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Constituents From the Stem Bark of *Cinnamomum zeylanicum* Welw. (Lauraceae) and Their Inhibitory Activity Toward *Plasmodium falciparum* Enoyl-ACP Reductase Enzyme

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Abstract: A *trans* cinnamic anhydride (**1**) isolated for the first time from a natural source, along with the known compounds: ferrulic acid (**2**), (*E*) *p*-hydroxy cinnamic acid (**3**), clovanediol (**4**), squalene (**5**), and α-bisabolene (**6**) were isolated from the stem bark of *Cinnamomum zeylanicum*. Their structures were elucidated by interpretation of NMR and MS data, and by comparison of these data with those reported in the literature. Their inhibitory properties were evaluated against *Plasmodium falciparum* enoyl-ACP reductase (*Pf*ENR) enzyme. Compounds **1**, **5** and **6** showed moderate inhibitory activity against *P. falciparum* enoyl-ACP reductase (*Pf*ENR) enzyme.

Keywords: *Cinnamomum zeylanicum;* Lauraceae; *Plasmodium falciparum* enoyl-ACP reductase (*Pf*ENR) enzyme; antimalarial activity.

1. Introduction

Cinnamomum zeylanicum Welw. (Lauraceae) also known as "the cinnamon of commerce", is native to Sri Lanka and tropical Asia [1]. The tree occurs in South India up to altitudes of 500 m but mostly below 200 m [1]. The barks are commonly used in Cameroon as spices and for the treatment of cardiovascular diseases [2]. It has been found that cinnamaldehyde (75 %) is the major compound in the oil of the stem bark while camphor (56%) is the major compound in the root bark [3]. The bark oil is a flavoring ingredient used widely in confectionery, baked foods, pickles, meat seasonings, soft

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drinks (cola-type), pharmaceuticals, oral-care products, etc. [1]. They are also used in traditional medicine to treat male impotence, digestive disorders, and hypertension, but also to ease delivery in childbirth [2]. Previous phytochemical studies have revealed the presence of phenolic compounds in the fruits [4] and some chemical constituents such as (*E*)-cinnamyl acetate, *trans-alpha*-bergamoten and caryophyllen oxide in the flower oil [5].

As part of our ongoing search for antimalarial compounds from Cameroonian medicinal plants, a *trans* cinnamic anhydride (**1**) isolated for the first time from a natural source together with five other known compounds (**2**-**6**) were isolated from the CH_2Cl_2/MeOH (1:1) extract of the stem bark of *C. zeylanicum*. The isolation and the structural elucidation of (**1**) are herein reported as well as the enzyme-inhibitory properties of the isolated compounds toward *Plasmodium falciparum* enoyl-ACP reductase (*Pf*ENR) enzyme.

2. Materials and Methods

2.1. Plant material

The stem bark of *C. zeylanicum* was collected in Djombé (Littoral Region of Cameroon) in April 2007 and authentified in the Cameroon National Herbarium, Yaoundé where a voucher specimen was deposited under the reference SRFC/22309. The plant material was dried, finely powdered, and used for the successive extraction.

2.2. General experimental conditions

Melting points were obtained on a Gallenkamp melting point apparatus. UV spectra were recorded on a Hitachi UV 3200 spectrophotometer. A JASCO 320-A spectrophotometer was used for scanning IR spectroscopy using KBr pellets. 1D and 2D NMR spectra were run on a Bruker spectrometers operating at 400 and 500 MHz, where chemical shifts (δ) were expressed in ppm with reference to the solvent signals. Column chromatography was performed on silica gel 230-400 mesh (Merck). Fractions were monitored by TLC using precoated aluminum-backed silica gel 60 F_{254} sheets. Spots were visualized under UV light (254 and 365 nm) and then sprayed with ceric sulfate reagent followed by heating at 100 °C. The FT APCI MS spectrum was obtained with an LTQ-Orbitrap Spectrometer (Thermo Fischer, USA). The spectrometer was operated in positive mode (1 spectrum s^{-1}; mass range: 150-800; with nominal mass resolving power of 60 000 at *m/z* 400 at a scan rate of 1 Hz, spray voltage 5 kV, tube lens 80 V) with automatic gain control to provide high-accuracy mass measurements within 2 ppm deviation using an internal calibration standard; Bis(2-ethylhexyl)phthalate: *m/z* 391.284586. The spectrometer was equipped with a surveyor HPLC system (Thermo Fisher, USA) consisting of LC-pump, PDA detector, and auto-sampler (injection volume 10 μL). Nitrogen was employed as both the sheath (50 arbitrary units) and auxiliary (10 arbitrary units) gas and helium served as the collision gas. The capillary temperature for the TSQ was set to 190 °C. The vaporizer temperature was set to 400 °C. Sample was analyzed using a gradient program as follows: 95% C isocratic for 4 min, linear gradient to 0% C over 20 min, after 100% D isocratic for 13 min, the system returned to its initial condition (95% C) within 1 min, and was equilibrated for 7 min. The EIMS spectrum was recorded on Argilent Technologies 6890N Network GC System with 5975 Inert XL Mass Selective Detector GCMS spectrometer.

2.3. Extraction and isolation

The dried and powdered stem bark of *C. zeylanicum* (2 kg) was extracted three times (each time for 48 h) with the mixture of CH_2Cl_2/MeOH (1:1). The filtrate obtained was concentrated *in vacuo* to yield a dark brown residue (220 g). This extract was successively extracted with *n*-hexane and EtOAc, yielding 10 g and 12 g of respective extracts after evaporation to dryness. On the basis of TLC, *n*-hexane extract and EtOAc extract were combined to afford *n*-hexane/EtOAc extract (22 g). This extract was subjected to silica gel (63-200 μm) column chromatography, using increasing polarity (0-

100 %) of cyclohexane-EtOAc and EtOAc-MeOH, to give five main fractions (1-5). Fractions 1 (3.2 g), and 2 (1.8 g) submitted to GC-MS analysis revealed the presence of fatty acids and were not further investigated. Fraction 3 (2 g) was purified by column chromatography over silica gel using gradient of cyclohexane-EtOAc to yield compounds **4** (11 mg) and **5** (10 mg). Fraction 4 (2.5 g) was rechromatographed over Sephadex gel LH-20 with CH_2Cl_2/MeOH (9:1) as eluent to afford compounds **1** (20 mg) and **6** (16 mg). Fraction 5 (3 g) was subjected to column chromatography over silica gel using cyclohexane/CH_2Cl_2 (1:1) as solvent to afford compounds **2** (16 mg) and **3** (21 mg).

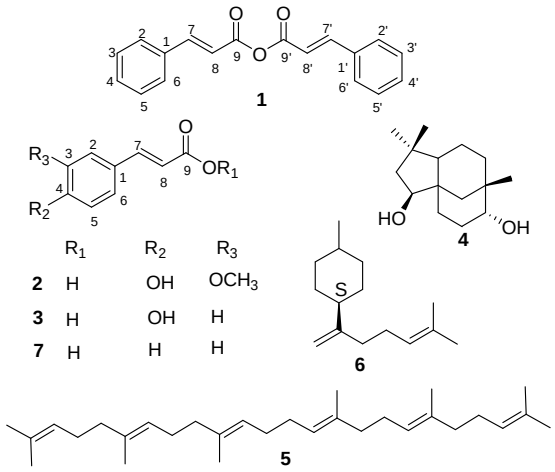

Figure 1. Chemical structures of compounds **1-7**.

*2.4. Acid hydrolysis of compound **1***

A solution of compound **1** (2 mg in 2 mL of pyridine in a 50 mL round flask) and 10 mL of HCl 5 % was refluxed at 60 °C for 14 hours. The solution was kept for 24 hours to evaporate pyridine. The remaining was concentrated and TLC indicated one spot compound. This was identified by analysis of its mass spectrum and ^{1}H NMR to *trans* cinnamic acid **7** (mp: 132-134 °C).

2.5. Biological activitiy

2.5.1. In Vitro P. falciparum Enoyl-ACP Reductase Assay

P. falciparum enoyl ACP reductase was over expressed, purified [6], and assayed as described earlier [7]. All chemicals and reagents were of analytical grade. *β*-NADH, crotonoyl CoA and DMSO were obtained from Sigma and the inhibitory activity was measured at 340 nm following the manufacturer's instructions. *Pf*ENR was kindly donated by Dr. Sean T. Prigge from Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland. All experiments were performed in 50 mM phosphate buffer (pH = 7.5), 100 mM NaCl. The isolates as well as triclosan were dissolved in DMSO and tested in 100 μL standard assay mixtures containing 0.1 mM NADH, 0.15 mM crotonoyl CoA and 170 nM *Pf*ENR in 96-well microtiter plates using SpectraMax micro plate spectrophotometer (Molecular Devices, CA, USA). The oxidation of NADH to NAD^+ was monitored at 25 °C for 10 min by following the change in absorbance at 340 nm. The inhibitory activity was further converted by SOFTmax PRO software (Molecular devices, Sunnyvale, CA) into percentage inhibition data using the following formula: % of inhibition = [(E - S)/E] X 100, where E is the activity of enzyme without test material and S is the activity of enzyme with test material. Percentage inhibition data were fitted to a log concentration curve of isolates using nonlinear regression.

3. Results and Discussion

3.1. Structure elucidation

The CH_2Cl_2/MeOH (1:1) extract of the stem bark of *C. zeylanicum* was fractionated and purified on silica gel and Sephadex LH-20 columns, successively, to afford 6 compounds: *trans* cinnamic anhydride (**1**), ferrulic acid (**2)** [8], (*E*) *p*-hydroxy cinnamic acid (**3)** [9], clovanediol (**4)** [10], squalene (**5**) [11], and α-bisabolene (**6)** [12]. Their structures (Figure 1) were elucidated by interpretation of NMR and MS data, and by comparison of these data with those reported in the literature.

Compound **1** was isolated as a white solid, mp 165-167 °C. The UV spectrum exhibited absorption bands at λ_{max} 228 and 212 nm characteristic of a conjugated aromatic system [13]. The IR spectrum showed absorption bands at ν_{max} 1682 (ester carbonyl), 1630 (Ar) and 1286 cm^{-1} (C-O). The molecular formula, $C_{18}H_{14}O_3$, with twelve degrees of unsaturation, was deduced from FT-APCI-MS data which showed a pseudomolecular ion peak $[M + H]^+$ at *m/z* 279.1014 (calcd 279.1021 for $C_{18}H_{15}O_3$). With the molecular formula $C_{18}H_{14}O_3$, and considering the fact that signals for only 7 protons and 9 carbons (instead of 14 protons and 18 carbons as per the mass spectrum) were observed in the respective ^1H and ^{13}C NMR spectra, let us to assume that compound **1** was symmetrical. This assumption was supported by the EIMS data where ion fragments were observed at *m/z* 147 [*M* - 131] and 131 [*M* - 147], respectively corresponding to $[M - C_9H_7O]^+$ and $[M - C_9H_7O_2]^+$, indicative of an oxygen atom to be the symmetric center. The ^1H-NMR spectrum (Table 1) exhibited signals of aromatic protons at δ_H 7.54 (4H, dd, *J* = 6.0; 2.4 Hz, H-2/2', H-6/6'), 7.39 (4H, overlapped, H-3/3', H-5/5'), 7.38 (2H, overlapped, H-4/4'), indicating the presence of two similar mono substituted aromatic rings. This spectrum also showed two doublets at δ_H 7.76 (2H, *J* = 16.0 Hz, H-7/7') and 6.43 (2H, *J* = 16.0 Hz, H- 8/8'), typical of deshielded *trans* olefinic protons of an α,β-unsaturated carbonyl group [14, 15]. These data suggested the cinnamic nature of compound **1**. This was confirmed by the ^{13}C-NMR spectrum (Table 1) which showed signals of aromatic carbons at δ_C 134.3 (C-1/1'), 131.0 (C-4/4'), 129.3 (C-3/3', C-5/5') and 128.6 (C-2/2', C-6/6'), signals of two olefinic carbons at δ_C 147.3 (C-7/7') and 117.3 (C-8/8') and signal of a typical anhydride carbonyl group at δ_C 159.1 (C-9/9'). The HRMS was used to confirm the dimeric structure of the compound. Compound **1** was thus characterized as the new naturally occurring *trans* cinnamic anhydride or (*E*)-2-phenyl-2-propenoic anhydride. Several cinnamic acid derivatives have being identified as constituent of oils from different parts of *Cinnamomum zeylanicum* [3, 5, 16].

In addition to the above new naturally occuring *trans* cinnamic anhydride (**1**), five known compounds (**2-6**) were isolated and acidic hydrolysis of (**1**) was carried out to furnish *trans* cinnamic acid (**7**). The structures of known compounds were established by comparison of their NMR and MS data with those reported in the literature.

Table 1. ^1H (400 MHz) and ^{13}C (100 MHz) NMR data of compound **1** in $CDCl_3$ [δ (ppm), *J* (Hz)]

Position	δ_C	δ_H
1/1'	134.3	-
2/2'	128.6	7.54 (2H, *dd*, *J* = 6.0; 2.4)
3/3'	129.3	7.39 (2H, overlapped)
4/4'	131.0	7.38 (2H, overlapped)
5/5'	129.3	7.39 (2H, overlapped)
6/6'	128.6	7.54 (2H, *dd*, *J* = 6.0; 2.4)
7/7'	147.3	7.76 (2H, *d*, *J* = 16.0)
8/8'	117.3	6.43 (2H, *d*, *J* = 16.0)
9/9'	159.1	-

Trans cinnamic anhydride (**1**): White solid; mp: 165-167 °C; IR (KBr) υ_{max}: 3024, 2973, 1682, 1630, 1286 cm^{-1}; ^1H and ^{13}C-NMR data, see Table 1; FT APCI MS: *m/z* 279.1014 (Calcd. for $C_{18}H_{15}O_3$: 279.1021**)**; EIMS: *m/z* 147 ($[M - C_9H_7O]^+$, 100), 131 ($[M - C_9H_7O_2]^+$, 22), 103 ($[C_8H_7]^+$, 54), 77 (39), 51 (31), 27 (3).

3.2. Plasmodium falciparum enoyl-ACP reductase enzyme inhibition activity

Malaria causes death of about 1 - 3 million people every year, mostly children below the age of 5 years in Sub-Saharan Africa [17]. Of the four species that cause human malaria, *P. falciparum* is responsible for most of the deaths [18]. The development of resistance to the existing drugs such as chloroquine, has created an urgent demand for new antimalarial agents [19, 20].

The parent CH_2Cl_2/MeOH (1:1) extract, the combined *n*-hexane-ethyl acetate-soluble fraction as well as compounds **1-7** were evaluated for their inhibitory properties against *P. falciparum* enoyl-ACP reductase enzyme, at 50 μM (Table 2). Compounds **1**, **5** and **6** showed moderate activity against *P. falciparum* as they inhibited the activity of the enzyme with 17.0, 24.3 and 33.3 %, respectively. Compounds **2**, **4** and **7** were not active while the CH_2Cl_2/MeOH (1:1) extract and the combined *n*-hexane-ethyl acetate-soluble fraction were weakly active. The activity of **1** may due to its anhydride nature compared to the acidic nature of compounds **2** and **3**. This may also justify the inactivity of *trans* cinnamic acid (**7**), obtained from compound **1** by acid hydrolysis. Thus the anti-plasmodial activity of the *n*-hexane/EtOAc extract from the stem bark of *C. zeylanicum* could be attributed to compounds **1**, **5**, **6**. The anti plasmodial activity was observed to decrease from the parent extract to the isolated compounds.

Table 2. *In vitro* inhibitory effect of compounds **1-7** and extracts on *Plasmodium falciparum* enoyl-ACP reductase enzyme (concentration 0.05 nM)

Compounds	**% inhibition**
1	17
2	Inactive
3	6.1
4	Inactive
5	33.3
6	24.3
7	Inactive
C/M*	3.1
H/AE**	3.7
Triclosan[b]	99.5

* CH_2Cl_2/MeOH (1:1) extract of the stem bark of *Cinnamomum zeylanicum*

**mixture of hexane and ethyl acetate extract of the stem bark of *Cinnamomum zeylanicum*

[b] Standard used for the assay

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP.

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