

UNIVERSITI SAINS MALAYSIA
GERAN PENYELIDIKAN UNIVERSITI PENYELIDIKAN
LAPORAN AKHIR

IN VIVO TERATOGENECITY AND ANTI-ALLERGIC OF
LIGNOSUS RHINOCEROS IN AN ASTHMATIC ROBERT MODEL
AND THEIR POSSIBLE BIOACTIVE CONSTITUENTS

PENYELIDIK

DR. NURUL ASMA ABDULLAH

PENYELIDIK BERSAMA

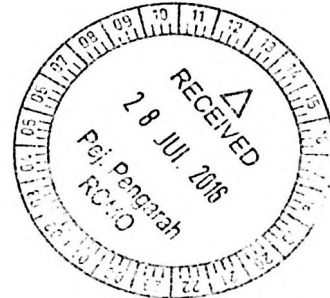
PROF. GAN SIEW HUA
DR. WAN EZUMI MOHD FUAD
DR. WAN MAJDIAH WAN MOHAMAD
DR. TENGKU AHMAD DAMITRI AL-ASTANI B TG. DIN
DR. FAEZAHTUL ARBAEYAH HUSEIN

2016

No. Rujukan : PPSK(R&D/LG(bil. 283)

Tarikh : 14 Julai 2016

Profesor Dr. Lee Keat Teong
 Pengarah
 Pejabat Pengurusan & Kreativiti Penyelidikan (RCMO)
 Bahagian Penyelidikan & Inovasi
 Tingkat 2, Bangunan Canselori 2
 Bangunan E42
 Universiti Sains Malaysia
 11800 Pulau Pinang
 (U/P En. Muhamad Nizam – Pegawai Sains)



Y.Bhg. Profesor Dr.,

LAPORAN PROJEK GERAN RUI

Dengan segala hormatnya saya merujuk kepada perkara di atas.

2. Bersama-sama ini saya sertakan 01 salinan laporan bagi projek penyelidikan yang berikut:-

| Bil. | Nama | Tajuk | Catatan |
|------|-------------------------|---|---------------|
| 1. | Dr. Nurul Asma Abdullah | In Vivo Teratogenecity and Anti-Allergic Effects of <i>Lignosus rhinoceros</i> in an Asthmatic Rodent Model and their Possible Bioactive Constituent (1001/PPSK/813065) | Laporan akhir |

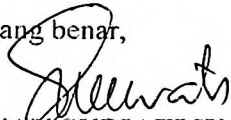
3. Diharapkan ianya mendapat perhatian untuk rekod pejabat Y.Bhg. Profesor Dr.

Sekian. Terima kasih.

BERKHIDMAT UNTUK NEGARA

Memastikan Kelestarian Hari Esok

Yang benar,


WAN SURIATI WAN NIK
 Pegawai Sains Kanan
 Unit Penyelidikan

s.k. Dr. Nurul Asma Abdullah

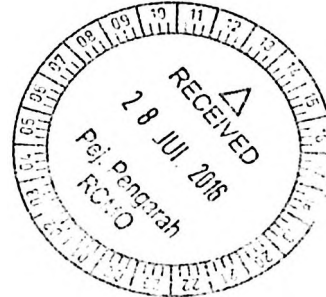
En. Iqram

Utk semakan dan tindakan
 lanjut. Tk.

Diana
 28/7.

No. Rujukan : PPSK(R&D/LG(bil. 283)

Tarikh : 14 Julai 2016

 Profesor Dr. Lee Keat Teong
 Pengarah
 Pejabat Pengurusan & Kreativiti Penyelidikan (RCMO)
 Bahagian Penyelidikan & Inovasi
 Tingkat 2, Bangunan Canselori 2
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 11800 Pulau Pinang
 (U/P En. Muhamad Nizam – Pegawai Sains)


Y.Bhg. Profesor Dr.,

LAPORAN PROJEK GERAN RUI

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2. Bersama-sama ini saya sertakan 01 salinan laporan bagi projek penyelidikan yang berikut:-


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| 1. | Dr. Nurul Asma Abdullah | In Vivo Teratogenicity and Anti-Allergic Effects of <i>Lignosus rhinoceros</i> in an Asthmatic Rodent Model and their Possible Bioactive Constituent (1001/PPSK/813065) | Laporan akhir |

3. Diharapkan ianya mendapat perhatian untuk rekod pejabat Y.Bhg. Profesor Dr.

Sekian. Terima kasih.

BERKHIDMAT UNTUK NEGARA
Memastikan Kelestarian Hari Esok

Yang benar,


WAN SURIATI WAN NIK
 Pegawai Sains Kanan
 Unit Penyelidikan

s.k. Dr. Nurul Asma Abdullah

 En. Iqram
 Utk semakan dan tindakan
 lanjut. Tk.
 Dina
 28/7.

Please use this checklist to self-assess your report before submitting to RCMO.
Checklist should accompany the report.

| NO. | ITEM | PLEASE CHECK (✓) | | |
|-----|--|------------------|-------|------|
| | | PI | JKPTJ | RCMO |
| 1 | Completed Final Report Form | ✓ | ✓ | ✓ |
| 2 | Project Financial Account Statement | | | ✓ |
| 3 | Asset/Inventory Return Form (Borang Penyerahan Aset/Inventori) | | | |
| 4 | A copy of the publications/proceedings listed in Section D(ii) (Research Output) | ✓ | ✓ | ✓ |
| 5 | Comprehensive Technical Report | ✓ | ✓ | ✓ |
| 6 | Other supporting documents, if any | | | |
| 7 | Project Leader's Signature | ✓ | ✓ | ✓ |
| 8 | Endorsement of PTJ's Evaluation Committee | | ✓ | ✓ |
| 9 | Endorsement of Dean/ Director of PTJ's | | ✓ | ✓ |



RU GRANT FINAL REPORT FORM

Please email a softcopy of this report to rcmo@usm.my



| | |
|------------|--|
| A | PROJECT DETAILS |
| i | <p>Title of Research:</p> <p><i>In vivo</i> teratogenicity and anti-allergic effects of <i>Lignosus rhinocerus</i> in an asthmatic rodent model and their possible bioactive constituents</p> |
| ii | <p>Account Number: 1001/PPSK/813065</p> |
| iii | <p>Name of Research Leader: Dr. Nurul Asma Abdullah</p> |
| iv | <p>Name of Co-Researcher:</p> <ol style="list-style-type: none"> 1. Prof. Gan Siew Hua 2. Dr. Wan Ezumi Mohd Fuad 3. Dr. Wan Majdiah Wan Mohamad 4. Dr. Tengku Ahmad Damitri Al-Astani B Tengku Din 5. Dr. Faezahtul Arbaeyah Husein |
| v | <p>Duration of this research:</p> <p>a) Start Date : 01/10/2012</p> <p>b) Completion Date : 31/03/2016</p> <p>c) Duration : 42 months</p> <p>d) Revised Date (if any) :</p> |
| B | ABSTRACT OF RESEARCH |
| | <p>(An abstract of between 100 and 200 words must be prepared in Bahasa Malaysia and in English. This abstract will be included in the Report of the Research and Innovation Section at a later date as a means of presenting the project findings of the researcher/s to the University and the community at large)</p> |

Bahasa Malaysia:

Lignosus rhinocerus (*L. rhinocerus*) atau lebih dikenali sebagai cendawan susu harimau, digunakan secara tradisional di kalangan masyarakat tempatan di Malaysia bagi merawat pelbagai penyakit termasuk asma. Kajian ini mengkaji jujuk mudah meruap daripada *L. rhinocerus* dengan menggunakan kromatografi gas-jisim spektrometri (GC-MS), kesan teratogenik dan kesuburan, sifat imunomodulatori dan kesan anti-asma *L. rhinocerus* di dalam model haiwan. Pengekstrakan berjujukan ke atas *L. rhinocerus* menggunakan lima pelarut telah mengenalpasti 44 jujuk. Sebatian daripada kumpulan asid lemak adalah komponen yang paling utama (68.58%) manakala kompaun yang paling utama adalah asid linoleik (49.39%). Kajian ini juga telah menunjukkan pemberian oral *L. rhinocerus* tidak menunjukkan sebarang tanda-tanda toksik yang berkaitan. Kajian ini juga mencadangkan pemberian oral *L. rhinocerus* tidak menunjukkan kesan teratogenik yang luar biasa di dalam tikus yang dikaji. Potensi anti-asma ekstrak *L. rhinocerus* telah dikaji ke atas tikus Sprague Dawley yang telah dicetuskan inflamasi pada saluran pernafasannya menggunakan ovalbumin (OVA). Rawatan dengan ekstrak *L. rhinocerus* telah berjaya menambah baik parameter yang berkaitan dengan asma di dalam tikus yang terarus. Rawatan dengan ekstrak *L. rhinocerus* telah berjaya mengurangkan jumlah eosinofil dalam BALF, IgE dalam serum, sitokin Th2 (IL-4, IL-5 dan IL-13) dalam BALF, infiltrasi eosinofil dalam paru-paru dan juga merubah bilangan sel T regulatori di dalam saluran pernafasan. Kesan imunomodulasi ekstrak *L. rhinocerus* telah menunjukkan penambahan populasi splenosit terutamanya populasi sel-sel CD3⁺ CD4⁺ dan CD3⁺ CD8⁺ dan juga proliferasi splenosit dan aktiviti fagositosis yang bertambah baik dan pengeluaran nitrik oksid (NO) yang terkawal. Kajian ini menyimpulkan bahawa *L. rhinocerus* mempunyai potensi untuk digunakan sebagai alternatif bagi rawatan alergik asma.

English:

Lignosus rhinocerus (*L. rhinocerus*) or locally known as Tiger's Milk mushroom, is traditionally used in the treatment of various diseases including asthma by indigenous communities in Malaysia. This study investigated volatile constituents of *L. rhinocerus* using gas chromatography mass spectrometry (GC-MS), teratogenic and male fertility, immunomodulatory properties and anti-asthmatic effects of *L. rhinocerus* in animal models. Sequential extractions of *L. rhinocerus* using five solvents demonstrated 44 constituents were identified. Compounds from the fatty acid group were the most predominant (68.58%) and the main constituent was linoleic acid (49.39%). This study also demonstrated that oral administration of *L. rhinocerus* did not noticeably show treatment-related signs of toxicity in both male and female rats. We also suggested that oral administration of *L. rhinocerus* extract did not result in remarkable teratogenicity in rats. The anti-asthmatic potential of *L. rhinocerus* extract was further investigated on ovalbumin (OVA)-induced airway inflammation Sprague Dawley rats. Treatments with *L. rhinocerus* extract significantly ameliorated related asthmatic parameters in the induced rats. Specifically, *L. rhinocerus* extract comparatively reduced eosinophils numbers in BALF, IgE in serum, Th2 cytokines (IL-4, IL-5 and IL-13) levels in BALF, eosinophil infiltrations in the lungs as well as modulate regulatory T cell numbers in the airways. Immunomodulatory effects of *L. rhinocerus* extract demonstrated improved splenocyte population mainly of CD3⁺CD4⁺ and CD3⁺CD8⁺ as well as improved splenocytes proliferation and phagocytosis activity and controlled nitric oxide (NO) production. In conclusion, *L. rhinocerus* has the potential to be used as an alternative for the treatment of allergic asthma.

| C | BUDGET & EXPENDITURE | | | | | | | | | | | | | | | | | | |
|-----------|---|-------------|--------------------|-------------|----------|--|----------|------|--|----------|---|---|----------|---|--|----------|---|---|----------|
| i | <p>Total Approved Budget : RM 220, 293.00</p> <p style="text-align: right;">Yearly Budget Distributed</p> <p style="text-align: right;">Year 1 : RM 85, 431.00</p> <p style="text-align: right;">Year 2 : RM 74, 431.00</p> <p style="text-align: right;">Year 3 : RM 60, 431.00</p> <p>Total Expenditure : RM 220, 544.43 *</p> <p>Balance : RM -251.43 *</p> <p>Percentage of Amount Spent (%) : 100%</p> <p>* Total expenditure and balance are not final.</p> <p><i># Please attach final account statement (eStatement) to indicate the project expenditure</i></p> | | | | | | | | | | | | | | | | | | |
| ii | <p>Equipment Purchased Under Vot 35000</p> <table border="1" data-bbox="100 840 1285 1056"> <thead> <tr> <th>No.</th> <th>Name of Equipment</th> <th>Amount (RM)</th> <th>Location</th> <th>Status</th> </tr> </thead> <tbody> <tr> <td></td> <td>None</td> <td></td> <td></td> <td></td> </tr> <tr> <td></td> <td></td> <td></td> <td></td> <td></td> </tr> </tbody> </table> <p><i># Please attach the Asset/Inventory Return Form (Borang Penyerahan Aset/Inventori) – Appendix 1</i></p> | No. | Name of Equipment | Amount (RM) | Location | Status | | None | | | | | | | | | | | |
| No. | Name of Equipment | Amount (RM) | Location | Status | | | | | | | | | | | | | | | |
| | None | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | |
| D | RESEARCH ACHIEVEMENTS | | | | | | | | | | | | | | | | | | |
| i | <p>Project Objectives (as stated/approved in the project proposal)</p> <table border="1" data-bbox="100 1261 1309 1746"> <thead> <tr> <th>No.</th> <th>Project Objectives</th> <th>Achievement</th> </tr> </thead> <tbody> <tr> <td>1</td> <td>To identify and quantify the major active chemical compounds of <i>Lignosus rhinocerus</i> extract</td> <td>Achieved</td> </tr> <tr> <td>2</td> <td>To investigate the <i>in vivo</i> teratogenicity effect of <i>Lignosus rhinocerus</i> extract in rats.</td> <td>Achieved</td> </tr> <tr> <td>3</td> <td>To determine the immunomodulatory effect of <i>Lignosus rhinocerus</i> extract.</td> <td>Achieved</td> </tr> <tr> <td>4</td> <td>To elucidate the anti-asthmatic effect of <i>Lignosus rhinocerus</i> extract in an asthmatic guinea pig model.</td> <td>Achieved</td> </tr> <tr> <td>5</td> <td>To make recommendation based on teratogenicity data and anti-asthmatic effect for the use of <i>Lignosus rhinocerus</i> as a potential therapeutic for allergic asthma.</td> <td>Achieved</td> </tr> </tbody> </table> | No. | Project Objectives | Achievement | 1 | To identify and quantify the major active chemical compounds of <i>Lignosus rhinocerus</i> extract | Achieved | 2 | To investigate the <i>in vivo</i> teratogenicity effect of <i>Lignosus rhinocerus</i> extract in rats. | Achieved | 3 | To determine the immunomodulatory effect of <i>Lignosus rhinocerus</i> extract. | Achieved | 4 | To elucidate the anti-asthmatic effect of <i>Lignosus rhinocerus</i> extract in an asthmatic guinea pig model. | Achieved | 5 | To make recommendation based on teratogenicity data and anti-asthmatic effect for the use of <i>Lignosus rhinocerus</i> as a potential therapeutic for allergic asthma. | Achieved |
| No. | Project Objectives | Achievement | | | | | | | | | | | | | | | | | |
| 1 | To identify and quantify the major active chemical compounds of <i>Lignosus rhinocerus</i> extract | Achieved | | | | | | | | | | | | | | | | | |
| 2 | To investigate the <i>in vivo</i> teratogenicity effect of <i>Lignosus rhinocerus</i> extract in rats. | Achieved | | | | | | | | | | | | | | | | | |
| 3 | To determine the immunomodulatory effect of <i>Lignosus rhinocerus</i> extract. | Achieved | | | | | | | | | | | | | | | | | |
| 4 | To elucidate the anti-asthmatic effect of <i>Lignosus rhinocerus</i> extract in an asthmatic guinea pig model. | Achieved | | | | | | | | | | | | | | | | | |
| 5 | To make recommendation based on teratogenicity data and anti-asthmatic effect for the use of <i>Lignosus rhinocerus</i> as a potential therapeutic for allergic asthma. | Achieved | | | | | | | | | | | | | | | | | |

ii Research Output

a) Publications in ISI Web of Science/Scopus

| No. | Publication (authors, title, journal, year, volume, pages, etc.) | Status of Publication (published/accepted/ under review) |
|-----|---|---|
| 1. | M. Johnathan, S. H. Gan , M. F. Wan Ezumi , A. H. Faezahtul and A. A. Nurul. Phytochemical profiles and inhibitory effects of Tiger Milk mushroom (<i>Lignosus rhinocerus</i>) extract on ovalbumin-induced airway inflammation in a rodent model of asthma. Johnathan et al. BMC Complementary and Alternative Medicine (2016) 16:167. | Published (IMPACT FACTOR 1.987, ISI-INDEXED), 21 ✓ |
| 2. | Johnathan M, Nurul AA, Ezumi MF, Gan SH. Gas Chromatography Mass Spectrometry Analysis of Volatile Compounds from <i>Lignosus rhinocerus</i> (Tiger milk mushroom). Research Journal of Pharmaceutical, Biological and Chemical Sciences. (2016), 7(4):5-16 | Published Accepted (SCOPUS INDEX) in press 2016, 2(4) July-Aug |
| | | |

b) Publications in Other Journals

| No. | Publication (authors, title, journal, year, volume, pages, etc.) | Status of Publication (published/accepted/ under review) |
|-----|---|--|
| | | |
| | | |

c) Other Publications

(book, chapters in book, monograph, magazine, etc.)

| No. | Publication (authors, title, journal, year, volume, pages, etc.) | Status of Publication (published/accepted/ under review) |
|-----|---|--|
| | | |

d) Conference Abstract/Proceeding

| No. | Conference (conference name, date, place) | Title of Abstract/Article | Level (International/National) |
|-----|---|--|-----------------------------------|
| 1. | Allergy School on Allergen Immunotherapy, 7-9th april 2016, Rungstedgaard, Denmark. | <i>Lignosus Rhinocerus</i> Extract Suppresses Airway Inflammation And Regulates Treg Imbalance In The Ovalbumin-Induced Asthmatic Model | International |
| 2. | Biomedical & Health Sciences Research 2015. The Everly Hotel Putrajaya. | Anti-Asthmatic Effects Of <i>Lignosus rhinocerus</i> (Tiger Milk Mushroom) Extracts on Ovalbumin-Induced Airway Inflammation In Sprague Dawley Model | International |
| 3. | International Conference on Natural Products 2015, 24th-25th March 2015, Johor Bahru, Malaysia. | Inhibitory effects of <i>Lignosus rhinocerus</i> (Tiger Milk mushroom) extracts on ovalbumin-induced airway inflammation | International |
| 4. | Immunology Symposium, Universiti Putra Malaysia. 16-17 th October 2014. | The inhibitory effects of tiger milk mushroom extract on airway inflammation in ovalbumin-induced allergic asthma. | National |

Please attach a full copy of the publication/proceeding listed above

| | |
|------------|---|
| iii | <p>Other Research Output/Impact From This Project (patent, products, awards, copyright, external grant, networking, etc.)</p> <ol style="list-style-type: none"> 1. Gold Medal at 25th International Invention, Innovation & Technology Exhibition (ITEX) for a product entitled "BreatheEzi (Tiger Milk Mushroom) for Management of Asthma". 2. Trademark granted for BreatheEzi™ (Natural alternative for the management of asthma). |
|------------|---|

E HUMAN CAPITAL DEVELOPMENT

a) Graduated Human Capital

| Student | Nationality (No.) | | Name |
|---------------|-------------------|---------------|----------|
| | National | International | |
| PhD | | | 1. 2. |
| MSc | | | 1. 2. |
| Undergraduate | | | 1. 2. |

b) On-going Human Capital

| Student | Nationality (No.) | | Name |
|---------------|-------------------|---------------|--|
| | National | International | |
| PhD | 1 | | 1. Johnathan Malagobadan (Thesis submitted – viva expected in August 2016) |
| MSc | 1 | | 1. Siti Zati Bayani Hamzah |
| Undergraduate | | | 1. 2. |

c) Others Human Capital

| Student | Nationality (No.) | | Name |
|----------------------|-------------------|---------------|--|
| | National | International | |
| Post Doctoral Fellow | | | 1. 2. |
| Research Officer | | | 1. 2. |
| Research Assistant | 2 | | 1. Siti Aminah Muhamad 2. Siti Nurshazwani Muhamad Sayuti |
| Others (.....) | | | 1. 2. |

| | |
|---|---|
| F | COMPREHENSIVE TECHNICAL REPORT |
| | <p>Applicants are required to prepare a comprehensive technical report explaining the project. The following format should be used (this report must be attached separately):</p> <ul style="list-style-type: none"> • Introduction • Objectives • Methods • Results • Discussion • Conclusion and Suggestion • Acknowledgements • References |
| G | PROBLEMS/CONSTRAINTS/CHALLENGES IF ANY |
| | <p><i>(Please provide issues arising from the project and how they were resolved)</i></p> <p>No major issues encountered during the research.</p> <p>The only problem is animal constraint since we had to wait several months in order to get the animals despite ordering made earlier.</p> |
| H | RECOMMENDATION |
| | <p><i>(Please provide recommendations that can be used to improve the delivery of information, grant management, guidelines and policy, etc.)</i></p> |

Project Leader's Signature:

 16/7/2016.

Name :

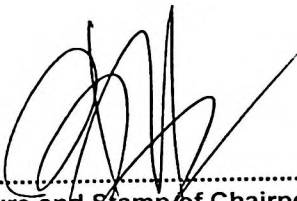
DR. NURUL ASMA ABDULLAH
Pensyarah Kanan

Date :

Pusat Pengajian Sains Kesihatan
Kampus Kesihatan USM
16150 Kubang Kerian, Kelantan

COMMENTS, IF ANY/ENDORSEMENT BY PTJ'S RESEARCH COMMITTEE

The researchers have achieved all (5) objectives; published one paper in a journal and another in Scopus. Aspect on Human Capital development was satisfactory - 1 PhD candidate is scheduled for his viva voce this year, and 1 MSc candidate is still active. Generally, the research outcomes are good.



Signature and Stamp of Chairperson of PTJ's Evaluation Committee

PROF. MADYA DR. LIM BOON HUAT
Timbuan Dekan

Name : Penyerikan dan Pengurusan Siswazah
Pusat Pengajian Sains Kesihatan
Kampus Kesihatan
Date : Universiti Sains Malaysia
16150 Kubang Kerian Kota Bharu, Kelantan



Signature and Stamp of Dean/ Director of PTJ

Name : PROFESOR AHMAD HJ. ZAKARIA
Dekan

Date : Pusat Pengajian Sains Kesihatan
Kampus Kesihatan 18/7/2016
Universiti Sains Malaysia
16150 Kubang Kerian, Kelantan

UNIVERSITI SAINS MALAYSIA
 JABATAN BENDAHARI
 KUMPULAN WANG UNIVERSITI PENYELIDIKAN (RU)
 PENYATA PERBELANJAAN SEHINGGA 31 MEI 2016

Jumlah Geran : RM 220,293.00 Ketua Projek : DR. NURUL ASMA ABDULLAH

Peruntukan OKT. 2012 : 85,431.00
 (Tahun 1) Tajuk Projek: IN VIVO TERATOGENICITY AND ANTI-ALLERGIC EFFECTS OF LIGNOSUS RHINOCEROS IN AN ASTHMATIC RODENT MODEL AND THEIR POSSIBLE BIOACTIVE CONSTITUENTS

Peruntukan OKT. 2013 : 74,431.00
 (Tahun 2) Tempoh : 3 Tahun (01/10/2012-30/09/2015)

Peruntukan OKT. 2014 : 60,431.00
 (Tahun 3) No. Akaun : 1001/PPSK/813065

| Kwgan | Akaun | PTJ | Projek | Peruntukan Projek | Perbelanjaan Terkumpul sehingga Tahun lalu | Peruntukan Semasa | Tanggungjawab Semasa | Bayaran Tahun Semasa | Belanja Tahun Semasa | Baki Projek |
|-------|-------|------|--------|-------------------|--|-------------------|----------------------|----------------------|----------------------|-------------|
| 1001 | 11000 | PPSK | 813065 | 54,843.00 | 33,537.63 | 21,305.37 | - | 720.35 | 720.35 | 20,585.02 |
| 1001 | 14000 | PPSK | 813065 | - | - | - | - | - | - | - |
| 1001 | 15000 | PPSK | 813065 | - | 1,000.00 | (1,000.00) | - | - | - | (1,000.00) |
| 1001 | 21000 | PPSK | 813065 | 14,904.81 | 9,095.25 | 5,809.56 | - | - | - | 5,809.56 |
| 1001 | 22000 | PPSK | 813065 | - | - | - | - | 100.00 | 100.00 | (100.00) |
| 1001 | 23000 | PPSK | 813065 | - | - | - | - | - | - | - |
| 1001 | 24000 | PPSK | 813065 | 450.00 | - | 450.00 | - | - | - | 450.00 |
| 1001 | 25000 | PPSK | 813065 | - | - | - | - | - | - | - |
| 1001 | 26000 | PPSK | 813065 | - | - | - | - | - | - | - |
| 1001 | 27000 | PPSK | 813065 | 129,095.19 | 125,981.18 | 3,114.01 | - | 33,398.91 | 33,398.91 | (30,284.90) |
| 1001 | 28000 | PPSK | 813065 | 2,000.00 | - | 2,000.00 | - | - | - | 2,000.00 |
| 1001 | 29000 | PPSK | 813065 | 9,000.00 | 13,946.46 | (4,946.46) | 754.72 | - | 754.72 | (5,701.18) |
| 1001 | 32000 | PPSK | 813065 | - | - | - | - | - | - | - |
| 1001 | 35000 | PPSK | 813065 | 10,000.00 | - | 10,000.00 | - | - | - | 10,000.00 |
| 1001 | 52000 | PPSK | 813065 | - | - | - | - | 2,009.93 | 2,009.93 | (2,009.93) |
| | | | | 220,293.00 | 183,560.52 | 36,732.48 | 754.72 | 36,229.19 | 36,983.91 | (251.43) |

Oral presentation in International Conference on
Biomedical & Health Sciences Research 2015 ,
Putrajaya, Malaysia
(25th – 27th January 2015)

ANTI-ASTHMATIC EFFECTS OF *LIGNOSUS RHINOCERUS* (TIGER MILK MUSHROOM) EXTRACTS ON OVALBUMIN-INDUCED AIRWAY INFLAMMATION IN SPRAGUE DAWLEY MODEL

M. Johnathan¹ S.H. Gan², H. Faezahtul², M.F. Wan Ezumi³, W.M. ,Wan Majdiah¹ and A. A. Nurul^{3*}

¹School of Dental¹, Medical² and Health³ Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia.

*Corresponding author: nurulasma@usm.my

Abstract

Background: *Lignosus rhinocerus* (*L.rhinocerus*) has been used as traditional medicinal plants in treating asthma by indigenous communities in Malaysia. However, no scientific studies have been carried out on its efficacy towards asthma. We investigated the effects of *L.rhinocerus* on OVA sensitized asthmatic Sprague Dawley rats to understand its potential anti-asthmatic role. **Material and methods:** Four groups of male Sprague Dawley rats (n=5), normal rats; sensitization plus OVA challenged rats; sensitization plus OVA challenged with *L.rhinocerus* treatment; sensitization plus OVA challenged with Dexamethasone treatment were used. **Results:** Our study showed *L.rhinocerus* extract to have significantly inhibited increases in total immunoglobulin E (IgE) in serum and cytokines IL-4, IL-5 and IL-13 levels in bronchoalveolar lavage fluid (BALF), and also effectively suppressed eosinophils numbers in BALF as well as attenuated eosinophil infiltration in lung. The results suggest that *L.rhinocerus* extract could be a potential therapeutic agent for treating asthma.

Keywords: *L. rhinocerus*, Asthma, Sprague Dawley, Interleukin-4, Interleukin-5, Interleukin-13, Immunoglobulin E, Bronchoalveolar lavage fluid

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Inhibitory effects of *Lignosus rhinocerus* (Tiger Milk mushroom) extracts on ovalbumin-induced airway inflammation

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Lignosus rhinocerus or locally known as Tiger Milk mushroom has been used traditionally by indigenous communities in Malaysia to treat allergic asthma. However, no scientific studies have been carried out on its efficacy towards asthma. We investigated the effects of *L.rhinocerus* using two extraction methods on ovalbumin sensitized allergic Sprague Dawley rats to understand its potential anti-asthmatic role. Five groups of male Sprague Dawley rats (n=5): normal rats; sensitization plus OVA challenged rats; sensitization plus OVA challenged with cold water extract of *L. rhinocerus* treatment; sensitization plus OVA challenged with hot water extract of *L. rhinocerus* treatment; sensitization plus OVA challenged with dexamethasone treatment were tested. Our study revealed that *L. rhinocerus* extracted with hot water extraction method significantly inhibited elevation in immunoglobulin E (IgE) in serum and level of Th2 cytokines i.e. IL-4, IL-5 and IL-13 in bronchoalveolar lavage fluid (BALF), and also effectively suppressed eosinophils numbers in BALF as well as attenuated eosinophil infiltration in lung region. The animals treated with cold water extract of *L. rhinocerus* showed slight reduction of some airways inflammation parameters; however the differences were not significant. The study indicate that *L. rhinocerus* extracted using hot water extract has significantly reduced allergic airway inflammation condition; thus suggesting a potential alternative for the management of allergic asthma.

CONFERENCES

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Inhibitory effects of Tiger Milk mushroom extract on airway inflammation in ovalbumin-induced allergic asthma

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Asthma is a chronic disorder that involves inflammation, swelling, and narrowing of the airways. Asthma is characterized by airways hyper-responsiveness, airways eosinophilia infiltration, excessive airways mucus production and increase of IgE and Th2 cytokines. Currently asthma is controlled by steroid-based medications; however steroid has many side effects. Therefore, alternative medication for management of asthma is needed. Tiger Milk mushroom (TMM) or scientifically known as *Lignosus rhinocerus* has been traditionally used to treat many illnesses by indigenous communities in Malaysia. However, no scientific studies have been carried out on its efficacy towards asthma. We investigated the effects of TMM on OVA sensitized asthmatic Sprague Dawley rats to understand its potential anti-asthmatic effect. Four groups of male Sprague Dawley rats (n=5), normal rats; sensitization plus OVA challenged rats; sensitization plus OVA challenged with TMM treatment and sensitization plus OVA challenged with Dexamethasone treatment were used. Our study showed that treatment of rats with TMM extract has significantly reduced asthmatic parameters i.e. total immunoglobulin E (IgE) in serum, Th2 cytokines IL-4, IL-5 and IL-13 levels in bronchoalveolar lavage fluid (BALF), and also effectively suppressed eosinophils number in BALF as well as attenuated eosinophil infiltration in lung. The results suggest that Tiger Milk mushroom extract could be a potential natural alternative for the management of allergic asthma.

RESEARCH ARTICLE

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Phytochemical profiles and inhibitory effects of Tiger Milk mushroom (*Lignosus rhinocerus*) extract on ovalbumin-induced airway inflammation in a rodent model of asthma

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Abstract

Background: *Lignosus rhinocerus* (*L. rhinocerus*), which is known locally as Tiger Milk mushroom, is traditionally used in the treatment of asthma by indigenous communities in Malaysia. However, to date, its efficacy on asthma has not been confirmed by scientific studies and there is also sparse information available on its active constituents. In this study, the volatile constituent of *L. rhinocerus* hot water extract was investigated using gas chromatography mass spectrometry (GC-MS). The potential effects of *L. rhinocerus* extract for anti-asthmatic activity was further investigated on ovalbumin (OVA)-sensitized asthmatic Sprague Dawley rats.

Methods: Sequential extraction using five solvents (petroleum ether, diethyl ether, hexane, ethyl acetate and methanol) was conducted prior to GC-MS analysis. Male Sprague Dawley rats were divided into the following four groups of five animals each: 1) normal rats, 2) sensitization plus OVA-challenged rats 3) sensitization plus OVA-challenged with *L. rhinocerus* treatment and 4) sensitization plus OVA-challenged with dexamethasone treatment. The levels of immunoglobulin E (IgE) in the serum and T-helper 2 cytokines, including interleukin (IL)-4, IL-5 and IL-13, in bronchoalveolar lavage fluid (BALF), as well as eosinophil infiltration in the lungs, were investigated.

Results: GC-MS analysis revealed the presence of five main groups (alkane, fatty acids, benzene, phenol and dicarboxylic acid) with a total of 18 constituents. Linoleic acid (21.35 %), octadecane (11.82 %) and 2,3-dihydroxypropyl elaidate (10.47 %) were present in high amounts. The extract significantly ameliorated the increase in total IgE in serum and IL-4, IL-5 and IL-13 levels in BALF and also effectively suppressed eosinophils numbers in BALF while attenuating eosinophil infiltrations in the lungs.

Conclusion: *L. rhinocerus* hot water extract has the potential to be used as an alternative for the treatment of acute asthma.

Keywords: *Lignosus rhinocerus*, Airway inflammation, Asthma, GC-MS, Linoleic acid, Tiger milk mushroom

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Background

Asthma affects 300 million people worldwide and is a result of a complex interplay between genetic and environmental factors [1]. Allergic asthma is a chronic disease characterized by recurrent wheezing, shortness of breath, chest tightness and coughing. The airway disorder is commonly characterized by airway eosinophilia infiltration, increased serum immunoglobulin E (IgE) and T-helper 2 (Th2) cytokine levels such as interleukin (IL)-4, IL-5 and IL-13 [2], as well as excessive production of airway mucus.

The Th2-dominated response is initially triggered when an airway allergen is taken by the dendritic cells and presented to specific Th cells by means of major histocompatibility complex (MHC) class II, thus stimulating the immunological synapse. Activated allergen-specific Th cells then polarize into Th1 or Th2 effector populations, differentiate and expand into a Th2 subpopulation. The activated Th2 cells function in recruiting and activating cytokines, including IL-4, IL-5 and IL-13, following stimulation of eosinophils. IL-4 cytokines have been reported to cause class switching of B cells, resulting in IgE synthesis, which is involved in mast cell degranulation [3]. The key role of IL-5 cytokines includes their involvement in the growth and differentiation of bone marrow eosinophils, as well as their release into the peripheral circulation [4]. Additionally, IL-5 also increases the activity of eosinophil recruitment, activation and survival at the sites of inflammation [5]. IL-13, which is known as the “central mediator of allergic asthma,” plays an important role in directing the allergic response in asthmatic patients [6]. Various studies are therefore primarily focused on products that have the potential to reduce IgE, IL-4, IL-5, IL-13 and eosinophils as therapeutic targets for asthma treatment [7–10].

Current asthma medications are mainly based on steroids and other types of anti-inflammatory drugs [11]. Among the available drugs, inhaled corticosteroids (ICSs) are very effective, as they suppress chronic airway inflammation in patients with asthma [12]. ICSs are generally considered harmless, though persistent use of ICSs at higher doses often leads to various systemic and local side effects [13], some of which include candidiasis, osteoporosis, growth retardation, cataracts and pharyngitis [14]. Thus, a safer alternative for the management of asthma is needed.

Lignosus rhinocerus (*L. rhinocerus*) from the *Polyporaceae* family is a wild mushroom exclusive to Malaysia. Locally known as “cendawan susu rimau” or “Tiger milk mushroom”, this unique mushroom is one of the reported 38 available types of edible mushrooms in Malaysia used for medicinal purposes by rural and indigenous communities [15]. The tuber is purported to

contribute the most medicinal value, and the indigenous populations in Peninsular Malaysia utilise it to treat diseases such as asthma, fever, breast cancer, stomach cancer and food poisoning, as well as to heal wounds [15, 16]. Previous studies have demonstrated anti-proliferative activities [17] and immunomodulatory properties [18] of this mushroom sclerotial. A recent study by Lee et al. [19] reported anti-acute inflammatory properties of sclerotial powder of *L. rhinocerus* using carrageenan-induced paw oedema model in rats. In addition, the study also demonstrated a potent inhibition of TNF- α production by the high-molecular-weight fractions of the sclerotial powder of *L. rhinocerus*. However, to date, no studies have been reported on the anti-asthmatic properties of *L. rhinocerus* in airway inflammation models. Thus this study reported anti-asthmatic effects of *L. rhinocerus* sclerotial extract in ovalbumin-induced airway inflammation of the rodent model and the profile of volatile constituents of the mushroom extract by GCMS analysis.

Methods

Preparation of *L. rhinocerus* hot water extract

Sclerotia of *L. rhinocerus* cultivar TM02 was obtained in dried powdered form from Ligno Biotech Sdn. Bhd. (Selangor, Malaysia). The powder was subjected to hot water extraction using a soxhlet for 24 h and was further concentrated using a rotary evaporator (Unimax1010, Heidolph, Germany) before being freeze-dried in a freeze drier (Ilshin BioBase, Gyeonggi-do, Korea).

Liquid-liquid extraction

A sequential liquid-liquid extraction was performed using 1) petroleum ether, 2) diethyl ether 3) hexane, 4) ethyl acetate and 5) methanol. The selected solvents ranged in polarity starting from non-polar (petroleum ether, diethyl ether and hexane) to more polar solvents (ethyl acetate and methanol) to take advantage of their different properties.

Briefly, 1 ml of petroleum ether was added to the capped glass tube containing 1 g of *L. rhinocerus* extract. The mixture was then vortexed for 1 min using a vortex mixer (Westbury, New York, USA), followed by centrifugation (Centrifuge Universal 32R, HettichZentrifugen, Germany) at 700 \times g for 5 min. The supernatant was aspirated before being transferred (100 μ l) to a new autosampler vial for GC-MS injection. The residue was used for subsequent extraction using diethyl ether followed by hexane, ethyl acetate and finally, methanol, as previously described for petroleum ether. Following extraction by each solvent type, the samples were individually injected into the GC-MS system in duplicate. Each sample was analyzed against a blank organic solvent containing a

similar type of organic solvent used in the extraction process each time.

GC-MS analysis

GC-MS analysis was performed on an HP6890 GC coupled with a HP5973 mass spectrometer (Hewlett Packard, CA, USA). The column was a HP-5MS fused-silica capillary column (50 m x 0.25 mm i.d.; 0.25- μ m film thickness) with helium as the carrier gas, and it was run at a constant pressure of 9.78 psi. Injection was conducted using a splitless mode at an injector temperature of 250 °C. The oven temperature was ramped from 40 °C to 280 °C (1-min hold) at a rate of 25 °C/min. The oven temperature was held at 310 °C for 6 min for each analysis. The total run time for each sample was approximately 28 min. The GC-MS interface temperature was set to 280 °C. MS mode was used during analytical scanning from 20 to 650 atomic mass units (amu). The ion source temperature was set at 250 °C.

The blank was injected first each time, followed by the sample injection. The chromatograms obtained from the total ion current (TIC) were integrated without any correction for co-eluting peaks, and the results were expressed as total abundance. The TIC peaks and chromatograms were analyzed using an Agilent Technologies 7890A GC system with 5973 VL MSD. All peaks were identified based on mass spectral matching (≥ 90 %) from both the National Institute of Standards and Technology (NIST) and Wiley libraries. Only compounds with 90 % or greater spectral matching accuracy were reported.

Animals

Ethical approval was obtained from Animal Ethics Committee Universiti Sains Malaysia. Male Sprague Dawley rats (age between 6 and 8 weeks; 200–250 g in weight) were used for this experiment. The rats ($n = 20$) were housed in polystyrene cages in an air-controlled room. All animals were maintained at the animal house, and tap water was allowed ad libitum. The animals were acclimatized to the experimental environment prior to commencement of the study.

Sensitization, airway challenge and treatment

The rats were divided into the following four groups of five animals each: (1) normal rats, (2) sensitization plus 1 % OVA challenge or “untreated group,” (3) sensitization plus 1 % OVA challenge with oral *L. rhinoceros* treatment (500 mg/kg per body weight) and (4) sensitization plus 1 % OVA challenge with intraperitoneal (ip) dexamethasone treatment (3 mg/kg per body weight). Sensitization on days 1 and 14 was conducted with a 2 x 1 ml i.p. injection of 10 mg/ml OVA (Nacalai Tesque, Kyoto, Japan) + 100 mg/ml alum (aluminium hydroxide) (Sigma, MO, USA) in phosphate buffered saline (PBS) (based on the

modified method of Makhoulouf and Hafez, [20]). An additional 50 ng/ml of *Bordetella pertussis* (Sigma, MO, USA) was also prepared with the solution as adjuvant. On day 23, the rats were challenged with 1 % OVA aerosol for 20 min/day for 7 days using an ultrasonic nebulizer (Mabist mist, Illinois, USA) and orally administered by gavage with 500 mg/kg per body weight of *L. rhinoceros* extract for 7 days. On day 30, 24 h after the final challenge, the rats were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg), and blood was collected by cardiac puncture.

Eosinophils and inflammatory cell count

Following sacrifice, bronchoalveolar lavage fluid (BALF) was obtained using an endotracheal tube by instilling and aspirating the trachea with PBS solution. BALF was centrifuged at 500 x g for 5 min, and the pellet was re-suspended with PBS. The total inflammatory cell number was quantified by counting the number of cells from at least five squares of the haemocytometer after excluding dead cells stained with trypan blue.

Measurement of total IgE in BALF

The total IgE was measured with a specific rat ELISA kit (Abnova, Heidelberg, Germany) according to the manufacturer's instructions. IgE levels in each sample were measured from optical density readings at 450 nm followed by calculation based on a standard curve that was generated using recombinant IgE.

Measurement of Th2 cytokines in BALF

An enzyme-linked immunosorbent assays (ELISA) was performed according to the manufacturer's instructions. IL-4, IL-5 and IL-13 in BALF were measured using specific rat IL-4, IL-5 and IL-13 ELISA kits (CusaBio, Hubei, China). The bound enzyme that was proportional to the respective cytokines concentration was investigated at absorbance of 450 nm. Cytokine concentrations were calculated from standard curves that were generated using respective recombinant interleukins.

Lung tissue histology

The lungs were removed, rinsed with PBS, weighed and fixed in 10 % formalin overnight for immunohistochemistry analysis. The lung tissue was embedded in paraffin and cut into 4- μ m thickness sections, followed by haematoxylin and eosin staining for lung inflammation scoring. The tissue was subsequently mounted and cover-slipped with di-n-butyl phthalate in xylene (DPX) mounting medium. The infiltration intensity at peribronchial and perivascular inflammatory spaces was scored as the approximate number of cell layers around vessels or bronchioles following criteria adapted from Tournoy et al. [21]. Whole lung sections were assigned using a

reproducible scoring system developed for a murine model (Curtis, et al., [22]). The scoring system ranges between 0 to 4 as follows, indicating inflammation severity based on no inflammation, the presence of occasional cuffs, the presence of a thin layer or presence of inflammatory cells surrounding the peribronchial/perivascular space that are 1–5 cells thick or > 5 cells thick:

- 0: no inflammation
- 1: mild inflammation
- 2: moderate inflammation
- 3: severe inflammation
- 4: extreme inflammation

For each rat, randomly distributed airway sections in the lung were analysed, and their mean scores were calculated. The inflammation present was evaluated with a double-blinded scoring method. Quantitative analysis of the lung inflammation score was performed using a Mirax Image viewer (Carl Zeiss, Jenna, Germany).

Statistical analysis

Data are expressed as the mean \pm standard deviation (SD). Statistical significance ($p < 0.05$) was determined by a one-way ANOVA followed by Scheffe's post hoc correction and inflammation scores were analysed with Wilcoxon-Mann Whitney test using Statistical Program for Social Science (SPSS) version 22.0 (New York, USA).

Results

GC-MS analysis of the volatile constituent of *L. rhinocerus* Five major groups, including alkanes (53 %; estimated weight fraction of 53 mg) and fatty acids (36 %), were detected (Fig. 1). Overall, eighteen constituents were extracted by the five solvents (petroleum ether, diethyl ether, hexane, ethyl acetate and methanol) (Fig. 2).

The highest numbers of constituents (eight compounds) were reported from non-polar (hexane) extraction followed by ethyl acetate (6 compounds), petroleum ether (5

compounds), diethyl ether (3 compounds) and methanol (2 compounds) extractions (Table 1). The constituent with the greatest proportion in *L. rhinocerus* hot water extract was linoleic acid (21.35 %), followed by 2,3-dihydroxypropyl elaidate (11.82 %) and octadecane (10.47 %). The chemical structures and molecular formula for the detected volatile constituents are described in Table 2.

Inhibition effects of *L. rhinocerus* on airway inflammation

A preliminary study was conducted prior to the present investigation to optimize *L. rhinocerus* dosages and the dose used in the sensitization protocol that was suitable for the anti-asthmatic study. Based on this finding, a dose of 500 mg/kg of *L. rhinocerus* is more effective in reducing asthma-related parameters when compared to low (125 mg/kg) and medium (250 mg/kg) dosages (unpublished data) and was therefore selected for the study. A previous toxicity study indicated that 1000 mg/kg of *L. rhinocerus* extract fed orally to rats did not cause any adverse effects, and it is therefore considered to be safe [23]. The sensitization method was modified from a previous study [20] based on similar preliminary optimization study, hence i.p. sensitization with the additional adjuvant of Bordetella pertussis and 7 days of challenge or treatment was suitable to establish the expected allergic model.

Our study targeted serum IgE detection, while Th2 cytokine detection was conducted in BALF. As in asthmatic models, airway obstructions and difficulties in breathing are assumed to result from the responses of immunoglobulins and cytokine levels, which lead to worsening of asthma symptoms. Eosinophils are also found in large numbers within the submucosa and epithelium in the allergic model. Most allergic mediators are stimulated by IgE and are released into the blood stream in response to inflammatory reactions [9]. In addition, BALF biofluid is widely accepted as a reliable sample with which to determine the composition of secreted pulmonary proteins and the products of activated cells. Therefore, in this study we aimed to investigate OVA-induced IgE detection in serum, while Th2 cytokines were detected in BALF.

High numbers of inflammatory cells in BALF indicate inflammation and an allergic condition. The level of inflammatory cell eosinophil infiltration in BALF was significantly reduced in the *L. rhinocerus* and dexamethasone groups compared to the untreated group (Fig. 3). However, the reduction observed with dexamethasone was slightly more prominent when compared to *L. rhinocerus*. The untreated group had the highest level of eosinophils, indicating marked induction of allergic asthma. The level of eosinophils in the untreated group was also significantly elevated when compared to the normal group.

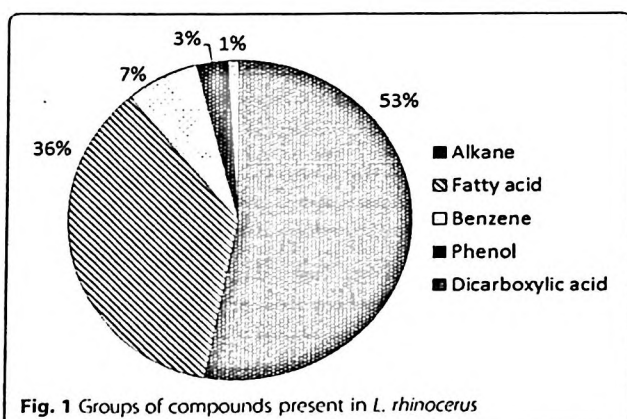


Fig. 1 Groups of compounds present in *L. rhinocerus*

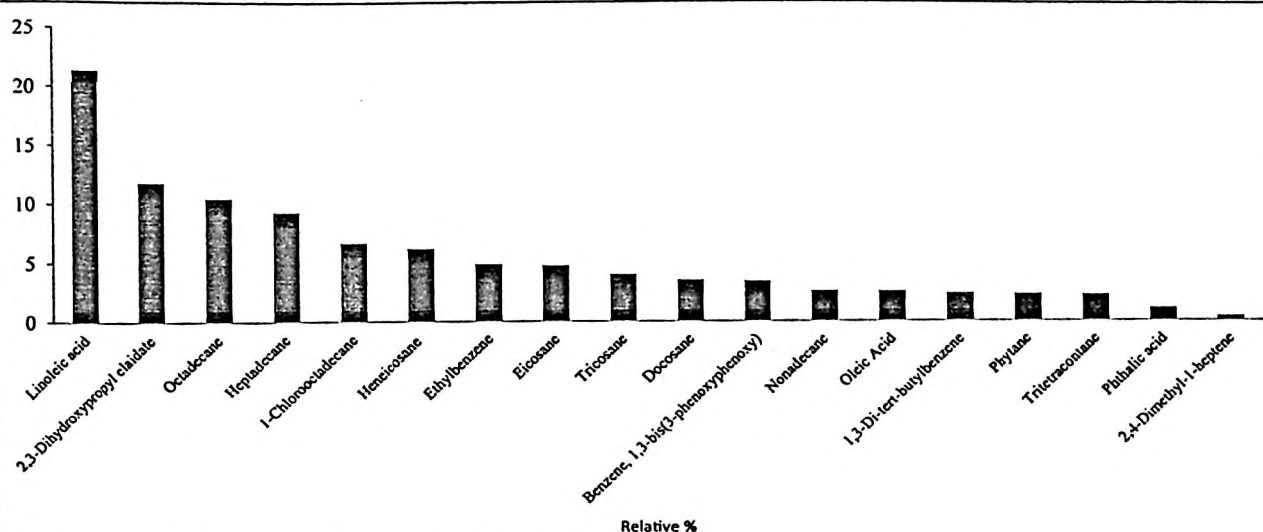


Fig. 2 Volatile compounds detected in *L. rhinocerus* and their relative percentages

Similarly, there was also a significant reduction in the mean serum IgE levels among animals receiving *L. rhinocerus* or dexamethasone when compared to the untreated group (Fig. 4). The reduction in the levels of IgE and Th2 cytokines indicate successful asthmatic treatment. In comparison, the IgE levels in the

untreated group were significantly elevated when compared to the normal group.

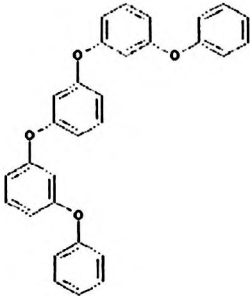


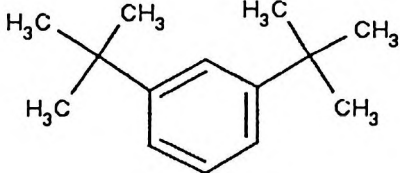
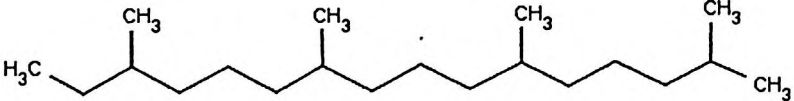
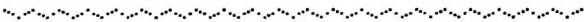
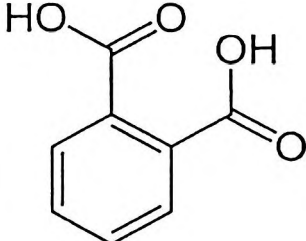
The levels of IL-4 (Fig. 5a) and IL-5 (Fig. 5b) were significantly reduced in the *L. rhinocerus* and dexamethasone groups when compared to the untreated group. There was also a reduction in the IL-13 level among animals

Table 1 Volatile compositions of *L. rhinocerus* following extraction using various solvents and their respective retention times, RT (min)

| Compounds | P.E | RT | D.E | RT | HX | RT | EA | RT | MTL | RT | Relative % |
|-------------------------------------|-----|-------|-----|------|----|-------|----|-------|-----|------|------------|
| Octadecane | ✓ | 10.31 | | | ✓ | 13.61 | ✓ | 9.43 | | | 10.47 |
| Heptadecane | ✓ | 12.76 | | | ✓ | 12.79 | ✓ | 8.99 | | | 9.29 |
| Benzene, 1,3-bis (3-phenoxyphenoxy) | ✓ | 3.18 | | | | | | | | | 3.43 |
| Phytane | ✓ | 9.86 | | | | | | | | | 2.30 |
| Docosane | ✓ | 12.05 | | | | | | | | | 3.54 |
| Phthalic acid | | | ✓ | 9.81 | | | | | | | 1.10 |
| 2,4-Dimethyl-1-heptene | | | ✓ | 3.88 | | | | | | | 0.36 |
| 1,3-Di-tert-butylbenzene | | | ✓ | 6.77 | ✓ | 6.76 | | | | | 2.38 |
| Heneicosane | | | | | ✓ | 14.5 | | | | | 6.22 |
| Tricosane | | | | | ✓ | 15.47 | | | | | 4.00 |
| 1-Chlorooctadecane | | | | | ✓ | 16.48 | | | | | 6.69 |
| Tritetracontane | | | | | ✓ | 10.83 | | | | | 2.25 |
| Eicosane | | | | | ✓ | 10.84 | ✓ | 10.32 | | | 4.77 |
| Nonadecane | | | | | | | ✓ | 9.86 | | | 2.61 |
| 2,3-Dihydroxypropyl elaidate | | | | | | | ✓ | 28.89 | | | 11.82 |
| Oleic acid | | | | | | | ✓ | 27.54 | | | 2.55 |
| Linoleic acid | | | | | | | | | ✓ | 3.20 | 21.35 |
| Ethylbenzene | | | | | | | | | ✓ | 4.08 | 4.87 |
| Total percentage | | | | | | | | | | | 100.00 |

PE petroleum ether, DE diethyl ether, HX hexane, EA ethyl acetate, MTL methanol

Table 2 Chemical structures of the major volatiles present in *L. rhinocerus* (Continued)

| | | |
|------------------------------------|--|--|
| Benzene, 1,3-bis(3-phenoxyphenoxy) | C ₃₀ H ₂₄ O ₄ |  |
| Nonadecane | C ₁₉ H ₄₀ |  |
| Oleic Acid | C ₁₈ H ₃₄ O ₂ |  |
| 1,3-Di-tert-butylbenzene | C ₁₄ H ₂₂ |  |
| Phytane | C ₂₀ H ₄₂ |  |
| Tritetracontane | C ₄₃ H ₈₈ |  |
| Phthalic acid | C ₈ H ₆ O ₄ |  |

receiving *L. rhinocerus* and dexamethasone. However, the reduction in IL-13 observed among animals receiving *L. rhinocerus* was not statistically significant when compared

to the untreated group (Fig. 5c). The levels of all Th2 cytokines in the untreated groups were significantly elevated when compared to the normal group.

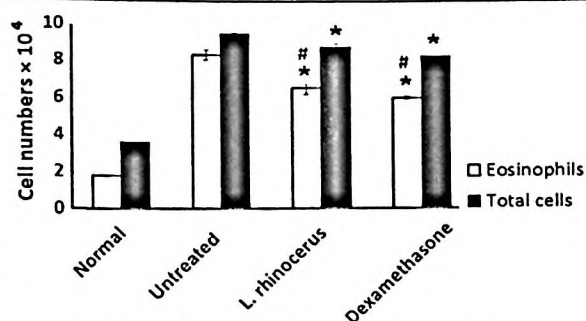


Fig. 3 The effect of *L. rhinocerus* extract on the recruitment of inflammatory cells in BALF. Normal; untreated, OVA-sensitized/challenged rats; *L. rhinocerus* extract + OVA-sensitized/ challenged rats; dexamethasone + OVA-sensitized/ challenged rats. Values are expressed as the means \pm SD ($n = 5$ /group). *Significantly different from untreated. #Significantly different within treatment

Haematoxylin and eosin staining was performed on the lung tissues to analyze the effects of *L. rhinocerus* extract on the histological feature of asthma. The lung tissue section of ovalbumin-induced asthmatic rats was characterized by the presence of dense peribronchial inflammation due to leukocyte infiltration when compared with the normal tissue (Fig. 6a). Substances that can attenuate this inflammation indicate that they are effective anti-asthmatic agents. Histopathological findings further confirmed that there was significant reduction in the severity of eosinophil cell inflammation with significantly reduced cells forming eosinophil layers/rings in both the *L. rhinocerus* and dexamethasone groups. In addition, the inflammation score indicated that both the *L. rhinocerus* and dexamethasone groups showed significantly reduced inflammation when compared to the untreated group (Fig. 6b).

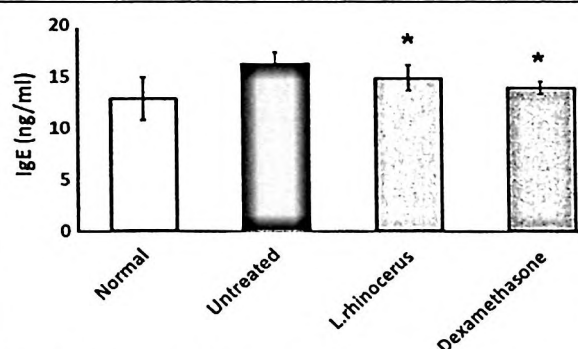


Fig. 4 The effect of *L. rhinocerus* extract on serum IgE levels. Normal; untreated, OVA-sensitized/challenged rats; *L. rhinocerus* extract + OVA-sensitized/challenged rats; dexamethasone + OVA-sensitized/ challenged rats. Values are expressed as the means \pm SD ($n = 5$ /group). *Significantly different from untreated

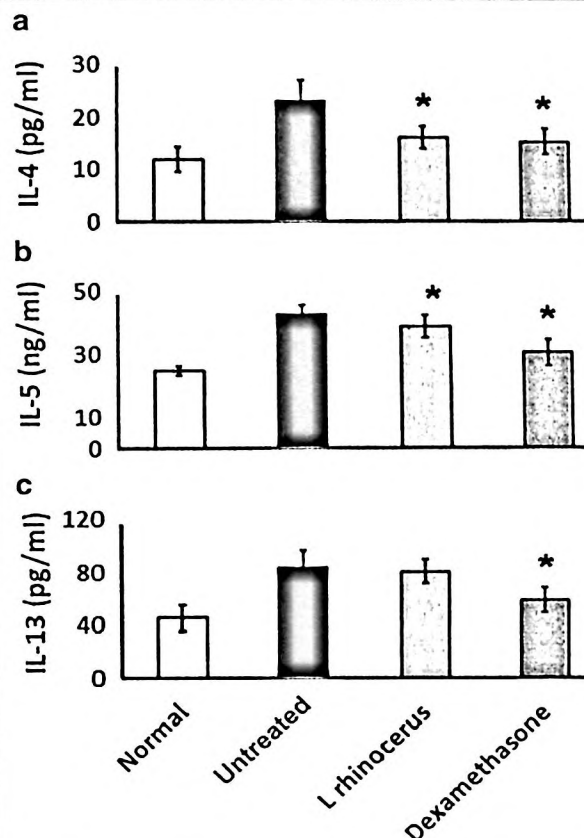


Fig. 5 The effect of *L. rhinocerus* extract on the cytokines (a) IL-4, (b) IL-5 and (c) IL-13 levels in BALF. Normal; untreated, OVA-sensitized/challenged rats; *L. rhinocerus* extract + OVA-sensitized/ challenged rats; dexamethasone + OVA-sensitized/ challenged rats. Values are expressed as the means \pm SD ($n = 5$ /group). *Significantly different from untreated

Discussion

Our study is the first to elucidate the composition of hot water extract of *L. rhinocerus* widely used in the traditional treatment of asthma. Alkanes were the major group found to be present, while linoleic acid was the major constituent detected. To our knowledge, our study is also the first to confirm the airway inhibition effects of *L. rhinocerus*.

The GC-MS analysis was conducted following a sequential extraction method that employs both polar and non-polar properties of solvents in separating compounds based on polarity. In this study, a simple liquid-liquid extraction method is beneficial because it does not involve a heating process, which minimizes damage to the volatiles and can prevent the formation of other artefacts [24, 25]. This sequential extraction conserves the amount of volatiles as well as selectively improves the process and the recovery of different types of extracts from analogous materials. The compounds were

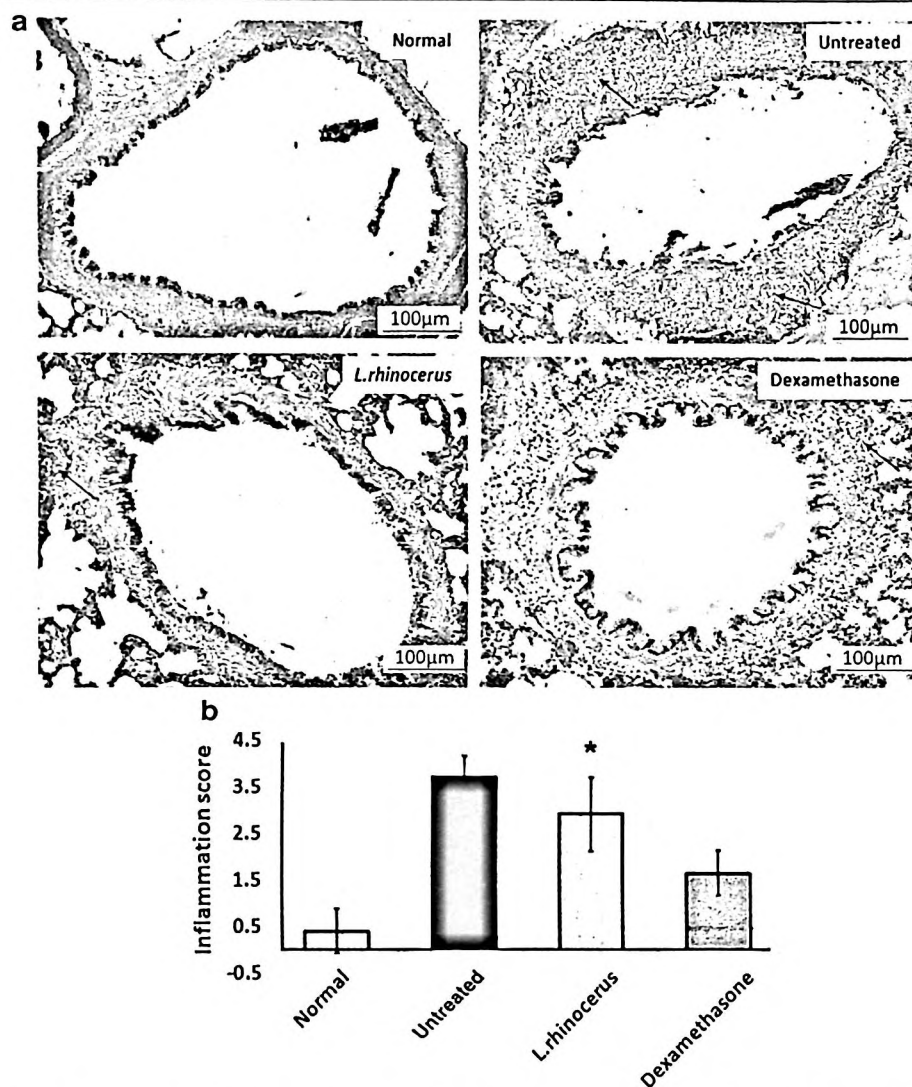


Fig. 6 The effect of *L. rhinocerus* on airway inflammatory leukocyte infiltration in the peribronchiole region and perivascular connective tissue. (a) Magnification 50x. Black arrows indicate the presence of infiltration with eosinophils/leukocytes surrounding the bronchiole. (b) Quantitative analysis on the inflammation score with a subjective scale of 0–4 used to assess leukocyte infiltration in the lungs

selected based on spectral library matching to the NIST and Wiley libraries.

Most of the compounds detected were from the alkane group. In comparison, a study of other types of medicinal mushrooms such as *Termitomyces* sp. and *Termitomyces microcarpus* prepared from petroleum ether crude extracts reported the presence of small amounts of octadecane (0.64 %) and heptadecane (1.01 %) [26], which are alkanes. Another researcher reported the presence of octadecane (0.15 %) in *Trichoderma harzianum* following GC-MS analysis [27]. However, our study detected the presence of higher amounts of octadecane (10.47 %) and

heptadecane (9.29 %) in *L. rhinocerus*. Similar to our study, a study on *Agaricus bisporus* comparing different strains of mushrooms (strains 310 and 342) reported high amounts of non-polar compounds such as octadecane (13.8 %) and nonadecane (14.4 %) in addition to eicosane and heptadecane [28]. In addition, several studies [28–30] have reported fatty acids to be the predominant constituent in mushrooms. Specifically, linoleic acid has been detected to be the major constituent (~65 %) of *Schizophyllum commune* and *Lentinus edodes* [31], which is also reported in our study as the most common compound (21.35 %). Similar to our study, the

fatty acid profile of several *Tricholoma* species, such as *T. portentosum* and *T. terreum*, also revealed the presence of linoleic acid (~28 %) [32].

Linoleic acid is a known precursor of 1-octen-3-ol (alcohol of fungi), which functions as the principal aromatic compound in most fungi [33–35]. A study on *L. rhinocerotis* comparing mycelium of shaken cultures and static cultures revealed the presence of 2,3-dihydroxypropyl elaidate compound (0.27–0.28 %) [36]. The presence of a similar compound was also detected in the spore lipids of *Ganoderma lucidum* (0.07 %) [37]. In our study, 2,3-dihydroxypropyl elaidate was reported to be present at approximately 11.82 % and constituted the second most common compound detected. In comparison, linoleic acid was compared in different parts of *A. bisporus* mushroom and was reported to be present in 12.2 % of the volume of the whole fresh sample, in 1.5 % of the stalks and in 0.8 % of the gills [38].

Linoleic acid was present in varying concentrations in different types of mushrooms, and unique properties were observed. In fact, such variations are key to understanding the aromatic and other medicinal properties. Nonetheless, GC-MS analysis of *L. rhinocerus* revealed compounds that were detectable and comparable with other mushrooms types and plants [29, 39, 40], making them both similar and different to plants of numerous species and genus.

This study is the first to report on the inhibition effects of *L. rhinocerus* on airway inflammation in asthmatic model. Asthmatic model of Sprague Dawley rat was used in the study and dexamethasone was used as a positive control as this compound is commonly used as a potent inhibitor of airway inflammation and remodelling. The administration of corticosteroids has been demonstrated to inhibit structural changes associated with airway fibrosis in animal models and is therefore widely utilized as a control in asthma studies [41]. In the context of the experimental design, all rats in this study (except for the normal group) underwent sensitization on days 1 (primary sensitization) and 14 (secondary sensitization). The purpose of an additional sensitization step on day 14 is to ensure that the levels of airway eosinophils do not revert back to the baseline, as well as to avoid the development of tolerance. In addition, these sensitizations were coupled with OVA along with alum and *Bordetella pertussis* to enhance Th2-dominated responses [42].

Generally, the activation, growth and differentiation of eosinophils are conducted by Th2-dominated responses [43]. It is reported that cytokines secreted by Th2 cells can help in the recruitment and activation of eosinophils in the nasal region. Eosinophil counts performed on BALF in this study revealed that treatment with hot water extract of *L. rhinocerus* has reduced the number of

eosinophils to 6.54×10^4 cells in BALF when compared to the untreated group, which contained 8.34×10^4 cells; this could indicate attenuated severity and possible withdrawal in eosinophil infiltration along the submucosa and epithelium regions. Groups of rats receiving hot water extract of *L. rhinocerus* and dexamethasone treatment had also significantly decreased the numbers of total inflammatory cells in BALF. These decreases are correlated with the level of cellular infiltration. Furthermore, the deterioration of inflammatory cell numbers in BALF was confirmed by lung tissue histology, thus possibly be valuable in controlling the inflammatory processes underlying exacerbation of allergic asthma. This result is in accordance with the previous study that demonstrated the reduction of eosinophils in OVA-treated animals after treatments with herbal extract [8].

An early finding indicated that in sensitized individuals, the nasal mucosa contains high level of IgE-binding mast cells [44]. Upon sensitization, increasing numbers of IgE-coated mast cells traverse the epithelium. During the phase of allergen exposure or challenge, the IgE-coated mast cells recognize the allergens deposited at mucosal regions, and the mast cells then degranulate. This leads to the release of mediators such as histamines and leukotrienes, which are responsible for blood vessel leakage to stimulate the sensory nerves leading to congestion, airway inflammation and obstruction as well as systemic reflexes such as sneezing [45]. Thus, reducing IgE levels has been the major focus in combating asthma. Accordingly, animals receiving hot water extract of *L. rhinocerus* had significantly reduced IgE levels (14.88 ng/ml) when compared to the untreated group (16.29 ng/ml). A recent study also reported that another type of mushroom polysaccharide from *A. camphorata* has similar anti-asthmatic effects shown by reducing IgE levels in an animal model of asthma [46].

In our study, it was noted that hot water extract of *L. rhinocerus* significantly reduced IL-4 levels in BALF. This is supported by a study using murine allergen challenged models where intranasal chitin polysaccharide components of fungal cell decreased IL-4 (16.22 pg/ml) production compared to untreated group (23.07 pg/ml) *in vivo* [47, 48]. Moreover, Th2 cytokine concentration levels of IL-5 (38.82 ng/ml) and IL-13 (80.54 pg/ml) were also reduced when hot water extract *L. rhinocerus* was used to treat sensitized rats in comparison with untreated groups (42.79 ng/ml and 83.69 pg/ml), respectively. Similarly, both IgE levels in the serum and Th2 cytokines in BALF were also reduced by dexamethasone. However, the reduction observed in the IL-13 levels due to *L. rhinocerus* administration was not significant, which may illustrate the varying effects of *L. rhinocerus* extract on IL-13 regulation. Interestingly, IL-13 has been reported to potentially have independent roles from