Author's response to reviews

Title: In vitro anti-Herpes simplex virus activity of crude extract of the roots of Nauclea latifolia Smith (Rubiaceae)

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Version: 2 Date: 23 August 2013

Author's response to reviews: see over
Dear Editor,

We are pleased to hear that our manuscript was found to be of interest by the Reviewers. It is our opinion that their comments have provided us with the opportunity to improve it. We hope that the revised manuscript is now suitable for publication in *BMC Complementary and Alternative Medicine*.

Please find below a point by point reply to the Reviewers’ comments.

**REVIEWSER 1:**

1) “It would be risky to identify compounds based on UV absorption, retention time and molecular ion peaks. Authors must identify these natural products with the aid of NMR and mass fragmentation patterns.”

The referee is right. For a reliable identification NMR is fundamental. Here we considered the identification a tentative on the basis of the phytochemical analysis of the extract without isolating the pure components and comparing the results of the spectroscopic (UV) and spectrometric (MS) data to those reported in the literature. We are now working on the isolation of the pure components because the results on the antiviral activity are very promising, and we have decided to publish the first part of our investigation in consideration of the possible interest of virological results. An in depth phytochemical study is under way.

We have modified the abstract giving more evidence to the fact that component identification is, at present, a tentative. The following sentence was included in the Abstract under the Results section: “Strictosamide, vincosamide and pumiloside were tentatively identified together with quinovic acid glycoside” (page. 3, lines 17-18).

Table 1 has been modified with the introduction of a missing mass fragment representative of the glycosidic nature of strictosamide: 3337 [M+H-162].

2) “The IC50 and CH2Cl etc should be corrected through out the manuscript. IC50 50 should subscript. CH2Cl2 2 should be subscript.”

In the original Word file we submitted, all but one IC₅₀ and CH₂Cl₂ were written properly (50 and 2 subscript) throughout the manuscript. Probably, they were modified during the conversion into a pdf file.

3) “Authors should also identify the active lead compound(s). This would help them to determine if the bioactivity is due to the individual natural products or synergistic effect of all compounds.”

As stated above, we are working on the isolation of pure components following the strategy of the bioassay guided fractionation, but, to date, we have not positive results in the direction of
ascribing the biological activity to specific components. We are now evaluating whether the antiviral activity is related to the synergic effect of more than one component in the total extract. However, in our opinion, this study is beyond the scope of the present paper which is intended to report on the antiviral activity of the *N. latifolia* crude extract.

REVIEWER 2:

Abstract

1- “In the last line of Background part of the Abstract, authors mentioned about the impact of HSV-2 in HIV transmission. However, this statement in the present format is not accurate as it could be confusing for the readers as there is no real relationship between HSV-2 and HIV incidence. I agreed that because HSV-2 is more prevalent between sex workers or people who does not have safe and protected safe and on the other hand the incidence of HIV is also among the same population is higher than the others BUT it is not because of relationship between biology of those viruses so it is strongly recommended to remove that sentence.”

The Referee’s concern refers to the following sentence in the Abstract: ” In this study, HSV-2 was chosen as a viral model because of its strong impact on HIV transmission and acquisition.” We are surprised by the Referee’s comment because our sentence reports the state of the art. Moreover, this sentence in the Abstract is well supported by several sentences and references in the Introduction (page 4): “The incidence of HSV recurrence is increased in people with an impaired immune system, such as HIV-seropositive individuals and in transplant recipients [5, 6]. On the other hand, genital herpes may increase the risk of HIV acquisition by disrupting epithelial cells, with induction of local inflammation and production of cytokines and chemokines that activate and recruit CD4+ HIV target cells [7]. In a systematic review including a meta-analysis of longitudinal studies, prevalent HSV-2 infection was associated with a three-fold increased risk of HIV acquisition in both men and women, suggesting that, in areas of high HSV-2 prevalence, a high proportion of HIV infection is attributable to HSV-2 [8]. Therefore, strategies that can prevent or treat HSV infections are expected to reduce rates of sexual HIV transmission [9].”

Moreover, if we look at the very recent literature we easily find further support. Below please find a selection of recent papers along with the relevant conclusions from their abstracts:


“Simulations reinforce these analytical results and demonstrate epidemiological synergy between HSV-2 and HIV. In particular, numerical results show that HSV-2 favors the invasion of HIV, may dramatically increase the peak as well as reducing the time-to-peak of HIV prevalence, and almost certainly has exacerbated HIV epidemics”


“These data support a role of HSV-2 infection in enhancing HIV transmissibility.”

Thurman AR, Doncel GF. Herpes simplex virus and HIV: genital infection synergy and novel approaches to dual prevention. Int J STD AIDS. 2012 Sep;23(9):613-9

“Sexual transmission of HIV-1, in the absence of co-factors, is poorly efficient. Data support that herpes simplex virus type-2 (HSV-2) may increase a woman’s susceptibility to HIV-1.”


“Our findings suggest that HSV-2 may be both a biological and risk-associated cofactor for HIV-1 acquisition. In resource-limited countries, where both infections are prevalent efforts at symptomatic and diagnostic screening and treatment of HSV-2 should be part of HIV-1 prevention programs.”

For all these reasons we completely disagree with the Referee on this matter and are willing to keep our sentence in the Abstract because it is informative for the readers.

2- “The methods part, under Abstract needs to be rewrite because of some grammatical errors.”

The Referee is right. The following words were missing: “it was subjected to” and are now included in the revised version (page 3, line 9)

3- “Statistical program name is GraphPad PRISM not PRISM alone. The other question is: why they used version 4 as now a days version 6 is available.”

As suggested by the Referee the name “PRISM” has been replaced by “GraphPad PRISM” throughout the manuscript in the revised version. As stated in Material and Methods we used version 4: this does not affect the quality of the statistical analyses.

Background:
1- “Page 4: Name of the antiviral drugs should not be started with capital letter.”

According to the Referee’s suggestion the capital letters in the antiviral drugs names were removed.

2- “Why there is no citation to the other available herbal medications for HSV such as, Aloe vera or red algae creams or gels?there are too many approved and studied herbal medications for HSV infections. Therefore, authors must include some studies related to those plants as well not just African plants.”
The Referee is right. The fact that there are many herbal medications for HSV infections should be mentioned to improve the manuscript. Since it is impossible to give the reader an overview just by citing some papers we decided to cite the following review (ref. 13):


Moreover, the following sentence has been included in the Introduction: “In this context, natural products from medicinal plant extracts are very important source of anti-HSV agents and several extracts and pure compounds from herbal medicines have been reported to exert an anti-HSV activity [13].” (page 6, lines 3-5)

Methods:

1- “Authors must cite to the method which they used for development of drug resistant strain of HSV-2.”

The method used is now cited in the Materials and Methods section of the revised version. (Ref n. 22).


2- “Is heparin the only reagent that authors used? If no please include all reagents under the same subheading.”

Acyclovir was added

3- “HSV Inhibition Assay is not a correct subheading so it must change to the proper term.”

The subheading “HSV Inhibition Assay” has been changed into “HSV antiviral assays” (page 8)

4- “The MOI is different from pfu but authors mixed them together so it must be Corrected.”

We know very well that pfu is different from MOI therefore we never mixed the two terms. The definition of MOI (Multiplicity of Infection) is the number of pfu per cell (pfu/cell). Consequently, each time we describe a condition of infection we write for instance: “cells were infected with an MOI of 0.01 pfu/cell). This is the standard description virologists use in the scientific literature. We do not understand the Referee comment.

5- “Why authors used just one concentration for virucidal activity?”

We used 33 µg/ml because it corresponds approximately to the IC₉₀. This has been explicated in the new version by modifying a sentence as follows: ” To explore if the extract exerts a direct virus-inactivating activity, a virucidal assay was performed with an extract concentration that reduces almost completely virus infection (IC₉₀).” (page 13, line 3)
6- “Attachment assay is somehow similar to the virucidal assay. It would not recommended to pre-treat the virus itself with the extract for attachment assay. On the other hand after 4 degrees incubation they MUST allow virus to enter to the cells for 1 h at 37 degrees with medium not with CMC containing medium. The method in this part is not acceptable.”

Actually the virus was not pre-treated for one our but just mixed with the extract at 4° C and then used for the infection. It was a mistake in the description of the protocol and we are grateful to the Referee for having pointed it out. In the new version this point has been corrected. As for the rest of the protocol, this is a standard procedure reported in many papers. The presence of CMC neither prevents entry nor subsequent infections. We just added two citations to show that his protocol is widely used (ref 25 and 26).

7- “In entry assay, authors allowed the viruses for entry for 3 h, but for post adsorption assay they adjust the whole process for adsorption and entry for 2 h. It is not consistent.”

3 h are necessary because cells have to go from 4°C to 37°C while in post adsorption assay cells are at 37°C so the binding and entry of the virus can directly start. Again, these are standard procedures reported in the literature as can be seen in the cited references (25, 26)

Results:
1- “Authors must mention the exact percentage of DMSO. In the present format of the manuscript they wrote, below 1%!!!!it could be 0.8% or 0.5% or...?It must be clear.”

As written in the manuscript the concentration of DMSO is always below 1% so it is not toxic. The exact concentration is not always written because it depends from assay to assay but the untreated well has always the same volume of DMSO as the treated well so that DMSO effect is excluded.

2- “On page 11, authors mentioned that non of extracts’ concentration shows cytotoxicity against Vero cells then they wrote that CC50 is above 100 microgram per ml so how it could be concluded??”

In the Results section we stated that “… the extract did not affect Vero cell viability at any concentration tested. The CC50 value was above 100 µg/ml, indicating that the antiviral activities observed were not due to cytotoxicity.” If 100 µg/ml is the higher dose tested and it does not show cytotoxicity it is obvious that CC50 value is above 100 µg/ml.

3- “If the exact CC50 was not calculated the how they calculated SI as SI can be calculated based on defined IC50 and CC50.”

Again we did not show any exact SI value but we stated that it is >68.4

4- “Authors have used 33 microgram per ml for virucidal activity and on the other hand they mentioned that they diluted the mixture of virus and extract at the time of cell infection below than the concentration of the extract which showed antiviral activity BUT they did not mention about the results. It is totally unclear and it is not acceptable in this format.”
Indeed the results are mentioned in the following sentences of the Results section: “To explore if the extract exerts a direct virus-inactivating activity, a virucidal assay was performed. To this aim, HSV-2 aliquots were incubated with 33 µg/ml of extract at 4°C or 37°C. After incubation, the samples were titrated on Vero cells at high dilutions to reduce the sample doses below the antiviral concentrations. As reported in Table 2, this treatment did not produce a significant loss of HSV-2 infectivity, even at a concentration higher than its IC₅₀.”

Perhaps the following sentence is not clear: “After incubation, the samples were titrated on Vero cells at high dilutions to reduce the sample doses below the antiviral concentrations.” Therefore we modified it as follows in the revised version of the manuscript: “After incubation, the samples were titrated on Vero cells at high dilutions at which the extract was not active” (page 13, lines 4-5)

Again, the virucidal assay used in this study is a standard one as can be seen in references 25, 26

5- “There is a major problem in this manuscript, authors mentioned in the first part of the antiviral assays in the method under subheading:HSV inhibition assay that they did the treatment starting from the time of virus adsorption and even after adsorption continuously BUT there is no available data for that treatment in result part!!!!!!on the other hand they just mentioned that post adsorption treatment gave them the best result so what about the continuous treatment that they mentioned earlier.”

We are grateful to the Referee for having pointed out that our description was non clear giving rise to a misunderstanding. The dose-response curves shown in Fig 2 refer to the treatment for which the Referee says there are no results. Indeed, after the removal of the virus inoculum the serial dilutions of extracts were added again to the infected cell cultures, as stated in the Material and Methods section. To make this point more clear we added the following sentence to the Results: “The serial dilutions of plant extract were added again after the removal of the virus inoculum.” (page 12, lines 4-5) This is the “continuous treatment” mentioned by the Referee

6- “They did not mention about any difference or similarities of their results for sensitive or drug resistant HSV-2 strains”

We do not understand this comment. Why the Referee says that we did not mentioned any similarities or differences? The following sentences in the Results sections presents the results and say that both strains were sensitive to the plant extract: “The dose–response curves shown in Fig. 2 demonstrate that the extract exerts a remarkable antiviral activity which is independent on the virus sensitivity to acyclovir. The IC₅₀ values for the HSV-2 acyclovir-sensitive or resistant strain are 7.17 µg/ml (95% CI: 5.36 to 9.59) and 5.38 µg/ml (95% CI: 4.15 to 6.99) respectively.”