mM aqueous formic acid at 40 min. This ratio was maintained for 10 min where after the solvent ratio was changed back to the initial starting conditions. The HPLC data of 1 and 2 were compared to those of the investigated species using Empower® software.

3. Results

3.1. Identification of isolated compounds

The mass spectrum of 1 has M⁺ at m/z 314 that corresponds to a molecular formula of C₁₇H₁₄O₆. A literature search revealed that the NMR data of 1 were similar to those of
Cirsimaritin (5,4’-dihydroxy-6,7-dimethoxyflavone, Fig. 1) isolated from *Microtea debilis* (Hasrat et al., 1997). The mass spectrum of 2a,b has M+ at m/z 334 of that corresponds to a molecular formula of C$_{20}$H$_{30}$O$_4$. From the NMR spectra of 2a,b, it was clear that the material was a mixture of two isomeric compounds. Compound 2a,b was found to be the labdane diterpene, 12S,16S/R-dihydroxy-ent-labda-7,13-dien-15,16-olide (Fig. 1), also isolated and identified from *Alomia myriadenia* (Zani et al., 2000). As also mentioned in the literature, the compound exists as an inseparable epimeric mixture.

The isolated compounds were subjected to nuclear magnetic resonance (NMR) spectroscopy analysis for identification. NMR spectroscopy experiments on 1 and 2a,b were performed on a Varian Inova 2000 300 MHz spectrometer. Chemical shifts were recorded in ppm referenced to tetramethylsilane or the solvent shift. Electron ionization MS of the compounds was performed by direct inlet at 70 eV on the GCMS-QP2010 gas chromatography-mass spectrometer.

Cirsimaritin (1) had $\delta_H$ (DMSO-d$_6$) 7.96 (2H, d, H-2’,6’), 6.93 (2H, d, H-3’,5’), 6.91 (1H, s, H-8), 6.84 (1H, s, H-3), 3.91 (3H, s, OMe), 3.72 (3H, s, OMe); $\delta_C$ 182.6 (C-4), 164.5 (C-2), 161.8 (C-4’), 159.0 (C-7), 153.1 (C-5), 152.5 (C-8a), 132.3 (C-6), 129.0 (C-2’,6’), 121.5 (C-1’), 116.5 (C-3’,5’), 105.5 (C-4a), 103.1 (C-3), 92.1 (C-8), 60.6 (OMe), 57.0 (OMe); m/z 314.

(12S,16S)-(12S,16R)-Dihydroxy-ent-labda-7,13-dien-15,16-olide (2a,b) (major isomer) had $\delta_H$ (CDCl$_3$) 6.13 (1H, s, H-16), 6.08 (1H, s, H-14), 5.51 (1H, s, H-7), 4.8 (1H, m, H-12), 1.79 (3H, s, H-17), 0.86 (3H, s, H-18), 0.87 (3H, s, H-19), 0.80 (3H, s, H-20); $\delta_C$ 173.0 (C-15), 172.0 (C-13), 133.9 (C-8), 123.5 (C-7), 117.0 (C-14), 98.3 (C-16), 68.6 (C-12), 49.8 (C-5, 9), 42.1 (C-3), 39.0 (C-1), 36.2 (C-10), 33.9 (C-12), 33.0 (C-4, 19), 23.8 (C-6), 22.3 (C-17), 21.8 (C-18), 18.7 (C-2), 13.6 (C-20); m/z 334.
3.1.2. HPLC analysis

Compound 1 was found in the NIST® (version 2) library and was also identified at m/z 314 as cirsimaritin, which matched with the NMR data, whereas 2a,b was not found in the NIST® library. The results of the HPLC analysis of 1 and 2a,b were found to be: (1) Rt: 31.4; UV (λ<sub>max</sub>): 214.4, 275.8, 334.1 nm; [M<sup>+</sup>] 314; (2a,b) Rt: 35.2; UV (λ<sub>max</sub>) 229.7 nm; [M<sup>+</sup>] 333.9.

The HPLC-UV-MS and the library data confirmed the presence of 1 in all the investigated species. The HPLC-UV-MS data also revealed that 2a,b may be present in the five taxa. Furthermore, α-tocopherol (vitamin E) was detected in the acetone extract of V. obovata ssp. wilmsii by reference to the NIST® library data (Rt: 51.3; UV [λ<sub>max</sub>]: 205.1, 293.4 nm).

3.2. In vitro biological activities

Vitex extracts, together with the isolated compound 2a,b were subjected to all biological assays with the exception of the anti-oxidant activity. Due to insufficient quantities compound 1 was excluded from biological testing.

3.2.1. Antimicrobial activity

The MIC assay (Table 1) revealed that all the species showed better activity towards the Gram-positive bacteria (0.02 mg/ml to 1.00 mg/ml), apart from Enterococcus faecalis which was found to be less sensitive (0.5 mg/ml to 8 mg/ml), and generally had lower activity against the Gram-negative bacteria and the yeast (0.50 mg/ml to 8.00 mg/ml). Escherichia coli was shown to be the most resistant pathogen to both extracts and compound 2a,b. V. rehmannii and V. obovata ssp. obovata extracts having similar activity, exerted superior
sensitivity to all the tested pathogens with the exception of *V. rehmannii* exhibiting more pronounced activity towards *Salmonella typhimurium*.

### 3.2.2. Anti-oxidant activity

Both the acetone and methanol extracts of *V. rehmannii*, *V. obovata* ssp. *obovata*, *V. obovata* ssp. *wilmsii* and *V. zeyheri* methanol extract showed free radical scavenging activity ranging from 22.14 to 30.56 µg/ml. Whilst *V. pooara* did not exhibit any anti-oxidative activity at 100 µg/ml for both the methanol and acetone extracts. The results of the *in vitro* assay revealed that the acetone extracts possessed slightly greater anti-oxidative properties than those of the methanol extracts.

### 3.2.3. Anti-inflammatory activity

All the extracts were found to be inactive at the starting concentration of 100.00 µg/ml, while NDGA (control) had an IC$_{50}$ of 5.00 µg/ml.

### 3.2.4. Antimalarial activity

The South African *Vitex* spp showed significant activity against *P. falciparum* chloroquine-resistant FCR-3, with IC$_{50}$ values ranging from 9.16 ± 1.37 µg/ml to 16.02 ± 3.07 µg/ml. 2a,b was the most active amongst the test compounds, with an IC$_{50}$ value of 2.39 ± 0.64 µg/ml.

### 3.2.5. Toxicity

The results of the toxicity assay (Table 1) revealed that the test compounds were toxic, with 2a,b being the most toxic (IC$_{50}$: 1.27 ± 0.21 µg/ml). *V. pooara* was shown to be the least
toxic with IC$_{50}$ value of 34.10 ± 6.86 µg/ml, while *V. rehmannii* and *V. obovata* ssp. *obovata* displayed the highest toxicity among the five taxa.

4. Discussion

4.1. Antimicrobial activity

The results of the MIC assay (Table 1) show that the extracts exhibited antimicrobial activity ranging from 0.02 mg/ml to 8.00 mg/ml. A MIC value < 8.00 mg/ml is considered as a significant antibacterial activity for plant extracts (Fabry et al., 1998) and based on this value, we can conclude that the *Vitex* extracts generally exerted some antimicrobial activity against all the tested pathogens, apart from *Salmonella typhimurium* towards which all the species, except *V. rehmannii* (MIC: 1.00 mg/ml), showed very low activity (MIC: 8.00 mg/ml). All the extracts exhibited higher activity towards Gram-positive bacteria than Gram-negative bacteria, a trend that was also observed in previous *in vitro* studies with plant extracts (Grosvenor et al., 1995; Martin, 1995; Valsaraj et al., 1997). The bioactivity-guided fractionation revealed that the antimicrobial activity as exhibited by *Staphylococcus aureus* for *V. rehmannii* was associated with compound 2a,b, which was identified as an inseparable epimeric mixture of the labdane diterpene 12S,-16S/R-dihydroxy-ent-labda-7,13-dien-15,16-olide. To the best of our knowledge, this is the first report on the isolation of 12S,16S/R-dihydroxy-ent-labda-7,13-dien-15,16-olide (2a,b) and cirsimaritin (1), from the genus *Vitex*. The broad *in vitro* antimicrobial activity observed for this labdane diterpene is in agreement with a previous study (Singh et al., 1999) in which interesting antimicrobial activity was reported for other labdane diterpenes.
HPLC analyses showed that compound 2a,b is present in all the investigated species, and, therefore, it can be concluded that 12S,16S/R-dihydroxy-ent-labda-7,13-dien-15,16-olide (2a,b) contributes (in part) to the antimicrobial activity of the South African Vitex spp. Various authors have reported that 12S,16S/R-dihydroxy-ent-labda-7,13-dien-15,16-olide (2a,b) is cytotoxic (Zani et al., 2000; Scio et al., 2003; Souza-Fagundes et al., 2003), and it is probable that its antimicrobial activity is associated with general cytotoxicity. To verify this, the toxicity profile of this compound was also investigated and the results are presented in Table 1.

Our results are in accordance with previous reports, which also revealed the antibacterial activities of other Vitex spp, such as V. trifolia (Hossain et al., 2001), V. negundo (Samy et al., 1998), V. doniana (Taiwo et al., 1999). The antimicrobial activity exhibited by the investigated species, especially their greater activity against Staphylococcus aureus, which is one of the Gram-positive bacteria responsible for skin infections in humans, may provide a scientific basis for the ethnomedical use of Vitex spp.

4.2. Anti-oxidant activity

The results presented in Table 1 show that both the acetone and methanol extracts of all the investigated Vitex spp exhibited some DPPH scavenging activity. According to Cuvelier and co-authors (1996), cirsimaritin was one of the eight compounds found in the anti-oxidant extracts. Thus cirsimaritin may contribute to the anti-oxidant activity observed with the acetone and methanol extracts. Moreover, vitamin E, one of the major non-enzymatic anti-oxidants (Thabrew et al., 2001), was detected in the acetone extract of V. obovata ssp. wilmsii.

From the above-mentioned findings, we propose that the anti-oxidant activities exerted by the various Vitex spp may be related to the presence of flavonoids, such as cirsimaritin,
which together with other anti-oxidant compounds such as vitamin E and the diterpenes contribute to the overall of the activity. Our findings on the anti-oxidant properties of these South African indigenous *Vitex* spp are in agreement with those reported for *V. rotundifolia* (Ono et al., 1999) and *V. negundo* (Dharmasiri et al., 2003).

4.3. Anti-inflammatory activity

None of the extracts were active in the 5-LO assay. However, previous studies (You et al., 1998; Alam and Gomes, 2003; Dharmasiri et al., 2003) revealed the anti-inflammatory activity of other *Vitex* spp. Furthermore, cirsimaritin (1) present in the five investigated taxa has been shown to be a potent inhibitor of 5-LO (92% of the activity) at 1 µM using a TLC method (Yoshimoto et al., 1983; Yamamoto et al., 1984). Thus, due to the presence of cirsimaritin, the studied species were presumed to possess anti-inflammatory activity. However, it should be remembered that the lack of activity could be attributed to the low concentration used in this assay. In addition, the anti-inflammatory pathway comprises not only of the 5-LO enzyme but of at least six others (cyclo-oxygenases [COX]-1, -2 and -3; 12- and 15-LO and cytochrome P450 epoxygenase) (Belton and Fitzgerald, 2003). Thus although these five *Vitex* spp are not specific inhibitors of the 5-LO enzyme, they may have activity against any of the other inflammatory enzymes. This variable specificity is best illustrated by different affinity of two iridoids, pedunculariside and agnuside, both isolated from *V. peduncularis* stem bark, towards COX-1 and COX-2 (Suksamrarn, 2002). Although it has been reported that *V. obovata* ssp. *wilmsii* is used in Zulu traditional medicine for its anti-inflammatory properties (Watt and Breyer-Brandwijk, 1962), this effect could be obtained if substantially more of the plant material is used by the traditional healers or if the plant is targeting another enzyme in the inflammatory pathway.
4.4. Antimalarial activity

All the investigated *Vitex* spp and 2\textsubscript{a,b} showed promising anti-plasmodial activity. However, given the general cytotoxicity of 12\textsubscript{S},16\textsubscript{S}/R-dihydroxy-\textit{ent}-labda-7,13-dien-15,16-olide (Zani et al., 2000; Scio et al., 2003; Souza-Fagundes et al., 2003) and its possible presence in all the investigated species, the antimalarial activity exerted by the extracts of the South African *Vitex* spp could primarily have been due to the cytotoxic properties of 12\textsubscript{S},16\textsubscript{S}/R-dihydroxy-\textit{ent}-labda-7,13-dien-15,16-olide (2\textsubscript{a,b}) rather than specific antimalarial properties.

4.5. Toxicity

The results of the MTT assay on transformed human kidney epithelium cells (Table 1) show that the test extracts are toxic. This is confirmed by the low values of the safety index which vary from 0.50 to 2.59 µg/ml, with 12\textsubscript{S},16\textsubscript{S}/R-dihydroxy-\textit{ent}-labda-7,13-dien-15,16-olide (2\textsubscript{a,b}) being the most toxic. Our results are in agreement with previous studies in which the cytotoxicity of 2\textsubscript{a,b} was reported (Zani et al., 2000; Scio et al., 2003; Souza-Fagundes et al., 2003). Based on the highest toxicity profile of the labdane 2\textsubscript{a,b} (safety index: 0.5), it can be concluded that 12\textsubscript{S},16\textsubscript{S}/R-dihydroxy-\textit{ent}-labda-7,13-dien-15,16-olide is one of the compounds responsible for the toxicity of the South African *Vitex* spp. However, it is necessary to determine the levels of 12\textsubscript{S},16\textsubscript{S}/R-dihydroxy-\textit{ent}-labda-7,13-dien-15,16-olide (2\textsubscript{a,b}) present in *Vitex* spp and correlate this to amount of plant material administered in traditional herbal preparations to establish efficacy and toxic levels of the labdane diterpene.
Acknowledgments

The National Research Foundation (Indigenous Knowledge Systems) and the Medical Faculty Research Endowment Fund, University of the Witwatersrand are gratefully acknowledged for financial support. We are also thankful to Robertet (Grasse, France) for assistance in setting up the 5-lipoxygenase assay, and we are indebted to Dr C.L. Bredenkamp and Ms P.M. Burgoyne (NBI) for the identification and assistance with the collection of the plant material.

References


Fig. 1. Structures of cirsimaritin (1) and 12S,16S/R-Dihydroxy-ent-labda-7,13-dien-15,16-olide (2a,b).
Table 1

Pharmacological assays on *Vitex* extracts and isolated compound (n = 3).

<table>
<thead>
<tr>
<th>Vitex extracts and compound</th>
<th>Antimicrobial activity: MIC values (mg/ml)</th>
<th>Antioxidant activity: IC50 (µg/ml) ± s.d</th>
<th>Antimalarial activity: IC50 (µg/ml) ± s.d</th>
<th>Toxicity: IC50 + safety index values</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V. obovata</em> sub ssp. Obovata</td>
<td>0.02</td>
<td>0.02</td>
<td>0.50</td>
<td>4.00</td>
</tr>
<tr>
<td><em>V. obovata</em> ssp. Wilmsii</td>
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<tr>
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<tr>
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<td>0.02</td>
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<tr>
<td><em>V. zeyheri</em></td>
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<td>1.00x10^-3</td>
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</tr>
<tr>
<td>Controls</td>
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<td>6x10^-5</td>
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<td>(a)</td>
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</tr>
</tbody>
</table>

n = number of repetitions; s.d = standard deviation; S.a = *Staphylococcus aureus*; B.c: *Bacillus cereus*; E.c = *Escherichia coli*; E.f = *Enterococcus faecalis*; S.t = *Salmonella typhimurium*; C.n = *Cryptococcus neoformans*; Ac = acetone extract; Me = methanol extract; a = ciprofloxacin; b = amphotericin B; c = vitamin C; d = chloroquine; e = quinine; nd = not determined due to insufficient quantities