European Review for Medical and Pharmacological Sciences 2011; 15: 1163-1171

Anti-*Candida albicans* activity and brine shrimp lethality test of *Hevea brasiliensis* latex B-serum

K.M.A. DARULIZA, K.L. YANG*, K.L. LAM, J.T. PRISCILLA, E. SUNDERASAN**, M.T. ONG

Institute for Research in Molecular Medicine (INFORMM), University Sains Malaysia, Pulau Pinang (Malaysia)
*Centre for Drug Research (CDR), University Sains Malaysia, Pulau Pinang (Malaysia)
**Rubber Research Institute of Malaysia, Malaysian Rubber Board, Kuala Lumpur (Malaysia)

Abstract. – *Background and Objectives:* *Hevea brasiliensis* extracts could potentially be employed as a relatively low cost resource for various anti-fungal activities due to the simplicity of the extract preparation and its abundance especially in the tropical region. Latex B-serum was reported to have anti-cancer property and its specificity in anti-fungal property has not been elucidated. The present study was conducted to determine the anti-fungal activity of Hevea latex B-serum against *Candida (C.) albicans* (a rounded cell fungus) and *Aspergillus (A.)* niger (a filamentous fungus).

Methods and Results: **The results showed that the anti-fungal activity of latex B-serum was specific to *C. albicans* but not to *A. niger*. The MIC value of latex B-serum for *C. albicans* was found to be 2.5 mg/ml. The time-killing profile showed that the growth of *C. albicans* was inhibited and the inhibition was prolonged throughout the tested time period. Brine shrimp toxicity test showed an LC_{50} of 461.0 mg/ml with latex B-serum, indicating the non-toxicity of the serum.**

Conclusion: **Further purification and identification of the crude extract should point the way to bioactive compound(s) responsible for anti-*Candida* activity.**

Key Words:

Hevea brasiliensis, Latex B-serum, *Candida albicans*, *Aspergillus niger*, Anti-fungal, Brine shrimp lethality test.

Introduction

Candida (C.) albicans is a member of the normal flora of the gastrointestinal tract that often causes serious oral and vaginal mucosa invasion and systemic disease in hosts with impaired immune defenses. *C. albicans* is the most causative agent associated with serious fungal infection, accounting for more than 90% of candidiasis cases[1,2], known as the most common invasive fungal infection in critically ill non-neutropenic patients[3].

The management of *Candida* infections faces a number of challenges including limited number of effective anti-fungal drugs, toxicity level of the available anti-fungal drugs, resistance of *Candida* to commonly used anti-fungal drugs, relapse of *Candida* infections and the high cost of anti-fungal drugs[4-7]. The difficulties associated with the management of *Candida* infections necessitate the discovery of new anti-fungal agents in order to increase the spectrum of activity against *Candida* and combat strains expressing resistance to the available anti-fungal drugs.

Hevea 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 nature[8-12]. While some of the diverse inherent constituents of *Hevea (H.)* have been acknowledged and further developed, research on the disease prevention and therapeutic potential of the plant, however, remained scarcely explored. In addition to its industrial applications, *Hevea* leaf hydroxynitrile lyase has been employed to synthesize active cyanohydrins for use as pharmaceuticals[13]. Recently it was reported that a sub-fraction of latex B-serum exerted specific anti-proliferative properties against a cancer-origin cell line[14].

H. brasiliensis sera could potentially be employed as a relatively low cost resource for various anti-microbial activities due to the simplicity

Corresponding Author: Ming Thong Ong, DSc; e-mail: omt@usm.my (also ong.mt1@gmail.com)

of the method of preparation and the abundance of the source material in rubber producing regions. However, the anti-fungal property of the latex sera has either not been extensively explored or the results obtained were not conclusive (as in latex B-serum) so far. Latex serum had been reported to be anti-microbial due to the presence of hevein, a chitin binding protein, and a number of hydrolytic enzymes [15-17], which are contained mainly in latex B-serum. Nevertheless, it has been suggested that latex B-serum alone was not sufficient to cause an inhibition of yeast growth[18]. Other reports tried to attribute the anti-fungal property of latex B-serum to the enzymatic activities[19]. The present study was aimed at examining the anti-fungal property of latex B-serum based on the difference between *C. albicans* (a rounded cell fungus) and *Aspergillus niger* (a filamentous fungus).

Materials and Methods

Preparation of Latex B-Serum

Latex was collected from field-grown RRIM 600 trees at the Rubber Research Institute of Malaysia Research Station, Sungai Buloh. To prepare latex B-serum, fresh latex collected in chilled flasks was fractionated by centrifugation at 44,000 g at 4ºC. The latex separates into three distinct parts upon high-speed centrifugation[20]. Latex B-serum was prepared from this based on a method previously described[21]. Briefly, after removal of the rubber cream and C-serum, the sediment at the bottom of the centrifuge tube was collected and re-suspended in 0.4 M mannitol to aid the removal of remnant C-serum while retaining the lutoids intact. The cleansed bottom fraction was recovered after another centrifugation and subjected to alternate freezing and thawing cycles to rupture the lutoids. The fluid from the lutoids, the B-serum was recovered by centrifugation and lyophilized for subsequent use. Lyophilized powder of latex B-serum was reconstituted with 1X phosphate buffered saline (PBS). 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 *et al.*[30]. Brine shrimp (*Artemia salina*) eggs (Sanders™ Great Salt Lake, Brine Shrimp Company L.C., Utah, USA) were hatched in artificial sea water prepared from commercial sea salt (38 g sea salt per litre deionized water) with constant light source and oxygen supply after 24 hours of incubation. Latex B-serum was used to prepare serial diluted working concentrations of 2-2000 µg/ml in sea water respectively. Each concentration had three replicates and (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 (LC_{50}) for brine shrimp with 95% confidence level was determined by *Probit analysis*[22] on a Finney computer program BioStat™ 2009 (AnalystSoft Inc., Vancouver, Canada). Percentage mortalities 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[22].

Preparation of Fungal Inoculums

C. albicans (ATCC 10231) and *A. niger* (ATCC 9142) were used as the test organisms and was obtained from the laboratory stock culture. Fungal culture was cultured on potato dextrose agar (PDA) at 28ºC for 48 to 96 hours. The stock culture was maintained on the slant agar at 4ºC.

Screening for Anti-Fungal Activity

The anti-fungal activities of the extracts were determined following the method described by National Committee for Clinical Laboratory Standard (NCCLS, 2001) with slight modification.

Disc Diffusion Assay

The extracts were tested for anti-fungal activity by the disc diffusion method according to the NCCLS (2001). 100 µl of suspension of the tested microorganism that has been adjusted according to 0.5 McFarland standard (10^6 CFU/ml) was used in this assay. Whatman filter paper No. 1 disks of 6 mm diameter were used to screen the anti-microbial activity. Each sterile disk was impregnated with 20 µl of the extract (50 mg/ml of crude extract), amphotericin B (10 µg/ml, as positive control), latex B-serum (10 mg/ml) and phosphate buffer saline (PBS, as negative con-

trol), before it was placed on the surface of the seeded plates. The plates were incubated at 28ºC for 48 to 96 hours and examined for zones of growth inhibition.

Minimum Inhibitory Concentration (MIC)

A 16-hour culture was diluted with a sterile physiologic saline solution (PS; 0.85 % (w/v) sodium chloride) with reference to the 0.5 McFarland standards to achieve inoculums of approximately (10^6 CFU/ml). A serial dilution was carried out to achieve final concentrations between 0.625-20.00 mg/ml for latex B-serum. The tubes were inoculated with 20 µl of the bacterial suspension per milliliter nutrient broth, homogenized, and incubated at 28ºC. The minimum inhibition concentration (MIC) value was determined as the lowest concentration of the serum in the broth medium that inhibited the visible growth of the test microorganism.

Time-Killing Profile

The time-killing profile of *Candida albicans* with 0.5 MIC, MIC and 2 MIC concentrations over time was plotted to assess the fungicidal effect. The latex B-serum was added to an aliquot of 25 ml of potato dextrose broth (PDB; Difco, Detroit, MI, USA) at 37ºC 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 potato dextrose agar plate and incubated at 28ºC for 18 hours. The growth of *Candida albicans* was monitored by counting the number of colonies (CFU: Colony Forming Unit) after overnight incubation. The growth of *Candida albicans* was measured every 6 hours for 48 hours.

Statistical Analysis

Statistical analysis was performed using one-way ANOVA and p values less than 5% were considered statistically significant ($p < 0.05$).

Results

Brine Shrimp Lethality Test (BSLT)

The data from brine shrimp lethality test were used to conduct Probit analysis[22], from where the lethal concentration (LC_{50}) for brine shrimp with 95% confidence level was determined with BioStat™ 2009 (AnalystSoft Inc., Vancouver, Canada). The final results are shown in Table I and Figure 1.

The LC_{50} value for latex B-serum was 461.0 mg/ml. The positive control in which potassium dichromate was used showed an LC_{50} of 482.7 µg/ml (Table II and Figure 2). According to the classification by Meyer et al[23] and Parra et al[24], crude extracts and pure substances with LC_{50} value lower than 1000 µg/ml are considered bioactive in toxicity evaluation of plant extracts by BSLT. Hence, the latex B-serum could be considered non-toxic against brine shrimp according to the results obtained from the BSLT conducted,

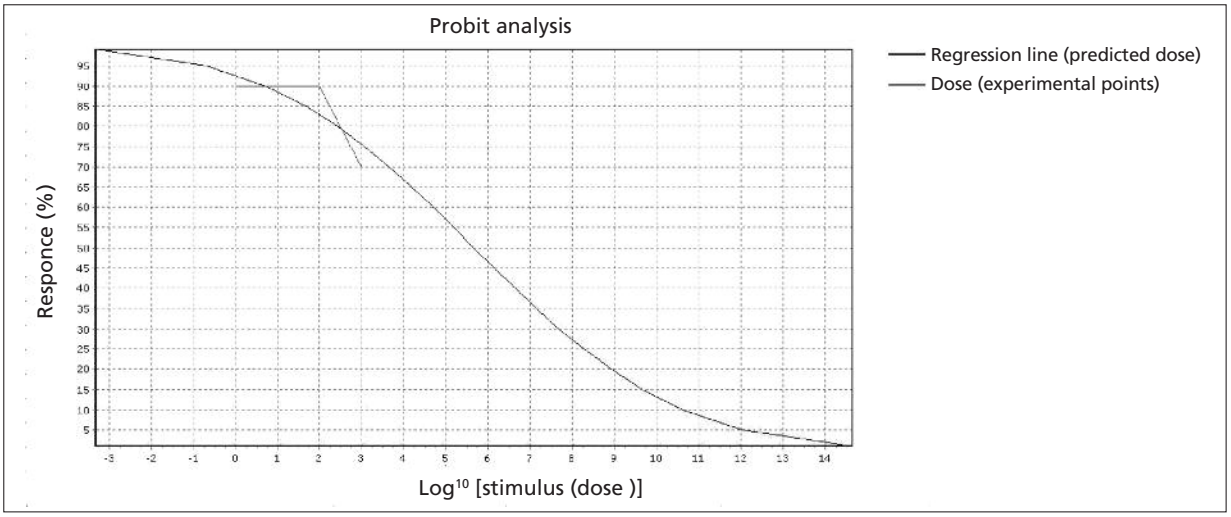

Figure 1. Probit analysis for brine shrimp lethality test with latex B-serum.

Table I. Probit analysis for brine shrimp lethality test with latex B-serum.

Alpha value (for confidence interval) 0.05							
Probit analysis – Finney method [lognormal distribution]							
Log^{10} [dose (stimulus)]	Actual percent (%)	Probit percent (%)	N	R	E®	Difference	Chi-square
0	0.9	0.9289	10	9	9.2894	-0.2894	0.0090
1	0.9	0.8866	10	9	8.8662	0.1338	0.0020
2	0.9	0.8288	10	9	8.2884	0.7116	0.0611
3	0.7	0.7550	10	7	7.5503	-0.5503	0.0401
Chi-square							
Chi-square	0.1122						
Degrees of freedom		2.0000					
p-level	0.9454						
Dose (stimulus) percentile							
Percentile	Probit (Y)	Log^{10} [Dose (stimulus)]	Standard error	Dose (stimulus)	Standard error		
1	2.6732	14.6412	5.7092	437,704,568,883,762.0000	112,041,963,610,936,000,000.0000		
5	3.3548	12.0115	4.5250	1,026,749,456,678.9400	17,195,295,311,633,500.0000		
10	3.7183	10.6090	3.8908	40,648,003,306.5317	158,065,030,831,314.0000		
16	4.0056	9.5005	3.3874	3,166,086,834.9178	3,862,995,447,814.0200		
20	4.1585	8.9103	3.1183	813,462,574.4982	534,077,436,287.5780		
25	4.3258	8.2650	2.8228	184,064,091.3663	61,193,587,437.9320		
30	4.4760	7.6855	2.5560	48,473,180.6825	8,718,336,648.4940		
40	4.7471	6.6396	2.0692	4,361,516.3931	255,720,983.6743		
50	5.0000	5.6637	1.6043	461,046.8548	9,263,501.1601		
60	5.2529	4.6879	1.1161	48,736.3071	316,502.5296		
70	5.5240	3.6420	0.4935	4,385.1920	6,127.3941		
75	5.6742	3.0625	0.3521	1,154.8380	1,042.4026		
80	5.8415	2.4172	0.6131	261.3079	504.2247		
84	5.9944	1.8270	0.6789	67.1378	153.2461		
90	6.2817	0.7185	0.4955	5.2294	7.3481		
95	6.6452	-0.6840	0.8536	0.2070	0.7244		
99	7.3268	-3.3137	2.1605	0.0005	0.0351		
Regression statistic							
LD_{50}	**461,046.8548**	**LD_{50} standard error**	**840,957,387.9416**				
LD_{50} LCL	0.0481	LD_{50} UCL	4,419,576,089,008.1400				
Log^{10} [LD_{50}]	5.6637	Standard error	3.5621				
Beta	-0.2592	Intercep	6.4679				
Beta standard error	0.2279						

Table II. Probit analysis for brine shrimp lethality test with potassium dichromate (positive control).

Dose (stimulus) percentile					
Percentile	Probit (Y)	Log^{10} [Dose (stimulus)]	Standard error	Dose (stimulus)	Standard error
1	2.6732	-0.8920	0.1423	0.1282	0.0428
5	3.3548	-0.7233	0.1074	0.1891	0.4720
10	3.7183	-0.0633	0.0894	0.2326	0.4820
16	4.0056	-0.5623	0.0758	0.2739	0.4810
20	4.1585	-0.5645	0.0690	0.2989	0.4770
25	4.3258	-0.4831	0.0618	0.3288	0.0470
30	4.4760	-0.4460	0.0559	0.3581	0.4620
40	4.7471	-0.3789	0.4690	0.4179	0.4520
50	5.0000	-0.3163	0.0415	0.4827	0.4620
60	5.2529	-0.2538	0.0403	0.5575	0.5180
70	5.5240	-0.1867	0.0440	0.6506	0.0660
75	5.6742	-0.1495	0.4790	0.7087	0.0783
80	5.8415	-0.1082	0.0533	0.7795	0.0960
84	5.9944	-0.0703	0.5910	0.8505	0.1161
90	6.2817	0.0008	0.0713	1.0017	0.1652
95	6.6452	0.0907	0.0883	1.2322	0.2522
99	7.3268	0.2593	0.1223	1.8167	0.5184
Regression statistics					
LD^{50}	**0.4827**	**LD_{50} Standard error**	**0.0462**		
LD_{50} LCL	0.3933	LD_{50} UCL	0.5721		
Log^{10} [LD_{50}]	-0.0316	Standard error	0.0415		
Beta	4.0422	Intercept	6.2787		
Beta standard error	0.7635				

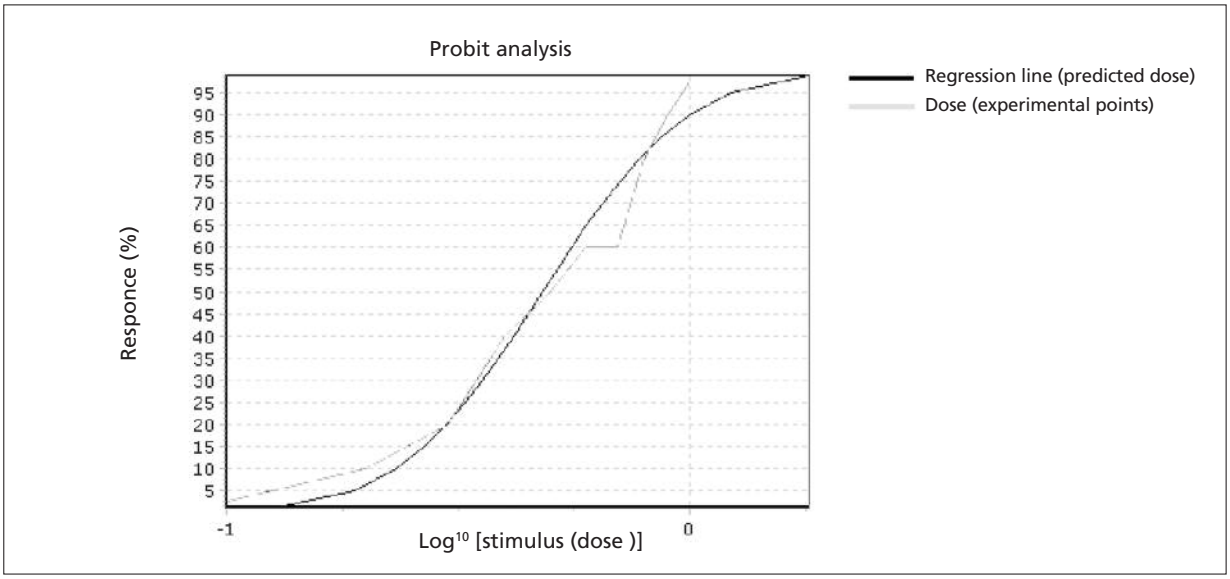

Figure 2. Probit analysis for brine shrimp lethality test with potassium dichromate (positive control).

whereas the LC_{50} for potassium dichromate clearly indicated the high cytotoxicity of the compound.

Disc Diffusion Assay

The results of anti-*C. albicans* and anti-*Aspergillus niger* activities of latex B-serum by the use of disc diffusion method were represented in Table III; amphotericin B, a standard anti-fungal drug served as positive control in the study.

It was discerned in the disc diffusion assay that latex B-serum had growth inhibitory effects against *Candida albicans*. The diameter of growth inhibition zone of B-serum (10 mg/disc) and was 10.8 mm (Table III). As for the standard anti-fungal drug, amphotericin B (0.05 µg/disc) that was used in the test, the inhibition zone was 35 mm. The negative control (PBS) did not show any growth inhibition on *Candida albicans*. A similar assay conducted against *Aspergillus niger* yielded a negligible inhibition zone of 6.0 mm with latex B-serum (Table IV), a value similar to that of the negative control (PBS), showing that there was no anti-*A. niger* activity exerted by latex B-serum. Statistical analysis confirmed that latex B-serum significantly affects growth of *Candida albicans*, but not *Aspergillus niger* (Table V).

In view of the results obtained from the disc diffusion method, subsequent tests were performed to determine the minimum inhibitory concentration (MIC) values of latex B-serum against *C. albicans*.

Table III. Disc Diffusion Assay conducted on *Candida albicans*.

Latex B-serum (10 mg/ml)	Amphotericin (10 µg/ml)	PBS
10.8 mm	35.0 mm	6.0 mm

Table IV. Disc Diffusion Assay conducted on *Aspergillus niger.*

Latex B-serum (10 mg/ml)	Amphotericin (10 µg/ml)	PBS
6.0 mm	35.0 mm	6.0 mm

Determination of Minimum Inhibitory Concentration (MIC) and Time Killing Profile

The potency of anti-*Candida albicans* activity of latex B-serum was assessed quantitatively by determining the MIC, as given in Table VI. The MIC value against *C. albicans* for latex B-serum was found to be 2.5 mg/ml, whereas that against amphotericin B was 0.0625 mg/ ml.

The MIC values confirmed the inhibitory effects of latex B-serum on *C. albicans* tested in the study. The growth profile of *C. albicans* in PDB in the presence of latex B-serum at 0.5 MIC, MIC, 2 MIC and control is shown in Figure 3.

In the cases of one and two fold MIC concentrations, the growth of *C. albicans* was inhibited by latex B-serum within 6 hours and subsequent recovery of growth was not seen (Figure 3). The growth profile suggested that the extracts significantly inhibited the growth of *C. albicans* and also prolonged anti-*Candida* activity against the

Table V. Statistical analysis showed that latex B-serum significantly affects a single test organism, namely *Candida albicans*, but not *Aspergillus niger* and the control (PBS). Both the inhibition zone for *A. niger* and the control (PBS) is not visible (6.0 mm).

DDA (mm)				
			Subset for alpha = 5	
Test organism		N	1	2
Tukey HSD[a]	PBS (control)	3	6.000	
	Aspergillus niger	3	6.200	
	Candida albicans	3		10.800
	Sig		.669	1.000
Duncan[a]	PBS (control)	3	6.000	
	Aspergillus niger	3	6.200	
	Candida albicans	3		10.800
	Sig		.410	1.000

Table VI. Minimum Inhibitory Concentration determined using *C. albicans* cultured in the presence of latex B-serum and of amphotericin.

Latex B-serum (mg/ml)	Amphotericin (µg/ml)
2.50	0.0625

organism as determined by the growth curves. The MIC concentrations altered the normal growth profile for *C. albicans* when compared to the control (with no extract present). The finding confirmed the fungicidal effect of latex B-serum on *C. albicans* at the MIC concentration.

Discussion

The search for safer and more effective anti-fungal agents is important with the increasing number of resistant strains of *C. albicans* to a number of anti-fungal agents[25,26]. Hence, there is a need for developing wider variety of anti-fungal agents for the treatment of fungal diseases.

The present study was aimed at the investigation of anti-fungal activity of latex B-serum against *C. albicans* (a rounded cell fungus) and *A. niger* (a filamentous fungus). The results obtained from the diffusion method, showed that latex B-serum exhibited favorable anti-fungal activity selectively against *C. albicans* but not against *A. niger*. The MIC values obtained for the serum against *C. albicans* also supported the findings of the disc diffusion assay. Furthermore, the MIC for latex B-serum suggested that its use to treat *C. albicans* infection might be possible due to its non-toxicity demonstrated in BSLT. The possibility was further strengthened by the alteration of the normal growth profile of *C. albicans* by the extracts at the MIC concentration.

Interestingly, the anti-fungal property observed in our study suggested that latex B-serum sourced from fresh field latex alone is sufficient to cause an inhibition of yeast growth. Earlier investigations using ammoniated *Hevea* latex and sera fractions, however, did not yield measurable anti-fungal activity[18,27].

The BSLT represents a rapid, inexpensive and simple bioassay for testing plant extracts bioactivity which in most cases correlates reasonably well with cytotoxic and anti-tumor properties. The degree of lethality was found to be directly proportional to the concentration of the extract[23]. BSLT results revealed that toxicity level for latex B-serum was considerably low. It is also interesting to note that latex B-serum showed significant high levels of anti-yeast property without affecting the survival of *A. salina*.

When compared to amphotericin B, the anti-fungal property of latex B-serum was not as strong as the standard anti-fungal agent. This might be due to the inhibition of certain anti-

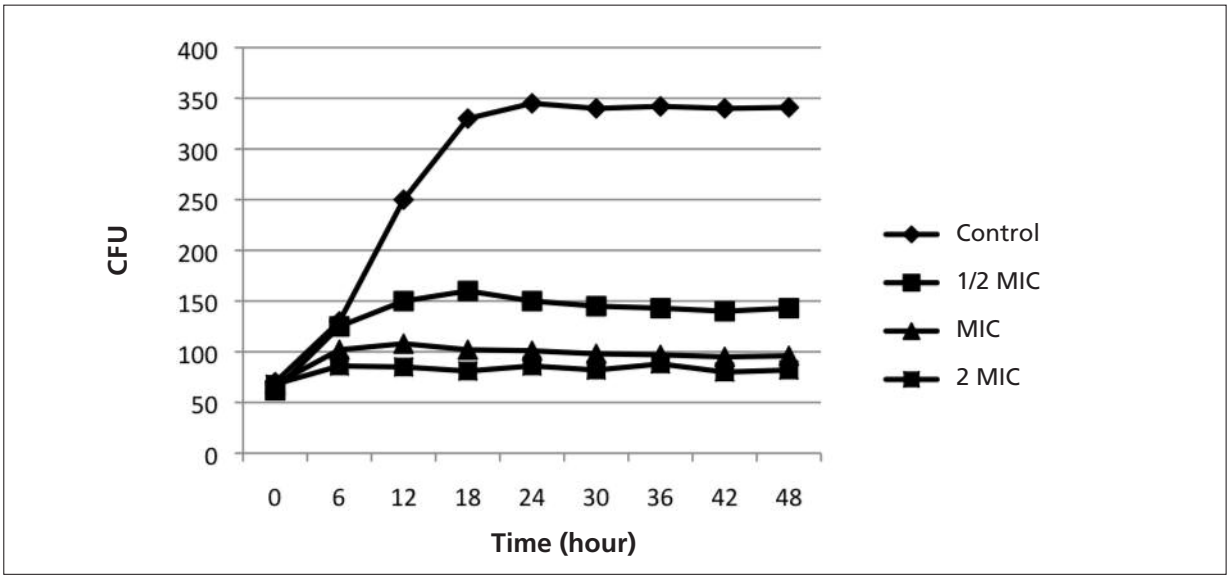

Figure 3. Growth profile for *Candida albicans* for the first 48 hours in PDB in the presence of various concentrations of latex B-serum: control (0), ½ MIC (1.25 mg/ml), MIC (2.5 mg/ml) and 2 MIC (5.0 mg/ml).

fungal compound(s) by the other molecules in the extracts. For latex B-serum, this phenomenon had been observed in the anti-proliferation assay conducted previously with the sub-fractions of the B-serum, wherein the BHM sub-fraction exerted the anti-proliferation property against HeLa cells, but not the BLM sub-fraction[14]. It is expected that further fractionating the B-serum into more specific sub-fractions may result in more effective anti-fungal properties.

If proven safe in further *in vivo* tests, the serum or its active fraction(s) may be used as an anti-fungal agent in known dosages, especially in rural communities where conventional drugs are unaffordable or unavailable and the health facilities are inaccessible. Our results indicate that the natural products analyzed seemed to be a good choice for the development of new strategies to treat *C. albicans* infection.

Nevertheless, caution should be exercised due to the allergenic proteins contained in latex B-serum[17, 28], precautious measures should be taken if used on human, and its use should be limited to those who are not affected by latex allergens.

Patients undergoing chemotherapy are vulnerable to opportunistic infections due to the partial impairment of their immune system. As *C. albicans* is an opportunistic disease causative agent that attacks the hosts with impaired immune system, the anti-fungal property of the latex B-serum could mean an additional advantage in anti-cancer treatment using the serum. If used in cancer treatment, at the same time of application of the serum in anti-proliferation treatment for cancer cells, the serum itself could exert its anti-fungal properties without having the need to introduce additional anti-fungal agents to prevent the opportunistic infection of *C. albicans*.

Interestingly, the expected presence of chitinases, β-1,3-glucanases and lysozyme in latex B-serum[15,19], which are among the potential anti-fungal/ anti-microbial agents of the B-serum, had little effect on *A. niger*. Our findings indicated that the latex B-serum exhibited an anti-fungal activity against *C. albicans*, but was inactive against *A. niger* (Table IV). It is possible that the mode of anti-*C. albicans* action of B-serum compound(s) leading to the results observed in this study was ineffective in the case of *A. niger*. The reason could be the constituents of the envelope of the two fungi are different. It had been reported that the difference in dry weight of chitin present on the envelope of filamentous fungus was ten times higher than that on rounded cell fungus (yeast)[29]. Chitin microfibrils have enormous tensile strength and significantly contribute to the overall integrity of the cell wall. If the anti-fungal activity of latex B-serum relied principally in the disruption of the chitin structure, the difference in the composition of chitin on the envelope of the organisms would play a crucial role in the outcomes. Alternatively, the envelope of *A. niger* might have additional composition that prevent the activity of the enzymes from acting on it.

Conclusion

Although the substances contained in latex B-serum should be further studied to analyze its effectiveness as a species-specific anti-fungal agent and its potential synergistic action with other anti-fungal agents, the results of this study have demonstrated that latex B-serum might offer great prospects for the production of new species-specific anti-fungal agent. These findings would probably add value to the existing therapeutic properties of the plant extract such as anti-proliferation property of latex B-serum in cancer-origin-cell-line specific manner[14].

Acknowledgements

We thank the Malaysian Rubber Board and University Sains Malaysia Incentive Grant (Grant number: 1001/CIPPM/822111) for funding the research. Daruliza K.M.A., Lam K.L. and Yang K.L. are recipients of the Fellowship from University Sains Malaysia and Lam K.L. and Yang K.L. are also recipients of Postgraduate Research University Research Grant from University Sains Malaysia.

References

1) Douglas LJ. *Candida* biofilms and their role in infection. Trends Microbiol 2003; 11: 30-36.
2) Edwards JE, Jr. *Candida* species, p. 2289-2306. In: G.L. Mandell, J.E. Bennett, and R. Dolin (ed.), Mandell, Douglas, and Bennett's Principles and practice of infectious diseases. 4th ed. Churchill Livingstone, Inc., New York, N.Y, 1995.
3) Eggimann P, Garbino J, Pittet D. Epidemiology of *Candida* species infections in critically ill nonimmunosuppressed patients. Lancet Infect Dis 2003; 3: 685-702.

4) KHAN ZU, CHANDY R, METWALI KE. *Candida albicans* strain carriage in patients and nursing staff of an intensive care unit: a study of morphotypes and resistotypes. Mycoses 2003; 46: 476-486.

5) KLEPSER ME. Antifungal resistance among *Candida* species. Pharmacotherapy 2001; 21: 124S-32S.

6) MUSTAFA NK, TANIRA MOM, DAR FK, NSANZE H. Antimicrobial activity of Acacia nilotica sub spp, Nilotica fruit extracts. Pharm Pharmacol Commun 1999; 5: 583-586.

7) RUNYORO DKB, MATEE MIN, NGASSAPA OD, JOSEPH CC, MBWAMBO ZH. Screening of Tanzanian medicinal plants for anti-*Candida* activity. BMC Com Alternat Med 2006; 30: 6-11.

8) CHOW CK, DRAPER HH. Isolation of gamma-tocotrienol dimers from Hevea latex. Biochemistry 1970; 9: 445-450.

9) BEALING FJ. Quebrachitol synthesis in Hevea brasiliensis. J Rubb Res Inst Malaysia 1981; 29: 111-112.

10) BURTON GW, WEBB A, INGOLD KU. A mild, rapid and efficient method of lipid extraction for use in determining vitamin E/ lipid ratios. Lipids 1985; 20: 29-39.

11) WAJANT F, FOSTER S. Purification and characterisation of hydroxyl nitrile lyase from Hevea brasiliensis. Plant Sci 1996; 115; 25-31.

12) DAS G, ALAM B, RAJ S, DEY SK, SETHURAJ MR, SEN-MANDI S. Over-exploitation associated changes in free radicals and its scavengers in Hevea brasiliensis. J Rubb Res 2002; 5: 28-40.

13) EFFENBERGER F. Hydoxynitrile lyases, interesting biocatalysts in stereo-selective organic syntheses. Int. Conf. Biodiversity and Bioresources: Conservation and Utilisation, Phuket, Thailand 1999; p. 38.

14) ONG MT, YANG KL, LAM KL, ONG GA, SUNDERASAN E. Susceptibility of HeLa (Cancer-origin) Cells to a Sub-fraction of Latex B serum. J Rubb Res 2009; 12: 117-124.

15) MARTIN MN. The latex of Hevea brasiliensis contains high levels of both chitinases and chitinases/lysozymes. Plant Physiol 1991; 95: 469-476.

16) JAN PARIJS J, BROEKAERT WF, GOLDSTEIN IJ, PEUMANS WJ. Hevein: an antifungal protein from rubber-tree (Hevea brasiliensis) latex. Planta 1991; 183: 258-264.

17) SUNDERASAN E, SAMSIDAR H, SHARIFAH H, WARD MA, YEANG HY, CARDOSA MJ. Latex B-serum β-1,3-glucanase (Hev b II) and a component of the microhelix (Hev b IV) are major latex allergens. J Nat Rubb Res 1995; 10: 82-99.

18) GIORDANI R, GACHON C, BUC J, REGLI P, JACOB JL. Antifungal action of Hevea brasiliensis latex. Its effect in combination with fluconazole on *Candida albicans* growth. Mycoses1999; 42: 465-474.

19) JACOP J-L, D'AUZAC J, PREVOT J-C. The composition of natural latex from Hevea brasiliensis. Clin Rev Allergy 1993; 11: 325-337.

20) MOIR GFJ. Ultracentrifugation and staining of Hevea Latex. Nature 1959; 184: 1626.

21) HSIA RCH. Oxygen Absorption by Hevea brasiliensis Latex. Trans Instn Rubb Ind 1958; 34: 267.

22) FINNEY DJ. Probit analysis. 1962 University Press, Cambridge.

23) MEYER BN, FERRIGNI NR. Brine shrimp: a convenient general bioassay for active plant constituents. Planta Med 1982; 45: 31-34.

24) PARRA AL, YHEBRA RS, SARDINAS IG, BUELA LI. Comparative study of the assay of Artemia salina L. and the estimate of the medium lethal dose (LD_{50} value) in mice, to determine oral acute toxicity of plant extracts. Phytomedicine 2001; 8: 395-400.

25) DANNAOUI E, BOREL E, MONIER MF, PIENS MA, PICOT S, PERSAT F. Acquired itraconazole resistance in Aspergillus fumigatus. J Antimicrob Chemother 2001; 47: 333-340.

26) MARR KA, WHITE TC, VAN BURIK JAH, BOWDEN RA. Development of fluconazole resistance in *Candida albicans* causing disseminated infection in patient undergoing marrow transplant. Clin Infect Dis 1997; 25: 908-910.

27) GIORDANI R, REGLI P, BUC J. Antifungal effect of Hevea brasiliensis latex with various fungi. Its synergistic action with amphotericin B against *Candida albicans*. Mycoses 2002; 45: 476-481.

28) YEANG HY, SITI ARIJA MA, FARIDAH Y, SUNDERASAN E. Allergenic proteins of natural rubber latex. Methods 2002; 27: 32-45.

29) BOWMAN SM, FREE SJ. The structure and synthesis of the fungal cell wall. BioEssays 2006; 28: 799-808.

30) MCLAUGHLIN JL, ROGERS LL. The use of biological assays to evaluate botanicals. Drug Inf J 1998; 32: 51.