

## Human Immunodeficiency Virus Type 1 (HIV-1) Reverse Transcriptase Inhibitory Effect of *Cymbopogon Nardus* Essential Oil

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**Abstract :** *The essential oil (EO) obtained from Cymbopogon nardus is used frequently as ingredients of anti-AIDS medication in Africa by indigenous people who rely mainly on traditional medicinal plant therapies. However, its mechanism still remains obscure. The present study investigated the biological effects of the EO derived from C. nardus collected in Benin on the activities of human immunodeficiency virus reverse transcriptase (HIV-1 RT) and Escherichia coli DNA polymerase I (DNA-POL I). Biochemical experiments showed that C. nardus EO inhibited the activity of HIV-1 RT, whereas it had no effect on DNA-POL I. To investigate the specificity of these biological effects of C. nardus, six other representatives EOs were assayed in the same way in order to determine their effects on HIV-1 RT and DNA-POL I. None of the other six EOs had significant effect on the activities of these two enzymes. Gas chromatography-mass spectrometry analysis of the EO obtained from the aerial parts of C. nardus by steam distillation identified among other constituents: (S)-β-citronellol, (R)-linalool, (R)-limonene, geraniol and (R)-citronellal. In particular, (S)-β-citronellol inhibited HIV-1 RT in vitro biochemical assay where the same concentration as C. nardus EO was used in the experiment.*

**Keywords:** *(S)-β-citronellol, Cymbopogon nardus, Poaceae, DNA polymerase I, Escherichia coli, essential oil, HIV-1 reverse transcriptase.*

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### 1. INTRODUCTION

Early in 1981, acquired immunodeficiency syndrome (AIDS) emerged suddenly among homosexuals and injected drug users in the United States (Gottlieb *et al.* 2006, Sharp *et al.* 2011). Subsequently, AIDS spread rapidly to become a global public health pandemic. Montagnier *et al.* isolated human immunodeficiency virus (HIV) from the blood of an AIDS patient in 1983 and identified it as the etiological agent of this devastating disease. Thus, many HIV-1 enzymes and proteins have been targeted in anti-HIV drug research (Barré-Sinoussi *et al.* 1983, Sepkowitz *et al.* 2001, Bär *et al.* 2004, Bauman *et al.* 2008, Sarafinos *et al.* 2009). Many antiretroviral drugs have been designed and classified according to their targets, including reverse transcriptase (RT) inhibitors, integrase inhibitors, protease inhibitors, and entry inhibitors (De Clercq 2009, Sayana *et al.* 2009, Chen *et al.* 2013, Dijk *et al.* 2013, Yu *et al.* 2013).

The standard cocktail therapy (highly active antiretroviral therapy, HAART) used recently in developed countries is very effective in suppressing viral replication and it has significantly reduced the mortality rate of HIV/AIDS patients, as well as improving their quality of life (Antonella *et al.* 2000, Chou *et al.* 2005). However, HAART has only reached a fraction of people in Sub-Saharan Africa where up to two-thirds of the world's total HIV/AIDS patients experience many treatment

problems (Cohen *et al.* 2008, World Health Organization 2011, World Health Organization 2012). These problems include a major lack of adequate therapy due to the high costs of anti-AIDS drugs, the unavailability of anti-AIDS drugs and various complex side effects, such as the emergence of anti-AIDS drug resistance (Hawkins 2006, Rosen *et al.* 2007, Park *et al.* 2009, History of HIV and AIDS in Africa 2013, Ziske *et al.* 2013). In this problematic background, african communities have developed many effective natural medicines from traditional therapeutic plants (Leteane *et al.* 2012). *Cymbopogon nardus* (*C. nardus*) is a perennial grass in the Poaceae family, which grows in tropical areas such as Sri Lanka, Africa, and Java and it is one of the most frequently used plants in traditional anti-AIDS drug preparations. The essential oil (EO) obtained from the leaves and stems of *C. nardus* by steam distillation has also been used to treat infections in the past in Africa (Wei *et al.* 2013). However, the mechanism that underlies its therapeutic efficacy still remains obscure.

In the present study, an *in vitro* biochemical investigation showed that the EO derived from *C. nardus* inhibited HIV-1 RT activity in a dose-dependent manner. Gas chromatography-mass spectrometry (GC-MS) analysis of this EO sample identified several constituents and their activities were assayed against HIV-1 RT and *Escherichia coli* DNA polymerase I (DNA-POL I). The results showed that (*S*)- $\beta$ -citronellol might be one of the constituents of *C. nardus* with inhibitory effects on HIV-1 RT.

## 2. MATERIALS AND METHODS

### 2.1. Reagents

*C. nardus* EO, *Ocimum gratissimum* EO, *Lippia multiflora* EO, *C. citratus* EO, *Melaleuca viridiflora* leaf EO and *Eucalyptus obliqua* leaf EO were all steam distilled extracts from these plants, which were purchased from Aronat + (Benin). Commercial (*R*)-citronellal (purity >90% by GC), (*R*)-linalool (purity >98% by GC), geraniol (purity >97% by GC) and (*R*)-limonene (purity >95% by GC) were purchased from Wako. (*S*)- $\beta$ -citronellol (purity >95% by GC) was purchased from Tokyo Chemical Industry Co. Ltd. ddTTP (purity 99% by HPLC) was purchased from Amsterdam Biosciences inhibition assay. Neomycin trisulfate salt hydrate (potency > 600  $\mu$ g per mg) was purchased from Sigma-Aldrich. The RT assay colorimetric kit, biotin-16-dUTP, digoxigenin deoxyuridine triphosphate (DIG-dUTP), DNA-dependent DNA-POL I from *Escherichia coli* lysogenic NM 964, deoxynucleoside triphosphate set (PCR grade) and hexanucleotide mix (10 $\times$  concentration) were purchased from Roche. Single-stranded DNA from calf thymus (purity >65%) was purchased from Sigma-Aldrich.

### 2.2. Evaluation of HIV-1 RT Inhibitory Effect

The quantitative determination of the HIV-1 RT inhibitory effect of EO used a colorimetric enzyme immunoassay, which involved the incorporation of DIG-and biotin-labeled dUTP into native DNA. The assay was performed with an RT assay colorimetric kit, according to the manufacturer's instructions with slight modifications. Briefly, 60  $\mu$ L of a reaction mixture containing final concentrations of 46 mM Tris-HCl, 266 mM KCl, 27.5 mM MgCl<sub>2</sub>, 9.2 mM dithiothreitol (DTT), 10  $\mu$ M biotin-16-dUTP/DIG-dUTP, 2.2  $\mu$ g/mL PolyA:oligo (dT) 15 template/primer hybrid, 1.7 ng/mL HIV-1 RT and various concentrations of sample (0.1–10 mg/mL) was incubated at 37°C for 15 h. The reaction mixture was transferred to the surface of streptavidin-coated ELISA plate wells. After washing away any unbound substances, an antibody directed against DIG conjugated to peroxidase (anti-DIG-POD) was added that bound to DIG-11-dTTP. After one more wash, 2,2-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) was added. The absorbance (OD<sub>405nm</sub>) of the color that developed in the presence of ABTS was evaluated at 405 nm using an Enzyme-Linked Immunosorbent Assay (ELISA) microplate reader (SpectraMax 340PC). A reaction solution where the inhibitor was replaced with dilution buffer was used as the control. A sample without RT solution was used as the negative control and a sample with 2  $\mu$ M ddTTP was used as the positive control. EOs were dissolved in ethanol to prepare a dilution series that ranged from 0.1 mg/mL to 10 mg/mL. The final ethanol concentration in the solutions was <1%, which had no effect on the assay results. The inhibitory effects of RT inhibitors were calculated as the percentage inhibition relative to the control, as follows:

$$\text{Control relative percentage of inhibition} = 100 - [ ( \text{Sample OD}_{405\text{nm}} - \text{Negative control OD}_{405\text{nm}} ) / ( \text{Control OD}_{405\text{nm}} - \text{Negative control OD}_{405\text{nm}} ) ] \times 100.$$

### 2.3. Evaluation of DNA-POL I Activity

Evaluations of the effects of EOs on the activity of DNA-POL I were performed as described by Ducancelle *et al.*, with slight modifications (Ducancelle *et al.* 2007). The DNA-POL I activity was assayed by measuring the incorporation of DIG-dUTP into the complementary DNA chain. Calf thymus DNA was used as the template with a mixture of hexanucleotides designed as random primers. Briefly, 20  $\mu$ L of a reaction mixture containing final concentrations of 50  $\mu$ g/mL calf thymus DNA, 50 mM Tris-HCl (pH7.2), 10 mM MgCl<sub>2</sub>, 0.1 mM DTT, 0.2 mg/mL Bovine serum albumin (BSA), dATP, dGTP, dCTP, dTTP, DIG-dUTP, biotin-16-dUTP and various concentrations of inhibitor (0.1–10 mg/mL) was incubated at 37°C for 15 h. The subsequent steps were performed in the same manner as the “Evaluation of HIV-1 RT inhibitory effect” described above. A sample without DNA-POL I solution was used as the negative control and a sample with 10 mM Neomycin trisulfate salt hydrate was used as the positive control. The ethanol concentration in the reaction mixtures was <1%, which had no effect on the activity of DNA-POL I.

### 2.4. Identification of the Major Constituents of *C. Nardus* EO

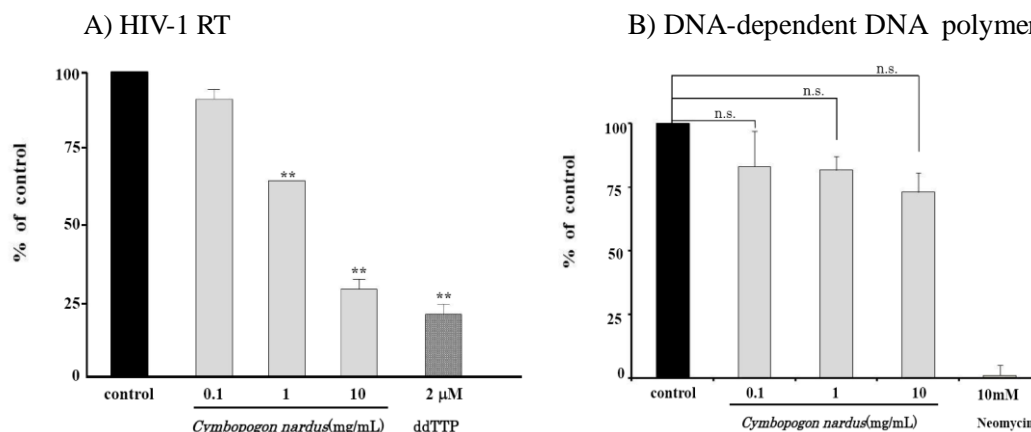
*C. nardus* EO was analyzed by GC-MS, as described by Kudo *et al.* (2009), using an Agilent 6890 GC combined with an Agilent 5973 MS. NAGINATA software was provided by Nishikawa. A HP-5ms fused silica capillary column (30 m x 0.25 mm i.d., 0.25  $\mu$ m film thickness, Agilent) coated with 5% phenylmethyl-silicone stationary phase was used. The GC-MS data analysis was performed using NIGINATA software based on the “free-drug database” and the “drug database with acetylating reagents”.

### 2.5. Statistical Analysis

The results were analyzed using the Student's *t*-test. Differences were considered significant at  $p < 0.05$

## 3. RESULTS

Efficacy and toxicity are two indispensable parts of EO activity evaluation, so the biological effects of *C. nardus* EO on HIV-1 RT were examined to assess its efficacy, whereas its effects on DNA-POL I were assessed to determine its toxicity. *C. nardus* EO had a dose-dependent inhibitory effect (IC<sub>50</sub> value of 1.2 mg/mL) against HIV-1 RT at concentrations ranged from 0.1 to 10 mg/mL (Fig. 1A). With  $\leq 10$  mg/mL of *C. nardus* EO, the activity of HIV-1 RT was significantly inhibited by up to 72.4% compared with the control ( $p < 0.001$ ). This inhibitory effect was similar to that produced with the positive control (2  $\mu$ M 2',3'-dideoxythymidine-5'-triphosphate; ddTTP). In addition, *C. nardus* EO had no major biological effects on DNA-POL I, while 10 mM Neomycin as a positive control showed clearly that effect. This enzyme retained up to 91.5% of its activity compared with the control when the same concentration as in HIV-1 RT assay of *C. nardus* EO (10 mg/mL, Fig. 1B) was used.



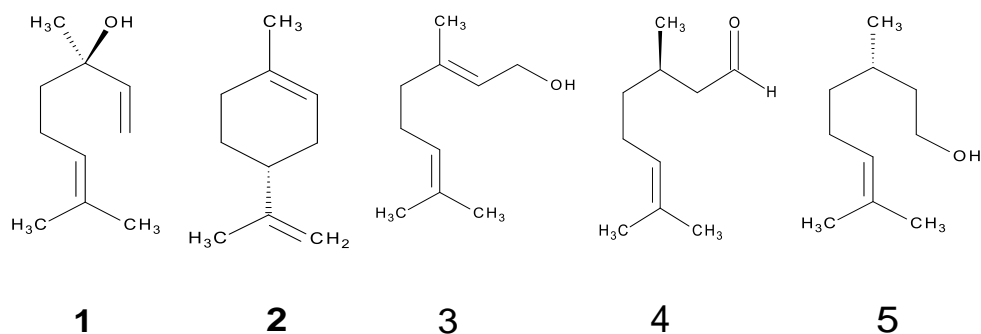
**Fig1.** Biological effects of *Cymbopogon nardus* essential oil on HIV-1 RT and DNA-dependent DNA polymerase I.

\* $p < 0.005$ , compared with control. \*\* $p < 0.001$ , compared with control. n.s.: not significant

To investigate the specificity of the inhibitory activity produced by *C. nardus* EO on HIV-1 RT and the inhibitory effect against DNA-POL I, six other representatives EOs were assayed in the same way.

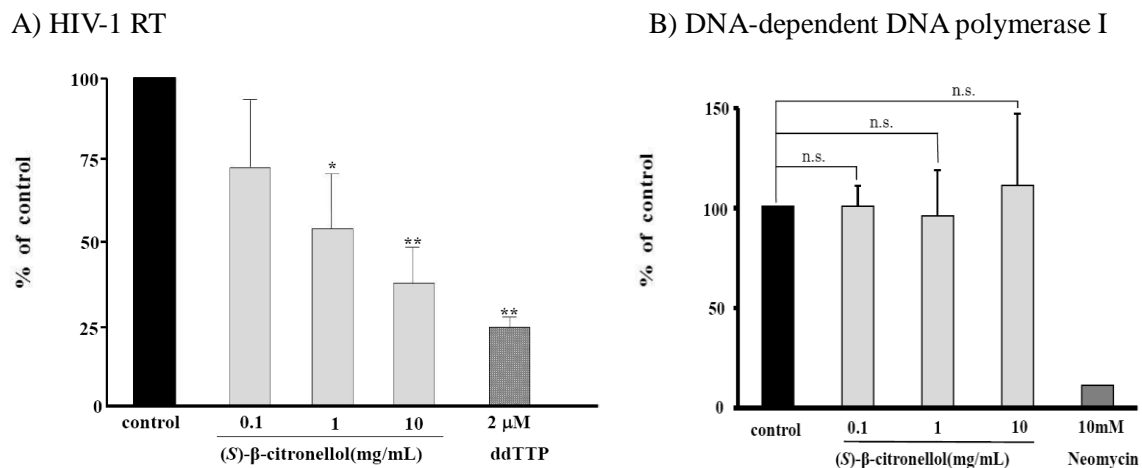
The results are shown in Table 1. *Cymbopogon citratus* EO (HIV-1 RT: IC<sub>50</sub> > 10 mg/mL; DNA-POL I: IC<sub>50</sub> = 9.6 mg/mL), *Ocimum gratissimum* EO (HIV-1 RT: IC<sub>50</sub> > 10 mg/mL; DNA-POL I: IC<sub>50</sub> > 10 mg/mL) had no inhibitory effects on the two enzymes. *Eucalyptus obliqua* leaf EO (HIV-1 RT: IC<sub>50</sub> = 4.5 mg/mL, DNA-POL I IC<sub>50</sub> > 10.0 mg/mL), *Melaleuca viridiflora* leaf EO (HIV-1 RT: IC<sub>50</sub> = 1.8 mg/mL, DNA-POL I: IC<sub>50</sub> > 10.0 mg/mL), and *Lippia multiflora* EO (HIV-1 RT: IC<sub>50</sub> = 5.5 mg/mL; DNA-POL I: IC<sub>50</sub> = 9.6 mg/mL) had effects on HIV-1 RT and DNA-POL I but they were weak compared with the effects of *C. nardus* on the same enzymes. Thus, none of the other six EOs had the same effects on these enzymes as *C. nardus* EO.

*C. nardus* EO was subjected to a quantitative GC-MS for analyzing the constituents. The results showed that about 27 peaks were identified (data not shown). Among these, the five commercial available constituents ((*R*)-linalool, (*R*)-limonene, geraniol, (*R*)-Citronellal and (*S*)- $\beta$ -citronellol) were selected for further experiments. Then, the activities of these constituents against HIV-1 RT and DNA-POL I were assayed. The results are shown in Table 2 and the chemical structures of the assayed compounds are shown in Fig. 2. (*R*)-linalool (HIV-1 RT: IC<sub>50</sub> > 10 mg/mL; DNA-POL I: IC<sub>50</sub> > 10 mg/mL), (*R*)-limonene (HIV-1 RT: IC<sub>50</sub> > 10 mg/mL; DNA-POL I: IC<sub>50</sub> > 10 mg/mL), geraniol (HIV-1 RT: IC<sub>50</sub> = 6.5 mg/mL; DNA-POL I: IC<sub>50</sub> = 5.6 mg/mL) and (*R*)-citronellal (HIV-1 RT: IC<sub>50</sub> > 10 mg/mL; DNA-POL I: IC<sub>50</sub> = 3.6 mg/mL) had very weak effects against HIV-1 RT and DNA - POL I. As shown in Fig. 3A, (*S*)- $\beta$ -citronellol had a dose-dependent inhibitory effect on HIV-1 RT at concentrations ranged from 0.1 to 10.0 mg/mL (Fig. 3A). With 10 mg/mL (*S*)- $\beta$ -citronellol, the HIV-1 RT activity was significantly inhibited by up to 63.1% compared with the control ( $p < 0.001$ ). The positive control treated with 2  $\mu$ M ddTTP inhibited HIV-1 RT in almost the similar way as 10.0 mg/mL (*S*)- $\beta$ -citronellol. The data were obtained from three independent experiments. These results demonstrate that (*S*)- $\beta$ -citronellol specifically decreases the activity of HIV-1 RT in a significant manner.



**Fig2.** Chemical structures of some constituents identified in *Cymbopogon nardus* essential oil.

1. (*R*)-Linalool, 2. (*R*)-Limonene, 3. Geraniol, 4. (*R*)-Citronellal, 5. (*S*)- $\beta$ -Citronellol



**Fig3.** Biological effects of (*S*)- $\beta$ -citronellol on HIV-1 RT and DNA-dependent DNA polymerase I.

\* $p < 0.05$ , compared with control. \*\* $p < 0.01$ , compared with control. n.s.: not significant

## Human Immunodeficiency Virus Type 1 (HIV-1) Reverse Transcriptase Inhibitory Effect of *Cymbopogon Nardus* Essential Oil

**Table1.** Biological effects of various essential oils on HIV-1 RT and DNA-POL I

Essential oils	IC <sub>50</sub> (mg/mL)	
	HIV-1 RT	DNA-POL I*
<i>Cymbopogon nardus</i>	1.2	>10.0
<i>Eucalyptusobliqua</i>	4.5	>10.0
<i>Ocimumgratissimum</i>	>10.0	>10.0
<i>Melaleucaviridiflora</i>	1.8	>10.0
<i>Lippiamultiflora</i>	5.0	9.6
<i>Cymbopogoncitratus</i>	>10.0	9.6

\*DNA-POL I from *Escherichia coli* lysogenic NM964.

### 4. DISCUSSION

Recent anti-AIDS therapies prioritize the combination of different categories of antiretroviral drugs. Typical combinations usually include two nucleoside RT inhibitors (NRTIs) combined with one protease inhibitor (PI), or two NRTIs combined with one non-nucleoside RT inhibitor (NNRTI). NRTIs are nucleoside analogues with the capacity to competitively inhibit RT activity as chain terminators because of their lack of a 3'-OH group. NNRTIs non-competitively inhibit HIV RT activity by binding to one of its allosteric sites, thereby leading to molecular structure deformation (Balzarini 2004, De Clercq 2004). At present, the most widely used antiretroviral drugs that form the basis of anti-AIDS therapy are RT inhibitors. It is easy to understand that HIV-1 RT became the primary target of anti-AIDS drug research because this enzyme is responsible for generating double-stranded linear DNA from the single-stranded RNA packaged in HIV-1 virions. Previously, researchers focused mainly on chemically synthesized HIV-1 RT inhibitors. Studies of active antiretroviral compounds from natural resources have investigated different parts of many plants (aerial parts and roots, etc.) but no previous reports had considered the HIV-1 RT inhibitory effects of EOs derived from traditional medicinal plants except Bicchi *et al.* (2009) who reported that EOs from *Ridolfia segetum* and *Oenanthe crocata* inhibited HIV-1 RT in a biochemical assay. Bicchi *et al.* (2009) showed that the active constituents with inhibitory effects in these oils were dillapiol and myristicin. Similarly, the present study of *C. nardus* EO showed that the monoterpene (*S*)- $\beta$ -citronellol (IC<sub>50</sub> = 3.3 mg/mL) present in this EO also inhibited HIV-1 RT in a dose-dependent manner. This inhibitory effect of (*S*)- $\beta$ -citronellol on HIV-1 RT activity was specific or at least unique among the *C. nardus* EO constituents assayed in the present study (Fig. 3 and Table 2). These results suggest that the anti-HIV-1/AIDS effect of *C. nardus* EO in traditional African medicine may be attributable to the HIV-1 RT inhibitory effect of (*S*)- $\beta$ -citronellol contained in this EO. On the other hand, we reported for the first time that *Melaleuca viridiflora* leaf EO, *Eucalyptus obliqua* leaf EO and *Lippia multiflora* EO had shown inhibitory effects against HIV-1 RT activity but this inhibitory effect is weak as compared with that of *C. nardus* EO. Ramanoelina *et al.* reported that *Melaleuca quinquenervia* EO contains (E)-nerolidol, 1,8 cineole,  $\alpha$ -terpineol and viridiflorol as major constituents. Furthermore, *Lippia multiflora* EOs contain linalool, germacrene *p*-cymene, thymol, thymyl acetate and 1,8 cineole as described previously by Juliani *et al.* (2008). Sefidekon *et al.* (2007) reported that 1,8 cineol is the main constituent of the essential oil of all *eucalyptus* species. Although all these works used GC/MS analysis, (*S*)- $\beta$ -citronellol had not been reported in any of these EOs. We then thought worth to conclude that the inhibitory activity of *Melaleuca quinquenervia* EO, *Lippia multiflora* EO and *Eucalyptus obliqua* EO against HIV-1 RT should be attributable to an unknown compound different from (*S*)- $\beta$ -citronellol. Further studies will be needed to identify the active constituents in the above three EOs.

**Table2.** Biological effects of various chemical constituents of *Cymbopogon nardus* on HIV-1 RT and DNA-POL I

Chemical constituents	IC <sub>50</sub> (mg/mL)	
	HIV-1 RT	DNA-POL I*
( <i>R</i> )-Linalool	>10.0	>10.0
( <i>R</i> )-Limonene	>10.0	>10.0
Geraniol	6.5	5.6
( <i>R</i> )-Citronellal	>10.0	3.6
( <i>S</i> )- $\beta$ -Citronellol	3.3	> 10.0

\*DNA-POL I from *Escherichia coli* lysogenic NM964.

## 5. CONCLUSION

The present study provides an evidence base for the HIV-1 RT inhibitory effect of *C. nardus* EO in traditional African medicine. (*S*)- $\beta$ -citronellol may be a potential HIV-1 RT inhibitor and traditional medicines containing *C. nardus* EO at an appropriate dose may be useful for AIDS therapy.

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