DOI: 10.5567/pharmacologia.2015.244.257

Antimicrobial, Antibiotic Resistance Modulation and Cytotoxicity Studies of Different Extracts of *Pupalia lappacea*

[1]Christian Agyare, [2]John Antwi Apenteng, [1]Francis Adu, [3]Emelia Kesseih and [1]Yaw Duah Boakye

[1]Department of Pharmaceutics, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana
[2]Department of Pharmaceutical Sciences, Central University College, Accra, Ghana
[3]Institute for Pharmaceutical Biology and Phytochemistry, University of Muenster, Correnstrasse 48, D-48149, Muenster, Germany

ABSTRACT

Background: The use of antibiotics over the years has led to increased bacterial resistance. With the current menace of microbial resistance to already existing antibiotics, there is an urgent need to discover new antimicrobial agents with different and novel mechanism of actions for new and re-emerging infectious diseases. *Pupalia lappacea* (L.) Juss is an annual or perennial herb (Family Amaranthaceae) which is used for the treatment of boils, wounds and skin infections. **Objective:** To determine the antimicrobial potential and antibiotic resistance modulation of the chloroform (PLC), petroleum ether (PLP) and ethanol (PLE) extracts and evaluate their possible cytotoxic effects. **Methods:** These include the determination of Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) Minimum Fungicidal Concentration (MFC) and microbial time-kill kinetics of the extracts against *Pseudomonas aeruginosa* ATCC 4853, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* NTCC 10073, *Proteus vulgaris* ATCC 4175 and clinical strains of *Streptococcus pyogenes*, *Klebsiella pneumonia* and *Candida albicans*. The ability of the extracts at sub-MIC concentrations to modulate the activity of some selected antibiotics including amoxicillin, cloxacillin, tetracycline, erythromycin and ampicillin was evaluated. Influence of the extracts on viability, proliferation and Lactate Dehydrogenase (LDH) release of HaCaT keratinocyte cells were evaluated at various selected concentrations. **Results:** All the extracts of *P. lappacea* exhibited broad-spectrum antimicrobial activity. The PLC demonstrated the highest antimicrobial activity with MIC of 2.5 to 4.0 mg mL^{-1} and MBC/MFC value ranging from 4.0-8.0 mg mL^{-1}. Time-kill kinetics generally revealed a concentration and time-dependent rate of microbiocidal activity of the extracts and MBC/MIC ratio <4. The PLC and PLP at sub-MIC concentrations of 0.5 mg mL^{-1} caused a 20% reduction in the MICs of amoxicillin, erythromycin, ampicillin and cloxacillin against *K. pneumonia*. There was also a 96% reduction in the MIC of amoxicillin against *E. coli* and *P. vulgaris*. None of the extracts exhibited cytotoxic activity against HaCaT keratinocytes within the concentrations used. **Conclusion:** All extracts of *P. lappacea* exhibited broad-spectrum antimicrobial activity and sub-MIC concentrations of the extracts showed microbial resistance modulation potential among the selected antibiotics.

Key words: Cytotoxicity, time-kill kinetics, antibiotic resistance

Pharmacologia 6 (6): 244-257, 2015

INTRODUCTION

Many infectious diseases have been known to be treated with herbal remedies throughout the history of mankind. Natural products, either as pure compounds or as standardized plant extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity. It is estimated that about 80% of world's population depend on medicines of plant origin which may consist of the additives aside the active ingredients[1]. Antibiotic bacterial resistance is the ability of bacteria to survive at the exposure to a Minimum Inhibitory Concentration (MIC) of an antibiotic. The use of antibiotics over the years has led to increased bacterial resistance. With the

Corresponding Author: C. Agyare, Department of Pharmaceutics, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana Tel: +233246369803

© 2015 Science Reuters, UK

current menace of microbial resistance to already existing antibiotics, there is an urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action for new and re-emerging infectious diseases[2]. Considering the diversity of nature and the enormous potential of plants, research into plants as crude antimicrobial agents or sources of lead compounds is on the increase. Methanol fruit extract of *Aframomum melegueta* and other Ghanaian medicinal plants have been found to possess antimicrobial activity[3].

Pupalia lappacea (L.) Juss is an annual or perennial herb (Family Amaranthaceae) found growing in the tropics and sub-tropical regions including Ghana, Nigeria, Congo DR etc. It is used for treatment of boils, wounds and skin infections. *P. lappacea* has been found to possess antioxidant and anticancer[4,5], wound healing[6,7], antinociceptive and antipyretic[8] properties. In previous study, the influence of chloroform, petroleum ether and ethanol leaf extracts of *P. lappacea* on the rate of wound closure, histology of treated and untreated wound tissues in rats has been investigated and reported as well as the antioxidant properties of these extracts. The HPLC profiles and secondary metabolites present in extracts have also been determined[6]. In this current study, the anti-infective, antibiotic resistance modulation and cytotoxic activities of chloroform, petroleum ether and ethanol leaf extracts of *P. lappacea* have been reported.

MATERIALS AND METHODS

Collection of plant material: Leaves of *P. lappacea* were collected in May, 2012 at Kuntenase in the Bosomtwi District, Ashanti Region of Ghana. It was authenticated and a voucher specimen (KNUST/HMI/2013/L006) has been deposited at the Department of Herbal Medicine Herbarium, Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana.

Extraction of plant material: The plant sample was cleaned of foreign material and dried at room temperature (25-28°C). It was then milled into coarse powder in a lab mill machine (Christy and Norris, United Kingdom). A quantity of 600 g was weighed and divided into 200 g portions of three. The various portions were extracted separately by cold maceration using 1.0 L of chloroform (BDH, England), petroleum ether (BDH, England) and 70% ethanol (Scharlau, United Kingdom) for 72 h. After 72 h, the extracts with solvents were centrifuged (3000 g for 10 min). The extract obtained was concentrated using rotary evaporator (Buchi, Disendorf, Germany) at 40°C and lyophilized. The dried powdered extracts were stored at 4°C.

Test organisms: The test organisms were *Pseudomonas aeruginosa* ATCC 4853, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* NTCC 10073, *Proteus vulgaris* ATCC 4175 and clinical strains of *Streptococcus pyogenes*, *Candida albicans* and *Klebsiella pneumonia*.

Determination of antimicrobial activity: The antimicrobial activities of the extracts were screened using the agar-well diffusion method[9]. Concentrations of 12.5, 50, 100 and 200 mg mL^{-1} of extracts were prepared with 50% v/v methanol as solvent for the chloroform (PLC) and petroleum ether (PLP) extracts and sterile distilled water as solvent for the ethanol extract (PLE). Twenty millilitres of nutrient agar (Oxoid, United Kingdom) was melted, stabilized at 45°C and seeded with 100 μL of 1.0×10^5 colony forming unit (CFU)/mL of test organisms and four equidistant wells (diameter of 6 mm) made in the seeded agar. One hundred microliters of different concentrations of the extracts were introduced into the wells and allowed to diffuse adequately for 1 h. The nutrient agar plates were then incubated at 37°C for 24 h. Zones of growth inhibition were determined. The activity of 50% v/v methanol was determined and it was found to exhibit no activity against the all the test organisms. The above procedure was repeated for *C. albicans* using Sabouraud dextrose agar (Oxoid, United Kingdom) as the medium and incubated at 30°C for 48 h. Ciprofloxacin (10 μg mL^{-1}) and ketoconazole (400 μg mL^{-1}) (Sigma-Aldrich, Damstadt, Germany) were used as reference antibiotics for the bacteria and *C. albicans* respectively. The experiment was carried out in triplicates.

Determination of Minimum Inhibitory Concentration (MIC): The minimum inhibitory concentrations of the extracts were determined by micro-dilution method[10]. The micro-titre plates were filled with 100 μL of double strength nutrient broth and the appropriate concentrations of the extracts were aseptically introduced into the wells and 10 μL of 1.0×10^5 CFU mL^{-1} of test microorganisms added and then incubated at 37°C for 24 h. The MIC was detected as the lowest concentration of extract that inhibited

© 2015 Science Reuters, UK

growth which was indicated by the absence of purple coloration upon the addition of 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazoliumbromide (MTT) (Sigma-Aldrich, Damstadt, Germany) after incubating at 37°C for 24 h. The above procedure was repeated for *C. albicans* using Sabouraud dextrose broth as the medium and incubated at 30°C for 5 days. Ciprofloxacin and ketoconazole were used as reference antibiotics for test bacteria and *C. albicans*, respectively. The experiments were carried out in triplicates.

Determination of minimum bactericidal and fungicidal concentration: The minimum bactericidal (MBC) and fungicidal concentrations (MFC) were determined according to the method described by Pfaller *et al.*[11]. Appropriate volumes of different concentrations of the extracts were added to micro-titre plates containing test bacteria and nutrient broth and incubated at 37°C for 24 h. Aliquots were taken from the various concentrations after incubation and inoculated onto nutrient agar plates. The inoculated nutrient agar plates were incubated at 37°C for 24 h. The MBC was determined as the minimum or least concentration of extract which showed no visible growth of microorganisms on the surface of the nutrient agar. The procedure was repeated for *C. albicans* using Sabouraud dextrose agar medium and incubated the plates at 30°C for 3 days. The experiments were carried out in triplicates.

Determination of the time-kill kinetics of extracts: The time kill kinetics test was performed according to the modified method described by Wiegand *et al.*[10]. Concentrations of the MIC, 2X MIC and 4X MIC of each extract were prepared in test tubes containing 10 mL nutrient broth and inoculated with 100 μL of 1.0×10^5 CFU mL^{-1} of the test organisms and incubated at 37°C. Aliquots of 1.0 mL were taken at time intervals of 0, 1, 2, 3, 4, 5, 6 and 24 h onto 20 mL nutrient agar and incubated at 37°C for 24 h. A control test was performed with the test organisms in the medium without the extracts. The CFU was determined after incubating for 24 h. A graph of the log CFU mL^{-1} was then plotted against time. The experiment was carried out in triplicates.

Determination of microbial resistance modulation of extracts: The ability of the extracts at sub-MIC concentrations to modulate the resistance of some microorganisms to some antibiotics was also evaluated. This involved the determination of the MIC of the antibiotic against the microbes without the extract and determination of MIC of the antibiotic in the presence of sub-MIC concentration of the extract. The microbial resistance modulation tests were performed according to a modified method described by Wiegand *et al.*[10]. The wells of the 96-well micro-titre plates were filled with 100 μL of double strength nutrient broth. Various concentrations of antibiotics including cloxacillin, amoxicillin, ampicillin, tetracycline and erythromycin with their HPLC purities of >98% (Sigma-Aldrich, Damstadt, Germany) were used in the determination of MICs. The reference antibiotics were tested against *P. aeruginosa*, *E. coli*, *S. aureus*, *S. pyogenes*, *P. vulgaris* and *K. pneumonia*. The MICs of the antibiotics were determined after incubation for 24 h at 37°C and upon the addition of MTT. Sub-inhibitory concentrations of 0.50 and 1.0 mg mL^{-1} of extracts and various concentrations of the antibiotics (cloxacillin, amoxicillin, ampicillin, tetracycline and erythromycin) plus the same inoculum size were incubated at 37°C for 24 h and MICs of the antibiotics in presence of the extracts were determined according to the method described above.

Influence of extracts on cell viability of HaCaT keratinocytes: The influence of the extracts on HaCaT keratinocyte cells was evaluated according to the method described by Agyare *et al.*[12]. Extract concentrations of 0.1, 1.0, 10.0, 50.0 and 100.0 μg mL^{-1} were added to the cells (6×10^3 cells mL^{-1}) in a 96 well Micro-Titre Plate (MTP) containing HaCaT keratinocyte medium (comprising of Dulbecco's Modified Eagle Medium (DMEM) high glucose with glutamine, Foetal Calf Serum (FCS), non-essential amino acids and penicillin/streptomycin) and incubated at 35°C with 5% CO_2. The medium was then washed with 100 μL Phosphate Buffer Solution (PBS) per well and filled with 50 μL of MTT solution. This was then incubated at 35°C for 12 h. Five percent (5%) Foetal Calf Serum (FCS) was used as a positive control while untreated HaCaT keratinocytes served as negative control. The MTT solution was then removed and dissolved with 50 μL DMSO. The intensity of the violet colouration was measured with MTP reader (Corona Electric Co. Ltd, Japan) at 595 nm against 690 nm.

Influence of extracts on proliferation of HaCaT keratinocytes: The influence of the extracts on the proliferation of HaCaT keratinocytes was performed using ELISA cell proliferation test kit[13]. The HaCaT keratinocyte cells (6×10^3 cells mL^{-1}) were cultured in micro-titre plates containing concentrations of

© 2015 Science Reuters, UK

0.1, 1.0, 10.0, 50.0 and 100.0 μg mL^{-1} of extracts constituted in HaCaT keratinocyte medium and incubated at 35°C for 48 h. After incubation, 10 μL labelling solution was added to the cells in 96-well tissue culture plates and incubated at 35°C with 5% CO_2 for 24 h. The medium was later removed and the cells treated with 100 μL fixation-denaturation solution for 30 min at 20 to 25°C. The fixation-denaturation solution was later removed and the cells incubated with 50 μL antibody solution BrdU-POD (peroxidase-marked-mouse anti-BrdU-antibody) at 20 to 25°C for 90 min with constant shaking. The antibody solution was later washed out three times and 50 μL substrate solution was added to the wells. It was later incubated at room temperature between 20 to 25°C until the formation of a clear blue solution. The peroxidase reaction was terminated upon the addition of 10 μL 1M H_2SO_4 solution. The termination of the reaction resulted in a change of the solution from blue to yellow. The absorbance of the yellow solution was determined with MTP reader at a wavelength of 450 nm against 690 nm.

Influence of extracts on lactic dehydrogenase (LDH) release from HaCaT keratinocytes: This was determined according to the method described by Decker and Lohmann-Matthes[14]. HaCaT keratinocytes (6×10^3 cells mL^{-1}) were grown in 96-well micro-titre plates containing concentrations of 0.1, 1.0, 10.0, 50.0 and 100.0 μg mL^{-1} of extracts constituted in HaCaT keratinocyte medium and incubated at 35°C with 5% CO_2 for 24 h. After incubation, 25 μL of each test medium (supernatant) was transferred into a 96-well MTP. To the remaining medium, 25 μL cell lysis solution (10% Triton X-100 in 5% FCS) was added. This was incubated for 1 h 30 min at room temperature with constant shaking. Twenty-five microlitres reaction mixture were added to both the supernatant and lysed cell medium and incubated in the dark at 20°C for 30 min. The reaction was terminated by the addition of 10 μL HCl 1M solution to each well. The absorbance of the resultant solution was measured with MTP reader at 490 nm against 690 nm.

Statistical analysis: GraphPad Prism Version 5.0 for Windows (GraphPad Software, San Diego, CA, USA) was used for all statistical analyses. Data are presented as Mean±SEM (N = 5) and analysed by one-way ANOVA followed by Dunnett's multiple comparison test. *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

RESULTS

The antimicrobial screening was performed by the agar-well diffusion method against the different strains of Gram-negative and Gram-positive bacteria and a fungus. The extracts exhibited broad spectrum antimicrobial activity against the selected test organisms (Table 1). The antimicrobial activity of the extracts generally increased with increasing concentration of extract but not for all the test organisms.

Minimum inhibitory concentration (MIC) of extracts and reference antibiotics: PLC demonstrated the highest antimicrobial activity with lowest MIC range of 2.5 to 4.0 mg mL^{-1} against the test organisms. PLE had high MIC of 20 to 40 mg mL^{-1} against the test organisms and this indicates low antimicrobial activity (Table 2).

Table 1: Antimicrobial activity of chloroform, petroleum ether, ethanol leaf extracts and reference antibiotics against test organism using the agar well diffusion method

Concentration (mg mL^{-1})	*B. subtilis*	*S. aureus*	*E. coli*	*S. pyogenes*	*P. aeruginosa*	*C. albicans*
Chloroform						
200	21.67±0.33	28.33±0.33	12.00±0.00	11.33±0.67	15.33±0.33	22.33±0.33
100	19.00±0.58	25.00±0.00	14.33±0.33	13.00±0.58	14.33±0.33	18.67±0.33
50	14.33±0.33	21.00±0.58	13.33±0.67	13.00±0.58	0.00±0.00	15.00±0.0
25	15.67±1.67	18.33±0.33	na	na	na	na
Petroleum ether						
200	12.00±0.58	28.67±0.67	12.33±0.33	10.00±0.0	17.00±0.58	22.67±0.67
100	18.33±0.88	25.00±0.0	10.67±0.33	7.33±0.67	8.67±0.33	20.67±0.67
50	15.33±0.33	19.67±0.33	8.00±0.58	na	na	17.33±0.33
25	19.33±0.67	15.67±0.33	7.00±0.0	na	na	12.00±0.0
Ethanol						
100	11.00±0.58	8.33±0.88	na	na	na	8.67±0.67
50	9.67±0.33	7.67±0.67	na	na	na	8.33±0.33
25	9.33±0.33	7.67±0.67	na	na	na	8.00±0.0
12.5	9.33±0.33	8.33±0.33	na	na	na	na
Reference antibiotics						
Ciprofloxacin 10 μg mL^{-1}	29.33±0.67	20.67±0.67	26.67±0.40	29.67±0.33	22.33±1.45	nd
Ketoconazole 400 μg mL^{-1}	nd	nd	nd	nd	nd	13.67±0.88

nd: Not determined, na: No activity; SEM: Standard error mean, diameter of well: 6 mm

© 2015 Science Reuters, UK

Table 2: Minimum inhibitory concentration of *P. lappacea* extracts and reference antibiotics against test organisms

	Minimum inhibitory concentration (mg mL^{-1})				
Test organisms	PLC	PLP	PLE	Ciprofloxacin	Ketoconazole
S. aureus	3.00	5.00	40.0	0.250	nd
B. subtilis	4.00	4.00	40.0	0.030	nd
P. aeruginosa	2.50	10.00	20.0	0.250	nd
S. pyogenes	2.50	4.00	20.0	0.063	nd
E. coli	3.00	12.00	30.0	0.125	nd
C. albicans	3.00	12.00	30.0	nd	5.00

nd: Not determined, PLC: Chloroform, PLP: Petroleum ether, PLE: Ethanol leaf extracts of *P. lappacea*

Table 3: Minimum bactericidal (MBC) and fungicidal concentration (MFC) of *P. lappacea* extracts against test organisms

Test organisms	PLC	PLP	PLE
MBC (mg mL^{-1})			
S. aureus	8.0	16.0	40.0
B. subtilis	6.0	18.0	50.0
P. aeruginosa	7.0	18.0	50.0
S. pyogenes	5.0	16.0	50.0
E. coli	4.0	18.0	50.0
MFC (mg mL^{-1})			
C. albicans	8.0	16.0	50.0

PLC: Chloroform, PLP: Petroleum ether, PLE: Ethanol leaf extracts of *P. lappacea*

Table 4: Ratio of MBC/MIC of *P. lappacea* extracts against test organisms

	MBC/MIC ratio		
Test organisms	PLC	PLP	PLE
S. aureus	2.67	3.20	1.00
B. subtilis	1.50	4.50	0.13
P. aeruginosa	2.80	1.80	2.50
S. pyogenes	2.00	4.00	2.50
E. coli	1.33	1.50	1.67
C. albicans	2.67	1.33	1.67

PLC: Chloroform, PLP: Petroleum ether, PLE: Ethanol leaf extracts of *P. lappacea*

Minimum-bactericidal (MBC) and fungicidal concentration (MFC) of extracts: The MBC/MIC value was determined for the extracts against the various test organisms. This indicates the static or cidal activity against the test organism. PLC and PLE showed bactericidal and fungicidal activities whiles PLP displayed static activity against *E. coli.* PLC exhibited the lowest MBC/MFC ratio indicative of good microbial killing effect. PLE also had high MBC and MFC values, indicating poor microbial killing effects (Table 3 and 4).

Microbial time kill kinetics of ethanol leaf extract (PLE) of *P. lappacea*: PLE exhibited antibacterial and antifungal activities against the test organisms with reduction of initial viable cells and subsequent gradual increment after the sixth hour (Fig. 1a-f).

Microbial time-kill kinetics of pet ether leaf extract of *P. lappacea* (PLP): PLP demonstrated antibacterial and antifungal activities at the various concentrations and times tested. The microbial time killing effect was generally concentration-dependent with increasing concentration resulting in increased microbial destruction (Fig. 2a-f).

Microbial time-kill kinetics of chloroform leaf extract of *P. lappacea* (PLC): PLC demonstrated concentration dependent antibacterial and antifungal activities against the test bacteria and fungus. There was a gradual reduction in the initial inoculum size of the test organisms with increasing concentration of extract (Fig. 3a-f).

Antibiotic resistance modulation: The ability of the extracts at sub-MIC concentrations to modulate the activity of some antibiotics to which some microbes have developed resistance was evaluated. PLC and PLE extracts modulated the resistance of some microorganisms to the antimicrobial effects of some antibiotics. The PLC at sub-MIC concentration of 0.5 mg mL^{-1} reduced the MIC of tetracycline and amoxicillin from >100 μg mL^{-1} to <1 μg mL^{-1} against *S. pyogenes, K. pneumonia, P. aeruginosa, P. vulgaris* and *S. aureus.* It also caused

© 2015 Science Reuters, UK

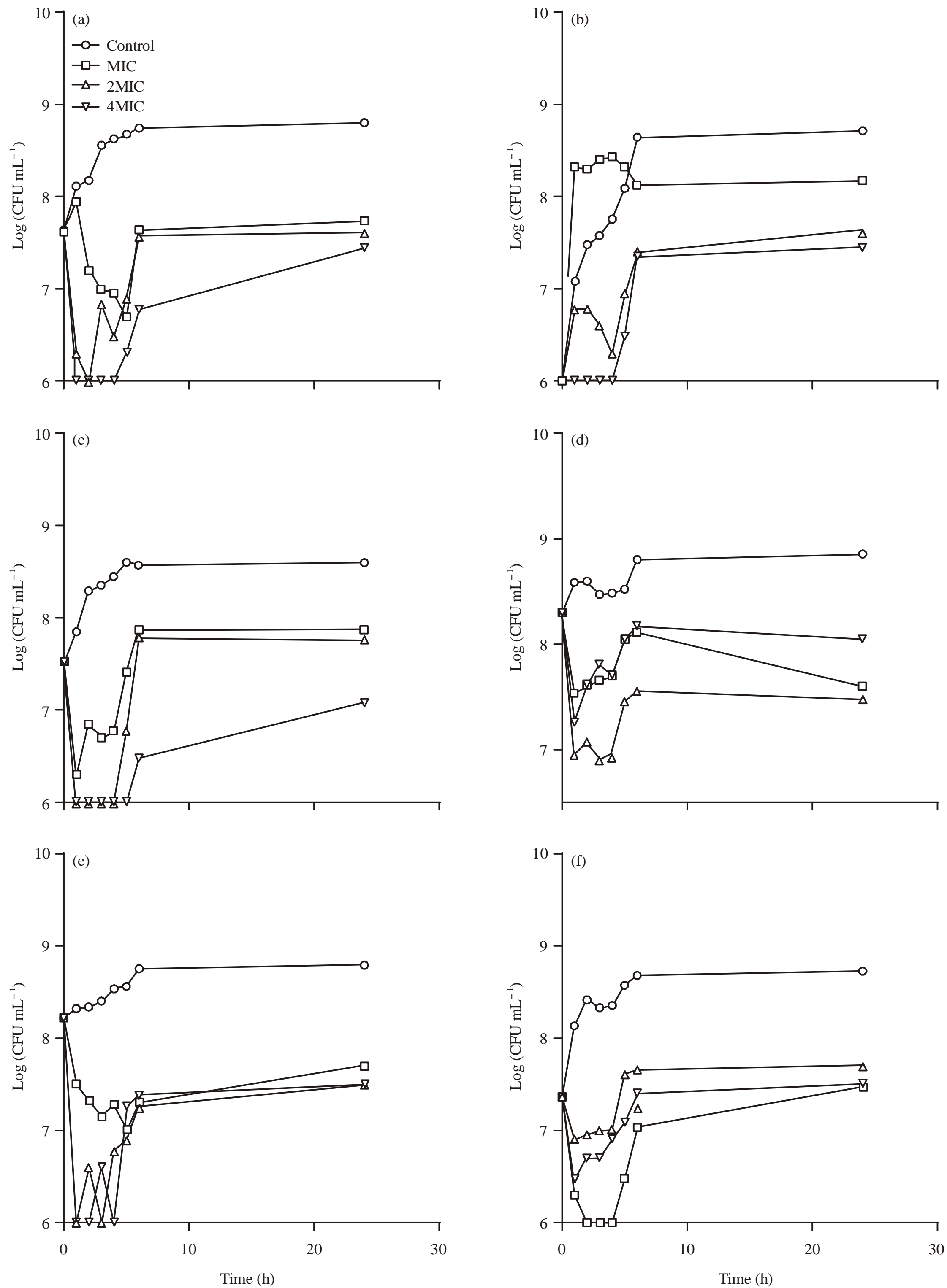

Fig. 1(a-f): Microbial time-kill kinetics of ethanol leaf extract of *P. lappacea* (PLE) against test organisms (a) *P. aeruginosa*, (b) *E. coli*, (c) *S. aureus*, (d) *S. pyogenes*, (e) *C. albicans* and (f) *B. subtilis*

© 2015 Science Reuters, UK

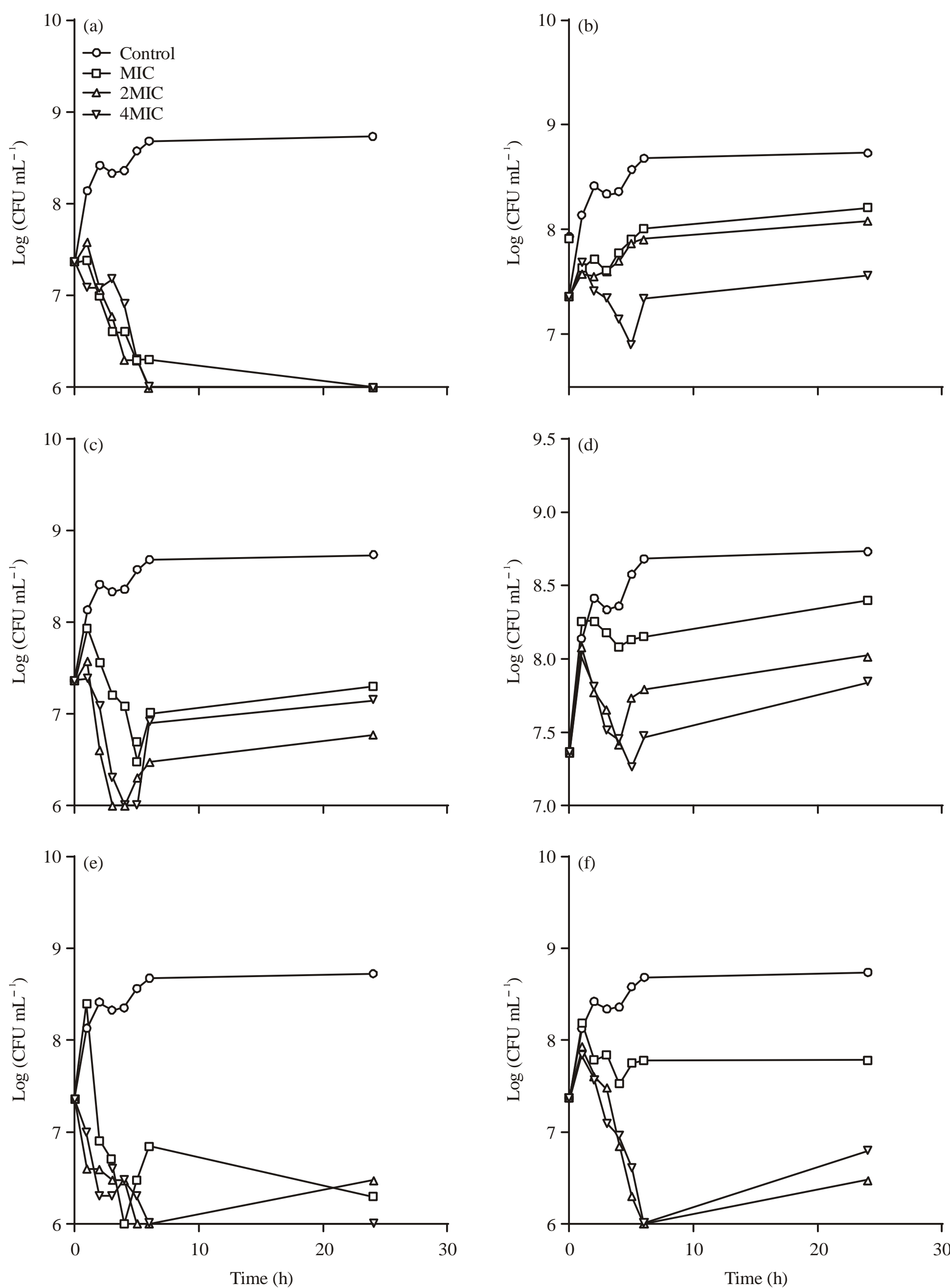

Fig. 2(a-f): Microbial time-kill kinetics of pet ether leaf extract of *P. lappacea* (PLP) against test organisms (a) *P. aeruginosa*, (b) *E. coli*, (c) *S. aureus*, (d) *S. pyogenes*, (e) *C. albicans* and (f) *B. subtilis*

a reduction in the MIC of cloxacillin on *E. coli* from >100 μg mL^{-1} to 2 μg mL^{-1} (98% reduction) resulting in an improved antibacterial activity (Table 5-6).

PLC further reduced the MICs of cloxacillin on *P. vulgaris* and *S. aureus* from >100 to 80 μg mL^{-1} (20% reduction in the MIC). The PLP extract at sub-MIC concentration of 0.5 mg mL^{-1} demonstrated resistance modulation effects on amoxicillin, cloxacillin, ampicillin and erythromycin against the test organisms. It caused a reduction in the MICs of amoxicillin, erythromycin, ampicillin and cloxacillin

© 2015 Science Reuters, UK

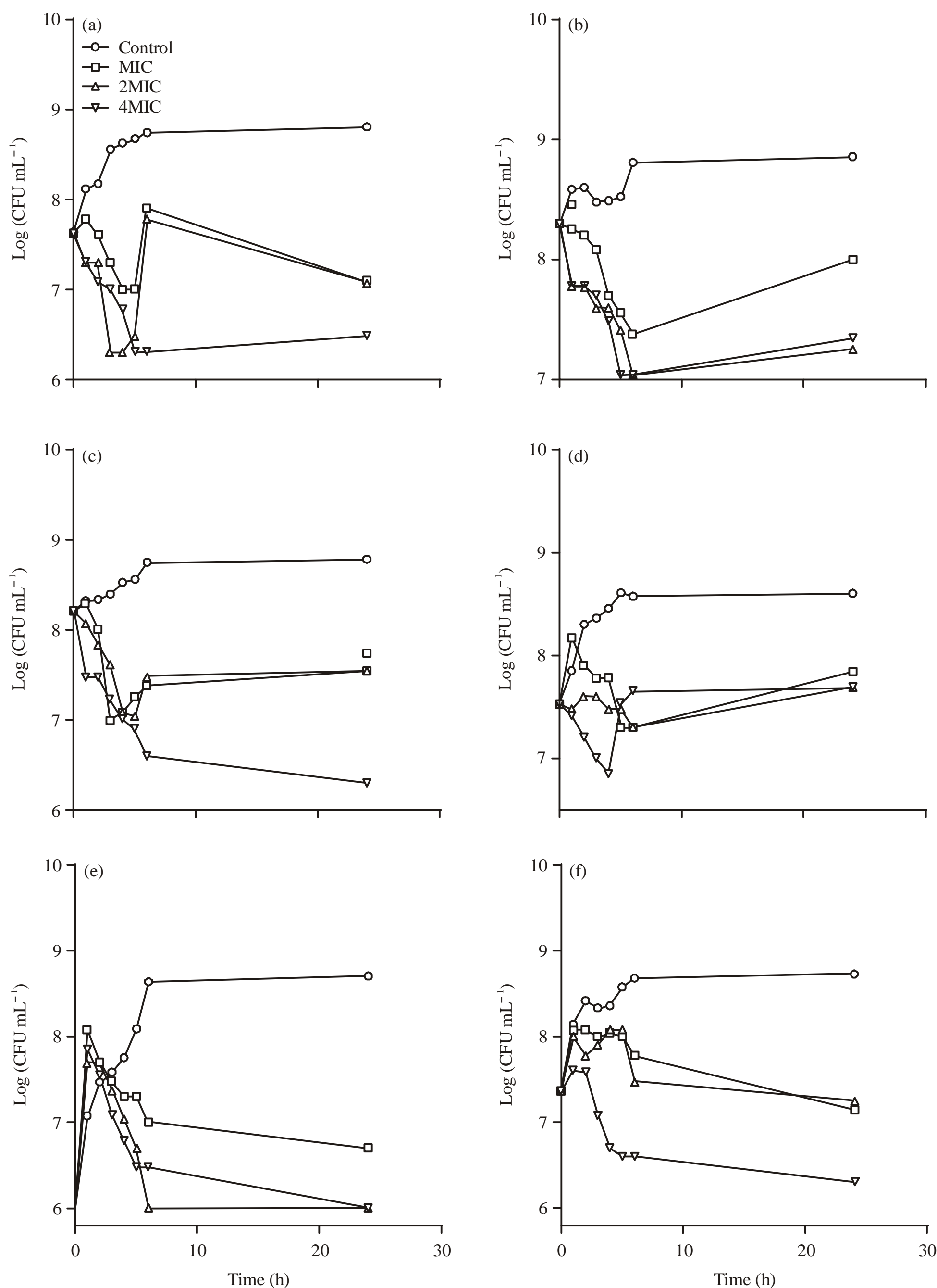

Fig. 3(a-f): Microbial time-kill kinetics of chloroform leaf extract of *P. lappacea* (PLC) against test organisms (a) *P. aeruginosa*, (b) *E. coli*, (c) *S. aureus*, (d) *S. pyogenes*, (e) *C. albicans* and (f) *B. subtilis*

against *K. pneumonia*, *P. aeruginosa*, *P. vulgaris* and *S. aureus*, respectively from >100 to 80 μg mL^{-1} (20% reduction in the MIC) and hence improving the antibacterial activities of these antibiotics (Tables 5 and 6).

Influence of extracts on viability, proliferation and LDH release of HaCaT keratinocytes: The influence of the PLC extract on the viability of cells was not statistically significant (p>0.05). The PLC extract had no cytotoxic effects at the concentrations tested

© 2015 Science Reuters, UK

Table 5: Effect of sub-MIC concentrations of PLC on MIC of tetracycline, amoxicillin and cloxacillin

		MIC (Antibiotic+Extract/$\mu g\ mL^{-1}$)	
Organisms	MIC (Antibiotic alone)/$\mu g\ mL^{-1}$	Extract (0.5 mg mL^{-1})	Extract (1.0 mg mL^{-1})
Tetracycline			
K. pneumonia	8	40	80
S. pyogenes	40	<1	10
P. aeruginosa	60	>100	>100
P. vulgaris	<1	1	60
S. aureus	40	>100	>100
E. coli	60	>100	>100
Amoxicillin			
K. pneumonia	>100	1	>100
S. pyogenes	>100	<1	>100
P. aeruginosa	>100	<1	>00
P. vulgaris	>100	<1	1
S. aureus	>100	<1	>100
E. coli	>100	2	>100
Cloxacillin			
K. pneumonia	>100	>100	>100
S. pyogenes	>100	>100	>100
P. aeruginosa	>100	>100	>100
P. vulgaris	>100	80	>100
S. aureus	>100	80	>100
E. coli	>100	100	>100

Table 6: Effect of sub-MIC concentrations of PLP on MIC of tetracycline, amoxicillin, cloxacillin, ampicillin and erythromycin

		MIC (Antibiotic+Extract/$\mu g\ mL^{-1}$)	
Test organisms	MIC (Antibiotic alone)/$\mu g\ mL^{-1}$	Extract (0.5 mg mL^{-1})	Extract (1.0 mg mL^{-1})
Tetracycline			
K. pneumonia	8	>100	>100
S. pyogenes	40	>100	>100
P. aeruginosa	60	>100	>00
P. vulgaris	<1	>100	>100
S. aureus	40	>100	>100
E. coli	60	>100	>100
Amoxicillin			
K. pneumonia	>100	80	>100
S. pyogenes	>100	>100	>100
P. aeruginosa	>100	>100	>100
P. vulgaris	>100	4	>100
S. aureus	>100	10	>100
E. coli	>100	4	>100
Cloxacillin			
K. pneumonia	>100	>100	>100
S. pyogenes	>100	>100	>100
P. aeruginosa	>100	>100	>100
P. vulgaris	>100	80	>100
S. aureus	>100	80	>100
E. coli	>100	100	>100
Ampicillin			
K. pneumonia	>100	0.8	>100
S. pyogenes	>100	1	>100
P. aeruginosa	>100	80	>100
P. vulgaris	>100	1	>100
S. aureus	>100	1	>100
E. coli	>100	1	>100
Erythromycin			
K. pneumonia	4	80	80
S. pyogenes	4	>100	80
P. aeruginosa	>100	80	60
P. vulgaris	4	60	80
S. aureus	6	60	80
E. coli	6	>100	100

© 2015 Science Reuters, UK

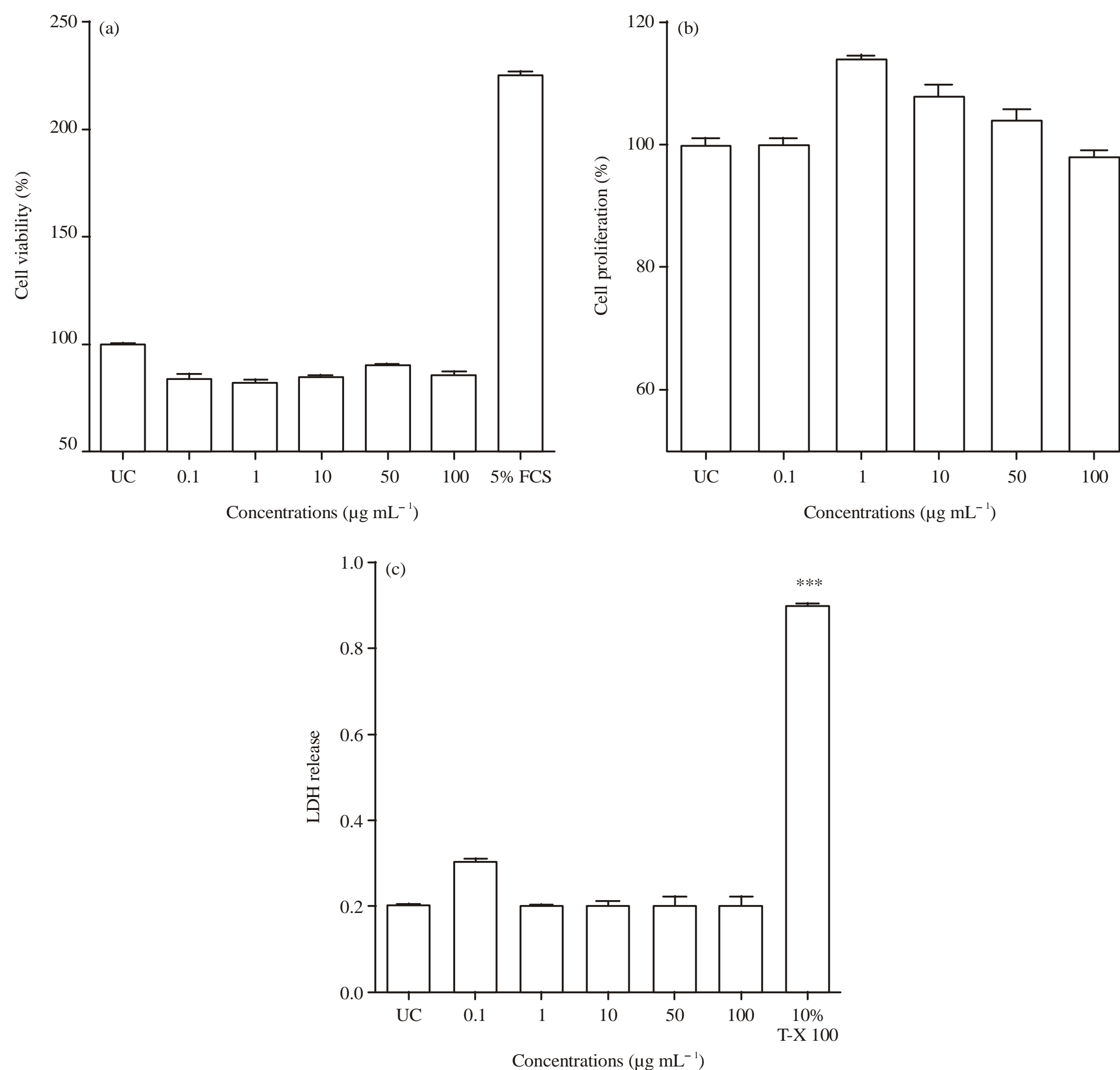

Fig. 4(a-c): Effect of PLC on (a) Proliferation, (b) Viability and (c) LDH release of HaCaT keratinocytes. UC: Untreated cells, FCS: Foetal calf serum, T-X100 = 10% Triton, *p<0.05, **<p 0.01, ***p<0.001

(Fig. 4). The PLE caused a reduction in cell proliferation of HaCaT keratinocytes at higher concentrations (50 to 100 μg mL^{-1}); however, there was increment in the cell viability with no cytotoxicity at the concentrations tested (Fig. 5). keratinocytes at higher concentrations (50 to 100 μg mL^{-1}); however, there was increment in the cell viability with no cytotoxicity at the concentrations tested (Fig. 5). The PLP (within the concentrations tested) caused statistically insignificant ($p>0.05$) reduction in cell proliferation and cell viability of the HaCaT keratinocytes (Fig. 6). No cytotoxic effect was observed for the various extracts used.

DISCUSSION

The leaf extracts of *P. lappacea* exhibited some level of biological activity. With respect to antimicrobial activity, the PLC and PLP extracts exhibited antimicrobial activity against Gram-positive bacteria (*B. subtilis*, *S. aureus* and *S. pyogenes*) and Gram-negative bacteria (*E. coli* and *P. aeruginosa*) and fungus (*C. albicans*) using the agar-well diffusion method. PLE however exhibited antimicrobial activity only against *C. albicans* and the Gram-positive organisms (Table 1). PLC exhibited low MIC values ranging from 2.5 to 4.0 mg mL^{-1} against the test organisms, PLP exhibited MIC values ranging from 4.0 to 12 mg mL^{-1}

© 2015 Science Reuters, UK

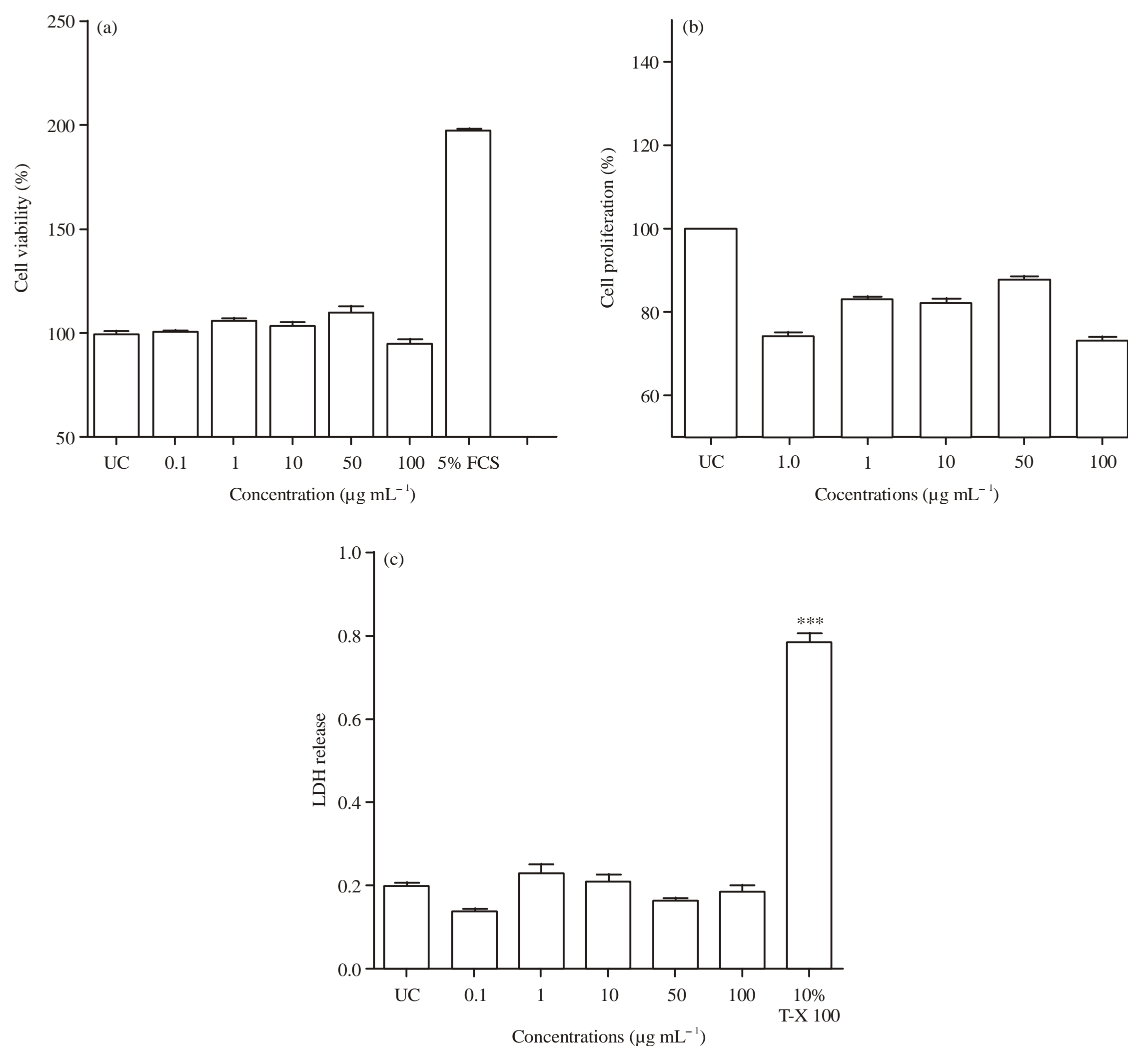

Fig. 5(a-c): Effect of PLE on (a) Viability, (b) Proliferation and (c) LDH release of HaCaT keratinocytes. UC: Untreated cells, FCS: Foetal calf serum, T-X100 = 10% Triton, *p<0.05, **<p 0.01, ***p<0.001

(Table 2). The antimicrobial activity of the extracts may be attributed to the presence of polyphenolic compounds and tannins[15] found in the different leaf extracts of *P. lappacea*[6]. The presence of other secondary metabolites such as saponins and alkaloids in the extracts may also contribute their antimicrobial activities[6,16]. The low MICs of PLC (<8 mg mL^{-1}) indicate strong antimicrobial activity against the test organisms[17]. In folkloric medicine, *P. lappacea* is used for the management of infections and wounds[18] and hence the activities of the extracts against some common pathogenic microbes (*S. aureus*, *S. pyogenes*, *P. aeruginosa*) that colonize wounds and cause infections leading to chronic, unhealed wounds or delay wound healing are very significant and of important observation. This may justify the use of the plant in folklore for the treatment of wounds and other infections. It may also give an indication of the presence of potent antimicrobials against organisms which can be isolated and purified for therapeutic use. The broad-spectrum antimicrobial activity exhibited by the extracts is also demonstrated in the MBC and MFC determinations (Table 3). According to Haas *et al.*[19], the type of antimicrobial action exhibited by an antimicrobial agent can be determined by calculating the ratio of MBC to MIC (MBC/MIC). MBC/MIC≥4 indicates bacteriostatic or fungistatic activity while MBC/MIC ratio 4 represents bactericidal or fungicidal activity. The PLC and PLE

© 2015 Science Reuters, UK

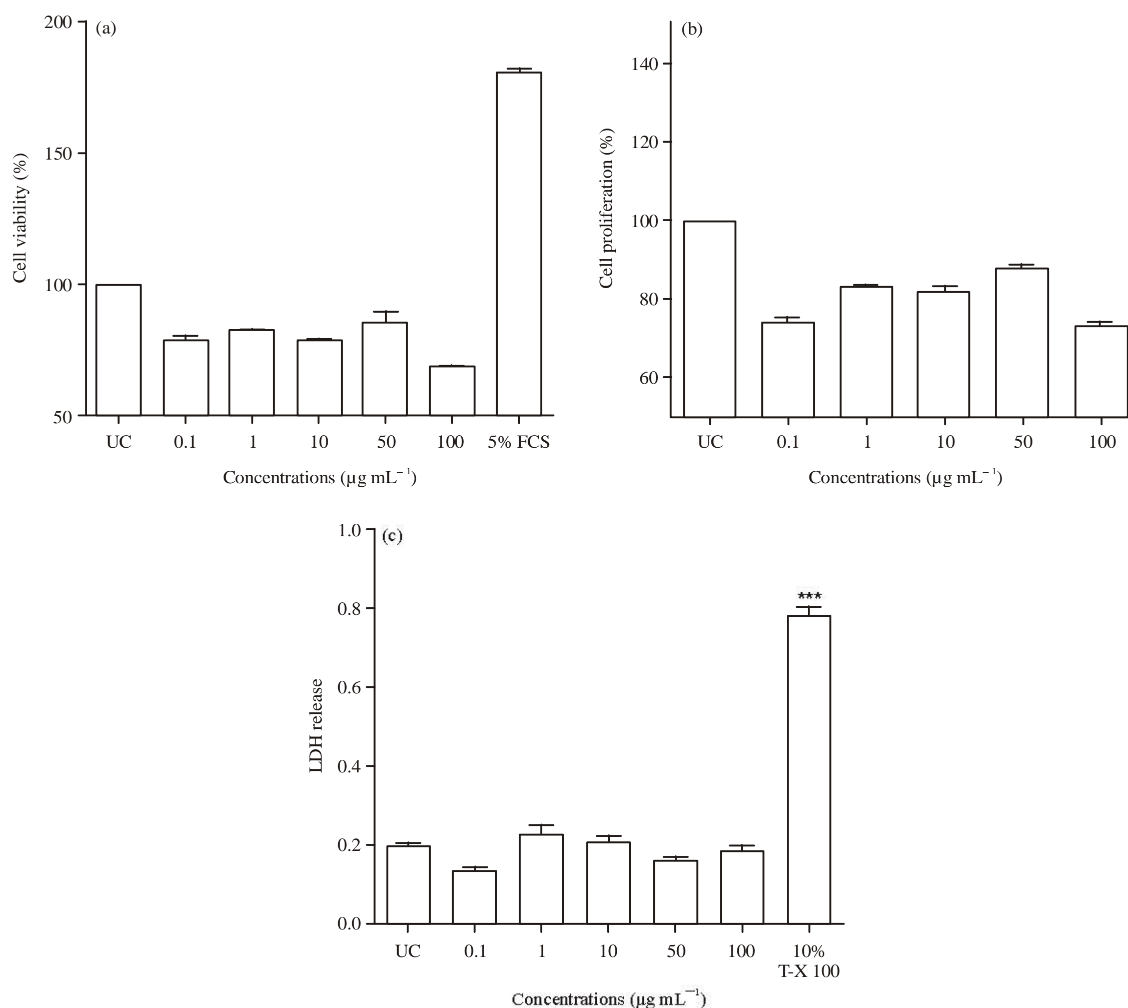

Fig. 6(a-c): Effect of PLP on (a) Viability, (b) Proliferation and (c) LDH release of HaCaT keratinocytes UC: Untreated cells, FCS: Foetal calf serum, T-X 100 = 10% Triton, *p<0.05, **<p 0.01, ***p<0.001

exhibited bactericidal and fungicidal activities against all the tested organisms since MBC/MIC ratios were less than 4. PLP showed bacteriostatic activity against *E. coli* and bactericidal and fungicidal activities against the other test organism (Table 4).

The time-kill kinetics of the extract of *P. lappacea* was performed to determine the rate of kill of the extracts and the extent of bactericidal or fungicidal action over 24 h. PLE exhibited time and concentration-dependent rate of kill against *S. aureus*, *P. aeruginosa* and *E. coli* with the 4 times the MIC concentration causing the highest reduction in the viable organisms. This situation was not similar with *B. subtilis* and *S. pyogenes* as the effect of the extract was not concentration dependent but only time dependent. PLP also exhibited concentration-dependent time kill kinetics with the 4X MIC and 2X MIC concentrations causing the most significant reduction in inoculum size against all the test organisms. This result revealed that extracts of *P. lappacea* exhibited a time and concentration dependent rate of microbial destruction with maximum reduction of the viable organisms within the first five hours and these finding were similar to other reports involving plant materials[3,20,21].

In most developing countries, most people who patronize herbal or traditional medicine may combine herbal remedies with orthodox medicine in order to enhance the activity and efficacy of these herbal preparations or orthodox medications especially the use of antibiotics for the treatment of skin infections[12,22]. The ability of the extracts of *P. lappacea* at sub-MIC concentrations to modulate the resistance of some

© 2015 Science Reuters, UK

microorganisms to certain antibiotics was also evaluated. According to Adu *et al*.[20], bacteria resistance modifying agents have the ability to reduce the MIC of an antibiotic to which resistance has occurred. Kamatou *et al*.[21] reported that antimicrobials from plants have been found to be synergistic enhancers; thus at sub-inhibitory concentrations, they can enhance the antimicrobial effects of standard antibiotics. Plants are also known to produce multi-drug resistance inhibitors (MDRIs) which enhance the activities of antimicrobial compounds[23]. The PLP extract at sub-MIC concentration of 0.5 mg mL^{-1} exhibited resistance modulation effects on amoxicillin, cloxacillin, ampicillin and erythromycin against the test organisms. It caused a reduction in the MICs of amoxicillin, erythromycin, ampicillin and cloxacillin against *K. pneumonia*, *P. aeruginosa*, *P. vulgaris* and *S. aureus*, respectively from 100 to 80 μg mL^{-1}. The MICs of amoxicillin against *P. vulgaris* and *E. coli* were also reduced by the extract from 100 to 4 μg mL^{-1} (96% reduction in the MIC). There was also a reduction in the MICs of ampicillin against *S. pyogenes*, *P. vulgaris, S. aureus, E. coli* by 0.5 mg mL^{-1} PLP extract from to 1 μg mL^{-1} (99% reduction in the MIC). These results show that the 0.5 mg mL^{-1} sub-MIC extract concentration of PLP and PLC modulated the resistance of the antibiotics better than the 1 mg mL^{-1} sub-MIC extract concentration. The ability of the extracts to modulate the resistance of the various organisms to the effects of the antibiotics may be due to a breaching of the various resistance mechanisms of the microbes which include efflux pump systems, the production of inactivating agents such as β-lactamases particularly to the penicillin antibiotic or altering antimicrobial binding sites[24]. The inhibition of efflux pumps or mechanism in a bacterial cell according to Adu *et al*.[20] could be a means of reversing resistance. The ability of these extracts to cause resistance modulation can be attributed to the phytochemicals in the plant particularly flavonoids which have been reported to reverse *S. aureus* resistance to many antibiotics[22]. These agents might have caused a reversal in the resistance mechanisms of these organisms hence causing the antibiotics to exert their effects. The extracts might have also enhanced the production of MDRIs resulting in the reduction in resistance. The screening of these resistance modulation agents in plants serves as a first step for the isolating and purification of compounds which can be combined with standard antibiotics to improve their activity.

Considering the extent of folkloric use of *P. lappacea* in the management of various ailments including wounds, skin infections, etc. and the various biological activities[4,5,18], reported and the possible cytotoxic effects of the plant extracts skin cells, the influence of the extracts on the viability, proliferation and LDH release on HaCaT keratinocyte cells ware evaluated. PLC increased the proliferation of the HaCaT keratinocytes at lower concentrations of 0.1 to10.0 μg mL^{-1} compared to the untreated cells. This observation was similar to other reports on the cytotoxicity studies of plant materials used for treatment of wound and other skin infections[12,25].

The effect of the extracts on LDH release from the cytoplasm of cells into extracellular fluids which indicates cell necrosis or apoptosis may be an indication of cytotoxic effects. The extracts (PLC, PLP and PLE) of *P. lappacea* did not exhibited significant ($p>0.05$) release of LDH from the HaCaT cells compared to the untreated cells and relative amount of LDH release less than 0.5 is considered as non-cytotoxic or necrotic[25] and hence no necrotic or toxic effects was observed for the extracts at the concentrations tested (Fig. 4-6). There is a need to isolate and characterize the bioactive compounds or principles responsible for the various biological activities exhibited by these extracts of *P. lappacea*.

CONCLUSION

The chloroform, ethanol and petroleum ether leaf extracts of *P. lappacea* exhibited broad-spectrum antimicrobial activity. The extracts showed concentration and time-dependent rate of microbial destruction against the test organisms. The petroleum ether and chloroform extracts at sub-MIC concentrations showed microbial resistance modulation potential among the selected antibiotics. The extracts (PLC, PLP and PLE) did not show cytotoxic effects within the concentrations used.

ACKNOWLEDGMENTS

Special thanks go to the technical staff of the Department of Pharmaceutics (Microbiology Section), Kwame Nkrumah University of Science and Technology, Kumasi, Ghana for their assistance, Mr. Eric Gyebi of Jachie, Ashanti Region, Ghana for the collection of plant material and Dr. Alex Asase, Department of Botany University of Ghana, for the identification of the plant.

REFERENCES

1. Nath, S., M.D. Choudhury, S. Roychoudhury, A.D. Talukdar and A.V. Sirotkin *et al*., 2011. Restorative aspect of castor plant on mammalian physiology: A review. J. Microbiol. Biotechnol. Food Sci., 1: 236-243.

© 2015 Science Reuters, UK

2. Parekh, J. and S.V. Chanda, 2007. *In vitro* antimicrobial activity and phytochemical analysis of some Indian medicinal plants. Turk. J. Biol., 31: 53-58.
3. Doherty, V.F., O.O. Olaniran and U.C. Kanife, 2010. Antimicrobial activities of *Aframomum melegueta* (Alligator pepper). Int. J. Biol., 2: 126-131.
4. Felix, A.A. and A.O. Domingo, 2008. Antioxidant activity and chemical constituents of *Pupalia lappacea* (L.) juss. Res. Biol. Sci., 3: 783-785.
5. Ravi, A., M. Alvala, V. Sama, A.M. Kalle, V.K. Irlapati and B.M. Reddy, 2012. Anticancer activity of *Pupalia lappacea* on chronic myeloid leukemia K562 cells. Daru, Vol. 20.
6. Apenteng, J.A., C. Agyare, F. Adu, G.A. Ayande and Y.D. Boakye, 2014. Evaluation of the wound healing potential of different leaf extracts of *Pupalia lappacea*. Afr. J. Pharm. Pharmacol., 8: 1006-1011.
7. Udegbunam, S.O., R.I. Udegbunam, C.C. Muogbo, M.U. Anyanwu and C.O. Nwaehujor, 2014. Wound healing and antibacterial properties of methanolic extract of *Pupalia lappacea* Juss in rats. BMC Complementary Alternat. Med., Vol. 14. 10.1186/1472-6882-14-157
8. Neeharika, V., H. Fatima and B.M. Reddy, 2013. Evaluation of antinociceptive and antipyretic effect of *Pupalia lappacea* Juss. Int. Curr. Pharm. J., 2: 23-28.
9. Serban, E.S., M. Ionescu, D. Matinca, C.S. Maier and M.T. Bojita, 2011. Screening of the antibacterial and antifungal activity of eight volatile essential oils. Farmacia, 59: 440-446.
10. Wiegand, I., K. Hilpert and R.E.W. Hancock, 2008. Agar and broth dilution methods to determine the Minimal Inhibitory Concentration (MIC) of antimicrobial substances. Nat. Protocol., 3: 163-175.
11. Pfaller, M.A., D.J. Sheehan and J.H. Rex, 2004. Determination of fungicidal activities against yeasts and molds: Lessons learned from bactericidal testing and the need for standardization Clin. Microbiol. Rev., 17: 268-280.
12. Agyare, C., M. Niehues, M. Lechtenberg, A. Deters and A. Hensel, 2009. Ethnopharmacological survey and *in vitro* confirmation of ethnopharmacological use of medicinal plants used for wound healing in Bosomtwi-Atwima-Kwanwoma area, Ghana. J. Ethnopharmacol., 125: 393-403.
13. Porstmann, T., T. Ternynck and S. Avrameas, 1985. Quantitation of 5-bromo-2-deoxyuridine incorporation into DNA: An enzyme immunoassay for the assessment of the lymphoid cell proliferative response. J. Immunol. Methods, 82: 169-179.
14. Decker, T. and M.L. Lohmann-Matthes, 1988. A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and Tumor Necrosis Factor (TNF) activity. J. Immunol. Methods, 115: 61-69.
15. De Melo, J.G., T.A. de Sousa Araujo, V.T.N. de Almeida e Castro, D.L. de Vasconcelos Cabral and M. do Desterro Rodrigues *et al*., 2010. Antiproliferative activity, antioxidant capacity and tannin content in plants of semi-arid northeastern Brazil. Molecules, 15: 8534-8542.
16. Kolapo, A.L., M.B. Okunade, J.A. Adejumobi and M.O. Ogundiya, 2009. Phytochemical composition and antimicrobial activity of *Prosopis africana* against some selected oral pathogens. World J. Agric. Sci., 5: 90-93.
17. Fabry, W., P.O. Okemo and R. Ansorg, 1998. Antibacterial activity of East African medicinal plants. J. Ethnopharmacol., 60: 79-84.
18. Neeharika, V., H. Fatima and B.M. Reddy, 2013. Evaluation of antinociceptive and antipyretic effect of *Pupalia lappacea* Juss. Int. Curr. Pharm. J., 2: 23-28.
19. Haas, W., C.M. Pillar, C.K. Hesje, C.M. Sanfilippo and T.W. Morris, 2010. Bactericidal activity of besifloxacin against staphylococci, *Streptococcus pneumoniae* and *Haemophilus influenzae*. J. Antimicrob. Chemother., 65: 1441-1447.
20. Adu, F., S.Y. Gbedema and K. Annan, 2009. Antimicrobial and resistance modulatory activities of *Coryanthe pachyceras*. Pharmacogn. Res., 1: 280-284.
21. Kamatou, G.P., R.L. Van Zyl, S.F. Van Vuuren, A.M. Viljoen and A.C. Figueiredo *et al*., 2006. Chemical composition, leaf trichome types and biological activities of the essential oils of four related Salvia species indigenous to southern Africa. J. Essential Oil Res., 18: 72-79.
22. Aiyegoro, O.A. and A.I. Okoh, 2009. Use of bioactive plant products in combination with standard antibiotics: Implications in antimicrobial chemotherapy. J. Med. Plant Res., 3: 1147-1152.
23. Stermitz, F.R., J. Tawara-Matsuda, P. Lorenz, P. Mueller, L. Zenewicz and K. Lewis, 2000. 5'-Methoxyhydnocarpin-D and Pheophorbide A: Berberis species components that potentiate berberine growth inhibition of resistant *Staphylococcus aureus*. J. Nat. Prod., 63: 1146-1149.
24. Tenover, F.C., 2006. Mechanisms of antimicrobial resistance in bacteria. Am. J. Med., 119: S3-S10.
25. Adu-Amoah, L., C. Agyare, E. Kisseih, P.G. Ayande and K.B. Mensah, 2014. Toxicity assessment of *Erythrophleum ivorense* and *Parquetina nigrescens*. Toxicol. Rep., 1: 411-420.

© 2015 Science Reuters, UK