Anti-fungal effect of *Hevea brasiliensis* latex C-serum on *Aspergillus niger*


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**Abstract.** – **Objectives:** *Hevea brasiliensis* extract could potentially be employed as a relatively low cost resource for various anti-fungal activities due to the simplicity of latex preparation and the abundance of latex that can be obtained in rubber producing regions. The present study was aimed at examining the species specific anti-fungal property of *H. brasiliensis* latex C-serum against *Aspergillus niger*.  

**Results:** The results showed that the latex C-serum exerted a specific antifungal activity against *Aspergillus niger*, but not *Candida albicans*. Low toxicity of the C-serum was demonstrated in Brine Shrimp Lethality Test (BSLT) with an LC50 value of 98.4 mg/ml.  

**Conclusions:** Pending further more elaborated investigations, *H. brasiliensis* latex C-serum, with its species specific anti-fungal and cancer-origin cell line specific anti-proliferation properties, would probably contribute in healthcare in addition to its traditional role in polymer industry.


**Introduction**

*Aspergillus (A.) niger* is a filamentous ascomycete fungus that is ubiquitous in the environment and has been implicated in opportunistic infections of humans1. As a saprophyte in soil, *Aspergillus niger* also causes economic loss to many agricultural plant products. Nevertheless, it is economically important as a fermentation organism used industrially to produce citric acid as well as being an organism used widely in other biotechnological productions.

Of more than 130 members of the genus, 16 species of *Aspergillus* have been documented as causative agents of human diseases5. However, only 3 species were reported to cause the vast majority of infections. These include *A. fumigates, A. flavus* and *A. niger* being the first, second and third most common causative species, respectively6. Human diseases caused by *A. niger* include aspergillosis and aspergilloma6,7. Medicinal plants represent a rich source of antimicrobial and anti-fungal agents5-7. However, the sources of anti-fungal agents from plants that are not commonly used for their medicinal properties, such as *Hevea (H.) brasiliensis*, remains scarcely explored.  

*H. brasiliensis*, although primarily cultivated for rubber elastomer contained in its latex, is also known for other valuable constituents. The latex itself contains proteins, lipids, quebrachitol, ribonucleic acids and organic salts in relatively small amounts, consistent with its cytoplasmic nature8-12. While some constituents of *H. brasiliensis*, such as hevein proteins from latex B-serum, have been acknowledged to have anti-fungal activities, the therapeutic application of the property may be limited due to the allergic effect on human being13-15. Other researches on the therapeutic potential of the plant, however, remained scarcely explored. It has been reported that *H. brasiliensis* leaf hydroxynitrile lyase could be employed to synthesize active cyanohydrins for use as pharmaceuticals16. More recently, it has been reported that the sub-fraction of latex B-serum and C-serum exerted specific anti-proliferative properties against cancer-origin cell line17. Research in the therapeutic use of *H. brasiliensis* extract could potentially lead to the employment of the extract as a relatively low cost resource for various anti-microbial and anti-
cancer activities due to the simplicity of latex preparation and the abundance of latex that can be obtained in rubber producing regions. The anti-microbial activity of the latex C-serum has not been previously demonstrated. The present study was aimed at examining the anti-Aspergillus niger activity as well as the cytotoxic effect of latex C-serum.

Materials and Methods

Preparation of Latex C-serum

Latex was collected from field-grown RRIM 600 H. brasiliensis at the Rubber Research Institute of Malaysia Research Station, Sungai Buloh. To prepare latex C-serum, fresh latex collected in chilled flasks was fractionated by centrifugation at 44,000 x g at 4°C for 1 hour. The latex separates into three distinct parts upon high-speed centrifugation. The upper layer (the rubber cream), consisting of rubber particles, was carefully removed and latex C-serum was prepared from the remaining supernatant based on a method previously described. The C-serum was then lyophilized for subsequent use. Lyophilized powder of latex C-serum was reconstituted with phosphate buffered saline (1× PBS; 10 mM Phosphate Buffer, 137 mM sodium chloride, and 2.7 mM potassium chloride, Amresco Inc. Ohio, USA). Serial dilutions of the serum were performed to prepare working concentrations ranging from 2-2000 µg/ml.

Brine Shrimp Lethality Test (BSLT)

The procedure for BSLT was modified from the assay described by McLaughlin (1998). Brine shrimp eggs, Artemia salina (Aquafauna Biomarine, Hawthorne, CA, USA), were hatched in artificial sea water prepared from commercial sea salt (38 g sea salt/litre deionized water) with constant light source and oxygen supply after 24 hours of incubation. Three hundred mg of latex C-serum was used to prepare serial diluted working concentrations of 2-2000 µg/ml in sea water. Each concentration had three replicates and control (PBS and sea water) in 2.4 ml sea water. Ten 48-hour-old nauplii were added into each concentration and adjusted to 4.8 ml sea water. Brine shrimp were then incubated for 24 hours under a constant light source and the number of living nauplii was counted the next day. Lethal concentration (LC₅₀) for Artemia salina with 95% confidence level was determined by Probit analysis on a Finney computer program BioStat™ 2009 (AnalystSoft Inc., Vancouver, Canada). Percent mortality observations were corrected for the natural mortality observed in the negative controls using Abbott’s formula, \(P = (p_i - C)/(1 - C)\), where \(p_i\) denotes the observed mortality rate and \(C\) means the natural mortality.

Test Organisms

Aspergillus (A.) niger (ATCC 9142) and Candida (C.) albicans (ATCC 10231) were cultivated on Potato Dextrose Agar (PDA; Difco, Detroit, MI, USA) at 28°C for 48 hours. The stock culture was maintained on nutrient agar slants at 4°C. A. niger and C. albicans were obtained from laboratory stock culture.

Disc Diffusion Method

The agar disc diffusion method was employed for the determination of anti-fungal activities of the latex C-serum against Aspergillus niger and Candida albicans. For determination of anti-fungal activity, fungal cultures were adjusted to 0.5 McFarland turbidity standard and 0.1 ml of the adjusted cultures was inoculated onto Potato Dextrose Agar (PDA; Difco, Detroit, MI, USA). Filter paper discs (6 mm in diameter) were impregnated with 20 µl of the serum (concentration: 10 mg/ml) and placed on the inoculated plates. These plates, after standing at 4°C for two hours, were incubated at 28°C for 48 hours. The diameters of the inhibition zones were measured in millimeters. All the tests were performed in triplicate. The test was also performed with amphotericin B (0.05 µg/disc) as positive control, and with PBS as negative control.

Determination of Minimum Inhibitory Concentration (MIC)

The inoculums of Aspergillus niger was prepared from 18 hours Potato Dextrose Broth (PDB; Difco, Detroit, MI, USA) and adjusted to 0.5 McFarland standards turbidity to achieve an inoculums size of approximately 10⁶ CFU/ml. A serial dilution was carried out to achieve final serum concentrations between 0.625-20.00 mg/ml. The tubes were inoculated with 20 µl of Aspergillus niger suspension per ml PDB. The procedure was repeated on the test organism using the standard anti-fungal as a positive control. A tube containing PDB only was seeded with the test organism as described above to serve as negative control. Tubes containing A. niger cultures...
were then incubated at 28°C for 48 hours. The fungal growth was then examined by observing the turbidity of the culture. The MIC value was determined as the lowest concentration of the crude extract in the broth medium that inhibited the visible growth of the test microorganism.

**Determination of Minimum Fungicidal Concentration (MFC)**

For the determination of MFC, 100 µl of the culture from the diluted sample of MIC assay was transferred onto PDA. The culture was spread on the plate and incubated at 28°C for 48 hours. The MFC was recorded as the lowest concentration of the latex C-serum that gave complete inhibition of colony formation for the tested *Aspergillus niger* at latter cultivation.

**Time-killing Profile of Aspergillus Niger**

The time-killing profile of *Aspergillus niger* with 0.5 MIC, MIC and 2 MIC concentrations over time was plotted to assess the fungicidal effect. The latex C-serum was added to an aliquot of 25 ml of PDB at 28°C in an amount which would achieve the concentration of 0 mg/ml (control) and the above mentioned MIC concentrations after the addition of the inoculums. Later, a solution of 1 ml inoculums was added to all MIC concentrations. Immediately after the addition of the inoculums, 100 µl of culture from each was inoculated onto a PDA plate and incubated at 28°C for 48 hours. The growth of *Aspergillus niger* was monitored by counting the number of colonies (CFU: Colony Forming Unit) after the incubation period. The growth of *Aspergillus niger* was measured every 6 hours for 48 hours.

**Results**

The results from brine shrimp lethality test are shown in Table I and Figure 1. The LC50 value for latex C-serum was 98.4 mg/ml. According to the classification by Meyer et al (1982), crude extracts and pure substances with LC50 value < 1000 µg/ml are toxic and LC50 value >1000 µg/ml are non-toxic.

The results of anti-fungal activity of the latex C-serum against *Aspergillus niger* and *Candida albicans* by the use of disc diffusion method were represented in Table II. Standard anti-fungal drug (amphotericin B) was used as positive control in the study.

In this study, it was demonstrated that the latex C-serum had inhibitory effects against *Aspergillus niger* but not *Candida albicans*. The diameter of growth inhibition zone of the C-serum was 15 mm and 6 mm for *A. niger* and *C. albicans* respectively (Table II). As to the standard anti-fungal drug used in the test, the inhibition zones of amphotericin B (0.05 µg/disc) was 35 mm. In view of the results obtained by the disc diffusion method, the MIC and MFC values of the latex C-serum were established for *A. niger* and the results were shown in Table III. The MIC values confirmed the existence of inhibitory effects on *A. niger* tested in the study, with MIC value of 2.5 mg/ml.

Therefore, data in the MIC results indicated an activity against *Aspergillus niger* for the latex C-serum. It should be noted that the latex C-serum tested by the disc diffusion method appeared less active than in the tests carried out in a liquid medium and this might be explained by the more limited diffusion of the serum in a solid medium. The MFC value of 5.00 mg/ml was observed for the latex C-serum, indicating the ability of the serum to kill the yeast cells.

The growth profile of *Aspergillus niger* in PDB at 0.5 MIC (1.25 mg/ml), MIC (2.5 mg/ml), 2 MIC (5.0 mg/ml) and control are shown in Figure 2. The growth profile was utilized in this study to confirm MIC findings and to evaluate the ability of latex C-serum to eliminate *A. niger* growth *in vitro*. In the case of 1 and 2 fold MIC concentrations (MIC and 2 MIC) the C-serum inhibited the fungal growth within 6 hours and subsequent regrowth was not seen (Figure 2). The growth profile thus suggested that the C-serum significantly inhibited the growth of *Aspergillus niger* and this activity was prolonged in time as determined by the growth curves.

**Discussion**

The results of BSLT revealed that the toxicity level for latex C-serum with LC50 at 98.4 mg/ml (Table I) was considerably low according to the classification by Meyer et al, without affecting the mortality rate of *Artemia salina*. This would be an additional beneficial property to the serum should it be use with its specific anti-proliferative properties against cancer-origin cells.
### Table I.

The results from Probit analysis. The LC$_{50}$ for A. salina with 95% confidence level was determined to be 98.4 mg/ml

<table>
<thead>
<tr>
<th>Alpha value (for confidence interval)</th>
<th>0.05</th>
<th>Probit analysis – Finney method [lognormal distribution]</th>
</tr>
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<td>Log 10 [dose (stimulus)]</td>
<td>Actual percent (%)</td>
<td>Probit percent (%)</td>
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</tr>
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<td>3</td>
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**Chi-square**

- Chi-square: 0.0174
- Degrees of freedom: 2.0000
- p-level: 0.9914

### Dose (stimulus) percentile

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<th>Percentile</th>
<th>Probit (Y)</th>
<th>Log 10 [dose (stimulus)]</th>
<th>Standard error</th>
<th>Dose (stimulus)</th>
<th>Standard error</th>
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</table>

**Regression statistics**

- **LD$_{50}$**: 98,428,8624
- **Standard error**: 23,499,414,4557
- **LD$_{50}$ LCL**: 0.5525
- **LD$_{50}$ UCL**: 17,534,636,125,4575
- **Log10(LD$_{50}$)**: 4.9981
- **Standard error**: 2.6790
- **Beta**: -0.2806
- **Intercept**: 6.4012
- **Beta standard error**: 0.2201
Anti-fungal effect of *Hevea brasiliensis* latex C-serum on *Aspergillus niger*

**Table II.** Zones of inhibition measured (in mm) from disc diffusion assay.

<table>
<thead>
<tr>
<th>Zones of inhibition (mm) on <em>Aspergillus niger</em> with:</th>
<th>Latex C-serum</th>
<th>Amphotericin B</th>
<th>PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<table>
<thead>
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<th>Zones of inhibition (mm) on <em>C. albicans</em> with:</th>
<th>Latex C-serum</th>
<th>Amphotericin B</th>
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<td>6.00</td>
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**Table III.** MIC and MFC values of latex C-serum on *A. niger*.

<table>
<thead>
<tr>
<th>MIC on <em>A. niger</em> with:</th>
<th>Latex C-serum (mg/ml)</th>
<th>Anti-fungal (µg/ml)</th>
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<tbody>
<tr>
<td></td>
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<td>0.0625</td>
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</table>

<table>
<thead>
<tr>
<th>MFC on <em>A. niger</em> with:</th>
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</table>

**Figure 1.** The regression line showing the predicted dose resulted from Probit analysis for brine shrimp lethality test with latex C-serum.

**Figure 2.** Growth profile for *A. niger* in PDB containing 0 mg/ml (control), 1.25 mg/ml (0.5 MIC), 2.5 mg/ml (MIC) and 5 mg/ml (2 MIC) of latex C-serum. CFU = Colony Forming Unit.
The latex C-serum had never been mentioned to have any anti-fungal properties. In our study, its specific anti-fungal activity against *Aspergillus niger*, but not *C. albicans*, has been shown the first time (Table II). This indicated that latex C-serum possessed potent anti-fungal bioactive compound(s) with high level of specificity towards *Aspergillus niger*. The anti-*A. niger* activity of latex C-serum was shown to be relatively weak compared to amphotericin B in disc diffusion assay. However, there was no agreement on the level of acceptance for plant when compared with standard anti-fungal drugs.

The results obtained from this assay merely indicated that the latex C-serum exerted an anti-fungal property against *Aspergillus niger*. The MIC and MFC assays (Table III), together with the time killing profile (Figure 2), clearly showed that the C-serum inhibited the growth of *Aspergillus niger* in a dose dependent manner and this effect was prolonged within the experimental time period of up to at least 48 hours post-treatment.

In this study, the whole C-serum was used. By further sub-fractionize the serum or separating the compounds in the serum, the active fraction or the active compound would exhibit a more potent activity against *A. niger*. Further work will be required to investigate the possibility of having the active anti-fungal sub-fraction or compound from the C-serum to be included in the therapeutic active sub-fractions of the latex sera\(^{17}\). This would be a value added to the therapeutic property of the latex serum.

The synergistic action of the anti-fungal active fraction or compound from latex C-serum with other anti-fungal agents should be assessed. This would allow the use of the existing anti-fungal agent by raising its anti-fungal effect in the presence of latex active sub-fraction.

Although the substances contained in the latex C-serum should be further studied to analyze their effectiveness as a species specific anti-fungal agent and potential synergistic action with other anti-fungal agents, the results of this study have demonstrated that the latex C-serum offer great prospects for the production of new species specific anti-fungal agent. This finding would probably add value to the existing cancer-origin cell line specific anti-proliferative property of the serum. With more findings on the biological activities of the serum, *H. brasiliensis* would contribute not merely to the polymer industry, but also in the pharmaceutical and healthcare sectors.

**Acknowledgements**

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**References**


12) **Das G, Alam B, Rai S, Dey SK, Sethuraj MR, Sennandi S.** Over-exploitation associated changes


