Author's response to reviews

Title: In vivo activity of terpinen-4-ol, the main bioactive component of Melaleuca alternifolia Cheel (tea tree) oil against azole-susceptible and -resistant human pathogenic Candida species

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Author's response to reviews: see over
RESPONSE TO THE REVIEWERS’ COMMENTS

Dear Editor

Thank you for sending us the reviewers’ comments, regarding our manuscript, and for allowing us to submit a revised version of our paper.

In revising the manuscript, we have taken into account all the referees’ remarks as described in the accompanying letter.

All changes have been made to the manuscript in accordance to the referees’ comments. In addition, a table with the Kovat’s Indices has been included as requested by one of the referees.

With all the suggested amendments now made together with other corrections, we believe the manuscript has been further improved, and hope that it will be accepted for publication.

I thank you again for the kind consideration given to our scientific effort.

REFEREE 1

The referee rightly stresses the need of assessing the biological activity of the minor constituents. While this certainly warrants further studies, we have elected to test primarily two of the most abundant constituents for which there are European Pharmacopeia Standards and preliminary information about their contrasting biological effects.

In fact, terpinen-4-ol had already been demonstrated to be active in vitro against Candida species. Therefore, our primary objective was to re-check in vitro activity with an improved methodology, and most of all, to demonstrate its in vivo activity for the first time.

Concerning the composition of Tea Tree Oil we fully agree on the need to show the Kovat Index of each component of the mixture in a table (now Table 1), specifically detailing these values. The table shows the Kovat Index as a percentage of each component of the mixture.

Furthermore, we have clarified in Materials and Methods section our reference to a specific bibliography for our use of the retention indices and the type of column used.

Finally, we have clarified in each specific section the number of the repeated determinations made for each test and the type of statistical assessment. This particularly applies to in vitro tests (see below, point by point reply).

One major clarification is due to animal investigation: we have used five rats for each experimental group. The results show a very low statistical deviation in each group. This approach has always been considered valid in all previous publications by our group. Also for ethical reasons we would never be allowed by our animal ethical Committee to repeat the same animal experiment under the above statistical consideration.

REFEREE 2

1. We have done our very best in editing the paper and improving its English, also with the help of an English-speaking colleague.

2. The comparison has been made with reference to drugs. Nonetheless, we wish to point out that some vaccines could also be used therapeutically.
3. According to well established microbiologically standards (CLSI, formerly NCCLS) a strain of *C. albicans* with a MIC greater than 64 micrograms/ml is a fluconazole-resistant strain. As often happens in the comparison between *in vivo* and *in vitro* activity of several drugs, the definition of resistance *in vitro* does not match completely with the *in vivo* investigations with animals, in which some residual activity persists. This is exactly the case of fluconazole.

4. The fungal load after the fourth week of infection was indeed determined, but has not been included in the figures, since after 28 days all animals healed completely from the infection.

**POINT-BY-POINT RESPONSE TO THE REFEREES’ SUGGESTION**

1. Page 1
   - line 19 - “GS: pino.salvatore@iss.it” amended to “GS: pino @iss.it”.

2. In “Abstract”:
   - Page 2
     - line 37, 38 - “(v/v)” changed to “(volume/volume)”.
     - line 41 - amended.
   - Page 3
     - lines 44, 45, 46 - all remarks have been amended accordingly.

3. In “Background”
   - Page 3
     - line 48 - According to our mother tongue English speaking colleague, the verbs must be in the present perfect tense.
     - lines 52, 53, 54 and 62 - all remarks have been considered and the text amended
     - lines 48, 50, 55 and 56 - the verbs must be in the present tense because this is factual statement.
     - lines 50, 55 and 56 - see above
     - lines 50, 51 - amended together with other changes

4. In “Methods”:
   - Page 4
     - lines 69,70,71 - have been changed according to the referees’ comments together with other changes.
     - line 69 - “with a low content of 1,8-cineole” text removed.
     - line 70 - information added after “as shown in the Results (see Table 1)”
   - lines 80, 81 - all the amendments have been made accordingly.
   - line 85 amended
   - lines 87, 88 - amended
   - line 94 - amended

   - Page 5
     - line 98 - “Table 1” and “see additional file 1” amended to “Table 2”.
   - line 107, 109 - amended

   - Page 6
     - line115, 117 and 128 - amended
• line 121 - the words, “Each experiment was performed in triplicate, independently” were added.

Page 7
• line 151 - amended.

5. In “Results”

Page 7
• line 156 - amended with reference [15].

Page 7, 8
• lines 159, 160, 161, 162 and 163 - amended to “The substances and their composition are shown in Table 1. The oil was a terpinen-4-ol type according to the European Pharmacopoeia [4] and the International Standard ISO 4730:1996 [5].”

Page 8
• line 167 - “Table 2” amended to “in Tables 2 and 3”.
• line 168 - “Table 2” renamed “Table 3”.
• lines 169, 170, 172, 173, 174, 175 and 183 - amended.
• line 180 - “Table 3” renamed “Table 4” and the words “the component” inserted
• line 187 - “In Table 2” amended to “In Table 3, MICs for”.

Page 9
• line 210 - amended.

6. In “Discussion”

Page 10
• line 220 - [15] changed to [16].
• lines 215, 216, 217, 223, 224, 225 and 228 - amended.
• line 229 - [13, 16] changed to “activity [13, 17]”.
• line 231 - [16-19] changed to [17-20].
• line 233 - amended.
• line 236 - [20] changed to [21].

Page 11
• line 238 - [21] changed to [22].
• line 242 - [22-24] changed to [23-25].
• line 244 - [25] changed to [26].
• lines 256 and 260 - amended

Page 12
• line 263 - amended.

7. In “References”

Page 14
Stream, Illinois; 1995” was added.
- line 324 - 16 changed to 17
- line 326 - 17 changed to 18
- line 329 - 18 changed to 19
- line 331 - 19 changed to 20
- line 334 - 20 changed to 21
- line 336 - 21 changed to 22

Page 15
- line 340 - 22 changed to 23
- line 344 - 23 changed to 24
- line 348 - 24 changed to 25
- line 351 - 25 changed to 26

8. In “Figures”

Page 16
Page 17
- line 377 - amended

9. In “Tables”

Page 18
- Tables were added.
- line 379 - “Table 1. Chemical composition of the Melaleuca alternifolia Cheel essential oil.” was added, “Table 1” renamed “Table 2”.
- line 382 - “Table 2” renamed “Table 3”.
- line 392 - “Table 3” renamed “Table 4”.
- lines 386, 387, 388, 390, 395, 396, 397, 398 and 400 - amended

10. In “Additional files”

Page 19
- “Additional files” were removed.

11. The “Figures files” were corrected as requested.

We again thank you for your kind assistance.

Kinds regards

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