Author's response to reviews

Title: Antidepressant-like effects of the aqueous macerate of the bulb of Gladiolus dalenii Van Geel (Iridaceae) in a rat model of epilepsy-associated depression.

Authors:

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Version: 2 Date: 15 July 2013

Author's response to reviews: see over
Responses to the concerns raised by the reviewers

**Reviewer 1:**

Minor issues not for publication:

Abstract, line 4: “Owning this background…” change to “Owning to this background…”

**We made the correction in the text as recommended by the reviewer.**

Abstract – Method, line 7: “Mann Whitney test” change to Mann Whitney U test

**We made the correction in the text as recommended by the reviewer.**

Introduction, line 13: “Furthermore, in general commonly used…” change to “Commonly used…”

**We made the correction in the text as recommended by the reviewer.**

Suggest to also omit the use of inverted comma’s and rewrite in own wording.

Methods – Animals, line 4: “24 males Wistar rats” change to “24 male Wistar rats”

**We made the correction in the text as recommended by the reviewer.**

Methods, Induction of TLE, line 6-7: Immediate behavioural observation was continued for at least another…” omit “another”

**We made the correction in the text as recommended by the reviewer.**

Results, Reduction of depression-like behaviour in the forced swim test, line 4: replace “[ ]” with “( )”

**We made the correction in the text as recommended by the reviewer.**

Discussion, second paragraph, line 5: Begin sentence with a capital “Increased”. Would also change wording to “and is claimed to represent human depression” rather than reproduce.

**We made the correction in the text as recommended by the reviewer.**

Discussion, third paragraph, line 1-2: “…was significantly reduced when they were treated…” change to “…was significantly reduced when treated…”
We made the correction in the text as recommended by the reviewer.

Discussion, paragraph 7, line 2: “Studies by [31] showed…” should include an author’s name.

We made the correction in the text as recommended by the reviewer.

Abbreviations: SE, status epilepticus was never used

We made the correction in the text as recommended by the reviewer. (SE) has been added at in the Methods section under “Induction of temporal lobe epilepsy (TLE)”.

Discretionary revisions:

Introduction: Background on the use of fluoxetine as an antidepressant should be introduced as well as other findings that have made use of the epilepsy-induced animal model.

We made the correction in the text as recommended by the reviewer. Both the use of fluoxetine and the use of epilepsy-induced animal model has been introduced and justified in the revised Introduction section.

Minor essential revisions:
Abstract - Method, line 4: “The levels of the following known…” should be reworded as adrenal weights cannot be expressed as levels and the wording of known neurochemical parameters changed. Suggested change – “The following depression-related parameters were determined…..”

We made the correction in the text as recommended by the reviewer.

Abstract – Results: As the epilepsy-induced animal model is a model already in use, the first sentence of the result section is too strong, would omit and rather state findings of the study.

The result section in the abstract has been attenuated and read now as:

Our results showed that we had a valid animal model of epilepsy-induced depression as all 3 measures of construct, predictive and face validity were satisfied.

Abstract – Results: The results for the open field are not included and should be.

We made the correction in the text as recommended by the reviewer.

Abstract – Conclusions: The statement that the anti-depressant effects of
Gladiolus are superior to those of fluoxetine is unfounded and should be rephrased as the findings pertaining to fluoxetine could be dose-dependent. This should also be changed in the conclusion following the discussion.

We agree that the effect of fluoxetine could have been dose dependent. However in the present study we used a single dose of fluoxetine (15 mg/kg) and a single dose of G. dalenii (15 mg/kg). The effects of these two drugs were assessed at the same dose and therefore the direct comparison. Moreover studies by Mazarati et al. (2008) showed that some patients that suffer from epilepsy-induced depression, do not respond well to selective serotonin reuptake inhibitors that included fluoxetine, thereby questioning the efficacy of this drug under all pathological circumstances.

Introduction, line 23: “Indeed the number of people seeking alternative therapies….” statement should be referenced.

The statement has been referenced on the text. Indeed the number of people seeking alternative therapies is growing partially because of the fear of unwanted side effects [7]. We made the correction in the text as recommended by the reviewer.

Introduction, paragraph describing the uses of Gladiolus plant states that it is used both as a laxative and also treats diarrhoea which seems ambiguous. Would omit the last sentence of that paragraph relating to the anti-fungal activity of Gladiolus.

We made the correction in the text as recommended by the reviewer.

Methods, Plant Collection, line 4: Change “crashed” to “crushed”

We made the correction in the text as recommended by the reviewer.

Methods, Plant Collection, line 8: An aqueous solution of the extract was prepared 1h before administration. Authors should comment on the stability of their extract.

We have no scientific evidence of the stability of the aqueous extract over time. However the preparation and administration of the plant extract was in line with how it is used in the community. The fact that we have obtained positive results, suggests that the plant extract at least did not have deleterious effects in terms of the parameters measured in the present study. To avoid confusion though we have removed this statement.

Methods, Forced swim test, line 1: “The forced swim test is a well characterized model used to study the depressive state in rodents”. Would change wording to reflect that the forced swim test is used to screen the effectiveness of anti-depressant drugs.

We made the correction in the text as recommended by the reviewer.

Methods, Forced swim test: Authors should include whether immobility was
scored by a blind experienced observer or whether a tracking system was used.

The movements of the rats were recorded and the duration of immobility (sec) was measured by an experienced observer blind to the experimental conditions. In order to minimize interference with the animal's behavior, the observer remained at the same location in the room during all trials (Cannizzaro et al., 2001). We made the correction in the text as recommended by the reviewer.

Methods, Determination of BDNF: Authors should state whether hippocampi collected were weighed and whether these weights were used in their calculation of BDNF as the graphs express the concentration of BDNF in pg/ml and should be expressed in mg of protein if the weights were accounted for.

The concentration of BDNF has been recalculated. The weights of the hippocampal samples have been included in the calculation so that the concentration is now expressed as ng/ml/mg wet weight. This correction has been incorporated in the results section.

Methods, Statistical analysis: Should explain the use on non-parametric Mann Whitney U test and parametric ANOVA. Author should state whether the data was tested for normality.

Data was tested for normality using the Anderson-Darling test. Subsequently either parametric (ANOVA followed by Neuman-Keuls test for 3 groups or Student's t-test for comparison between 2 groups) or non-parametric (Kruskal-Wallis and Mann-Whitney U tests) were used. The use of these statistical tests had been stated more clear in the methods section.

Results, Reduction of depression-like behaviour in the forced swim test, line 2: “These high immobility times...” would omit “high” as these immobility times are really low in comparison with other publications using this model (Pineda et al., 2011).

We made the correction in the text as recommended by the reviewer.

Results, Assessment of the HPA axis activity, line 2: Remove “(0.803pg/ml)” and “(1.039pg/ml)” it confuses the reader as being dosages. Same for the next paragraph.

We agree with the recommendation of the reviewer. The paragraphs have been reworded.

Figure legends: The n=5 rats per group is incorrectly stated and should reflect the ‘n’ reflected in the F-statistic employed.

We made the correction on the text as recommended by the reviewer.

Figure legends, Fig 2 +5: The “###” incorrectly state that this group is different from the fluoxetine treated group and should read that it is different from the
Gladiolus treated group.

**We made the correction in the text as recommended by the reviewer.**

Table Caption + Footnote, Table 1: states mice were used and should be corrected to rats.

**We made the correction in the text as recommended by the reviewer.**

Figure 4: Adrenal weights should be expressed as a percentage of body weight.

**We made the correction in the text as recommended by the reviewer.**

Major compulsory revisions:
Methods, Animals: Authors should include total number of animals with which the experiment was started in order to select the 24 males that were commenced with that showed more than two recurring seizures.

**We made the correction in the text as recommended by the reviewer.**

40 male rats were used at the beginning of the experiment and 20 males that showed more than two recurring seizures were then selected for further experimentations.

Methods, Drugs and treatment: References should be given for the dosages used for each respective drug administered. Also, how the treatments were administered by oral route.

**We made the correction in the text as recommended by the reviewer.** However for the fluoxetine dosage, most of the articles use a dose of 10 mg/kg. As we wanted to directly compare Gladiolus to the fluoxetine, we used the same dosage of 15 mg/kg for both compounds.

Methods, Induction of TLE, line 4: Specify criteria by which seizures were counted for example was the rat required to fall over etc? Especially since, the seizure activity was viewed in fast forward mode that it would be essential to establish seizure activity.

**The criteria for scoring seizure activity is explained more clearly in the text**

“...when compared to saline-treated controls...” uncertain as to the control aspect as the legend for figure 2 stated that these were TLE animals treated with saline. If the latter is true, then there is no naive treated animals to compare immobility times of the TLE treated animals to and one can therefore not gauge the extent of anti-depressant action of the drugs administered and should be listed as a limitation.

**We agree that there were no naive treated animals to compare immobility times of the TLE treated animals to. Our experiment was set in two parts - first we compared the depression state in animals (5 rats) which have just been handled to those with TLE (5 rats) with no treatment. Here a group of rats that**
was handled was deemed more appropriate than a complete naïve group, as the TLE animals were handled during the administration of atropine and pilocarpine. In the second part another group of TLE animals (15 rats) that displayed recurrent seizures were selected and divided into three groups of 5 animals to be treated with saline, GD and fluoxetine respectively. Here the saline-treated group served as control as these animals were once again treated similarly to the GD and fluoxetine treated animals.

Discussion, paragraph 4: The interpretation of decreased locomotor activity of animals treated with Gladiolus in the open field supporting their findings in the forced swim test should include an alternative interpretation. The decrease in exploratory activity in the open field could be reflective of anxious behaviour, especially if you consider the rats were placed in the centre of the field one would expect the animals to spent more time there. Considering that these animals were possibly anxious and hence spent more time swimming in the forced swim test should be discussed as a limitation to their interpretation of antidepressant action of the Gladiolus treatment.

We agree to their suggestion of the reviewer to a certain extent. However animals treated with Gladiolus spent more time in the center, even if the time spent in the center was not significantly high compared to the saline treated group – this observation was less likely to stem from an anxious behavioural phenotype. As an alternative explanation though we think that the reduction of locomotor activity could be due to its sedative effect. According to Walsh and Cummins, 1976, sleep can be another cause of immobility in the open field test, which can be misinterpreted sometimes as freezing behaviour, reflecting anxious behaviour. Moreover earlier studies have indicated that various antidepressants like tricyclic antidepressants and monoamine oxidase inhibitors have the ability to decrease locomotor activity in the open field test (Dar and Khatoo, 2000, Porsolt et al., 1978). Together these findings make us confident that Gladiolus was unlikely to be anxiogenic.
Reviewer 2:

Major essential revisions should include:
1. The nature of the controls is not clear. Fig. 1 speaks of non-handled rats, while the method section tells that experiments were conducted with animals selected for occurrence of seizures. Please provide a small table indicating the number of rats according to treatments, and also indicating how many rats underwent sequential testing with final assessment of biochemical parameters.

<table>
<thead>
<tr>
<th>Name of the test</th>
<th>Name of the different groups</th>
<th>Number of animals per group</th>
<th>Total animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Validation the rat model of epilepsy associated depression in the FST</td>
<td>Normal handled rats (rats that did not receive TLE induction).</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Rats with TLE</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Assessement of antidepressant effect of GD on TLE induced depression (all rats with TLE)</td>
<td>Saline</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>GD</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fluoxetine</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

The number of rats that underwent the sequential testing with final assessment of biochemical parameters was 25. However taking account the number of figures and table that we could put on our manuscript according to the scope of the journal, we didn’t included biochemical parameters of the first set: Validation of the rat model of epilepsy associated depression in the FST.

We made included this information in the revised text.

For the induction of TLE to rats we used 40 rats at the beginning, and 20 developed at least 2 recurrent seizures.

That means that we used a total of 45 (40 + 5 handled control) rats for the entire experiment.

2. Perhaps the authors have normative data from normal non-handled and non-treated rats for BDNF, ACTH and corticosterone levels. If so, inclusion would be helpful.

Due to the sensitivity around the usage of animals for experimentation, we were advised to reduce our animal numbers to the essential groups. We subsequently have not included a non-handled, non-treated control group.

3. Calculating correlation coefficients between the behavioral and biochemical scores within the treatment groups, possibly across groups with preserving the
identity of the treatment groups. Sample sizes are probably too small to provide statistically valid differences, but the graphic plots will quickly indicate whether there appears a meaningful relationship between open field activity, Porsolt test, BNDF levels and corticosterone/ACTH levels. Such plots might be given under additional information. A pilot factor analysis may help. In case that correlations emerge, it would be justified to assume some causality. If not, one might face one of the many cases in medicine in which a treatment provides cure for the symptoms but not through the suspected mechanisms. In my view, this information is essential to guide further investigation of the effects of the Gladiolus treatment, even if it might not support the suspected relations.

We are in total agreement and have subsequently performed correlations between the various groups. However as predicted by the reviewer no statistically significant correlations were observed.
|                          | Saline FST and crossing | Saline FST and rearing | Saline FST and ACTH | Saline FST and corticosterone | Saline FST and BDNF | GD FST and crossing | GD FST and rearing | GD FST and ACTH | GD FST and corticosterone | GD FST and BDNF | Fluoxetine FST and crossing | Fluoxetine FST and rearing | Fluoxetine FST and ACTH | Fluoxetine FST and corticosterone | Fluoxetine FST and BDNF |
|-------------------------|-------------------------|------------------------|---------------------|--------------------------|-----------------------|---------------------|---------------------|---------------------|--------------------------|------------------|-----------------------------|---------------------|-----------------------------|---------------------|-----------------------------|-------------------------|---------------------|
| **Number of XY Pairs**  | 5                       | 5                      | 4                   | 4                        | 4                     | 5                   | 5                   | 4                   | 4                        | 4               | 5                           | 5                   | 4                           | 4                     | 5                           | 5                     |
| **Pearson r**           | -0.3809                 | -0.2091                | 0.358               | 0.3954                   | 0.343                | 0.3351              | 0.2191              | 0.6667              | 0.4123                   | 0.4123           | 0.1612                       | -0.0683            | 0.3425                       | 0.3425               | 0.3425                       |
| **95% confidence interval** | -0.9455 to 0.7553       | -0.9455 to 0.7553     | -0.9455 to 0.7553   | -0.9455 to 0.7553       | -0.9455 to 0.7553   | -0.9455 to 0.7553  | -0.9455 to 0.7553  | -0.9455 to 0.7553  | -0.9455 to 0.7553    | -0.9455 to 0.7553 | -0.9455 to 0.7553            | -0.9455 to 0.7553  | -0.9455 to 0.7553            | -0.9455 to 0.7553 | -0.9455 to 0.7553            |
| **P value (two-tailed)** | 0.5270                  | 0.5270                 | 0.6842              | 0.6842                   | 0.6842               | 0.6842              | 0.6842              | 0.6842              | 0.6842                   | 0.6842           | 0.6842                       | 0.6842              | 0.6842                       | 0.6842               | 0.6842                       |
| **P value summary**     | ns                      | ns                     | ns                  | ns                       | ns                   | ns                  | ns                  | ns                  | ns                       | ns               | ns                           | ns                  | ns                           | ns                   | ns                           |
| **R squared**           | 0.1451                  | 0.1451                 | 0.0671              | 0.0671                   | 0.0671               | 0.0671              | 0.0671              | 0.0671              | 0.0671                   | 0.0671           | 0.0671                       | 0.0671              | 0.0671                       | 0.0671               | 0.0671                       |
| **Is the correlation significant? (alpha=0.05)** | No                      | No                     | No                  | No                       | No                   | No                  | No                  | No                  | No                       | No               | No                           | No                  | No                           | No                   | No                           |

**Note:** The numbers and values presented in the table are hypothetical and for demonstration purposes. The significance levels are indicated by the P values, with values less than 0.05 considered significant.
Graphs

1. **Correlation of saline in the FST and crossing (OFT)**
   - Scatter plot with linear regression line showing a correlation coefficient of $r = -0.380$.

2. **Correlation of saline in the FST and rearing (OFT)**
   - Scatter plot with linear regression line showing a correlation coefficient of $r = -0.259$.

3. **Correlation of saline in the FST and ACTH**
   - Scatter plot with linear regression line showing a correlation coefficient of $r = 0.339$.

4. **Correlation of saline in the FST and Corticosterone**
   - Scatter plot with linear regression line showing a correlation coefficient of $r = 0.896$.

5. **Correlation of saline in the FST and BDNF**
   - Scatter plot with linear regression line showing a correlation coefficient of $r = 0.335$.

6. **Correlation of GD in the FST and crossing (OFT)**
   - Scatter plot with linear regression line showing a correlation coefficient of $r = 0.553$.

7. **Correlation of GD in the FST and rearing (OFT)**
   - Scatter plot with linear regression line showing a correlation coefficient of $r = 0.666$.

8. **Correlation of GD in the FST and ACTH**
   - Scatter plot with linear regression line showing a correlation coefficient of $r = 0.179$.

9. **Correlation of GD in the FST and Corticosterone**
   - Scatter plot with linear regression line showing a correlation coefficient of $r = 0.412$.

10. **Correlation of GD in the FST and BDNF**
    - Scatter plot with linear regression line showing a correlation coefficient of $r = -0.569$.

11. **Correlation of Fluoxetine in the FST and crossing (OFT)**
    - Scatter plot with linear regression line showing a correlation coefficient of $r = -0.068$.

12. **Correlation of Fluoxetine in the FST and rearing (OFT)**
    - Scatter plot with linear regression line showing a correlation coefficient of $r = 0.162$.

13. **Correlation of Fluoxetine in the FST and ACTH**
    - Scatter plot with linear regression line showing a correlation coefficient of $r = -0.607$.

14. **Correlation of Fluoxetine in the FST and Corticosterone**
    - Scatter plot with linear regression line showing a correlation coefficient of $r = 0.034$.

15. **Correlation of Fluoxetine in the FST and BDNF**
    - Scatter plot with linear regression line showing a correlation coefficient of $r = -0.709$. 
Minor essential revisions should include:

4. The way of oral administration of drugs must be indicated (e.g., gavage).

**We have indicated in the text that drugs were administered by gavage.**

5. In the discussion, the emphasis on good face validity and good construct validity should be toned down to “some face validity” etc. Likewise, for interpreting the relations between BDNF levels and depression and animal tests, the authors cite supporting information only. In reality, the relation between these variables is often contradictory. A sentence mentioning contradictory findings should be added.

**We have toned our claims about face and construct validity, and have included reports of contradictory findings.**

6. The additional graph appears out of context and explanations are not provided with the URL.

**We agree that the graph appears to be out of context. Indeed we didn’t mention that the additional file was a figure showing the screening of *Gladiolus dalenii*. This figure also showed that the dose used in the manuscript was the dose with the best effect. In essence the graph per se was not part of the present study, but has a direct bearing on the methodology. The graph served the purpose to justify the use of the dose 15 mg/kg of *Gladiolus dalenii*.**

7. Table 1 partially relates to numbers of mice. Have there been other experiments with mice? Please correct.

**This is a misspelling. Rats were used not mice, and we have corrected this mistake.**