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Antimicrobial activity of *Bulbothrix setschwanensis* (Zahlbr.) Hale lichen by cell wall disruption of *Staphylococcus aureus* and *Cryptococcus neoformans*

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ABSTRACT

In the present study, antimicrobial activity of a common Himalayan lichen *viz. Bulbothrix setschwanensis* (Zahlbr.) Hale extract in three common solvents (acetone, chloroform and methanol) was evaluated against six bacterial and seven fungal clinical strains. The acetone extract showed promising antimicrobial activity against *S. aureus* (1.56 mg/mL) and *C. neoformans* (6.25 mg/mL). Further, GC-MS analysis revealed 2,3-bis(2-methylpentanoyloxy)propyl 2-methylpentanoate and Ethyl 2-[(2R,3R,4aR,8aS)-3-hydroxy-2,3,4,4a,6,7,8,8a-octahydropyrano [3,2-b]pyran-2-yl]acetate as the predominant compounds. The combination of acetone extract with antibacterial drugs [kanamycin (KAN), rifampicin (RIF)] and antifungal drugs [amphotericin B (Amp B) and fluconazole (FLC)] showed lysis of *S. aureus* and *C. neoformans* at non-inhibitory concentration (FICI values were 0.31 for KAN, 0.18 for RIF, 0.37 for Amp B and 0.30 for FLC, respectively). Notably, the acetone extract confirmed cell wall damage of both *S. aureus* and *C. neoformans* cells and was clearly visualized under scanning electron microscopy (SEM), flow cytometry and confocal microscopy. Besides this, the three extracts also have less significant cytotoxic activity at MIC concentrations against mammalian cells (HEK-293 and HeLa). This study for the first time suggests that the chemical compounds present in the acetone extract of *B. setschwanensis* could be used against *S. aureus* and *C. neoformans* infections.

1. Introduction

In recent past, the treatment of bacterial and fungal disease caused by pathogenic microorganisms has become more difficult due to rapid emergence of microbial strains that have become resistant to drugs [1]. Further, this microbial resistance is responsible for morbidity and mortality in burn, HIV infection (AIDS), steroid therapy, cancer chemotherapy and organ transplant patients [2]. This rapidly increasing antimicrobial drug resistance has posed a serious threat to public health and become a major challenge to the scientific community for the search of novel antimicrobial agents with a distinct mode of action [3]. In disparity to the steep growth in microbial infection cases, only a few clinically approved antimicrobial drugs have been developed over the past 5-6 decades. These drugs having several drawbacks such as development of drug resistance in the pathogens, acute and chronic effects (e.g., amphotericin B), limited clinical efficacy (e.g., terbinafine) owing to the poor pharmacokinetics, lack of potency, complex drug interaction with the host cells, poor bioavailability. These drugs have led for the discovery of new antimicrobial agents [4] and this become more important recently because well-known antimicrobial drugs (*viz.* chloramphenicol, amphotericin B, fluconazole, rifampicin, penicillin) have became less effective against some common bacterial pathogens (e.g., *Staphylococcus aureus* and *Escherichia coli*) and fungal pathogens (e.g., *Candida albicans*, *Candida glabrata* and *Cryptococcus neoformans*) [5,6]. Therefore, there is an urgent need to explore new antimicrobial compounds with effective microbicidal activity, minimum hemolysis, mammalian cell toxicity, target specific, rapid killing, multiple mechanisms of action and no interaction towards clinically available antimicrobial drugs [7]. In this regard, the search for new antimicrobial agents from natural resources has increased as an alternative to well-known antimicrobial drugs [8] and over the last decade, the pharmaceutical industry has shown a great interest in natural products for the discovery of new leads [9].

Natural products produced from microorganisms, fungi, marine organisms and plants have served as a rich source of novel bioactive compounds [10,11] and about 44% of new antimicrobial compounds

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have been synthesized from them [12,13]. Lichens which are complex organisms consisting of a symbiotic association of a fungus and algal partner and being used in folk medicines for centuries [14] also not lag far behind as they produce several secondary metabolites having antibiotic, antimycotic, antiviral, anti-inflammatory, analgesic, antiproliferative and cytotoxic effects [15,16]. More than 20,000 lichens species are present throughout the world representing 8% of the total terrestrial ecosystem on earth [17] of which only few have been screened for their antimicrobial efficacy. The main aim of the present study was to evaluate the antimicrobial activity of a foliose lichen *viz. B. setschwanensis* extract against human bacterial and fungal pathogens, which is well known for the reduction of tyrosine enzyme activity [18]. The present study revealed synergistic effect of *B. setschwanensis* extract with known antibacterial (rifampicin and kanamycin) and antifungal agents (fluconazole and amphotericin B) against *S. aureus* and *C. neoformans* and also describes the killing kinetics and mechanistic studies of *B. setschwanensis* extract against these two pathogens.

2. Materials and methods

2.1. Chemicals, microorganisms and growth media

All the solvents (acetone, chloroform and methanol) were purchased from SD fine chemicals, Mumbai, India, while the standard antibiotics (kanamycin, rifampicin, amphotericin B and fluconazole) were purchased from HiMedia, Mumbai, India. The microbial strains used in the present study were procured from National Collection of Pathogenic Fungi (NCPF), Post-Graduate Institute of Medical Education and Research (PGIMER), Chandigarh and Microbial Culture Collection Centre (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India (Supplementary Table S1). The bacterial strains were cultured in Mueller Hinton broth (HiMedia, India), while the fungal strains in yeast extract-peptone-dextrose [YEPD broth and YEPD agar, (HiMedia, India)] and RPMI 1640 media (HiMedia, India). For agar plates, 2.5% (w/v) bacteriological agar (HiMedia, Mumbai) was added to the medium. The strains were stored with 15% glycerol at −80 °C as frozen stocks. The cells were freshly revived on respective agar plates from the stock before the commencement of each experiment.

2.2. Collection, identification and extraction of compounds from lichen material

The lichen specimen (*B. setschwanensis*) was collected from Western Himalayan Region (Narayan Ashram, Pithoragarh district, Uttarakhand) in September 2014 and is deposited at the herbarium of CSIR-National Botanical Research Institute (LWG 17-031326), Lucknow and Department of Botany, S.S.J. Campus, Kumaun University, Almora (KU) for future reference. The sample was sorted and cleaned from any debris and air dried in shady place. After drying, the sample was ground into a fine powder and then 2 g of it was suspended in 20 mL of different organic solvents (acetone, chloroform and methanol) in shaking incubator (REMI-CIS 24 Plus) at 37 °C for 24 h [19]. The extracts were then filtered through Whatman No. 1 filter paper and the filtrates were then subjected to membrane filtration by using 0.45 μM pore size filter for concentrating [20,21]. All the extracts were stored at 4 °C.

2.3. GC-MS analysis of lichen extracts

The extract was subjected to GC-MS (GC-Thermo trace-1300GC) equipped with TSQ8000 MS (triplequardrapole) detector. The chromatography was performed by using the TG5-MS column (30 m × 0.25 mm, 0.25 μm) and the injection temperature was 250 °C. Helium flow was 1 mL/min. After a 5 min solvent delay time at 70 °C; the oven temperature was increased at 5 °C/min to 310 °C, 1 min isocratic and cooled to 70 °C, followed by the additional 5 min delay. Total running time of GC-MS was 35 min. The relative percentage of the extract constituents was expressed as percentage with peak area normalization. For the identification of compounds in the extracts, retention indices (RI) and mass spectra fragmentation pattern of these were compared with those stored in the database of National Institute Standard and Technology (NIST), version 2015. The homologous alkane series (Sigma, St. Louis, USA) was used as internal standard [22].

2.4. Antimicrobial activity

2.4.1. Antibacterial activity

Antibacterial activity was performed against all the six bacterial strains [*Enterococcus faecalis* (*E. faecalis*), *Escherichia coli* (*E. coli*), *Listeria monocytogens* (*L. monocytogens*), *Staphylococcus aureus* (*S. aureus*), *Streptococcus pyogens* (*S. pyogens*), *Vibrio cholera* (*V. cholera*)] as per guidelines of Clinical and Laboratory Standards Institute [23]. All these bacterial strains were grown overnight and diluted in Mueller-Hinton broth (MHB) to a cell density of 10^5 Colony Forming Unit (CFU)/mL. Bacterial cells (100 μl) and extracts (from 50 mg/mL to 0.095 mg/mL) were added into the 96-well flat-bottomed microtitre plate (HiMedia, India) which was then incubated at 37 °C without shaking for 24 h. The visual and optical density at 600 nm was measured using microplate reader (Thermo scientific, Model 680). The minimum inhibitory concentration (MIC), which is defined as the concentration of the drug that inhibits > 99% growth, was measured and rifampicin (RIF), a well-known standard antibacterial drug was used as a positive control [24,25].

2.4.2. Antifungal activity

Antifungal activity was performed against all the seven fungal strains [*Candida albicans* (*C. albicans*), *Candida glabrata* (*C. grabrata*), *Candida krusei* (*C. krusei*), *Candida parapsilosis* (*C. parapsilosis*), *Candida keyfer* (*C. keyfer*), *Candida tropicalis* (*C. tropicalis*), *Cryptococcus neoformans* (*C. neoformans*)] as per the guidelines of Clinical and Laboratory Standards Institute (23) in RPMI 1640 medium using broth microdilution method. The concentration of extracts (acetone, chloroform and methanol) ranged between 50 mg/mL and 0.095 mg/mL. The 96-well flat-bottom microtiter plates were incubated without shaking at 30 °C for 48 h and the visual and optical density was determined at 600 nm using a microplate reader (Thermo Scientific, Model 680) for analyzing growth inhibition. The MIC was measured and amphotericin B (Amp B), a well-known standard antifungal drug was used as a positive control [26,27].

2.5. Synergy of acetone extracts with known antimicrobial agents

The interaction of acetone extract with well-known antimicrobial kanamycin (KAN) rifampicin (RIF), amphotericin B (Amp B) and fluconazole (FLC) was evaluated by the checkerboard method and is expressed as the fractional inhibitory concentration index (FICI) i.e. sum of the FIC for each agent. FIC value of the most effective combination was used in calculating FICI [28]. FICI = FIC of X + FIC of Y = $C_X^{comb}/MIC_X^{alone} + C_Y^{comb}/MIC_{Y,}^{alone}$ where, MIC_X^{alone} and MIC_Y^{alone} are the MICs of drug X and Y when acting alone and C_X^{comb} and C_Y^{comb} are concentrations of drugs X and Y at isoeffective combinations, respectively. The FICI was interpreted as synergistic when it was ≤0.5, as antagonistic when > 4.0, and any value in between as indifferent [29,30].

2.6. Time-kill kinetic assay

S. aureus cells (~1 × 10^5 CFU/mL) were inoculated at 37 °C, 200 rpm in MHB medium containing acetone extract (at 1.56 mg/mL conc.) and 100 μl aliquots of it were removed at pre-determined time points (0, 4, 8, 12, 16, 20 and 24 h). The aliquots were serially diluted in saline (10 folds) and plated on the MHA plates. The number of colonies were counted after incubating the plates at 37 °C for 24 h

Table 1
Phyto-chemical identification in acetone extract of *B. setschwanensis*.

S. No.	% Peak area	Peak RT (min.)	Compound detected	CAS No.	Mol. formula	Mw
1	2.41	11.67	2,3-di(hexanoyloxy)propyl hexanoate	621-70-5	$C_{21}H_{38}O_6$	386.52
2	2.41	11.67	Ethyl 5-oxodecanoate	2051-49-2	$C_{12}H_{22}O_3$	214.30
3	2.41	11.67	Hexadecanethioic S-acid	2432-81-7	$C_{16}H_{32}OS$	272.49
4	26.72	12.93	Ethyl 2-[(2R,3R,4aR,8aS)-3-hydroxy-2,3,4,4a,6,7,8,8a-octahydropyrano[3,2-b]pyran-2-yl] acetate	Unknown	$C_{12}H_{20}O_5$	244.28
5	1.44	15.69	Dimethyl 2-ethylidenepropanedioate	85547-53-1	$C_7H_{10}O_4$	158.15
6	0.63	16.05	1,2,3-Propantriyl-tripentanoate	125476-47-3	$C_{18}H_{32}O_6$	344.44
7	0.63	16.05	*Tert*-butyl 3-(hydrazinecarbonyl)azetidine-1-carboxylate	Unknown	$C_9H_{17}N_3O_3$	215.24
8	0.48	16.47	1-(3-acetyl-2,4,6-trihydroxyphenyl)ethanone	34874-90-3	$C_{10}H_{10}O_5$	210.18
9	0.48	16.47	(1aR,3aS,7aS)-3a-hydroxy-1a,4,4-trimethyl-2H-oxireno[2,3-c][1]benzofuran-7-one	34874-75-4	$C_{11}H_{14}O_4$	210.22
10	64.31	22.42	2,3-bis(2-methylpentanoyloxy)propyl 2-methylpentanoate	56554-55-3	$C_{21}H_{38}O_6$	386.50
11	2.11	27.78	β-D-Glucopyranuronic acid, 1-(2-propylpentanoate)	56687-69-5	$C_{14}H_{24}O_8$	320.33

[31,32]. Similarly *C. neoformans* cells (~1 × 10^4 CFU/mL) were inoculated in RPMI 1640 medium containing acetone extract (at 6.25 mg/mL conc.). The test tubes were incubated at 30 °C, 200 rpm and 100 µl aliquots were removed at pre-determined time points (0, 4, 8, 12, 16, 20 and 24 h). The aliquots were serially diluted in saline water (10 folds) and plated on YEPD agar plates. The numbers of colonies were counted after incubating the plates at 30 °C for 48 h [31].

2.7. Confocal microscopy and flow cytometry

The permeabilization effect of acetone extract on *S. aureus* and *C. neoformans* cell wall were checked by using a membrane impermeant dye *viz.* propidium iodide (PI). Overnight grown colonies of *S. aureus* (~1 × 10^5 CFU/mL) and *C. neoformans* cells (~1 × 10^4 CFU/mL) were suspended in MHB and RPMI 1640 medium containing acetone extract at MIC (1.56 mg/mL for *S. aureus* and 6.25 mg/mL for *C. neoformans*) and PI (1.42 µg/mL). After incubation for 8 h at 37 °C (*S. aureus*) and 30 °C (*C. neoformans*) with constant shaking (200 rpm), the cells were harvested by centrifugation and suspended in phosphate buffer saline (PBS, pH 7.4) and examined under confocal microscope (Olympus Fluoview™ FV1000 SPD; Olympus, Tokyo, Japan) with a wavelength > 560 nm for PI. Both (*S. aureus* and *C. neoformans*) cells without treatment of acetone extract served as a control [24].

For flow cytometry analysis, *S. aureus* (~1 × 10^5 CFU/mL) and *C. neoformans* cell (~1 × 10^4 CFU/mL) were first harvested at log phase and suspended in MHB and RPMI 1640 medium, respectively. The cells were then incubated at 37 °C and 30 °C for 8 h with PI (1.42 µg/mL) and acetone extract at MIC concentrations (1.56 mg/mL for *S. aureus* and 6.25 mg/mL for *C. neoformans*). After incubation, the cells were harvested by centrifugation and suspended in PBS. Flow cytometry was performed via FACS Calibur flow cytometer (Becton-Dickinson, San Jose, CA, USA). Untreated *S. aureus* and *C. neoformans* cell served as a control [32].

2.8. Scanning electron microscopy

The suspensions of *S. aureus* and *C. neoformans* cells were prepared from overnight grown cultures in MHB and RPMI 1640 medium (pH 7), respectively. 1.56 mg/mL of the acetone extract was added to *S. aureus* cells (~1 × 10^5 CFU/mL) which was then incubated at 37 °C for 24 h, while 6.25 mg/mL was added to *C. neoformans* (~1 × 10^4 CFU/mL) which was incubated for 16 h at 30 °C. After treatment the samples were observed under SEM (JEOL2100F, JEOL) [33–35].

2.9. Mammalian cells toxicity

The mammalian cells toxicity of extracts (acetone, chloroform and methanol) against normal human embryonic kidney cells (HEK-293) and cervical cancer cells (HeLa) was performed by MTT (3-(4,5)-dimethylthiazol-2-yl)-2,5-(diphenyl tetrazolium bromide) assay. The cells (5 × 10^4/well) were cultured in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum (FBS) in a 96-well microtiter plate at 37 °C for overnight. The next day, extracts of acetone, chloroform and methanol (at 50 mg/mL) were added to the cells in separate wells and incubated at 37 °C for 18 h. The cells were further incubated at 37 °C for 3–4 h in 20 µl of MTT solution (5 mg/mL) in PBS. The supernatant (120 µl) was removed and 100 µl DMSO was added to it, and the resulting suspension was mixed to dissolve the formazan crystals. The percent viability of cells was calculated by the ratio of $O.D._{570}$ of treated cells to the $O.D._{570}$ of untreated cells. Untreated cells and 10% Dimethyl sulfoxide (DMSO) were taken as negative and positive controls, respectively [36].

3. Results and discussion

3.1. GC-MS profile of B. setschwanensis extract

The GC-MS profile of *B. setschwanensis* extract revealed the presence of 11 different compounds which were characterized and identified by comparison of their mass fragmentation pattern with similar in NIST library database (Table 1 and Supplementary Fig. S1). Among the identified compounds, 2,3-bis (2-methylpentanoyloxy) propyl 2-methyl pentanoate (64.31%) and Ethyl 2-[(2 R,3R,4aR, 8aS)-3-hydroxy-2,3,4,4a,6,7,8,8a-octahydropyrano [3,2-b] pyran-2-yl] acetate (26.72%), were found to be present in major constituents, while, 2,3-di (hexanoyloxy) propyl hexanoate (2.41%), Ethyl 5-oxodecanoate (2.41), Hexadecanethioic S-acid (2.41%), β-D-Glucopyranuronic acid, 1-(2-propylpentanoate) (2.11%), Dimethyl 2-ethylidenepropanedioate (1.44%), 1,2,3-Propantriyl-tripentanoate (0.63%), *Tert*-butyl 3-(hydrazinecarbonyl)azetidine-1-carboxylate (0.63%), 1-(3-acetyl-2,4,6-trihydroxyphenyl)ethanone (0.48%), (1aR,3aS,7aS)-3a-hydroxy-1a,4,4-trimethyl-2H-oxireno [2,3-c] [1]benzofuran-7-one (0.48%) were present in traces. The ester containing compounds made up the largest component of the extracts (91.14%) and shows antimicrobial activity. Our results corroborate with earlier reports mentioning that ester containing compounds, isolated from natural resources have potent antifungal, antibacterial, UV stabilizing and antioxidant activities [37].

3.2. Antimicrobial activity of acetone extract

The results of antimicrobial activity of acetone, chloroform and methanol extracts ranged from 50 mg/mL to 0.95 mg/mL and are summarized in Tables 2 and 3. Interestingly, the acetone extract has been found more potent against *S. aureus* (at 1.56 mg/mL conc.) and *C. neoformans* (at 6.25 mg/mL conc.) among all tested extracts. These findings agreed with previous reports that the substances responsible for the antimicrobial activity are mainly non-polar in nature. The control solvent (DMSO) did not have any effect on the viability of *S. aureus* and *C. neoformans* cells at concentrations equivalent to MIC concentrations of *B. setschwanensis* extracts.

Table 2
Antibacterial activity of different solvent extracts and control antibiotics (rifampicin) against different clinical pathogenic bacteria.

Strains	Antibacterial activity (MIC values) of *B. setschwanensis* extracts			
	Control (RIF)[a] (μg/mL)	Acetone (mg/mL)	Chloroform (mg/mL)	Methanol (mg/mL)
E. coli	04.00	6.25	50.00	50.00
S. aureus	05.00	01.56	6.25	50.00
E. faecalis	62.50	50.00	50.00	25.00
V. cholera	62.50	25.00	50.00	6.25
S. pyogens	62.50	06.25	06.25	25.00
L. monocytogens	125.00	50.00	25.00	25.00

[a] RIF = Rifampicin (Standard antibacterial drug).

Table 3
Antifungal activity of different solvent extracts and control antifungal antibiotics (amphotericin B) against different clinical pathogenic fungi.

Strains	Antifungal activity (MIC values) of *B. setschwanensis* extracts			
	Control (Amp B)[a] (μg/mL)	Acetone (mg/mL)	Chloroform (mg/mL)	Methanol (mg/mL)
C. albicans	00.72	50.00	50.00	50.00
C. glabrata	00.36	25.00	50.00	> 50.00
C. krusei	00.72	> 50.00	25.00	50.00
C. parapsilosis	00.72	> 50.00	25.00	50.00
C. keyfer	11.50	50.00	25.00	25.00
C. tropicalis	00.72	25.00	50.00	12.60
C. neoformans	01.44	6.25	50.00	50.00

[a] Amp B= Amphotericin B (Standard antifungal drug).

3.3. Synergy of extract with known antibacterial and antifungal agents

The combination of new lead compounds with known antimicrobials is an effective way to increase their antimicrobial efficacy [27,38]. Keeping this in mind, the potency of acetone extract was evaluated by checkerboard dilution assay with known marketed antibacterial and antifungal compounds against *S. aureus* and *C. neoformans* (Table 4). Results indicate that the combination of the sub-inhibitory concentration of acetone extract resulted in a decrease in MIC concentration of both kanamycin and rifampicin by ~8.0 folds (MIC of 2.3 mg/mL to 0.28 mg/mL for KAN, and MIC of 4.10 mg/mL to 0.51 mg/mL for RIF) against *S. aureus*. Similarly, the MIC for amphotericin B was lowered by ~2.8 folds with acetone extract (MIC of 0.72 mg/mL to 0.25 mg/mL) against *C. neoformans*, while a sharp decline of ~10 folds was noticed for fluconazole (1.25 mg/mL to 0.124 mg/mL) against *C. neoformans*.

Table 4
Combinatorial effect of acetone extract with antibacterial (kanamycin and rifampicin) and antifungal agents (amphotericin B and fluconazole).

MIC of Antibacterial agent (μg/mL)	MIC of acetone extract (mg/mL)	Sub MIC conc. of antibacterial agent (μg/mL) and acetone extract (mg/mL) in combination	FIC index	Effect
Synergy of antibacterial agents with acetone extract against *S. aureus*				
02.30 (KAN)	01.56	0.28 (KAN) + 00.18 (acetone extract)	00.31	Synergy
04.10 (RIF)	01.56	0.51 (RIF) + 00.09 (acetone extract)	00.18	Synergy
Synergy of antifungal agents with acetone extract against *C. neoformans*				
00.72 (Amp B)	6.25	0.25 (Amp B) + 00.06 (acetone extract)	0.37	Synergy
01.25 (FLC)	6.25	0.12 (FLC) + 00.12 (acetone extract)	0.30	Synergy

3.4. Time-kill kinetics of S. aureus and C. neoformans

To find out the efficacy and killing rate of antimicrobial compounds, time-kill kinetics was performed and under this process both the bacterial and fungal cells were assessed by plating out treated cells suspension and counting the number of viable cells at different time points. Colony forming units (CFUs) of *S. aureus* and *C. neoformans* were rapidly reduced after treatment with the acetone extract (at MIC = 1.56 mg/mL for *S. aureus* and at MIC = 6.25 mg/mL for *C. neoformans*) in comparison to untreated control. Maximum killing with acetone extract was observed after 24 h for *S. aureus*, while for *C. neoformans* after 16 h (Fig. 1). Therefore, it can be interpreted that the acetone extract is successfully inhibiting the growth of Gram positive bacterium *S. aureus* and the fungal strain *C. neoformans*.

3.5. Mechanistic study

3.5.1. Membrane permeabilization of S. aureus and C. neoformans

To determine whether the acetone extract is causing membrane instability, we added PI to the treated cells, because PI is a positively charged nucleic acid binding dye and gives red fluorescence at 530 nm excitation after getting associated with DNA. Hence, after acetone extract treatment, the cells of *S. aureus* and *C. neoformans* were observed under confocal microscopy and flow cytometry for permeabilization. The results revealed an increase in the PI fluorescence in treated *S. aureus* and *C. neoformans* cells in comparison to untreated cells, and the presence of PI fluorescence confirmed that permeabilization is occurring in *S. aureus* and *C. neoformans* cells (Fig. 2ab and Supplementary Fig. S3).

3.5.2. Morphological changes

Effect of acetone extract on the outer surface morphology of *S. aureus* and *C. neoformans* was investigated by scanning electron microscopy (SEM) and it was observed that there was a distinct change in cell morphology of both *S. aureus* and *C. neoformans* when treated with acetone extract at MIC concentrations (1.56 mg/mL for *S. aureus* and 6.25 mg/mL for *C. neoformans*). The cell wall roughness and disruption was observed within 8 h of incubation (Fig. 3 and Supplementary Fig. S4 a-b) that leads to cell death. The results of the present study corroborate with those of Gupta et al. (2012) who also demonstrated that the lichen crude extract containing polyphenolic compounds can destabilize the morphological structure of *S. aureus* cells [39].

3.6. Mammalian cell toxicity

The mammalian cell [human cervical cancer cells (HeLa cells) and human normal embryonic kidney cells (HEK-293)] were used to determine the safety profile of acetone, chloroform and methanol extract at 50 mg/mL concentration. Our study shows that the extract have less significant toxicity at concentration of 50 mg/mL. The acetone, chloroform and methanol extract have toxicity against HEK-293 cells (~25%, ~21%, ~20%) and HeLa cells (~19%, ~25%, 25%) (Fig. 4, Supplementary Table S2).

4. Conclusion

The secondary metabolites of lichens have attracted scientists across the world in the search for new antimicrobial substances. In the present paper the extracts of *B. setschwanensis* in three different solvents (acetone, chloroform and methanol) showed broad spectrum antimicrobial efficacy against clinical strains of six bacterial and seven fungal pathogens and have less significant mammalian cell cytotoxicity. The acetone extract was found to exhibit a promising level of

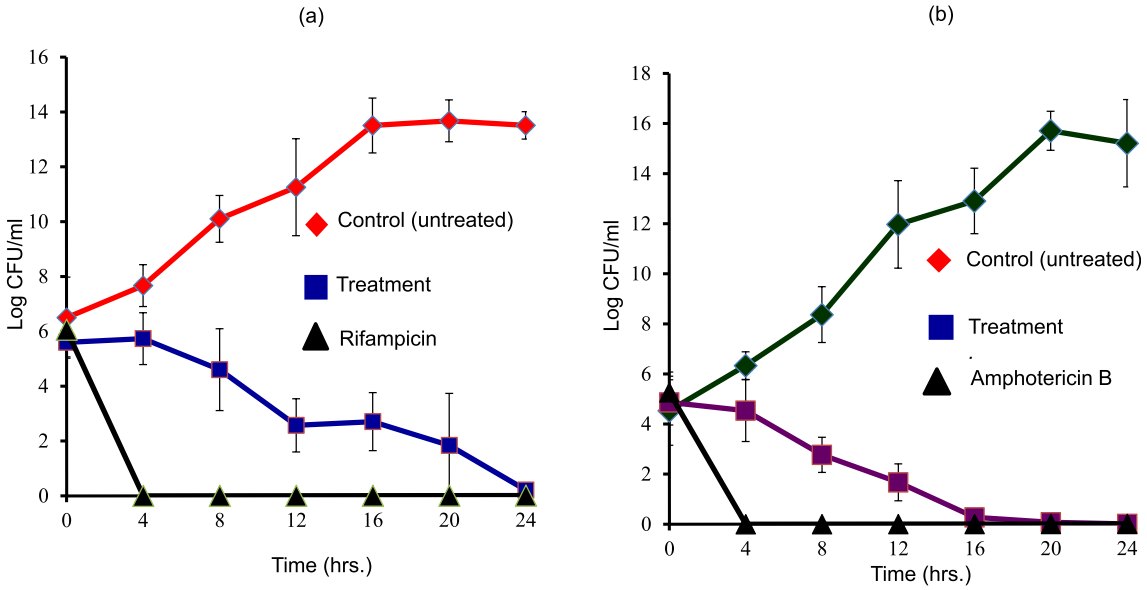

Fig. 1. Time-kill kinetics of *S. aureus* and *C. neoformans* by acetone extract of *B. setschwanensis* at MIC conc. (a). Untreated control (♦), treated cells (■), rifampicin control (▲). (b). Untreated control (♦), treated cells (■), amphotericin B control (▲) Samples withdrawn at indicated times were evaluated for colony forming units. Assays were performed in MHB and RPMI-1640 medium and incubated at 37 °C (*S. aureus*) and 30 °C (*C. neoformans*) for 24 h and 48 h respectively. Standard deviation of three independent experimental data are plotted.

antimicrobial efficacy against *S. aureus* and *C. neoformans* and also demonstrated remarkable synergistic activity in nature with well-known marketed antibacterials (rifampicin and kanamycin) and anti-fungals (amphotericin B and fluconazole) at their non-cidal concentrations. Further GC-MS analysis of the acetone extract resulted in the identification of phytochemical compounds; 2,3-bis (2-methyl-pentanoyloxy) propyl 2-methyl pentanoate (64.31%) and Ethyl 2-[(2 R,3R,4aR, 8aS)-3-hydroxy-2,3,4,4a,6,7,8,8a-octahydropyrano [3,2-

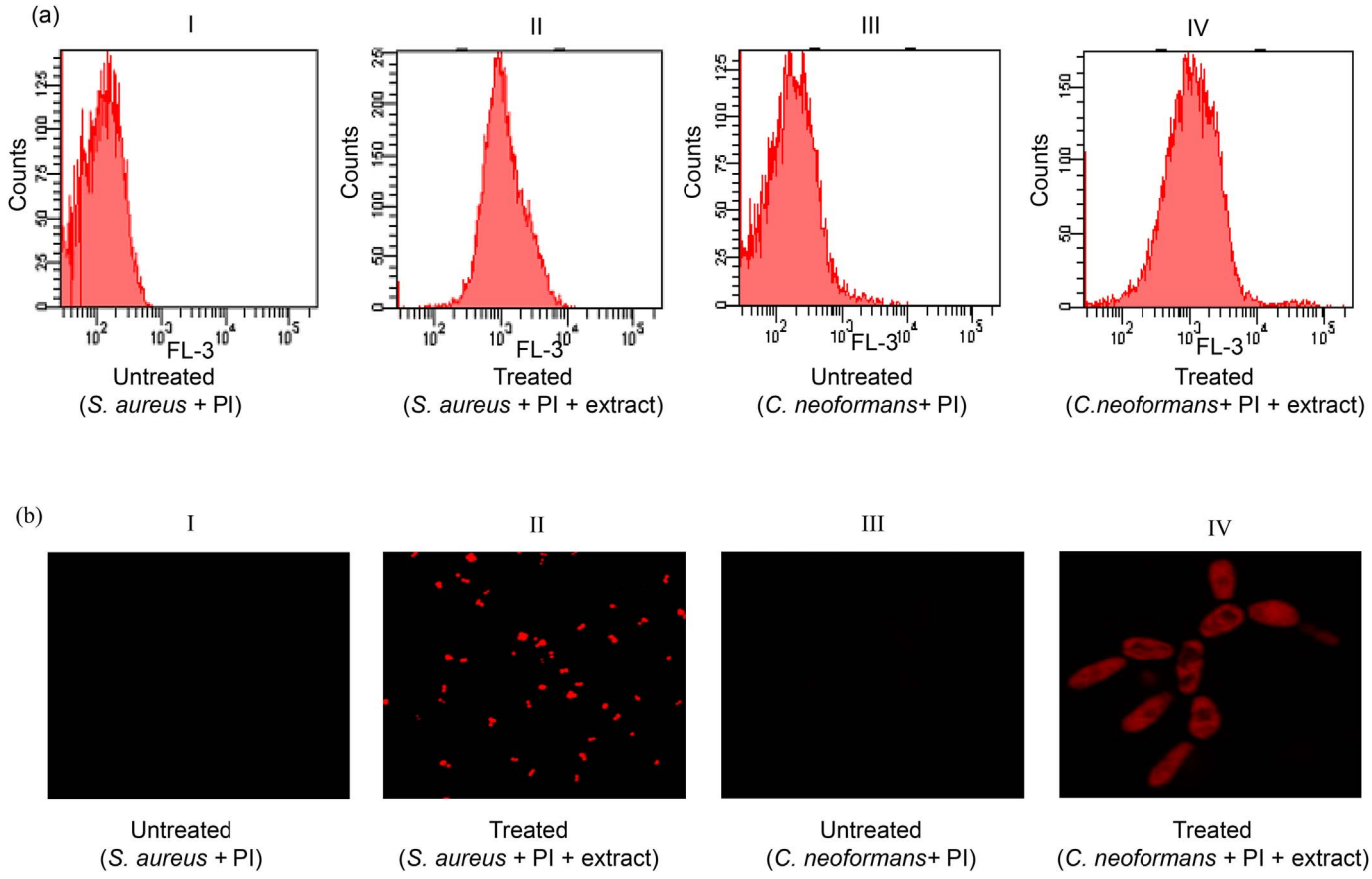

Fig. 2. (a) Flow cytometry analysis of membrane permeabilization assay by propidium iodide (PI). (I) untreated *S. aureus* cells, (II) *S. aureus* (~10^5 cells) with acetone extract (1.56 mg/mL) incubated for 8 h at 37 °C in MHB medium, (III) untreated *C. neoformans* cells, (IV) *C. neoformans* cells with acetone extract (6.25 mg/mL) incubated for 8 h at 30 °C in RPMI 1640 medium. (b) Confocal microscopy analysis of membrane permeabilization assay. (I) untreated *S. aureus* cells, (II) *S. aureus* cells (~10^5) with acetone extract (1.56 mg/mL) incubated for 8 h at 37 °C in MHB medium, (III) untreated *C. neoformans* cells, (IV) *C. neoformans* cells with acetone extract (6.25 mg/mL) incubated for 8 h at 30 °C in RPMI 1640 medium.

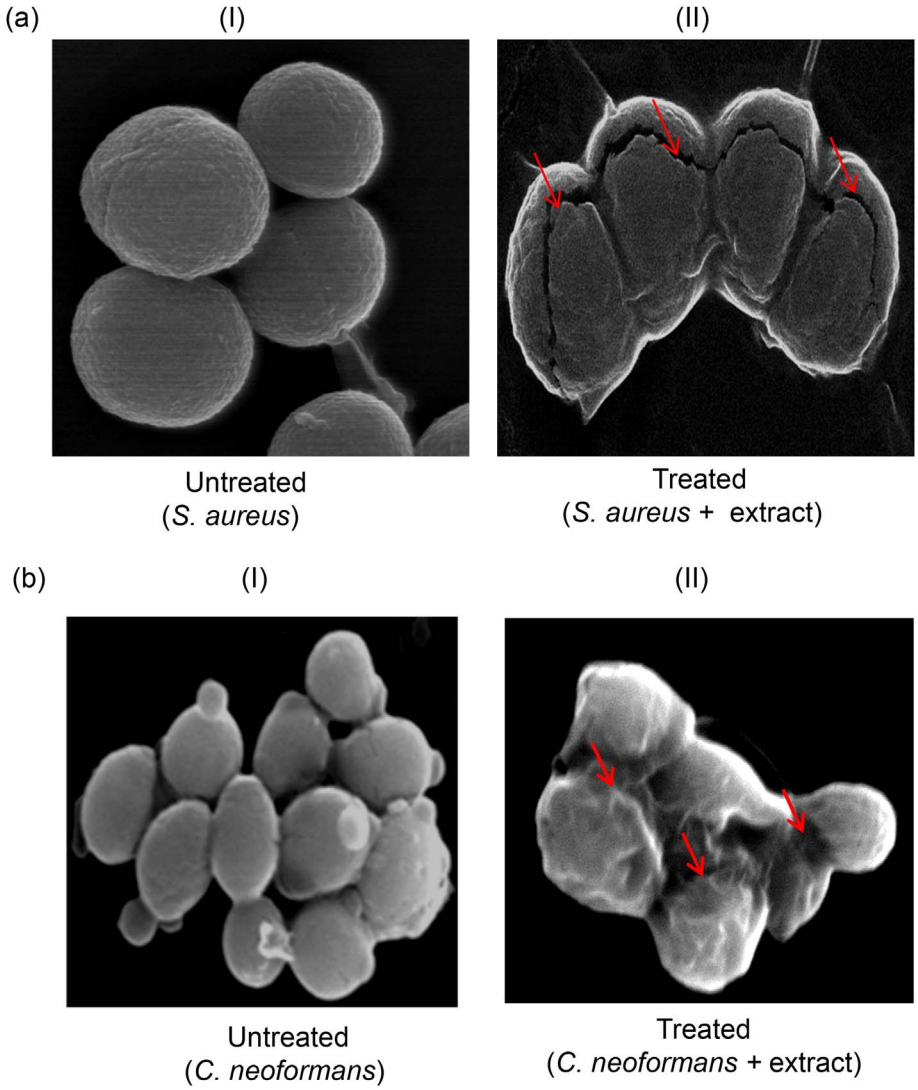

Fig. 3. Scanning electron microscopy images; (a) *S. aureus*, (I) Untreated cells (15.0 K × 6 mm), (II) Treated cells with acetone extract (15.0 K × 6 mm), (b) *C. neoformans*, (I) Untreated cells (5 K × 24 mm), (II) Treated cells with acetone extract (5 K × 24 mm). Arrows indicating damages which *S. aureus* and *C. neoformans* cells are facing.

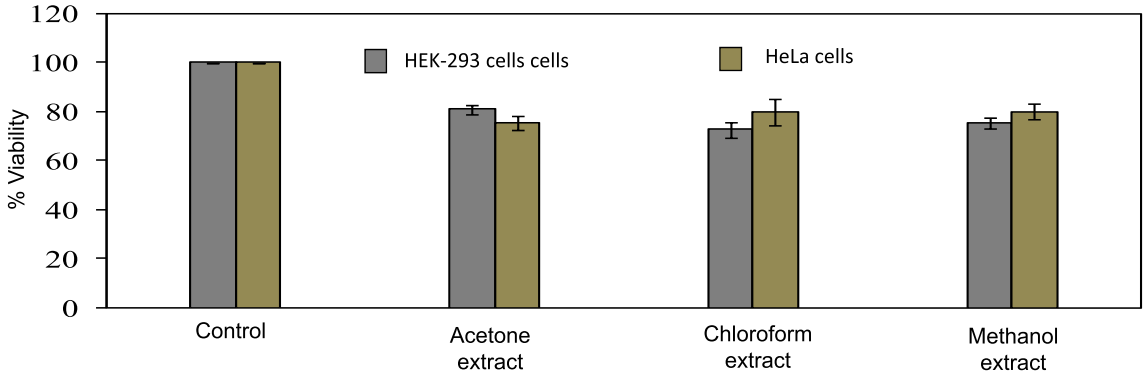

Fig. 4. Mammalian cell cytotoxicity of acetone, chloroform and methanol extract (at MIC conc.) in term of viability of HEK 293 and HeLa cells. Standard deviation of three independent experimental data are plotted.

b] pyran-2-yl] acetate (26.72%). As observations of cell wall disruptions caused by acetone extracts suggest that the antimicrobial activity of acetone extract of *B. setschwanensis* might be a multistep process involving initial cell wall damage, leading to action on the intracellular components of the cells, which ultimately leads to cell death.

Conflicts of interest

The authors wish to confirm that there is no conflict of interest in this work.

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Abbreviations

CLSI Clinical Laboratory Standard Institute
FACS Fluorescence activated cell sorting
SEM Scanning Electron Microscopy (SEM)
CLSM Confocal Laser Scanning Microscopy
MTT assay (3-(4, 5)-dimethylthiazol-2-yl)-2,5-(diphenyl tetrazolium bromide) assay
RIF Rifampicin
KAN Kanamycin
Amp B Amphotericin B
FLC Fluconazole
MIC Minimum Inhibitory Concentration
FICI Fraction Inhibitory Concentration Index
CFU Colony Forming Units
HeLa cells Human cervical cancer cells
Hek 293 cells Human embryonic kidney cells
DMSO Dimethyl sulfoxide

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.micpath.2017.12.015.

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