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Chemical composition and *in vitro* antitrypanosomal activity of fractions of essential oil from *Cymbopogon nardus* L.

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Abstract. Essential oil from *Cymbopogon nardus* was evaluated for activity against *Trypanosoma brucei brucei* BS221 (IC_{50} = 0.31 ± 0.03 µg/mL) and cytotoxic effect on normal kidney (Vero) cells (IC_{50} = >100 µg/mL). The crude essential oil was subjected to various chromatography techniques afforded active sub fractions with antitrypanosomal activity; F4 (IC_{50} = 0.61 ± 0.06 µg/mL), F6 (IC_{50}= 0.73 ± 0.33 µg/mL), F7 (IC_{50} = 1.15 ± 0 µg/mL) and F8 (IC_{50} = 1.11 ± 0.01 µg/mL). These active fractions did not exhibit any toxic effects against Vero cell lines and the chemical profiles investigation indicated presence of α-and γ-eudesmol, elemol, α-cadinol and eugenol by GC/MS analysis.

INTRODUCTION

Human African Trypanosomiasis (HAT) is a fatal disease and commonly called sleeping sickness. It is transmitted by the bite of an infected tsetse fly (genus *Glossina*). Human sleeping sickness is caused by subspecies *Trypanosoma brucei rhodesiense* (east to South Africa) and *Trypanosoma brucei gambiense* (West and Central Africa). *Trypanosoma brucei brucei* is closely related to these subspecies. Sleeping sickness currently affects about half a million people in sub-Sahara Africa and estimated 60 million people are at risk contracting this disease, which is fatal if untreated (WHO, 1998; Barret, 1999). To date, existing drugs to treat HAT are expensive, toxic, causing severe and fatal side effects and have to cross the blood-brain barrier. Moreover, the appearance of drug resistant trypanosomes makes the problem even worse, which calls for an urgent development of alternative and efficient drugs either synthetically or from plant origin.

Essential oils can be used as alternatives or adjuncts to current anti parasitic therapies. Only few studies have addressed the effect of essential oil and their components against trypanosomes. *Cymbopogon nardus* L. is known for their rich essential oil content and widely used in food or drinks, mosquito repellent, perfumery and health and care products (Jantan & Zaki, 1999). Various reports on the bioactive constituent and bioactivity of this plant including antimalarial (Tchoumbouugnang *et al.*, 2005), anti fungal (Matasyoh *et al.*, 2011), antibacterial (Dorman *et al.*, 2000), antiviral (Nurulaini *et al.*, 2006), antioxidant (Cheel *et al.*, 2005) and larvicidal (Zaridah *et al.*, 2003) are available. In this study, we focused on the trypanocidal and cytotoxic activities of the essential oil of *C. nardus* and its fractions against *T. brucei brucei* strain, BS221 and Vero cell. In addition, chemical profiling of all active fractions were carried to determine their active chemical constituents by GC/MS analysis.

MATERIALS AND METHODS

Plant material

The whole plant of *C. nardus* was collected from Muar, Johor. The plant was authenticated by a botanist in FRIM. The voucher specimen was prepared and stored at FRIM.

Extraction and Fractionation

Fresh whole plant materials of *C. nardus* (1.06 kg) were subjected to hydrodistillation for 16 h using Clevenger-type apparatus. The essential oil (4.3 g, 0.41%) was obtained after drying over $MgSO_4$ anhydrous. The crude essential oil was subjected to vacuum liquid chromatography on silica gel 60 (230–400 mesh) using n-hexane, *n*-hexane-EtOAc (9:1, 7:3, 4:6), and MeOH as eluents to obtain seven fractions, EOCN1-EOCN7. The combined active fractions, EOCN(3-5) (2.4 g) were chromatographed using silica gel 60 (70-230 mesh) with eluent system; n-hexane, *n*-hexane-EtOAc (9:1, 7:3, 4:6) and MeOH afforded eight subfractions, F1 (5.3 mg, 0.0005%), F2 (4.8mg, 0.0004%), F3 (8.4 mg, 0.0008%), F4 (23.7 mg, 0.0024%), F5 (18.3 mg, 0.0018%), F6 (295 mg, 0.029%), F7 (326 mg, 0.033%) and F8 (83 mg, 0.008%).

Gas chromatography / mass spectrometry analysis

The GCMS analysis was performed on a Agilent GCMS 7890A/5975C Series MSD (70eV direct inlet) equipped with HP-5MS fused silica capillary column (30m x 0.25 mm; 0.25µm film thickness). The oven temperature was programmed from 60ºC (10 minutes) to 230ºC (1 minute) at a rate of 3ºC/min. Injector and detector temperature were set up at 220ºC and 280ºC, respectively. The mass range was 50-550 in the full scan mode with a rate of 2.91 scans/ sec. Helium was the carrier gas and the volume of oil injected was 0.1µl. Identification of the chemical components was based on the comparison of their mass spectral data with the Wiley, HPCH 2205.L and NIST05a.L mass spectral database. In addition GC analysis was carried out on a Shimadzu GC-2010 gas chromatograph equipped with a flame ionization detector (FID) using fused silica capillary column CBP-5 (25m x 0.25mm; 0.25µm film thickness). Helium was used as carrier gas and the injector and detector temperature were set up at 220ºC and 280ºC, respectively. The oven temperature was programmed from 60ºC to 230ºC at 3ºC/min and finally held at 230ºC for 10 minutes. The peak areas and retention times were measured by electronic integration.

In vitro antitrypanosomal assay

Primary, secondary and tertiary *in vitro* antitrypanosomal screening assays against *T. b. brucei* were conducted in this study (Hitomi & Kazuhiko, 2005). In the primary assay, two concentrations of extracts (1.56 µg ml^{-1} and 12.5 µg ml^{-1}l) were used for the testing. Extracts at each concentration dilution were tested in duplicate 96-well plates. Extracts that showed positive for antitrypanosomal activity in both or either one of the concentration well were selected for the secondary and tertiary assays, which consisted of seven final extract concentrations (12.5 to 0.05 µg/mL). Extracts were tested in duplicate plates for secondary assay and triplicate plates for tertiary assay. All assays were performed in a flat bottom 96-well microtiter plate. *In vitro* anti-trypanosomal activity of test sample was estimated by a dose response curve using alamar blue sensitivity assay according to the method of Raz *et al.* (1997). Standard trypanocidal drug, pentamidineisothonine (SIGMA) was dissolved in 5% dimethyl-sulfoxide (DMSO) and included in the assay as positive control. Negative control-solvent (5% DMSO and 25% ethanol) as well as negative control-blank (Sterile MiliQ water) were used as negative controls in the experiment as negative controls. Five micro litre of the pre-dilution of the extracts, standard drug and negative control were added to each well of a 96-well microtiter plate. Then, 95 µl of the trypanosomes suspension at a density of 20000-25000 trypanosomes ml^{-1} was inoculated to each well except control-blank well. After incubation for 72 hours at 37ºC under 5% carbon dioxide, 10 µl of fluorescent dye alamar blue was added to each well and incubation was further continued for 3-6 hours until colour change was observed. All

tests were performed two to three times independently. Plates were analysed using Tecan Infinite M200 plate reader at an excitation wavelength of 530 nm and an emission wavelength of 590 nm. Absorbance data were transferred into a graphic program (Excel) and were evaluated to determine the IC_{50} values (Hitomi & Kazuhiko, 2005).

Cytotoxicity and selectivity index

In vitro cytotoxicity assay was carried out using normal kidney (vero) cells according to the procedure described by Kohana & Otoguro (1999) and Malebo *et al.* (2009). Cells were grown in standard media according to the method reported by Siti Syarifah *et al.* (2011). Serial dilution of extracts was prepared to produce six final concentrations (100 to 0.1 µg ml^{-1}) and 10 µl of pre-dilution of extracts, standard drugs, and negative control was added to each well of a 96-well microtiter plate containing of 40000 cells ml^{-1}. Standard drug pentamidine was used as positive control, ethanol and DMSO as negative control-solvent and sterile MiliQ water as negative control-blank. The plates were incubated at 37°C under 5% carbon dioxide for 72h and assayed using the alamar blue assay as previously described with shortened incubation time. After incubation, plates were read using an excitation wavelength of 530 nm and an emission wavelength of 590 nm. Data were transfer into Excel program to calculate the IC_{50} value. IC_{50} was defined as the concentration of extract required to reduce a 50% of cell growth compared to control cultures. Based on the cytotoxicity results, calculation of selectivity index (SI) value was performed to select extracts that were very selective to trypanosome parasites and had low toxicity effects on normal cells by using the formula below.

$$\text{Selectivity Index (SI)} = \frac{IC_{50} \text{ value (cytotoxicity)}}{IC_{50} \text{ value (antitrypanosomal activity)}}$$

RESULTS AND DISCUSSION

Composition of essential oil

The major chemical compositions of essential oil determined in this study is in accordance with previous reports (Dugo *et al.*, 1998; Mahalwal & Ali, 2003; Nakahara *et al.*, 2003; Koba *et al.*, 2009), mainly: [citronellal (35.5%), geraniol (28%) and citranellol (11%)] as major components. This oil consisted of two monoterpene hydrocarbons (1.9%), seven oxygenated monoterpenes (79%), five sesquiterpenes (12.3%) and four oxygenated sesquiterpenes (2.6%) including elemol, β-eudesmol, γ-eudesmol and citronellyltiglate.

Chemical Fractionation and Identification

Essential oil of *C. nardus* exhibited strong antitrypanosomal activity (0.31 ± 0.03 µg/mL) and good selectivity index (SI>323). Thus, the crude essential oil was subjected to vacuum liquid chromatography to afford seven fractions (EOCN1-EOCN7). Evaluation of all fractions on anti trypanosomal activity resulted in three active fractions; EOCN3 (IC_{50}= 0.44 µg/mL, SI> 227), EOCN4 (IC_{50}= 0.30 µg/mL, SI> 333.3) and EOCN5 (IC_{50}= 0.59 µg/mL, SI> 169.5) as listed in Table 1. The combined active fractions EOCN (3-5) were then rechromatographed and afforded eight sub fractions, (F1-F8) as described in Figure 1. Anti trypanosomal and cytotoxic activities of all sub-fractions (Table 1) namely F4 (IC_{50}= 0.61 µg/mL, SI= 127), F6 (IC_{50}= 0.73 µg/mL, SI> 137), F7 (IC_{50}= 1.15 µg/mL, SI> 87) and F8 (IC_{50}= 1.11 µg/mL, SI> 90) were determined as active sub fractions against *T. b. brucei*, BS221. In the present study, the active subfractions F4, F6, F7 and F8 were subjected to chemical profiling using GC/MS (Table 2). The analysis showed γ-eudesmol and α-cadinol as major constituents in subfractions F4 and F6 meanwhile elemol is one of the major compound in subfraction F7 and F8. Other compounds such as geranial and citronellol were listed as minor constituents as shown in Table 2.

Anti trypanosomal and cytotoxic activities

To our knowledge, there is no report on the *in vitro* antitrypanosomal activity of active constituents from *C. nardus*. A few reports claimed on the antiprotozoal activity of

diverse essential oils affecting protozoa such as *Leishmania*, *Trypanosoma and Plasmodium* (Mikus *et al*., 2000; Rosa *et al*., 2003; Tchoumbougnang *et al*., 2005; Ueda-Nakamura *et al*., 2006; Santoro *et al*., 2007; Wink & Nibret, 2010). Thus in our study, we focused on antitrypanosomal, cytotoxic activities and chemical investigation of active fractions of the essential oil. In our screening, activity scores of the selectivity index (SI) values were separated into three categories: score 1-not selective (1< SI < 10), score 2-moderate (10< SI < 100) and score 3-selective (SI $\geq$ 100). Table 1 showed fractions EOCN (3-5) demonstrated strong antitrypanosomal activity with SI values

Table 1. *In vitro* antitrypanosomal activity against *Trypanosoma brucei brucei* BS221 and cytotoxic activity of fractions from essential oil of *C. nardus* against vero cells

Fraction	IC_{50} (µg/mL)		Selectivity Index (SI) Vero/BS221
	Antitrypanosomal activity, BS221	Cytotoxicity Vero	
C. nardus essential oil	0.31 ± 0.03	>100	>323
Main Fraction			
EOCN1	ND	>100	–
EOCN2	3.94 ± 0.7	>100	>25.4
EOCN3	0.44 ± 0.01	>100	>227.3
EOCN4	0.30 ± 0.01	>100	>333.3
EOCN5	0.59 ± 0.1	>100	>169.5
EOCN6	1.46 ± 0.04	>100	68.5
EOCN7	3.26 ± 1.58	>100	30.7
Sub fractions			
F1	> 12.5	ND	–
F2	> 12.5	ND	–
F3	> 12.5	ND	–
F4	0.61 ± 0.06	77.41	127
F5	> 12.5	ND	–
F6	0.73 ± 0.33	>100	>137
F7	1.15 ± 0	>100	>87
F8	1.11 ± 0.01	>100	>90
Anti trypanosomal drug			
Pentamidine	0.00438	0.01883	4.29

ND = Not Determined

*SI values were determined for samples showing strong active ($IC_{50} \leq 1.56$ µg/mL) and moderate active (1.56 µg/mL $\leq IC_{50} \leq$ 12.5 µg/mL) antitrypanosomal activity

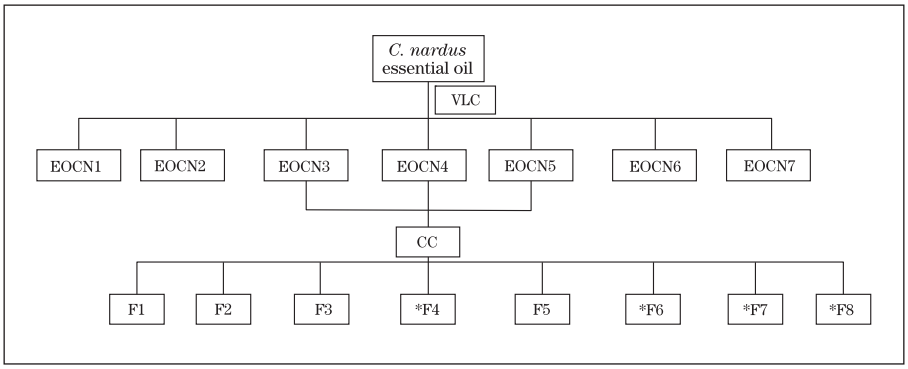

Figure 1. Extraction and fractionation of *Cymbopogon nardus* essential oil for antitrypanosomal activity

VLC = Vacuum Liquid Chromatography. CC = Column Chromatography. *F = Active sub fraction

Table 2. Chemical composition ofactive fractions from *Cymbopogon nardus* essential oil, determined by GC/MS

Subfraction	Compound	Percentage (%)
F4	γ-eudesmol	21.6
	α-cadinol	36.9
	eugenol	21
	geranial	3.2
F6	α-cadinol	49.6
	γ-eudesmol	34.1
	elemol	2.9
F7	γ-eudesmol	34.9
	α-eudesmol	25.8
	elemol	22.4
	α-cadinol	7.5
F8	α-cadinol	46.9
	elemol	39.6
	γ-eudesmol	5.5
	citronellol	2.4

≥ 100. These active fractions, EOCN (3–5) were subjected to further fractionation using column chromatography to obtain eight subfractions, F1 to F8. Subfractions F1, F2, F3 and F5 showed no activity (IC_{50} > 12.5 µg/mL) against *T. b. brucei* BS221 meanwhile sub fractions F4, F6, F7 and F8 demonstrated strong activity with good SI value (score 3). In our chemical investigation, α-eudesmol, γ-eudesmol and eugenol appeared in active sub fractions which exhibited anti-trypanocidal properties *in vitro*. This is in accordance with report by Otoguro *et al.* (2011) where α-eudesmol is effective against *T. b. brucei* strain GUTat 3.1, with EC_{50} values 0.10 µg/mL (0.45 µM).

In vitro anti trypanosomal activity of essential oil of *C. nardus* and its fractions were evaluated against *T. b. brucei* strain BS221. Active sub fractions, F4, F6, F7 and F8 showed strong anti trypanosomal activity and selective against trypanosome parasites. Oxygenated monoterpenes such as geranial, eugenol, citronellol and oxygenated sesquiterpenes; α-eudesmol, γ-eudesmol, elemol and α-cadinol are present in active fractions of this oil.

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