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PROSPECTING THE BIOACTIVE POTENTIAL OF *Callistemon lanceolatus* (Sm.) LEAVES AND ITS PHYTOCONSTITUENTS

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ABSTRACT

The present study explores the antimicrobial potential of *Callistemon lanceolatus* leaves. Different extracts of *Callistemon lanceolatus* leaves revealed its broad-spectrum antimicrobial potential where *Staphylococcus aureus* and *Klebsiella pneumoniae* 1 were the most sensitive. Cardiac glycosides were most abundant and showed maximum antimicrobial activity closely followed by phytosterols. Both ethyl acetate extract and phytoconstituents showed lower minimum inhibitory concentration as compared to gentamicin and were microbicidal in nature. The results of antimicrobial screening, minimum inhibitory concentration, viable cell count and post antibiotic effect corroborated well with each other. Antiproliferative attribute of *C. lanceolatus* ethyl acetate extract and phytoconstituents against MCF 7 cell line is being reported for the first time and were found to be biosafe as revealed by Ames and 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay. The current exploration of *C. lanceolatus* leaves holds a promising antimicrobial as well as anticancer attribute of the plant extract as well as its phytoconstituents. The biosafe nature of the above also adds importance to the study to be taken up further.

Keywords: Antimicrobial, *Callistemon*, Phytoconstituents, Antiproliferative, Biosafety

1. INTRODUCTION

The endless war between the mankind and the pathogenic microorganisms has been going on since the discovery of first antibiotic i.e. penicillin. This led to the development of several prevalent antibiotics from various sources i.e. bacterial, fungal, actinomycetes and plants. But that wasn't enough as the microorganisms surpassed the race with the emergence of resistance, thereby excluding the beneficial prophecy of antibiotics. Now this problem has affected the whole mankind in several ways thereby letting multidrug pathogens to cause diseases which are beyond the spectrum of various antibiotics. The indiscriminate use of antibiotics has posed a major threat to mankind in the form of multidrug resistance acquired by many microorganisms viz. MRSA (Methicillin resistant *Staphylococcus aureus*), VRSA (Vancomycin resistant *Staphylococccus aureus*), VRE (Vancomycin resistant *Enterococci*) and SRE (Streptomycin resistant *Enterococci*) etc. This problem resulted in the discovery of several novel antimicrobial compounds with good potency but only a few of them could succeed as the pathogens regained the ability of resistance by various mechanisms, thereby resulting in the development of several serotypes of resistant pathogenic microorganisms by genetic and biochemical mechanisms **[1]**. The key to burgeoning problem of resistance liesin further exploration of nature particularly the plants which have remained the least explored of all the natural resources in the field of healthcare and medicine **[2]**. The ancient, Indian as well as Chinese, medicinal system utilized plants for different therapeutic purposes which have taken a leap since several decades **[3, 4]**. Despite the extensive use of medicinal plants in traditional medicine and current usage, still there is a paucity of data to provide scientific validation to such medicinal plants. There is a need to look for better antimicrobials with better efficacy and lesser side effects. The use of plant based extracts and phytochemicals, could be significant in the field of healthcare and medicine **[5, 6]**.

Over 25% pharmaceutical products today are obtained directly or indirectly from plants. The studies on plant based drugs have been exploited in various medicinal systems such as Ayurveda, Unani, Homeopathy, Siddha etc. The genus *Callistemon* belonging to the family Myrtaceae comprises of over 30 species. The name *Callistemon* was given by Robert Brown, and its species are distributed in wet tropical regions such as Australia, south America and tropical Asia but now

they have expanded to all over the world **[7]**. *Callistemon* spp. are used mainly in forestry, essential oil production, windbreak farming, degraded and reclamation and for ornamental purposes **[8, 9]**. However, they are also used in weed control **[10]** and even as bioindicators for environmental management **[11]**. The nectar of the flowers are also used for making soft drinks, thus showing their relevance in food industry as well. Vimidione isolated from essential oils of *C. viminalis* have been evaluated to possess fumigant, contact toxicity and even insecticidal property, therefore have been pivotal in pesticidal industry **[12]**. In addition to *Callistemon* sp. antimicrobial activity of essential oils from other plants has also been reported previously **[13]**.

In vitro and In vivo investigations of plant derived phytoconstituents have resulted in promising molecules showing anticancerous properties. Vinca alkaloids (vincristine and vinblastine), epipodophyllotoxin derivatives, camptothecin etc. demonstrated themselves as promising derivatives showing anticancer potential **[14]**. But the toxic nature of these compounds and resistance conferred by some tumor cells justify the need for better therapeutics. Cancer is the leading cause of mortality in the world, and the incidences has progressed to a great extent in the past few decades, which can be attributed to changes in lifestyle, pollution, improper medication practices etc. **[15]**. Thus, the identification of new compounds and natural sources can help significantly in the prevention and treatment of cancer.

Keeping in mind the pharmaceutical potential the present work has been carried out to explore the antimicrobial and anticancer activity of organic extracts and phytoconstituents from *C. lanceolatus* leaves. The essential oils of various species of *Callistemon* have been reported to possess antimicrobial activity **[16, 17, 18]**. However, *C. lanceolatus* leaves still remain untapped as a potential candidate. The present study provides scientific validation to the antimicrobial potential of the ethyl acetate extract of *C. lanceolatus* leaves and its bioactive phytoconstituents. It reports on phytochemical screening and their quantification, which were then tested for their antimicrobial potential on the basis of agar well diffusion assay (ADA), Minimum inhibitory concentration (MIC), viable cell count (VCC) and Post antibiotic effect (PAE). Further synergistic behavior of ethyl acetate extract and phytoconstituents with standard antibiotics has also been ascertained. The antiproliferative potential of both ethyl acetate extract of *C. lanceolatus*

leaves and the isolated phytoconstituents (cardiac glycosides and phytosterols) have been tested against Human Breast cancer cell lines i.e. MCF 7.Keeping in mind their future potential, the biosafety evaluation by Ames test and MTT assay has also been carried out.

2. METHODS

2.1 Test organisms

The reference strains of test bacteria and yeast were procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, Indiaviz. *Enterococcus faecalis* (MTCC 439), *Staphylococcus aureus* (MTCC 740) and *Staphylococcus epidermidis* (MTCC 435) as Gram positive bacteria; *Escherichia coli* (MTCC 119), *Klebsiella pneumonia* 1 (MTCC 109), *K. pneumoniae* 2 (MTCC 530), *Pseudomonas aeruginosa* (MTCC 741), *Salmonella typhimurium*1 (MTCC 98), *Salmonella typhimurium* 2 (MTCC 1251) and *Shigella flexneri* (MTCC 1457) as Gram negative bacteria and yeast strains, *Candida albicans* (MTCC 227) and *Candida tropicalis* (MTCC 230). The cultures were maintained on nutrient agar slants with regular sub culturing *Enterococcus faecalis, Candida tropicalis* and *Candida albicans* were maintained on trypticase soya agar (TSA), Sabouraud dextrose agar and yeast malt agar (YMA) respectively. The glycerol stock of the cultures was preserved at -80°C **[19]**.

2.2 Incoculum preparation

A loopful of microbial culture (bacterial/yeast) taken aseptically in 5ml suitable broth was incubated for 4 hours at 37 ºC and 25ºC respectively. The incoculum was incubated or diluted further to meet the turbidity standard of 0.5 McFarland units **[20]**.

2.3 Plant material

The *C. lanceolatus* leaves used in this study were procured from the campus of Guru Nanak Dev University, Amritsar, India and deposited in the Herbarium of Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar vide accession number 572/ Botanical &Environment Science The plant material was surface sterilized using 1% mercuric chloride and rinsed with sterilized distilled water 3-4 times and thereafter it was dried at 40 °C for overnight **[19]**. The plant material was grounded to powder form using electric grinder.

2.4 Determination of best organic extractant

Five organic solvents i.e. chloroform, hexane, ethyl acetate, butanol and methanol were used for the extraction of bioactives from the plant material. The aqueous extract

(15%) together with equal volume of solvent was shaken vigorously twice in a separating funnel. The organic layer obtained was pooled and concentrated using rotary evaporator at 40-50 °C and the dried residue obtained was weighed and reconstituted in 30% Dimethyl sulfoxide (DMSO). The extract, thus obtained was screened for its antimicrobial activity against thirteen test organisms by agar well diffusion assay **[21]**.

2.5 Phytochemical analysis of *C. lanceolatus*leaves

2.5.1 Qualitative analysis

The powdered plant material was assayed for the presence of phytoconstituents viz. alkaloids, flavanoids, saponins, tannins, cardiac glycosides, anthranol glycosides, phytosterolsby standard methods with some modifications as described earlier **[19]**.

2.5.2 Quantitative isolation of phytoconstituents

Quantitative isolation of the detected phytoconstituents was done by standard methods as described earlier **[21, 22]**. The phytoconstituents showing positive antimicrobial activity in agar well diffusion assay ADA were further studied for MIC, VCC and PAE studies **[23]**.

2.6 Minimum Inhibitory Concentration (MIC)

Both ethyl acetate extract and isolated phytoconstituents showing antimicrobial efficacy on ADA were further used for determining their MIC by agar dilution method. The stock solutions (10 mg mL^{-1}in case of ethyl acetate extract and 30 mg $mL^{-1}$for both cardiac glycosides/phytosterols) were diluted to a range of concentrations varying from 0.1-5 µg $mL^{-1}$and 0.05-0.1 µg $mL^{-1}$for ethyl acetate extract and phytoconstituents, respectively. The MICs so obtained were compared with those of standard antibiotics, *i.e.*, gentamicin and amphotericin B as positive controls. The concentration at which different organisms did not show any growth or complete inhibition was considered as MIC.

2.7 Viable cell count (VCC)

The antimicrobial action (microbicidal or microbistatic) of ethyl acetate extract of leaves was evaluated by VCC studies where stock solutions of 6X MIC of the ethyl acetate extract as well as both isolated phytoconstituents i.e. cardiac glycosides and phytosterols were mixed respectively with equal volume of the diluted inoculum 10^{-3}. After every 2h, starting from 0-24h, 100µl of this mixture was spread onto sterilized agar plates. The mean number of colonies were counted and compared with that of control in which the ethyl acetate extractor isolated

phytoconstituuents of *C. lanceolatus* leaves were replaced with 30% DMSO.

2.8 Post antibiotic effect (PAE)

The PAE of ethyl acetate extract and phytoconstituents was determined as described earlier **[24]** with slight modifications. The various MIC values (as discussed in **Table 2** of results) were applied in the VCC, for both ethyl acetate extract and the isolated phytoconstituents, were mixed in a concentration of 6X with the respective test organisms (in equal volume) broth diluted upto 10^{-3}. After 2 h of incubation, the drug activity was stopped by diluting (upto 10^{-3}) the mixed suspension with sterilized double strength broth. After a regular interval of 2 h the mixture was spreaded onto sterilized agar plates upto 24 h and incubated at respective temperature. The mean number of colonies were counted and compared to that of control. The PAE was calculated as follows:

$$\mathbf{PAE = T - C}$$

where T represents the time required for the count in the test culture to increase 1 $\log_{10}$ CFUmL^{-1}above the count observed immediately after drug removal and C represents the time required for the count of the untreated control tubes to increase by 1 $\log_{10}$ CFU mL^{-1}.

2.9 Synergistic behavior of ethyl acetate extract and phytoconstituents with standard antibiotics

The synergistic or antagonistic action of ethyl acetate extract and phytoconstituents (cardiac glycosides/ phytosterols) with that of standard antibiotics i.e. gentamicin and amphotericin B was done according to Braga *et al*. 2005 **[25]** with some modifications. The test extract (i.e. ethyl acetate extract and isolated phytoconstituents) and standard antibiotics were tested at different range of concentrations i.e. 1X MIC, 1/5th MIC and 2X MIC and mixed together in equal concentration in the nutrient medium viz. nutrient agar for bacterial cultures, yeast malt agar for *Candida albicans* and sabaraoud agar for *Candida tropicalis*. The organisms showing no growth were indicative of the positive results which were compared to that of control where a plate with diluent was used instead of extract, was considered as negative control and plate with only antibiotic or test extract (at the concentration similar to that of above mentioned) were positive controls. The organism that showed no visible growth or inhibition at 1X, 1/5thX or 2X MIC were considered showing synergistic effect. On the other hand the organism showing growth at combined 2X MIC of ethyl acetate extract or phytoconstituents and standard antibiotic which otherwise got inhibited in controls at the respective concentrations were considered to show antagonistic behavior.

2.10 Antiproliferative cell culture studies on MCF 7cell line

Human Breast cancer cell line MCF 7 was procured from the National Centre for Cell Science, Pune, India. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with streptomycin (100 U mL^{-1}), gentamicin (100 µgmL^{-1}), amphotericin B(0.25 µg mL^{-1}) and 10% fetal bovine serum (Himedia) in a CO_2 incubator (5% CO_2; 90 % Relative Humidity) at 37°C. The *in vitro* cytotoxicity of powder samples was determined against MCF 7cell line by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide) assay **[26]**. 5 x 10^3 cells were added in each well of 96 well plate and incubated at 37°C, 5% CO_2 for 24 h. The cells were treated with powder sample (ethyl acetate extract and isolated phytoconstituents) for 48 h, washed and 100 µl of fresh medium with 20 µl MTT solution (5 mg ml-1)was added to each well. The cells were incubated at 37°C, 5% CO_2 for 4 h. After incubation the medium was removed and formazan product was dissolved in 100 µl of DMSO (Dimethyl sulfoxide) and shaken for 10 min **[21]**. The optical density was measured at 550 nm by microplate reader. Percentage of cell growth inhibition was calculated by using formula:

% cell growth = (OD of treated cells/ OD of control) X 100

2.11 Biosafety evaluation

2.11.1 MTT assay

The biosafety of ethyl acetate extract and isolated phytoconstituents (Cardiac glycosides and phytosterols) was evaluated using MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] cellular toxicity assay **[28]** as described earlier **[20]**.

2.11.2 AMES mutagenecity assay

Ethyl acetate extract and the two selected phytoconstituents (cardiac glycosides and phytosterols) were subjected to plate incorporation method of Ames test to evaluate their mutagenecity as described earlier **[21]**.

3. RESULTS

3.1 Qualitative and Quantitative analysis of Phytochemicals

The qualitative screening of various phytochemicals in *C. lanceolatus* leaves bystandardized methods demonstrated the presence of saponins, tannins, cardiac glycosides and phytosterols as the major groups in variable abundance with cardiac glycosides were the most prevalent (44mg $g^{-1}$of plant material) followed by tannins (35.67 mg g^{-1} of plant material). Phytosterols and saponins were invariably present almost in similar quantity (31 and 28.33mg $g^{-1}$of plant material) **(Table 1)**.

3.2 Screening for the best extractant

Ethyl acetate was the best solvent and demonstrated maximum antimicrobial potential as evident from ADA. It was the best extractant not only in terms of inhibition zone (IZ) but also in terms of spectrum of activity. Rest of the solvents closely followed in the order butanol (But) > hexane (Hex) > chloroform (Chl). Among various pathogenic bacteria tested, *S. aureus* among gram positive and *K. pneumoniae* 1 among gram negative showed maximum sensitivity with an IZ of 27.33and 24.66 mm respectively. The clinical isolate, MRSA also showed an IZ of 22.33 mm However, *S. typhimurium*1,ethyl acetate extract did not show any activity but Hex supported the best activity (IZ of 15.55mm) followed by But (IZ of 14.66 mm) and Chl (IZ of 14 mm) respectively. *E. coli* was sensitive only to ethyl acetate extract (IZ of 15.5 mm) and But extract (IZ of 16.66 mm) while *E. faecalis* and *S. flexneri* were sensitive to ethyl acetate extract only. Even in case of yeast i.e. *C. albicans*, ethyl acetate supported the best antimicrobial activity (IZ of 28 mm) closely followed by But, Hex and Chl **(Figure 1)**.The antimicrobial activity in case of ethyl acetate extract showed significant difference as compared to rest of the extractants when evaluated by two way ANOVA where the *f*-ratio value 38.62879 and *p*-value of 0.000042 was observed. Most of the results showed significant difference at $p < .05$ and <0.001.*C. tropicalis* on the other hand was resistant to all the extracts.

3.3 Screening of phytoconstituents for antimicrobial activity

Screening of various phytoconstituents revealed cardiac glycosides and phytosterols to possess maximum antimicrobial activity whereas tannins and saponins were less potent both in terms of inhibition zone as well as spectrum of activity. In case of cardiac glycosides as well as phytosterols, MRSA (IZ 34 mm in cardiac glycosides and 32 mm in phytosterols) closely followed by *S. epidermidis* (IZ 32.33 mm in cardiac glycosides and 31.66 mm in phytosterols) showed maximum sensitivity respectively, whereas in case of gram negative, *K. pneumoniae* 1 remained most sensitive showing an IZ of 23.33 mm for both cardiac glycosides and phytosterols. Similarly, both cardiac glycosides and phytosterols showed comparable activity in case of *C. albicans* with an IZ of 15 and 14.33 mm, respectively **(Figure 2)**. However, *S. typhimurium* 2 showed marginally better sensitivity with tannins (IZ 20.33mm) as compared to cardiac glycosides and phytosterols. The antimicrobial activity in case of cardiac glycosides and tannins differ significantly

when evaluated by two way ANOVA where a significant difference at $p < .05$ and in some cases $p<0.001$ was observed.

3.4 Minimum inhibitory concentration

The microorganisms found sensitive in ADA experimentation were further subjected to find out the MIC of ethyl acetate extract and the isolated phytconstituents (cardiac glycosides and phytosterols) by agar dilution method. In case of ethyl acetate extract of leaves, *S. aureus* showed the lowest MIC of 0.5 µg mL^{-1} followed by *S. epidermidis* at 1 µg mL^{-1}among gram positive bacteria whereas *K. pneumonia* and *S. flexneri* showed an MIC of1 µg mL^{-1}among gram negative bacteria. The yeast strain i.e. *C. albicans* showed relatively higher MIC of 5 µg mL^{-1} **(Table 2)**.

Similarly, both the phytoconstituents i.e. cardiac glycosides and phytosterols showed similar MIC against *S. aureus* and MRSA i.e. 0.5 µg mL^{-1}among gram positive whereas in case of gram-negative lowest MIC was observed against *K. pneumoniae*1 and *S. flexneri* i.e. 1 µg mL^{-1}. However, yeast*, C. albicans* showed relatively higher MIC ranging from 5- 7 µg mL^{-1} **(Table 2)**.

3.5 Viable Cell count

The ethyl acetate extract of *C. lanceolatus* leaves demonstrated bactericidal nature as *K. pneumoniae1* got killed instantaneously at 0h among gram negative bacteria whereas among gram positive *S. aureus,* MRSA *and S. epidermidis* got completely killed in 2h. On the other hand *E. faecalis, E. coli, K. pneumoniae2, P. aeruginosa* and *S. flexneri* got killed between 4-6h. Yeast strain i.e. *C. albicans* took 4 h for complete killing to take place **(Figure 3 a)**.

Similarly, cardiac glycosides isolated from *C. lanceolatus* leaves resulted ininstantaneous killing of *S. epidermidis* and MRSA, while *K. pneumoniae* 1 showed a cellular viability of 7.2% at zero hour and got completely killed in 2h of duration while rest of the tested microorganisms i.e. *E. faecalis, S. aureus, E. coli, S. typhimurium2, K. pneumoniae* 2, *P. aeruginosa, S. typhimurium1*and *S. Flexneri* got completely killed between 4-6 h. *S. typhimurium1,* took 8 h for complete killing **(Figure 3b)**. On the other hand, *C. albicans* took 6 h for complete killing to take place.

Phytosterols isolated from *C. lanceolatus* leaves also showed a similar pattern to that of cardiac glycosides as among gram positive *S. epidermidis* and MRSA got killed instantaneously, and among gram negative it took 2 h for complete killing of *K. pneumoniae* 1 (showing a viability of 4.6% at 0 h) and *S. flexneri*. While *E. faecalis*, *S. typhimurium*2, *S. aureus* and *P. aeruginosa*

got completely killed in 4-6 h. It took 6 h for complete killing of *C. albicans* **(Figure 3c)**.

3.6 Post antibiotic effect (PAE)

PAE ranging from 2-6 h was sustained by ethyl acetate extract of *C. lanceolatus* leaves. The extract was equally effective against both gram positive and gram negative bacteria, where it showed PAE of 6h against *S. epidermidis* and *K. pneumoniae* 1. However, yeast strain, *C. albicans* and the clinical isolate MRSA showed a PAE of 2 and 4 h respectively. Rest of the tested microorganisms showed a PAE of 2 h each **(Figure: 4a)**.

In case of cardiac glycosides, the PAE increased to 8 h against MRSA followed by *S. epidermidis* and *K. pneumoniae* 1 which showed PAE of 6 h each. *S. aureus, P. aeruginosa, S. typhimurium 2, S. flexneri, E. faecalis, E. coli, K. pneumoniae* 2 and *C. albicans* showed a PAE ranging from 2-4 h **(Figure: 4b)**.

Despite relatively narrow spectrum of activity phytosterols showed better PAE as compared to both ethyl acetate extract and cardiac glycosides; MRSA, *S. epidermidis, S. typhimurium* 2 and *P. aeruginosa* showed a PAE of 6 h, and *S. aureus, K. pneumoniae* 1, *S. flexneri, E. faecalis, C. albicans* showed a PAE ranging from 2-4 **(Figure: 4c)**.

3.7 Synergistic behavior of extract and phytoconstituents with standard antibiotics

The ethyl acetate extract and isolated phytoconstituents of *C. lanceolatus* leaves when added to standard antibiotics i.e. gentamicin and amphotericin B revealed synergistic action with lowered MIC values as well as increased spectrum of activity. *S. aureus, S. epidermidis, K. pneumoniae* 1, *S. epidermidis, C. albicans* and *C. tropicalis* were taken into consideration for such studies. Synergistic action was observed in ethyl acetate extract as well as phytoconstituents where they showed inhibition at 1/5th MIC (mentioned above) when used in combination with standard antibiotic i.e. gentamicin and amphotericin B at 1/5th MIC as well. Similarly, *C. tropicalis* which was otherwise resistant to the both the extracts (ethyl acetate extract and the isolated phytoconstituents) but when used in combination with amphotericin B it showed sensitivity even at 1/5th MIC. However, In contrast to above, *S. aureus* showed an antagonistic behavior with Phytosterol as it showed growth at 2X MIC when used in combination with gentamicin **(Table 3)**.

3.8 Antiproliferative cell culture studies on MCF 7 cell line

The cellular response on the MCF 7 cell lines is represented in **Figure 5** where the cardiac glycosides demonstrated the best antiproliferative potential followed by phytosterols and ethyl acetate extract which in turn showed better potential as compared to curcumin. The proliferation of the cells in case of cardiac glycosides was 21.90% at minimal testing concentration of 31.25 µg mL^{-1}which further reduced to 14.694% at 125 µg mL^{-1} **(Figure 5)**. In case of phytosterolsand ethyl acetate extract at 125 µg mL^{-1}.The proliferation of MCF 7 cell lineremained 14.42 and 17.007% only. The antiproliferative potential of both ethyl acetate extract and phytoconstituents showed better efficacy as compared to the positive control included i.e.curcumin which showed 48.84% inhibition at 100 µM.

Table 1: Distribution of phytoconstituents in leaves of *C. lanceolatus*

Phytoconstituents	Yield (in mg/gram of plant material)
Cardiac Glycosides	44.00±1.15a
Tannins	35.67±1.20b
Phytosterols	31.00±1.15c
Saponin	28.33±0.88d

The result is significant at $p < .05$.

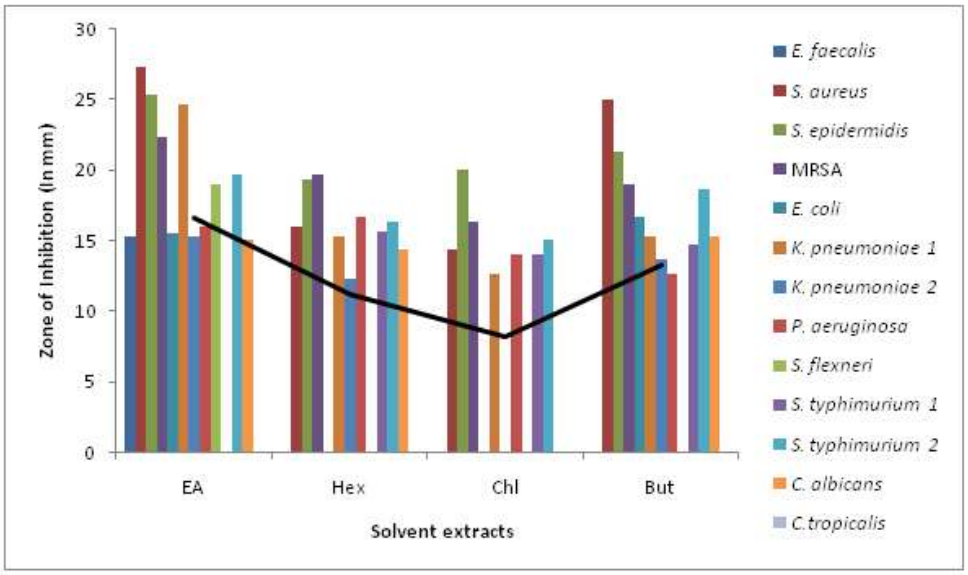

Fig 1: Comparison between antimicrobial activity of various organic extracts of *C. lanceolatus* leaves

EA- ethyl acetate, Hex- hexane, Chl- chloroform, But- Butanol

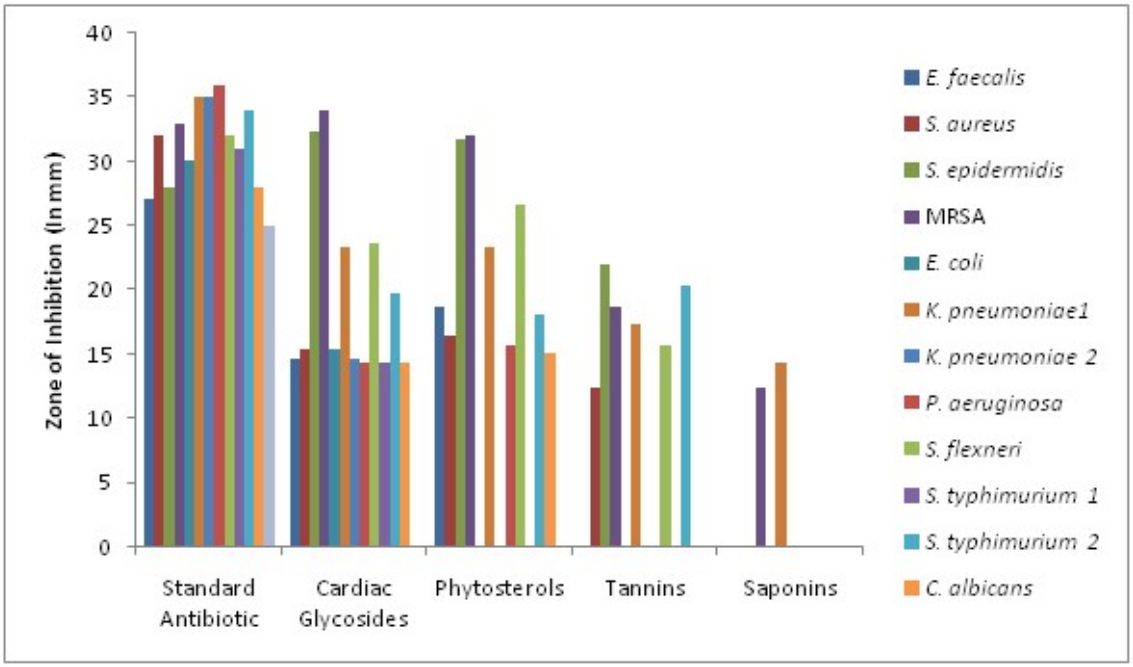

Fig 2: Antimicrobial activity of phytconstituents of *C. lanceolatus* leaves

Table 2: MIC of ethyl acetate extract and phytoconstituents of *C. lanceolatus* leaves

Microorganisms	Standard Antibiotic	Ethyl acetate	Cardiac Glycosides	Phytosterols
	MIC (µg/ml)			
E. faecalis	30*	5	5	5
S. aureus	5*	1	5	5
S. epidermidis	30*	1	0.5	0.5
MRSA	1*	5	0.5	0.5
E. coli	5*	5	5	ND
K. pneumoniae1	0.3*	1	1	1
K. pnemoniae2	0.5*	5	7	ND
P. aeruginosa	5*	5	7	5
S. flexneri	5*	1	1	1
S.typhimurium1	5*	ND	7	ND
S. typhimurium2	0.5*	1	5	5
C. albicans	0.5#	5	5	5

 # amphotericin B, * gentamicin

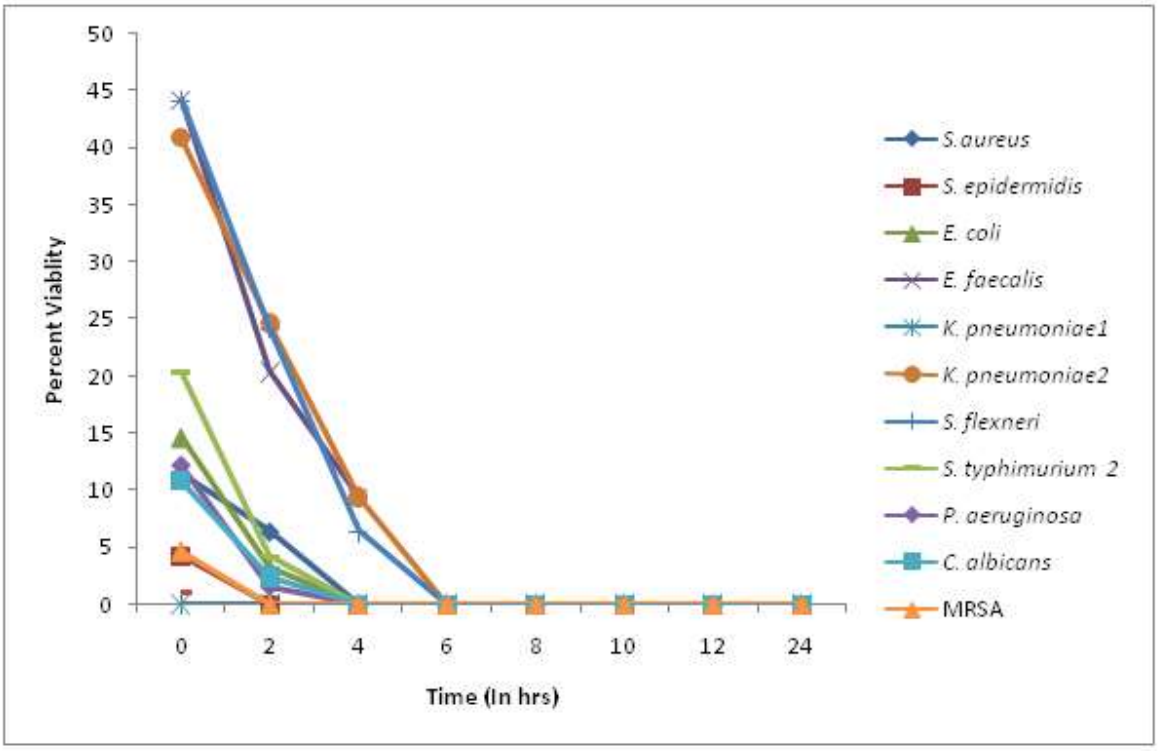

Fig 3 a: Viable cell count studies of Ethyl acetate extract of *C. lanceolatus* leaves

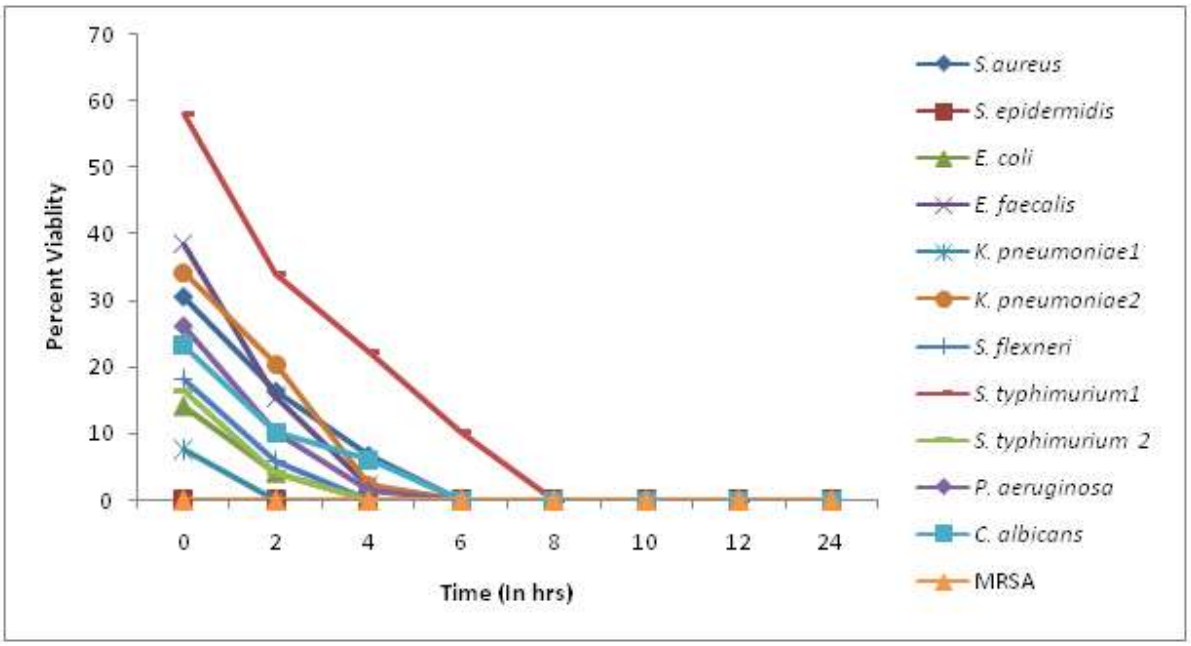

Fig 3 b: Viable cell count studies of cardiac glycosides extract of *C. lanceolatus* leaves

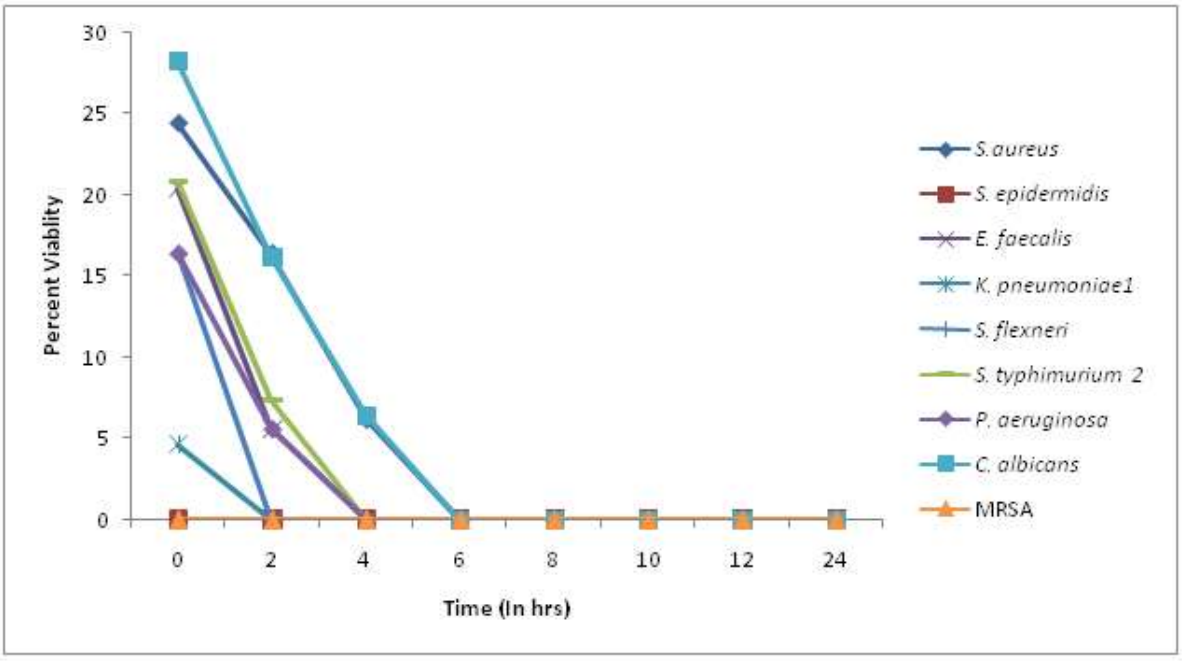

Fig 3 c: Viable cell count studies of phytosterols of *C. lanceolatus* leaves

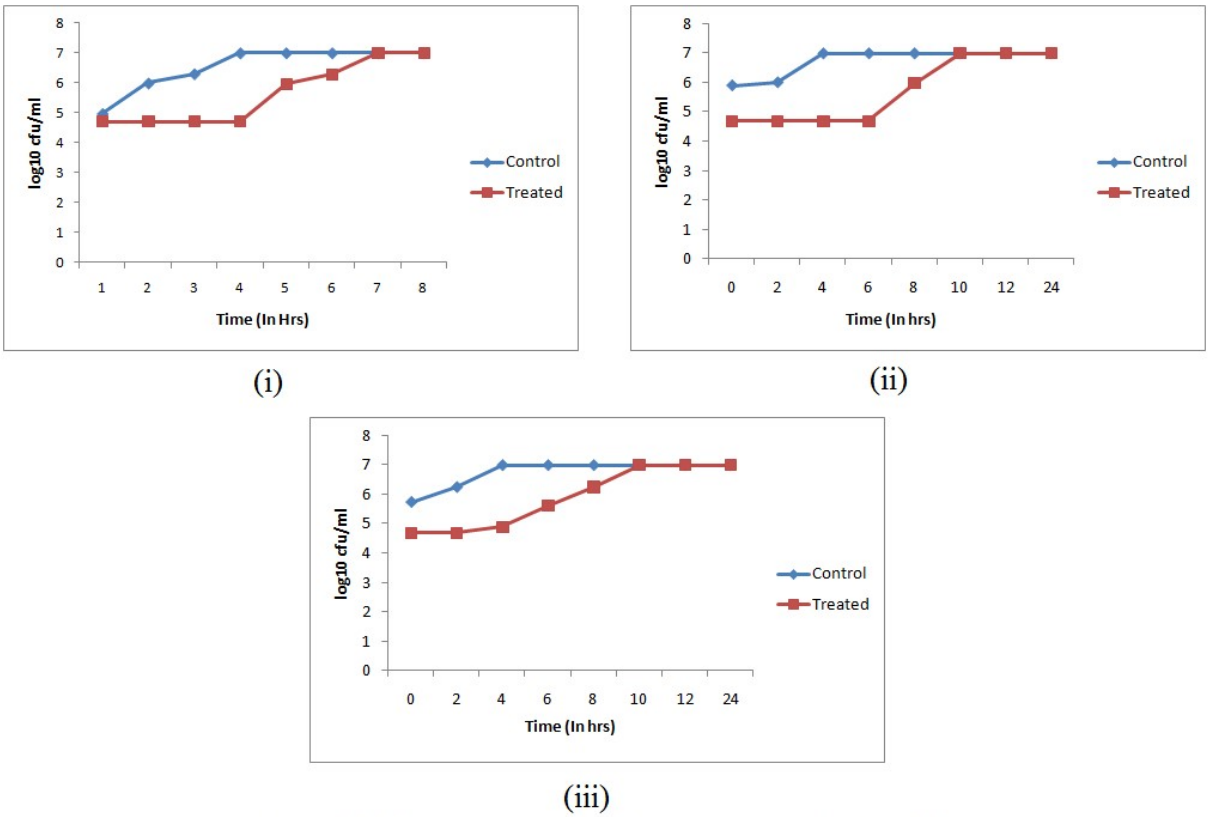

Fig 4 a: Growth curve showing post antibiotic effect of ethyl acetate extractof *C. lanceolatus* leaves against i- *S. epidermidis;* ii- *K. pneumoniae* 1; iii- MRSA

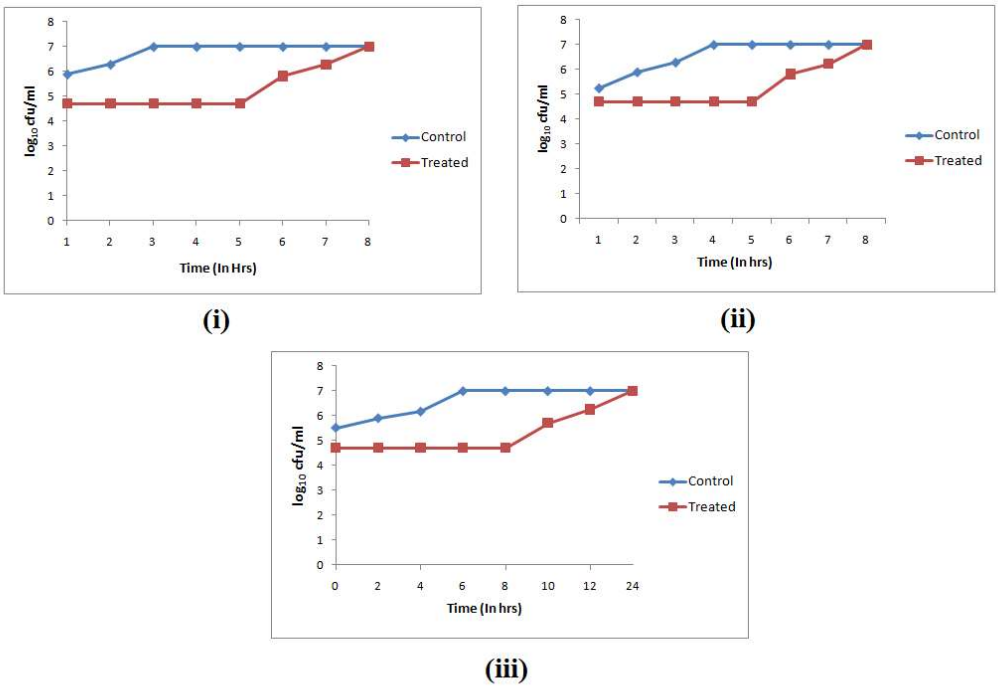

Fig 4 b : Growth curve showing post antibiotic effect of cardiac glycosidesof *C. lanceolatus* leaves against i- MRSA; ii- *S. epidermidis*; iii- *K. pneumoniae* 1

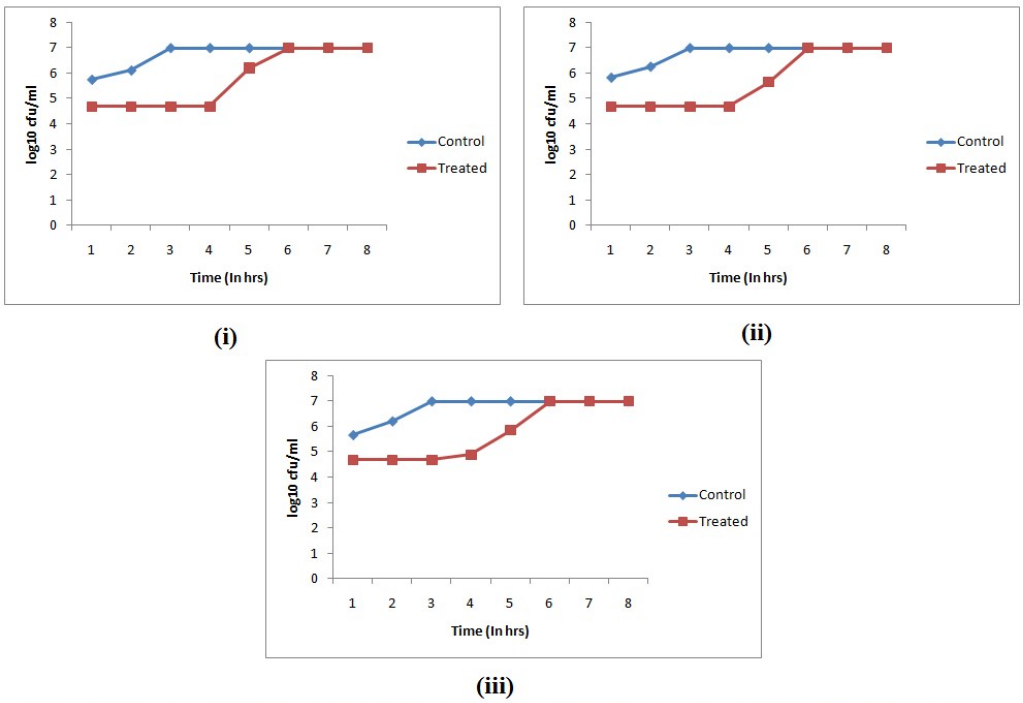

Fig 4 c: Growth curve showing post antibiotic effect of a- ethyl acetate extract, b- cardiac glycosides, c- phytosterols of *C. lanceolatus* leaves against i- MRSA; ii- *S. epidermidis*; iii- *P. aeruginosa*

Table 3: Synergistic antimicrobial effect of antibiotic with ethyl acetate extract and phytoconstituents of *C. lanceolatus* against some of the pathogens

S. aureus	1/5th MIC	MIC	2X MIC
Ethyl acetate extract	+	-	-
Cardiac glycosides	+	-	-
Phytosterols	+	-	+
Gentamicin	+	-	-
ethyl acetate extract + gentamicin	-	-	-
Cardiac glycosides + gentamicin	-	-	-
Phytosterols + gentamicin	-	-	-
K. pneumoniae 1			
ethyl acetate extract	+	-	-
Cardiac glycosides	+	-	-
Phytosterols	+	-	-
Gentamicin	+	-	-
ethyl acetate extract + gentamicin	-	-	-
Cardiac glycosides + gentamicin	-	-	-
Phytosterols + gentamicin	-	-	-
S. epidermidis			
ethyl acetate extract	+	-	-
Cardiac glycosides	+	-	-
Phytosterols	+	-	-
Gentamicin	+	-	-
ethyl acetate extract + gentamicin	-	-	-
Cardiac glycosides + gentamicin	-	-	-
Phytosterols + gentamicin	-	-	-
C. albicans			
ethyl acetate extract	+	-	-
Cardiac glycosides	+	-	-
Phytosterols	+	-	-
Amphotericin B	+	-	-
ethyl acetate extract + Amphotericin B	-	-	-
Cardiac glycosides + Amphotericin B	-	-	-
Phytosterols + Amphotericin B	-	-	-
C. tropicalis			
ethyl acetate extract	+	+	+
Cardiac glycosides	+	+	+
Phytosterols	+	+	+
Amphotericin B	+	-	-
ethyl acetate extract + Amphotericin B	-	-	-
Cardiac glycosides + Amphotericin B	-	-	-
Phytosterols + Amphotericin B	-	-	-

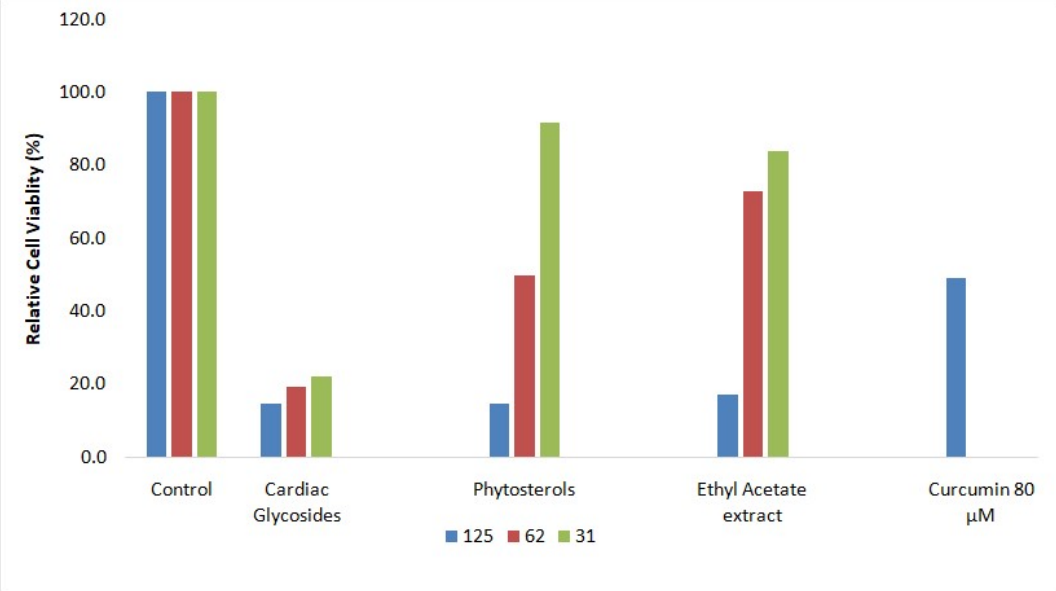

Fig 5: Antiproliferative potential of ethyl acetate extract and phytoconstituents of *C. lanceolatus* leaves

3.9 Biosafety Evaluation

3.9.1Ames test

Both ethyl acetate extract and isolated phytoconstituents were tested for their mutagenecity by Ames test which resulted in numerous revertant colonies (682) in positive control (sodium azide); while, the bacteria incubated with the ethyl acetate extract of *C. lanceolatus* leaves showed 12 revertant colonies whereas cardiac glycosides and phytosterols showed 14 and 13 revertant colonies, therefore were considered to be non-mutagenic.

3.9.2 MTT assay

Metabolic activity of the cells can be directly correlated with reduction of MTT dye, to formazen crystals. The ethyl acetate extract and phytoconstituents of *C. lanceolatus* leaves were found to be non-cytotoxic, as the absorbance of ethyl acetate extract and phytoconstituents (phytosterols and cardiac glycosides) treated cells was comparable with the untreated (control), 94.2% viable cells were observed in the ethyl acetate extract while phytosterols and cardiac glycosides showed 92.3 and 91.1 % viability.

3.10 Statistical analysis

Data were analyzed and treatments were compared using two way ANOVA with 95% confidence limits ($P<0.05$).

4. DISCUSSION

The deliberate and indiscriminate use of antibiotics has leadto the major problem of microbial resistance which is imparting a negative effect on human health. To answer this problem there has been a surge in the discovery of novel antimicrobials and natural resources have been the most promising because of their lesser side effects and better efficacy. The present study is an effort made in the same direction wherein,the ethyl acetate extract and phytoconstituents of *Callistemon lanceolatus* leaves were worked out for their antimicrobial potential. The initial screening was in line with the previous reports on associated medicinal plants viz. *C. citrinus, C. viminalis* **[7, 21, 29]**. The well-known pathogens *S. aureus, S. epidermidis, K. pneumoniae, C. albicans* and even the clinical isolate MRSA showing sensitivity to ethyl acetate extract and phytoconstituents provides credence to the study. Synergistic behavior with standard antibiotics i.e. gentamicin and amphotericin B has also been worked out. The biosafety of these as tested by AMES test and MTT assay highlight the significance, to pursue the studies to find out the lead compound and scaling up for clinical trials.

Ethyl acetate served as the best extractant to support the best antimicrobial activity in

terms of IZ as well as spectrum of activity in comparison to other organic solvents. Ethyl acetate extract was found to be closely comparable to gentamicin against some microorganisms, thus demonstrated its potential as alternative to the available antibiotics. The qualitative tests correlated well with the previous studies on *C. lanceolatus* leaves as the presence of similar phytoconstituents were reported **[30]**, the slight variation reported may be attributed to different plant parts and geographical locations. Cardiac glycosides were the most abundant phytoconstituent group followed by tannins which goes well in agreement with similar work on other plants and plant parts **[19, 30]**. The presence of other phytoconstituents followed the similar trend which showed consonance with the previous studies on various extracts of *C. lanceolatus* leaves **[31]**. The study encouraged as the antimicrobial activity followed a similar pattern to previous studies on *Moringa oleifera*as cardiac glycosides closely followed by phytosterols showed the broad-spectrum antimicrobial activity **[19, 32]**. The antimicrobial activity ofcardiac glycosides as well as phytosterols against gram positive bacteria was observed to be better as compared to gram negative bacteria pointing towards their probable mechanism of action as disrupting their cell wall or inhibiting the cell wall synthesis. The antimicrobial screening of ethyl acetate extract and isolated phytoconstituents by ADA revealed better antimicrobial efficacy of the cardiac glycosides and phytosterols as compared to ethyl acetate extract which can be attributed to the fact that actual compound responsible for antimicrobial potential got further purified as phytoconstituents.

The MIC values of ethyl acetate extract (1-5 µg mL^{-1}) and the phytoconstituents (0.5-7 µg mL^{-1}cardiac glycosides and 0.5-5 µg mL^{-1}phytosterols) supported well the results obtained from ADA and are better than obtained in previous studies on aqueous extract of *C. lanceolatus* seeds **[20]**, where the MIC ranged from 1- 5mgmL^{-1} and organic extracts of *Liquidambar orientalis, Vitis vinifera* ethanolic extracts 8- 14.2mg mL^{-1} **[33]**. Encouragingly, the MIC values of ethyl acetate extract was found to be lower as compared to gentamicin **(Table 2)** against some microorganisms viz. *E. faecalis, S. aureus, S. epidermidis* and *S. flexneri*, whereas comparable MIC was observed in case of *E. coli* and *P. aeruginosa.* Cardiac glycosides and phytosterols demonstrated antimicrobial potential particularly against *E. faecalis, S. epidermidis,* MRSA and *S. flexneri* where their MIC was lower in

comparison to gentamicin. Cardiac glycosides however gave MIC comparable to gentamicin against *S. aureus and E. coli*to gentamicin whereas phytosterols gave comparable MIC against *S. aureus* and *P. aeruginosa.*

Furthermore, viable cell count studies being a promising method was worked out to evaluate the antimicrobial action (microbicidal or microbistatic) which was depicted in terms of killing rate **[34]**. Some of the microorganisms got killed instantaneously while a few took a maxima upto 6 h to get killed and to add up further it is encouraging to mention here that no test organism showed re-growth in the present study using all the test extracts. Thus, the bactericidal nature can be useful for drug development purposes. Cardiac glycosides were found to be the more efficient as compared to phytosterols for most of the organisms which were potent as compared to ethyl acetate extract. The findings of VCC were correlating well with the results obtained from ADA and MIC. The study indicates that the phytoconstituents, cardiac glycosides and phytosterols were found to have better antimicrobial potential as demonstrated by ADA and MIC in comparison to ethyl acetate extract. The effectiveness of ethyl acetate extract and phytoconstituents is clearly indicated as *K. pneumoniae*1got killed instantaneously in the presence of ethyl acetate extract, while in case of phytoconstituents, *S. epidermidis* and MRSA got killed at 0 h with cardiac glycosides and phytosterol. The results obtained from VCC clearly demonstrate the phytoconstituents to possess better efficacy against gram positive as compared to gram negative bacteria. Furthermore, the synergistic action of ethyl acetate extract and phytoconstituents with standard antibiotics showed good synergy which correlated well with the previous studies on *Punica granatum* extracts **[25]**. In addition to this *C. tropicalis* which otherwise was resistant to both ethyl acetate extract as well as phytoconstituents showed synergistic effect with amphotericin B thereby got inhibited at 1/5th synergistic concentration. Hence leading to possible explanation that ethyl acetate extract and phytoconstituents might have altered the permeability of membrane in such a manner so as to allow the enhanced influx of antibiotic thus showing the inhibitory effect, also the pore formation ability of the amphotericin B thereby the inhibitory effect of both becomes more enhanced **[35]**. However, a unique observation was made when phytosterol in combination with gentamicin at 2X MIC

each was not effective against *S. aureus*, though it showed good sensitivity at 1X, 1/5X and 2X MIC whether in combination or alone. Hence, it was that synergistic behavior of antibiotic and phytoconstituents may undergo a reversal when tested at higher concentration.

Antimicrobial dosing regimens is based not only on the pathogen susceptibility but also various pharmacokinetic parameters such as drug concentration and/or route of drug administration. The ethyl acetate extract as well cardiac glycosides and phytosterol were found to be effective even after removing these and showed the PAE ranging from 2 to 6 h in case of ethyl acetate extract and phytosterol while cardiac glycosides gave a slightly longer PAE ranging from 2-8 h. The study reflects its coherence as the PAE results obtained are in line with the data obtained from ADA, MIC and VCC.

MCF 7 cell line is derived from human breast cancer cells **[36]**. Both the ethyl acetate extract and phytoconstituents revealed better antiproliferative efficacy against Human breast cancer cell line (MCF 7) as compared to curcumin. The efficacy of the ethyl acetate extract of *C. lanceolatus* leaves appears to be better in comparison to previous reports on ethanolic extracts of *Markhami atomentosa* leaves at 189 µg mL^{-1} **[37]**. Apparently, this is the first report on the potency of *C. lanceolatus* leaves against MCF 7 cell line. The proliferation of the MCF 7 cells was affected in such a way that growth rate of MCF 7 cell population declined when treated with ethyl acetate extracts as well its phytoconstituents of *C. lanceolatus* leaves. The biosafety of the extract was based on cellular viability of the living sheep blood cells in which enzyme-based assay of cellular activity was indicative of biosafe nature of the ethyl acetate extract and its phytoconstituents. The results obtained encourages for the selective inhibition ability of the extracts where they were inhibitory for the cancerous cells while ineffective for the living sheep blood cells. No doubt this necessitates further understanding for the mechanism of action towards both living cell and the cancer cell line.

To demonstrate the practical applicability of the data obtained it was pertinent to demonstrate the biosafety of test extract and/or the phytoconstituents It was very encouraging to note that the extracts used in this study were found to be non-cytotoxic and non-mutagenic as demonstrated by MTT assay and Ames test respectively. The safe profile of medicinal plants has also been demonstrated previously in literature **[19,**

20]. Similar study on biosafety profiling has been done in literature on fungi **[23, 38]**.

CONCLUSION

The study concludes on the grounds of bioactive potential of *Callistemon lanceolatus* leaves. The ethyl acetate extract and phytoconstituents showed potential against pathogens *viz. Staphylococcus epidermidis, Staphylococcus aureus, Klebsiella pneumoniae1, S. flexneri, Candida albicans* and clinical isolate i.e. Methicillin-resistant *Staphylococcus aureus* (MRSA). The results obtained from ADA showed a good coherence with the results obtained from MIC, VCC and PAE. Low killing time and MIC values are indicative of the antimicrobial potential of the test extract as well as phytoconstituents. Also, the better antiproliferative efficacy of ethyl acetate extract and phytoconstituents as compared to curcumin further endorses for its anticancer potential. The biosafety nature of both the test extracts (ethyl acetate extract and phytoconstituents) being non mutagenic as well as nontoxic credence the study for scaling up to commercial extent. These findings endorse for further exploration so as to find out the ld bioactive compounds and for clinical trials.

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Conflict of Interest

Both the authors declare no conflict of interest

Ethics approval and consent to participate

As the study does not involve any animal model system so no such approval is required.

Consent for publication

Both the authors hereby declare their consent for publication

Availability of data and materials

The study does not involve any such data and will be provided if required.

Competing interests

Both the authors declare that they have no competing interests.

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Author contributions

DSA, being the Ph.D supervisor contributed in analysis and drafting of the manuscript. Experimental work was done by Lovedeep Nim. The design of the experiments as well as the manuscript was contributed equally by both the authors.

Abbreviations

IMTECH: Institute of Microbial technology; MTCC: Microbial type culture collection; MRSA: Methicillin resistant *Staphylococcus aureus;* PBS: Phosphate buffer saline; ELISA: enzyme linked immunosorbent assay; YMA: Yeast malt agar; TSA: trypticase soya agar; CFU: colony forming units; ADA: agar well diffusion assay; MIC: minimum inhibitory concentration; VCC: viable cell count; MTT:3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide; DMSO: Dimethyl sulfoxide; IZ: Inhibition zone; ND: not determined; PAE: post antibiotic effect.

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