WILEY
Online Proofing System Instructions

The Wiley Online Proofing System allows proof reviewers to review PDF proofs, mark corrections, respond to queries, upload replacement figures, and submit these changes directly from the locally saved PDF proof.

1. For the best experience reviewing your proof in the Wiley Online Proofing System ensure you are connected to the internet. This will allow the PDF proof to connect to the central Wiley Online Proofing System server. If you are connected to the Wiley Online Proofing System server you should see a green check mark icon above in the yellow banner.


Connected


Disconnected

2. Please review the article proof on the following pages and mark any corrections, changes, and query responses using the Annotation Tools outlined on the next 2 pages.

3. Save your proof corrections by clicking the "Publish Comments" button in the yellow banner above. Corrections don't have to be marked in one sitting. You can publish comments and log back in at a later time to add and publish more comments before you click the "Complete Proof Review" button below.

4. If you need to supply additional or replacement files bigger than 5 Megabytes (MB) do not attach them directly to the PDF Proof, please click the "Upload Files" button to upload files:

Click Here

5. When your proof review is complete and all corrections have been published to the server by clicking the "Publish Comments" button, please click the "Complete Proof Review" button below:

IMPORTANT: Did you reply to all queries listed on the Author Query Form appearing before your proof?

IMPORTANT: Did you click the "Publish Comments" button to save all your corrections? Any unpublished comments will be lost.

IMPORTANT: Once you click "Complete Proof Review" you will not be able to add or publish additional corrections.

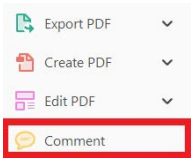
Click Here

WILEY

USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

Required software to e-Annotate PDFs: Adobe Acrobat Professional or Adobe Reader (version 11 or above). (Note that this document uses screenshots from Adobe Reader DC.)
The latest version of Acrobat Reader can be downloaded for free at: http://get.adobe.com/reader/

Once you have Acrobat Reader open on your computer, click on the Comment tab (right-hand panel or under the Tools menu).



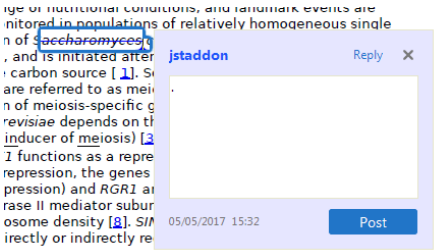
This will open up a ribbon panel at the top of the document. Using a tool will place a comment in the right-hand panel. The tools you will use for annotating your proof are shown below:

1. Replace (Ins) Tool – for replacing text.

Strikes a line through text and opens up a text box where replacement text can be entered.

How to use it:

- Highlight a word or sentence.
- Click on .
- Type the replacement text into the blue box that appears.

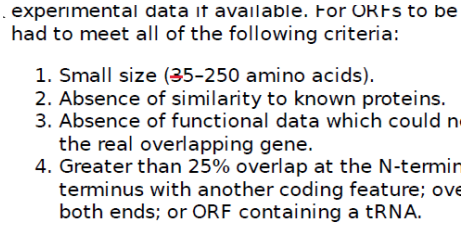


2. Strikethrough (Del) Tool – for deleting text.

Strikes a red line through text that is to be deleted.

How to use it:

- Highlight a word or sentence.
- Click on .
- The text will be struck out in red.

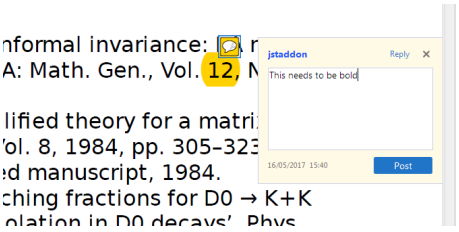


3. Commenting Tool – for highlighting a section to be changed to bold or italic or for general comments.

Use these 2 tools to highlight the text where a comment is then made.

How to use it:

- Click on .
- Click and drag over the text you need to highlight for the comment you will add.
- Click on .
- Click close to the text you just highlighted.
- Type any instructions regarding the text to be altered into the box that appears.

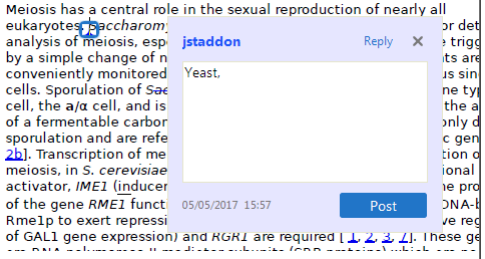


4. Insert Tool – for inserting missing text at specific points in the text.

Marks an insertion point in the text and opens up a text box where comments can be entered.

How to use it:

- Click on .
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the box that appears.



USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

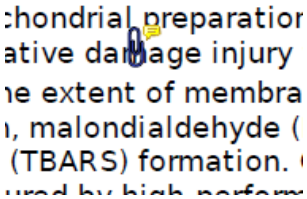
5. Attach File Tool – for inserting large amounts of text or replacement figures.

Inserts an icon linking to the attached file in the appropriate place in the text.

How to use it:

- Click on  .
- Click on the proof to where you'd like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.

The attachment appears in the right-hand panel.

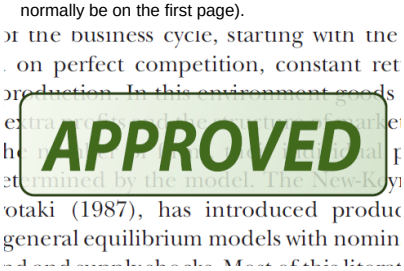


6. Add stamp Tool – for approving a proof if no corrections are required.

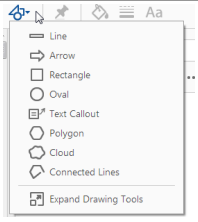
Inserts a selected stamp onto an appropriate place in the proof.

How to use it:

- Click on  .
- Select the stamp you want to use. (The Approved stamp is usually available directly in the menu that appears. Others are shown under *Dynamic*, *Sign Here*, *Standard Business*).
- Fill in any details and then click on the proof where you'd like the stamp to appear. (Where a proof is to be approved as it is, this would normally be on the first page).

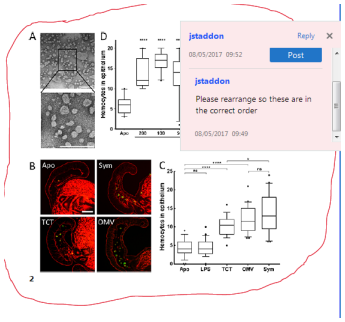


Drawing tools available on comment ribbon



7. Drawing Markups Tools – for drawing shapes, lines, and freeform annotations on proofs and commenting on these marks.

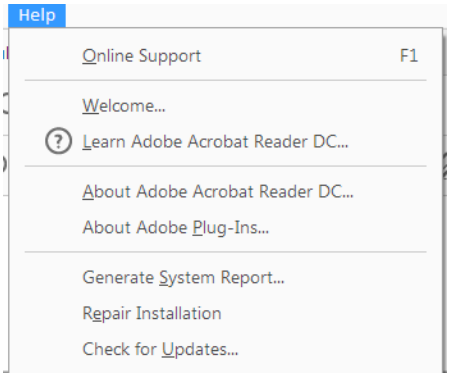
Allows shapes, lines, and freeform annotations to be drawn on proofs and for comments to be made on these marks.



How to use it:

- Click on one of the shapes in the Drawing Markups section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, right-click on shape and select *Open Pop-up Note*.
- Type any text in the red box that appears.

For further information on how to annotate proofs, click on the Help menu to reveal a list of further options:



WILEY

Author Query Form

Journal: CBDV

Article: 201700436

Dear Author,

During the copyediting of your manuscript the following queries arose.

Please refer to the query reference callout numbers in the page proofs and respond to each by marking the necessary comments using the PDF annotation tools.

Please remember illegible or unclear comments and corrections may delay publication.

Many thanks for your assistance.

Query reference	Query	Remarks
1	**AUTHOR: Please confirm that given names (red) and surnames/family names (green) have been identified correctly.**	
2	**AUTHOR: Please provide full postal address details for affiliations 1 and 2.**	
3	**AUTHOR: Please check that authors and their affiliations are correct.**	
4	**AUTHOR: As per journal style 5–8 keywords are allowed. Please check and provide.**	
5	**AUTHOR: Please provide the exact text in the place of "■ and ?".**	

C B D V | 201700436 | WILEY | Dispatch: 12.1.18 | CE: Ishwarya R
Journal Code | Manuscript No. | | No. of pages: 10 | PE: Revathi K.

CHEMISTRY & BIODIVERSITY

Chemical Composition of Essential Oils from *Thymus vulgaris*, *Cymbopogon citratus*, and *Rosmarinus officinalis*, and Their Effects on the HIV-1 Tat Protein Function

Giordana Feriotto,[a] Nicola Marchetti,[a] Valentina Costa,[a] Simone Beninati,[b] Federico Tagliati,[c] and Carlo Mischiati*[c]

[a]Department of Chemistry and Pharmaceutical Sciences, University of Ferrara, Ferrara, Italy
[b]Department of Biology, University "Tor Vergata", Rome, Italy
[c]Department of Biomedical Sciences and Surgical Specialties, University of Ferrara, via Luigi Borsari 46, 44121 Ferrara, Italy, e-mail: carlo.mischiati@unife.it

New drugs would be beneficial to fight resistant HIV strains, in particular those capable of interfering with essential viral functions other than those targeted by highly active antiretroviral therapy drugs. Despite the central role played by Tat protein in HIV transcription, a search for vegetable extracts able to hamper this important viral function was never carried out. In this work, we evaluated the chemical composition and possible interference of essential oil from *Thymus vulgaris*, *Cananga odorata*, *Cymbopogon citratus*, and *Rosmarinus officinalis* with the Tat/TAR-RNA interaction and with Tat-induced HIV-1 LTR transcription. GC/MS Analysis demonstrated the biodiversity of herbal species translated into essential oils composed of different blends of terpenes. In all of them, 4 – 6 constituents represent from 81.63% to 95.19% of the total terpenes. Essential oils of *Thymus vulgaris*, *Cymbopogon citratus*, and *Rosmarinus officinalis* were active in interfering with Tat functions, encouraging further studies to identify single terpenes responsible for the antiviral activity. In view of the quite different composition of these essential oils, we concluded that their interference on Tat function depends on specific terpene or a characteristic blend.

Keywords: antiviral activity, essential oils, trans-activating region-RNA, transcription

Introduction

The appearance of HIV strains resistant to different antiviral drugs increased between 2004 and 2010, as reported by the World Health Organization. High levels were observed in several countries, including Angola (16%), Argentina (10%), Botswana (10%), Cuba (22%), Mexico (9%), Papua New Guinea (16%), and South Africa (14%).[1] In light of this recrudescence, the identification of new drugs, active on HIV replication, returns to be a primary objective in the fight against acquired immune deficiency syndrome (AIDS).

Several vegetable extracts have inherent anti-HIV potential and represent a natural reservoir for future antiviral drugs. Many of them inhibit HIV-1 replication by not yet described mechanisms. For instance, *Litsea* species extracts affect viral replication at a concentration of 20 μg/mL without clearly visible cytotoxicity in a panel of human cell lines, and eight prototypic sesquiterpenes and litseaverticillols active against HIV were identified in *Litsea verticillata* extracts.[2] A general inhibitory effect on HIV replication was described in extracts prepared from *Cassine transvaalensis*,[3] *Avicennia marina*,[4] *Erythrina abyssinica*,[5] *Prunella vulgaris*,[6] *Cassia sieberiana* and *Cassia abbreviata*,[7] and *Rosmarinus officinalis*.[8] The methanol extracts of root, shoot, leaf and seed of *Thymus* species inhibited HIV replication at concentrations between 200 and 500 μg/mL[9] and an ethanol extract of *Rosa canina* (fruit), *Urtica Dioica* and *Tanacetum Vulgare* (leaf and stem) provoked a significant rise in CD4 count in HIV patients.[10] Moreover, other vegetable extracts exhibited specific activities on HIV viral proteins or functions, *i.e.* inhibiting the reverse transcriptase, protease or integrase activities or interfering with virus entries into cells. In this regard, *Sanguisorba officinalis*[11] and *Pelargonium sidoides*[12] extracts inhibited the entry of the virus into the cells. *Pometia pinnata*[13] and *Mimusops elengi*[14] leaf extracts can modulate the activity of HIV integrase and numerous vegetable extracts showed inhibitory potential on reverse transcriptase.[15][16]

In addition to crude extracts, essential oil (EO) isolated from aromatic plants by steam or hydro-

distillation showed specific antiviral activity.[17] Although EOs are complex natural mixtures of about 20 – 60 different components, a few major low molecular weight terpenes and terpenoids present at high concentrations determine the biological activity.[18]

New molecules, capable of interfering with essential viral functions different from those targeted by actual antiviral drugs, would be beneficial to fight resistant HIV strains. In this respect, the central role played by Tat (Trans-activator of transcription) protein in HIV transcription is considered an attractive target for antiviral drug development.[19] The Tat protein of HIV recruits the kinase activity of the P-TEFb complex to the short viral TAR (Trans-Activating Region) RNA, with a consequent strong increase of elongated viral transcripts and reappearance of HIV from the latent stage.[20] Therefore, compounds interfering with transcription could impair viral reactivation.[21] In this respect, chemically synthesized small molecules targeting the Tat/TAR-RNA complex were able to modulate HIV replication and Tat-dependent HIV promoter transcription.[22][23]

In this work, we analyzed the potential of EOs from different vegetable species to interfere with Tat/TAR-RNA interaction and Tat-induced activity of the HIV promoter.

Results

GC/MS Analysis

A few predominant terpenes were identified by GC/MS (*Table 1*). In *Rosmarinus officinalis*, 6 out of 21 constituents detected represent about 91% of the EO (α-pinene 10.11%, camphene 4.61%, β-pinene 7.71%, eucalyptol 50.63%, camphor 13.27%, β-caryophillene 4.62%). In *Cymbopogon citratus*, 5 out of 15 constituents detected represent 95.19% (β-pinene 22.47%, linalool 2.41%, *cis*-verbenol 6.14%, nerol 4.98%, *cis*-citral 59.19%). In *Thymus vulgaris*, 5 out of 34 compounds identified represent 81.64% (γ-terpinene 13.16%, linalool 3.31%, thymol 37.78%, isothymol 23.15%, β-caryophillene 4.24%). In *Cananga odorata*, 4 out of 26 constituents represent 91.64% (linalool 16.58%, α-gurjunene 7.10%, benzyl benzoate 18.71%, benzyl salicylate 49.25%).

Influence of EOs on the HIV-1 Tat/TAR-RNA Complex

We studied the effect of different EOs on the Tat/TAR-RNA complex by Electrophoretic Mobility Shift Assay (EMSA). After gel electrophoresis, the Tat/TAR-RNA complex can be easily distinguishable from the free TAR-RNA because the former has a slower migration. Initially, we evaluated the influence of EO presence on complex assembly. To this aim, we performed the assembly of Tat/TAR-RNA complex in the absence or presence of increasing amount of EO. When the EO concentration became effective, gel analysis showed conversion of the Tat/TAR-RNA band into a fast migrating band containing the free-TAR-RNA. Among the EOs examined, only those from *Thymus vulgaris*, *Cymbopogon citratus*, and *Rosmarinus officinalis* showed a marked inhibitory effect in the range of concentrations tested (*Figure 1,a*). EMSA analysis showed a marked inhibitory potential of EOs from *Rosmarinus officinalis* (from 0.25 to 0.5 μg/mL), *Thymus vulgaris* (from 3 to 6 μg/mL), and *Cymbopogon citratus* (from 6 to 12 μg/mL) on the binding of Tat to TAR-RNA. Another EO was inactive, *i.e.* EO from *Cananga odorata*, being ineffective on the Tat/TAR-RNA interaction at equivalent doses or even at higher than those tested above (from 7.5 to 240 μg/mL).

In addition, we examined whether the EOs could bind directly to TAR-RNA. For this purpose, the TAR-RNA was incubated in the absence or presence of increasing concentrations of EO to verify whether its gel migration could be slowed down by stable interactions with EO constituents (*Figure 1,b*). A distamycin derivative (compound 25)[23] and neomycin, two drugs that are able to bind the TAR-RNA and change its gel migration, were used as positive controls. The distamycin derivative forms covalent bonds with TAR-RNA and traps it in the loading well, while neomycin modifies TAR-RNA structure and only delays its migration. As reported in *Figure 1,b*, the presence of EO did not affect band intensity nor did it cause a delay in TAR-RNA migration even in the presence of high EO concentrations (from 30 to 240 μg/mL).

Finally, we studied the effect of EOs on stability of preformed Tat/TAR-RNA complex. To this aim, Tat protein was pre-incubated in the presence of TAR-RNA to allow the assembly of Tat/TAR-RNA complexes and then EO was added at incremental concentrations. As shown in *Figure 1,c*, EOs from *Thymus vulgaris*, *Cymbopogon citratus* and *Rosmarinus officinalis* compromised the stability of the Tat/TAR-RNA complex, as clearly visible by the appearance of free TAR-RNA band in the concentration ranges from 6 to 12 μg/mL, from 12 to 25 μg/mL and from 1 to 2 μg/mL, respectively. These concentrations were comparable to those observed in the previous investigation (*Figure 1,c*). Once again, the EO from *Rosmarinus officinalis* was the most efficient.

Taken together, these data highlight the specific potential of the EOs from *Thymus vulgaris*, *Cymbopogon*

Table 1. Composition of EOs analyzed by GC/MS

	Components[a]	*Rosmarinus officinalis*[b]	*Cymbopogon citratus*[b]	*Thymus vulgaris*[b]	*Cananga odorata*[b]
1	α-Pinene	10.11	0.08	1.22	0.51
2	Camphene	4.61	–	0.87	–
3	β-Pinene	7.71	22.47	0.32	0.38
4	Myrcene	0.68	–	1.46	0.04
5	β-Terpinene	0.06	–	–	–
6	α-Phellandrene	–	–	0.34	–
7	3-Carene	0.33	–	0.12	–
8	α-Terpinene	–	–	1.98	–
9	iso-Sylvestrene	–	–	0.66	0.16
10	Eucalyptol	50.63	0.47	1.71	0.11
11	*trans*-β-Ocimene	–	0.91	0.03	–
12	*cis*-β-Ocimene	–	0.52	0.06	–
13	Bornylene	–	–	–	0.16
14	γ-Terpinene	–	–	13.16	–
15	Terpinolene	0.24	–	0.33	0.10
16	Myrtenol	–	0.45	–	–
17	Perillene	–	0.26	–	–
18	Linalool	0.77	2.41	3.31	16.58
19	Myrtanal	–	0.32	–	–
20	iso-Camphane	–	–	–	0.37
21	Camphor	13.27	–	1.38	–
22	Photocitral a	–	0.83	–	–
23	β-Terpineol	–	–	–	0.13
24	β-Citronellal	–	0.48	–	–
25	Borneol	–	–	1.47	–
26	*p*-Menth-1-en-8-ol	0.55	–	–	–
27	*cis*-Verbenol	–	6.14	–	–
28	4-Terpineol	0.99	–	1.40	0.18
29	*trans*-Carveol	–	0.35	–	–
30	α-Terpineol	2.44	–	0.68	1.58
31	γ-Terpineol	–	–	–	0.49
32	Nerol	–	4.98	0.06	0.14
33	*cis*-Citral	–	59.19	–	–
34	Bornyl acetate	–	–	0.31	–
35	Thymol	–	–	37.78	–
36	iso-Thymol	–	–	23.15	–
37	α-Cubebene	0.10	–	–	–
38	Thymol acetate	–	–	0.21	–
39	Copaene	0.34	–	0.07	–
40	β-Bourbonene	–	–	0.10	–
41	Cadinene	–	–	–	0.19
42	α-Gurjunene	–	–	–	7.10
43	β-Cedrene	–	–	–	0.15
44	β-Caryophyllene	4.62	–	4.24	0.16
45	Sesquichamene	–	–	–	1.40
46	β-Cubebene	–	–	0.11	–
47	Alloaromadendrene	–	–	0.05	1.17
48	α-Caryophyllene	0.57	–	0.21	0.05
50	γ-Gurjunene	–	–	–	0.54
51	γ-Muurolene	0.27	–	–	0.27
52	Cuparene	–	–	–	0.14
53	γ-Cadinene	0.24	–	0.28	–
54	δ-Cadinene	0.31	–	–	–
55	β-Cadinene	–	–	0.50	–
56	Caryophyllene oxide	0.97	–	1.26	–
57	γ-Eudesmol	–	–	0.14	–

Table 1. (cont.)

	Components[a]	*Rosmarinus officinalis*[b]	*Cymbopogon citratus*[b]	*Thymus vulgaris*[b]	*Cananga odorata*[b]
58	δ-Cadinol	–	–	0.19	–
59	Benzyl benzoate	–	–	–	18.71
60	Benzyl salicylate	–	–	–	49.25

[a] Components were identified by comparing the MS data with the spectrum library and listed in increasing order of retention time. [b] % Peak area relative to total peaks area obtained from peak report.

citratus, and *Rosmarinus officinalis* in preventing the formation of the Tat/TAR-RNA complex and in facilitating its disruption.

Effects of EOs on Tat-dependent HIV-1 LTR Transcription

In order to study the effects of EOs on Tat-dependent HIV-1 long terminal repeat (LTR) transcription, we used the HL3T1 cell line as an experimental model mimicking viral latency reactivation. These cells contain an HIV-1 LTR-CAT cassette stably integrated into their genome that is transcribed at high level in the presence of Tat protein.

First, we determined for each EO the concentration exerting a similar effect on the proliferation of HL3T1 cells. Cells were cultured for three days in the absence or presence of increasing concentrations of EO and then cell viability was evaluated by determining oxidative metabolism activity by MTT assay. Cell growth in the presence of EO was expressed as a percentage with respect to untreated control. By expressing the cell growth as a function of EO concentration (*Figure 2,a*), we interpolated the concentration at which the EOs from *Rosmarinus officinalis*, *Thymus vulgaris*, *Cymbopogon citratus* or *Cananga odorata* inhibited cell growth by 50% compared to untreated control (IC_{50}), which was 0.18 μg/mL, 1.30 μg/mL, 0.61 μg/mL, and 0.60 μg/mL, respectively. Cisplatin was used as a positive controls for cytotoxic effect and the dose-activity dependence was evaluated.

Subsequently, the effects of EOs on Tat-induced HIV-1 LTR transcription activity were evaluated. In a preliminary experiment, addition of Tat to the HL3T1 cells significantly augmented ($P < 0.05$) the HIV-1 LTR transcription by 97.2 ± 5.9 fold ($n = 9$). A distamycin derivative (compound 25),[23] capable of inhibiting viral transcription induced by Tat, was used as a positive control in each experiment and inhibited the increase of transcription in a dose-dependent manner (*Figure 2, b*), with a concentration causing 50% Reduction of Transcription (RT_{50}) of 4.3 μM. In a following experiment, the effects of different EOs on Tat-induced HIV transcription were compared. As described above, EOs have different cytotoxicity. To exclude the possibility that modulation of transcription could solely be due to their effect on cell proliferation, rather than to specific mechanisms of inhibition of Tat/TAR-RNA interaction, concentrations of each EO producing similar effects on cell growth, expressed as multiples (1, 1.5, or 2 fold the IC_{50}) or sub-multiple (0.5 fold the IC_{50}) of their respective IC_{50} concentration, were compared (*Figure 2,c*). The results showed a strong inhibitory effect on viral transcription only for EOs that interfered with the binding of Tat to TAR-RNA (*Figure 1*). In particular, the EO of *Rosmarinus officinalis* (RT_{50} = 0.05 μg/mL) was the most interesting since it reduced viral transcription to 10% when administered at 0.09 μg/mL (0.5 fold the IC_{50}), a concentration at which 75% of the cells were still viable. Moreover, the EOs of *Thymus vulgaris* (RT_{50} = 0.83 μg/mL) and *Cymbopogon citratus* (RT_{50} = 0.54 μg/mL) showed good inhibitory potentials on viral transcription, because they reduced viral transcription to 52% and 60% when administered at 0.30 μg/mL and 0.65 μg/mL (0.5 fold the IC_{50}), respectively. Conversely, the EO from *Cananga odorata* (RT_{50} not determinable from the range of concentrations tested) had no effect on viral transcription in the presence of Tat, even when used at highly cytotoxic concentrations (1 – 2 fold the IC_{50}, 0.60 – 1.20 μg/mL). In addition, the Selectivity Index (*SI*) was calculated for each EO as the ratio of the IC_{50} value to the RT_{50} value ($SI = IC_{50}/RT_{50}$). Excluding EO from *Cananga odorata* (*SI* = not determinable), the others showed *SI* values greater than 1, *i.e. Cymbopogon citratus* (*SI* = 1.13), *Thymus vulgaris* (*SI* = 1.57), and *Rosmarinus officinalis* (*SI* = 3.6). By these SI values, even if still below the threshold value 5 – 10 considered acceptable to produce beneficial antiviral effects versus cytotoxicity in therapy, the EOs from *Cymbopogon citratus, Thymus vulgaris*, and *Rosmarinus officinalis* showed an effect on viral transcription even if it was not sufficiently high to indicate them as such in experimental antiviral therapy. However, their inhibitory effect on viral transcription and on binding of Tat to TAR-RNA reported above (*Figure 1*) suggested that they are a source from which to draw future antiviral drugs.

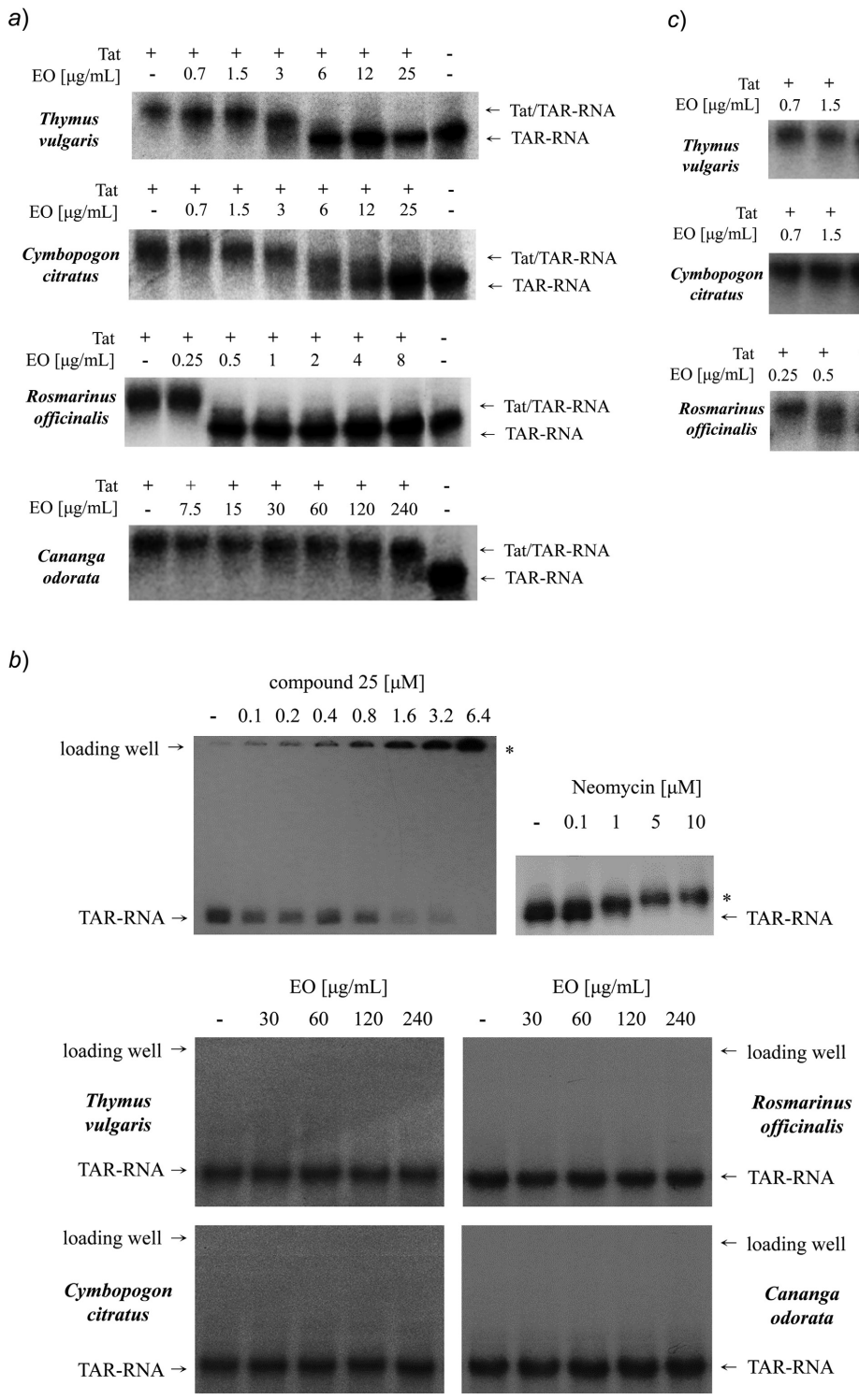


Figure 1. Dose-dependent effects of EOs on the interaction of Tat with TAR-RNA studied by EMSA. *a*) Assembly of the Tat/TAR-RNA complex was performed in the absence or presence of increasing amount of EO. *b*) Effect of EO on TAR-RNA migration. RNA binding drugs compound 25 and neomycin were used as positive control. Asterisks indicate TAR-RNA bands with modified migration rate. *c*) Tat protein was pre-incubated with ~~incremental EO concentrations prior to addition of TAR-RNA.~~

Discussion

The appearance of HIV strains resistant to different antiviral drugs has increased as reported by the World Health Organization.[1] In light of this recrudescence, the search for molecules capable of interfering with essential viral functions different from those targeted by highly active antiretroviral therapy drugs would be

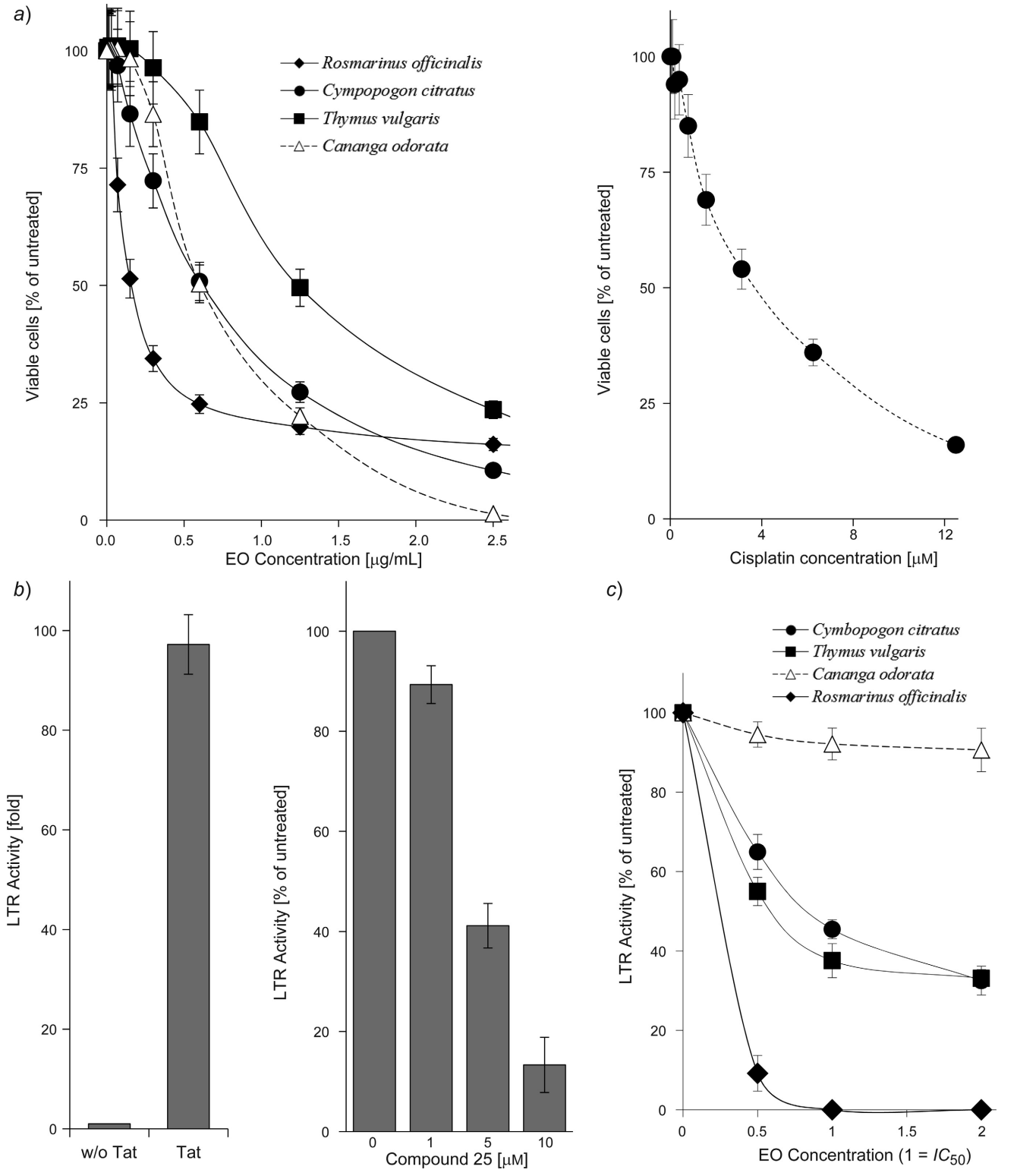


Figure 2. Effects of EOs on Tat-induced HIV-1 transcription in HL3T1 cells. *a*) Dose dependent effects of EOs on cell proliferation. Cisplatin was used as positive control of cytotoxicity. *b*) *c*) Tat-activated LTR-driven chloramphenicol acetyltransferase assay. The results were indicated as mean (n = 9) ± standard deviation. *b*) Induction of the LTR-driven transcriptional activity obtained in the presence of Tat compared to basal activity in its absence (fold) and dose-dependent effects of the positive control molecule compound 25 on Tat-induced viral transcription. The transcriptional activity in the presence of compound was expressed as percent to that observed in untreated cells. The results were indicated as mean (n = 9) ± standard deviation. w/o Tat = without Tat. *c*) Dose-dependent effects of EOs on Tat-induced transcription. The comparison of the inhibitory potential of each EO on viral transcription was performed using concentrations producing similar effects on cell growth to exclude the possible contribution of cytotoxicity. 0.5 = 0.5 fold the IC_{50}; 1 = IC_{50}; 1.5 = 1.5 fold the IC_{50}; 2 = 2 fold the IC_{50}. The results were indicated as mean (n = 9) ± standard deviation.

beneficial to fight resistant HIV strains. In this respect, the central role played by Tat protein in HIV transcription is one of the most attractive targets for antiviral drug development,[19] and molecules interfering with the Tat/TAR-RNA complex could impair HIV transcription and virus reactivation.[21] A previous work reported in the literature described the effects of a natural compound contained in tea leaves, *i.e.* tannic acid, which modulated HIV transcription by interfering with the binding of nuclear factors to a DNA element of 30-bp located in the neighborhood of the NF-kB element in the HIV promoter.[24] However, a study on plant compounds able to inhibit viral transcription by interfering with the interaction between Tat and TAR-RNA, has never been performed.

In this work, we first studied the interfering potential of EOs showing different terpene compositions (*Table 1*) on Tat/TAR-RNA interaction. Subsequently, EOs were tested as inhibitors of Tat-induced transcriptional activity of the HIV-1 promoter in HeLa cells. We believe that EOs with both characteristics should have anti-HIV potential for *in vivo* applications and could be an interesting source of antiviral drugs for use in patients who have become refractory to standard antiretroviral therapies. Among the EOs tested, we reported that only those of *Thymus vulgaris*, *Cymbopogon citratus*, and *Rosmarinus officinalis* affected the assembly of Tat/TAR-RNA complexes. It was reported in literature that molecules of vegetable origin bind to structured t-RNA, *i.e.* berberine and palmatine, perturbing its conformation.[25] In a similar manner, constituents in the active EOs could bind TAR-RNA inducing a change in its structure that prevents Tat binding. However, our EMSA (*Figure 1,b*) performed using a native gel indicated that EO components did not affect the structure of TAR-RNA because, in their presence, no additional bands with modified migration appeared beyond that of TAR-RNA. Therefore, the interference in Tat/TAR-RNA complex formation observed in *Figure 1,a* did not result by TAR-RNA structure modifications, but rather should be due to the direct activity of the EO constituents towards the Tat protein. This assumption was corroborated by data presented in *Figure 1,c* showing that addition of active EOs destabilized preformed Tat/TAR-RNA complexes.

EOs administration strongly reduced Tat-induced HIV-1 LTR transcription (*Figure 2,c*). We found that inhibitory effects of EOs on viral transcription directly correlated with their efficiency in inhibiting the interaction of Tat with TAR-RNA, suggesting that the antiviral effect of EOs occurred by targeting this complex. The transcriptionally active Tat protein is a monomer with a cysteine-rich domain, in which several CxxC motifs form intramolecular disulfide bonds required for transactivation activity.[26] Inasmuch as its activity dramatically decreased by pre-incubating the protein with strongly reducing agents,[26] likewise specific terpenes or a blend of them present within the active EO could reduce the cysteine disulfide bond of Tat, thus preventing its binding to TAR-RNA. However, not all the terpenes could be able to reduce Tat intramolecular disulfide bonds. In fact, even if the antioxidant activities observed in EOs of *Cananga odorata*, *Rosmarinus officinalis* and *Thymus vulgaris* were comparable,[27] their effects on Tat/TAR-RNA interaction (*Figure 1,a* and *1,c*) and Tat-induced transcription (*Figure 2,c*) were sharply different, with the EO of *Cananga odorata* being completely inactive with respect to the others even when used at highly cytotoxic concentrations. Therefore, biodiversity in the terpene composition, rather than a generic antioxidant activity of terpenes, could influence different bioactivity of the EOs. Although SI values do not recommend the use of EOs in experimental antiviral therapy, mainly because of their cytotoxicity, their intrinsic potential on Tat functions suggests that they could be reservoirs of future antiviral drugs.

We tried to identify the putative molecules responsible for the antiviral activity of the EOs. Usually, a few major components of the EO are responsible for its biophysical and biological features.[18] GC/MS composition of the EOs of *Thymus vulgaris*, *Cymbopogon citratus* and *Rosmarinus officinalis* showing promising antiviral activities was compared with that of the inactive EO of *Cananga odorata*. The comparison did not reveal common predominant molecules (*Table 1*). Thus, we excluded that antiviral effect of active EOs could be due to a single common terpene. We believe that a blend of the major terpenes characteristic of each EO could likely determine their antiviral effects. However, as suggested by others,[28] we cannot exclude that the synergy of all terpenes, including those present in trace amounts, could contribute to the biological effects of the EOs herein studied.

Conclusions

EOs interfered with Tat functions, a feature that was never described before. The similar bioactivity associated with the sharp different chemical compositions of EOs from *Thymus vulgaris*, *Cymbopogon citratus*, and *Rosmarinus officinalis* make them a potential natural source of future anti-HIV drugs targeting Tat function.

Experimental Section

Essential Oils

The EO of *Cananga odorata* (density = 0.964 gr/mL) was from CTM, Verona, Italy. The EO of *Cymbopogon citratus* (density = 0.876 gr/mL) was from Fundacion Chankuap, Macas, Ecuador. The The EO of *Thymus vulgaris*, thymol chemotype (density = 0.895 gr/mL), was from Extrasynthese, Genay, France. The EO of *Rosmarinus officinalis* cineol chemotype (density = 0.932 gr/mL) was from Vitalis Dr. Joseph S.r.l., Brunico (BZ), Italy. The EOs were aliquoted and stored at −80 °C in the dark in a glass vial sealed with Teflon cap until their use.

GC/MS Analysis

The EOs were analyzed by GC/MS using an *Agilent 5973* Network quadrupole mass selective detector coupled with an *Agilent GC 6850 Series II* Network Trace gas chromatograph. A *HP-5MS* capillary column containing 5%-phenyl-methylpolysiloxane (30 m × 0.25 mm, film thickness, 0.25 μm) was employed. GC operating conditions were as follows: carrier gas, helium with a flow rate of 2 mL/min; column temperature program was from 45 °C to 100 °C at 1 °C/min, then from 100 °C to 250 °C at 5 °C/min; injector inlet temperature, 280 °C; volume injected, 1 μL of the EO in dichloromethane; split ratio, 1:40. MS operating parameters were as follows: ionization potential, 70 eV; ion source temperature, 230 °C; quadrupole temperature 150 °C, solvent delay 4.20 min, mass range 35 – 300 *m/z*. The GC retention and mass spectra of peaks obtained were compared with those of authentic standards from NIST/MST/EPA software database and mass spectra from the literature.[29 – 31]

Cell Cultures

The HL3T1 cell line is a HeLa derivative containing stably integrated, silent copies of the HIV-1 LTR promoter linked to the chloramphenicol acetyl transferase (CAT) gene.[32] The cells produce the CAT enzyme upon introduction of active Tat. The cells were cultured in DMEM (*Dulbecco* Modified Essential Medium) with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in 5% CO_2.

Labelling of the TAR-RNA

The labelling of HIV-1 TAR-RNA was performed as described previously.[33] Briefly, the plasmid pGA99 carrying the wild-type TAR-RNA sequence was linearized with HindIII. Labeling was performed in 20 μL of buffered solution containing 50 mM Tris-Cl pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, 0.1 mM EDTA pH 8, 1 μg of the linearized plasmid, 200 μM of each cold ATP, UTP, and CTP, 50 U of RNA-guard, 10 μCi [α^{32}P] GTP, and 20U of SP6 RNA polymerase. After 30-min incubation at 37 °C, the reaction was heated at 65 °C for 5 min and the labelled TAR-RNA was purified by phenol-extraction. Free nucleotides were removed by gel filtration on *Sephadex G150* column.

EMSA

The Tat peptide used in this study (HIV-1 Tat 37 – 72, EVA790.1) was obtained through CFAR (WHO-UNAIDS Repository) and donated by the World Health Organization – Joint United Nations Programme on HIV/AIDS (WHO-UNAIDS). The radiolabeled TAR-RNA was heated at 90 °C for 2 min in a solution containing 10 mM phosphate, 1 mM EDTA, 0.1 M NaCl at pH 7.0 and afterwards cooled to room temperature to allow it to assume the secondary structure. The folded TAR-RNA was diluted and added to the binding reaction. To test the direct interaction with TAR-RNA, a serial dilution of EO was performed in binding buffer (10 mM phosphate buffer pH 7.2, 1 mM EDTA, 0.1 M NaCl, 1 mM $MgCl_2$, 1 mM DTT, 170 μg/mL yeast tRNA and 10% glycerol)[34] and then TAR-RNA was added to the reaction mix in a final volume of 10 μL, followed by an additional 15 min incubation on ice. Neomycin (*Sigma–Aldrich*, Milan, Italy) was used as a positive control.[35] In a preliminary experiment, to determine the optimal peptide concentration necessary to obtain a clearly distinguishable shift of migration of the Tat/TAR RNA complex with respect to the free TAR-RNA, 1 ng of labelled TAR-RNA was incubated with increasing amounts of Tat 37 – 72 that ranged from 0 to 100 μM in binding buffer for 15 min on ice. In subsequent experiments, Tat was used at 0.25 μM. The interference of EOs with either assembly or disruption of Tat/TAR-RNA complexes was studied. To study the effect on assembly, Tat was pre-incubated for 15 min on ice with different concentrations of EO and then TAR-RNA was added to the reaction mix followed by an additional 15 min incubation on ice. Alternatively, to test the ability of EOs to disassemble preformed Tat/TAR-RNA complex, TAR-RNA was pre-incubated with Tat for 15 min on ice and then different concentrations of EO were added for an additional 15 min incubation on ice. Samples were fractionated by electrophoresis through native 12% polyacrylamide gel in 1 × TBE (90 mM TRIS/borate and 2 mM EDTA at pH

7.7) and the gel was dried under vacuum at 80 °C before autoradiographic procedure (*Biomax*, Kodak).

Cell Growth Assay

The effects of EOs on cell proliferation were tested on HL3T1 cells by the CellTiter 96 Cytotoxicity Assay kit (*Promega Italia*, Milan, Italy), following the indicated instructions. Cisplatin (*Sigma–Aldrich*, Milan, Italy) was used as positive control for cytotoxicity. Briefly, 100 μL of cells diluted in DMEM plus 10% FBS (*Sigma–Aldrich*, Milan, Italy) at a density of 13,000 cells/mL were seeded in each well of a 96-well plate and then grown for 72 h in the presence or absence of increasing amounts of EOs or cisplatin. After the addition of 25 μL of staining solution, the cells were incubated for 2 h at 37 °C whereupon 100 μL lysis solution were added and the plate was incubated over-night at 37 °C to homogenate the color. Color quantification was performed at 550 nm (OD_{550}) and 620 nm (OD_{620}). The sample values were compared with control values obtained in the absence of EO and expressed as percent of control. Percentages were graphed as a function of cisplatin or EO concentrations. The concentration required for 50% inhibition of the cellular proliferation, half maximal inhibitory concentration (IC_{50}), was calculated. Dose-response equations were built in to GraphPad-Prism software version 6.01 and analysed by nonlinear regression to obtain the IC_{50} values.

Cell Transfections and CAT Assay

HL3T1 cells were diluted in DMEM plus 10% FBS and seeded in a 24-well plate at 20,000 – 28,000 ~~cells?~~/ cm^2. After 2 h, necessary to allow complete cell adhesion to well bottom, the medium was replaced and the cells were incubated for 24 h in the presence of EO at different concentrations multiples or submultiple of the IC_{50}. EOs were diluted in DMEM plus 10% FBS. In our experimental setting, distamycin derivative (compound 25)[23] was used as a positive control of interference for Tat function. Then, after a wash with PBS, 0.5 mL fresh DMEM was added to the cells. The HIV-1 Tat (0.5 μg in 50 μL Opti-MEM) (*Invitrogen*, *Life Technologies*, Milan, Italy) was mixed with Lipofectamine 2000 (2 μL in 50 μL Opti-MEM) (Invitrogen, Life Technologies, Milan, Italy) and the Tat/liposome complexes obtained 5 min after were directly added into cell culture medium. Two hour later, FBS was added and cells were cultured for 72 h. Afterwards, the collected cells were frozen and thawed to obtain cytoplasmic extracts and subsequently assayed for CAT activity.

Statistical Analysis

The results were expressed as arithmetic mean ± standard deviation. All the experiments were performed three times in triplicate ($n = 9$). Statistical calculations were performed using a one-way ANOVA and the differences among groups were examined using the *Student*'s *t*-test. *P* value <0.05 was considered significant.

Acknowledgements

The Tat peptide used in this study (HIV-1 Tat 37 – 72, EVA790.1) was obtained through CFAR (WHO-UNAIDS Repository) and donated by the World Health Organization – Joint United Nations Programme on HIV/AIDS (WHO-UNAIDS). We thank Prof. *Gianni Sacchetti* (University of Ferrara) for providing us with the EOs.

Author Contribution Statement

M. C. conceived and designed the study, and wrote the draft; *G. F.* carried out EMSA and the experiments on Tat-induced transcription; *F. T.* carried out the cell culture and cytotoxicity experiments; *C. V.* carried out GC/MS analysis and *M. N.* carried out terpene profiling; *S. B.* contributed to the analysis of data and revision of the paper.

Disclosure Statement

This article does not contain any studies with human or animal subjects performed by any of the authors. The authors declare that they have no conflicts of interest.

References

[1] R. L. Hamers, K. C. Sigaloff, C. Kityo, P. Mugyenyi, T. F. R. de Wit, 'Emerging HIV-1 Drug Resistance after Roll-Out of Antiretroviral Therapy in Sub-Saharan Africa', *Curr. Opin. HIV AIDS* **2013**, *8*, 19 – 26.

[2] Y. Guan, D. Wang, G. T. Tan, N. Van Hung, N. M. Cuong, J. M. Pezzuto, H. H. Fong, D. D. Soejarto, H. Zhang, 'Litsea Species as Potential Antiviral Plant Sources', *Am. J. Chin. Med.* **2016**, *44*, 275 – 290.

[3] N. S. Mthethwa, B. A. Oyedeji, L. C. Obi, O. A. Aiyegoro, 'Anti-Staphylococcal, Anti-HIV and Cytotoxicity Studies of Four South African Medicinal Plants and Isolation of Bioactive Compounds from Cassine Transvaalensis (Burtt. Davy) Codd', *BMC Complement Altern. Med.* **2014**, *14*, 512.

[4] R. Namazi, R. Zabihollahi, M. Behbahani, A. Rezaei, 'Inhibitory Activity of *Avicennia marina*, a Medicinal Plant in Persian Folk Medicine, against HIV and HSV', *Iran. J. Pharm. Res.* **2013**, *12*, 435 – 443.

[5] M. M. Mohammed, N. A. Ibrahim, N. E. Awad, A. A. Matloub, A. G. Mohamed-Ali, E. E. Barakat, A. E. Mohamed, P. L. Colla, 'Anti-HIV-1 and Cytotoxicity of the Alkaloids of

Erythrina abyssinica Lam. Growing in Sudan', *Nat. Prod. Res.* **2012**, *26*, 1565 – 1575.
[6] C. Oh, J. Price, M. A. Brindley, M. P. Widrlechner, L. Qu, J. A. McCoy, P. Murphy, C. Hauck, W. Maury, 'Inhibition of HIV-1 Infection by Aqueous Extracts of *Prunella vulgaris* L', *Virol. J.* **2011**, *8*, 188.
[7] M. M. Leteane, B. N. Ngwenya, M. Muzila, A. Namushe, J. Mwinga, R. Musonda, S. Moyo, Y. B. Mengestu, B. M. Abegaz, K. Andrae-Marobela, 'Old Plants Newly Discovered: *Cassia sieberiana* D.C. and *Cassia abbreviata* Oliv. Oliv. Root Extracts Inhibit In Vitro HIV-1c Replication in Peripheral Blood Mononuclear Cells (PBMCs) by Different Modes of Action', *J. Ethnopharmacol.* **2012**, *141*, 48 – 56.
[8] O. I. Aruoma, J. P. Spencer, R. Rossi, R. Aeschbach, A. Khan, N. Mahmood, A. Munoz, A. Murcia, J. Butler, B. Halliwell, 'An Evaluation of the Antioxidant and Antiviral Action of Extracts of Rosemary and Provençal Herbs', *Food Chem. Toxicol.* **1996**, *34*, 449 – 456.
[9] M. Soleimani Farsani, M. Behbahani, H. Z. Isfahani, 'The Effect of Root, Shoot and Seed Extracts of the Iranian Thymus L. (Family: Lamiaceae) Species on HIV-1 Replication and CD4 Expression', *Cell J.* **2016**, *18*, 255 – 261.
[10] R. Zabihollahi, R. Namazi, M. R. Aghasadeghi, A. F. Esfahani, S. M. Sadat, M. H. Modarressi, 'The In Vitro Anti-Viral Potential of Setarud (IMOD™), A Commercial Herbal Medicine with Protective Activity against Acquired Immune Deficiency Syndrome in Clinical Trials', *Indian J. Pharmacol.* **2012**, *44*, 448 – 453.
[11] J. Liang, J. Chen, Z. Tan, J. Peng, X. Zheng, K. Nishiura, J. Ng, Z. Wang, D. Wang, Z. Chen, L. Liu, 'Extracts of Medicinal Herb *Sanguisorba officinalis* Inhibit the Entry of Human Immunodeficiency Virus Type One', *Yao Wu Shi Pin Fen Xi* **2013**, *21*, S52 – S58.
[12] M. Helfer, H. Koppensteiner, M. Schneider, S. Rebensburg, S. Forcisi, C. Müller, P. Schmitt-Kopplin, M. Schindler, R. Brack-Werner, 'The Root Extract of the Medicinal Plant *Pelargonium sidoides* is a Potent HIV-1 Attachment Inhibitor', *PLoS ONE* **2014**, *9*, e87487.
[13] A. Suedee, S. Tewtrakul, P. Panichayupakaranant, 'Anti-HIV-1 Integrase Compound from *Pometia pinnata* Leaves', *Pharm. Biol.* **2013**, *51*, 1256 – 1261.
[14] A. Suedee, S. Tewtrakul, P. Panichayupakaranant, 'Anti-HIV-1 Integrase Activity of *Mimusops elengi* Leaf Extracts', *Pharm. Biol.* **2014**, *52*, 58 – 61.
[15] A. T. Mbaveng, V. Kuete, B. M. Mapunya, V. P. Beng, A. E. Nkengfack, J. J. Meyer, N. Lall, 'Evaluation of Four Cameroonian Medicinal Plants for Anticancer, Antigonorrheal and Antireverse Transcriptase Activities', *Environ. Toxicol. Pharmacol.* **2011**, *32*, 162 – 167.
[16] H. S. Kwon, J. A. Park, J. H. Kim, J. C. You, 'Identification of Anti-HIV and Anti-Reverse Transcriptase Activity from *Tetracera scandens*', *BMB Rep.* **2012**, *45*, 165 – 170.
[17] K. R. Kurapati, V. S. Atluri, T. Samikkannu, G. Garcia, M. P. Nair, 'Natural Products as Anti-HIV Agents and Role in HIV-Associated Neurocognitive Disorders (HAND): A Brief Overview', *Front. Microbiol.* **2016**, *6*, 1444.
[18] F. Bakkali, S. Averbeck, D. Averbeck, M. Idaomar, 'Biological Effects of Essential Oils – A Review', *Food Chem. Toxicol.* **2008**, *46*, 446 – 475.
[19] S. Massari, S. Sabatini, O. Tabarrini, 'Blocking HIV-1 Replication by Targeting the Tat-Hijacked Transcriptional Machinery', *Curr. Pharm. Des.* **2013**, *19*, 1860 – 1879.
[20] K. A. Jones, B. M. Peterlin, 'Control of RNA Initiation and Elongation at the HIV-1 Promoter', *Annu. Rev. Biochem.* **1994**, *63*, 717 – 743.
[21] G. Mousseau, S. Mediouni, S. T. Valente, 'Targeting HIV Transcription: The Quest for a Functional Cure', *Curr. Top. Microbiol. Immunol.* **2015**, *389*, 121 – 145.
[22] M. Baba, 'Inhibitors of HIV-1 Gene Expression and Transcription', *Curr. Top. Med. Chem.* **2004**, *4*, 871 – 882.
[23] C. Mischiati, A. Finotti, A. Sereni, S. Boschetti, P. G. Baraldi, R. Romagnoli, G. Feriotto, K. T. Jeang, N. Bianchi, M. Borgatti, R. Gambari, 'Binding of Hybrid Molecules Containing Pyrrolo [2,1-c] [1,4] Benzodiazepine (PBD) and Oligopyrrole Carriers to the Human Immunodeficiency Type 1 Virus TAR-RNA', *Biochem. Pharmacol.* **2004**, *67*, 401 – 410.
[24] F. Uchiumi, T. Hatano, H. Ito, T. Yoshida, S. Tanuma, 'Transcriptional Suppression of the HIV Promoter by Natural Compounds', *Antiviral Res.* **2003**, *58*, 89 – 98.
[25] M. M. Islam, R. Sinha, G. S. Kumar, 'RNA Binding Small Molecules: Studies on t-RNA Binding by Cytotoxic Plant Alkaloids Berberine, Palmatine and the Comparison to Ethidium', *Biophys. Chem.* **2007**, *125*, 508 – 520.
[26] S. E. Koken, A. E. Greijer, K. Verhoef, J. van Wamel, A. G. Bukrinskaya, B. Berkhout, 'Intracellular Analysis of In Vitro Modified HIV Tat Protein', *J. Biol. Chem.* **1994**, *269*, 8366 – 8375.
[27] G. Sacchetti, S. Maietti, M. Muzzoli, M. Scaglianti, S. Manfredini, M. Radice, R. Bruni, 'Comparative Evaluation of 11 Essential Oils of Different Origin as Functional Antioxidants, Antiradicals and Antimicrobials in Foods', *Food Chem.* **2005**, *91*, 621 – 632.
[28] M. Dell'Agli, R. Fagnani, N. Mitro, S. Scurati, M. Masciadri, L. Mussoni, G. V. Galli, E. Bosisio, M. Crestani, E. De Fabiani, E. Tremoli, D. Caruso, 'Minor Components of Olive Oil Modulate Proatherogenic Adhesion Molecules Involved in Endothelial Activation', *J. Agric. Food Chem.* **2006**, *54*, 3259 – 3264.
[29] P. Sandra, C. Bicchi P, 'Capillary Gas Chromatography in Essential Oil Analysis', Hüthig Publishing, Heidelberg, 1987.
[30] R. P. Adams, 'Identification of Essential Oil Components by Gas Chromatography and Mass Spectrometry', Allured Publishing Corporation, Carol Stream, IL, 2001.
[31] NIST, 'Mass Spectra Search for the NIST/EPA/NIH Mass Spectral Library Version 2.0', National Institute of Standards and Technology, Gaithersburg, MD, 2005.
[32] B. K. Felber, G. Pavlakis, 'A Quantitative Bioassay for HIV-1 Based on Trans-Activation', *Science* **1988**, *239*, 184 – 187.
[33] C. Mischiati, K. T. Jeang, G. Feriotto, L. Breda, M. Borgatti, N. Bianchi, R. Gambari, 'Aromatic Polyamidines Inhibiting the Tat-Induced HIV-1 Transcription Recognize Structured TAR-RNA', *Antisense Nucleic Acid Drug Dev.* **2001**, *11*, 209 – 217.
[34] C. T. Rigl, D. H. Lloyd, D. S. Tsou, S. M. Gryaznov, W. D. Wilson, 'Structural RNA Mimetics: N3'→P5' Phosphoramidate DNA Analogs of HIV-1 RRE and TAR RNA form A-Type Helices That Bind Specifically to Rev and Tat-Related Peptides', *Biochemistry* **1997**, *36*, 650 – 659.
[35] S. Wang, P. W. Huber, M. Cui, A. W. Czarnik, H.-Y. Mei, 'Binding of Neomycin to the TAR Element of HIV-1 RNA Induces Dissociation of Tat Protein by an Allosteric Mechanism', *Biochemistry* **1998**, *37*, 5549 – 5557.

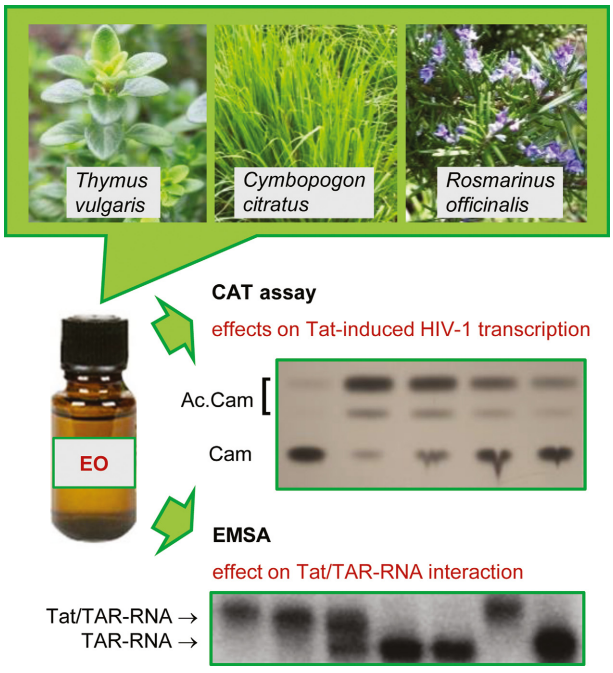
Received September 22, 2017
Accepted December 13, 2017

CHEMISTRY & BIODIVERSITY

Entry in the Table of Contents for Contribution »201700436«

Chemical Composition of Essential Oils from *Thymus vulgaris*, *Cymbopogon citratus*, and *Rosmarinus officinalis*, and Their Effects on the HIV-1 Tat Protein Function

*G. Feriotto, N. Marchetti, V. Costa, S. Beninati, F. Tagliati, C. Mischiati**



Keywords for Contribution »201700436«

antiviral activity
essential oils
trans-activating region-RNA
transcription

MARKED PROOF

Please correct and return this set

Please use the proof correction marks shown below for all alterations and corrections. If you wish to return your proof by fax you should ensure that all amendments are written clearly in dark ink and are made well within the page margins.

Instruction to printer	*Textual mark*	*Marginal mark*
Leave unchanged	··· under matter to remain	New matter followed by ⌐ or ⌐(x2)
Insert in text the matter indicated in the margin	⌐	
Delete	/ through single character, rule or underline or ⊢⊣ through all characters to be deleted	⌐/ or ⌐/(x2)
Substitute character or substitute part of one or more word(s)	/ through letter or ⊢⊣ through characters	new character / or new characters /
Change to italics	— under matter to be changed	⌐
Change to capitals	≡ under matter to be changed	≡
Change to small capitals	= under matter to be changed	=
Change to bold type	~ under matter to be changed	~
Change to bold italic	≈ under matter to be changed	≈
Change to lower case	Encircle matter to be changed	≠
Change italic to upright type	(As above)	⌐
Change bold to non-bold type	(As above)	⌐
Insert 'superior' character	/ through character or ⌐ where required	Y or Y under character e.g. Y^{3} or Y^{3}
Insert 'inferior' character	(As above)	⌐ over character e.g. Y_{2}
Insert full stop	(As above)	⊙
Insert comma	(As above)	,
Insert single quotation marks	(As above)	Y or Y and/or Y or Y
Insert double quotation marks	(As above)	Y or Y and/or Y or Y
Insert hyphen	(As above)	⊢
Start new paragraph	⌐	⌐
No new paragraph	⌐	⌐
Transpose	⌐	⌐
Close up	linking ⁀ characters	⁀
Insert or substitute space between characters or words	/ through character or ⌐ where required	Y
Reduce space between characters or words	| between characters or words affected	⊤