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Original Article

REACTIVE OXYGEN SPECIES GENERATION IN THE ANTIBACTERIAL ACTIVITY OF *LITSEA SALICIFOLIA* LEAF EXTRACT

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ABSTRACT

Objective: The present work was carried out to investigate the antibacterial activity of *Litsea salicifolia* leaf extract and to study whether there is a generation of oxidative stress in its mechanism of antibacterial action.

Methods: *L salicifolia* was screened for its antibacterial activity against the bacterial strains collected from the Microbial Type Culture Collection and gene bank (MTCC) viz. *Escherichia coli* MTCC 443 and *Staphylococcus aureus* MTCC 96. Disc diffusion method was used for screening. The preliminary screening was done with petroleum ether (PE), chloroform (CHF), methanol (MT) and aqueous (AQ) extracts of the *L. salicifolia* leaf. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined using macro-broth dilution method. In this work, oxidative stress on bacterial cells after exposure to plant extract was measured using nitroblue tetrazolium method (NBT).

Results: Experimental evidence indicated that the CHF extract is more efficient against *S. aureus* compared to the other extracts with MIC value of 0.076 mg/ml and MBC value of 0.4 mg/ml. Our results revealed that there was a generation of reactive oxygen species (ROS) in the treated bacterial cell cytoplasm. Transmission electron microscopy (TEM) revealed considerable damage in the cell envelope as well as morphological changes in the extract treated bacterial cells. There were also changes in DNA isolated from treated cells.

Conclusion: From the present study, we can conclude that the active constituents in the plant extract contribute in cell killing involving generation of free radical-induced oxidative stress, which possibly the cause or the consequence of the alteration of some other cellular mechanisms ultimately leading to cell death.

Keywords: Medicinal plant, Antibacterial activity, Oxidative stress

INTRODUCTION

In recent years, although various antimicrobial agents have been newly introduced the increasing development of drug resistance of the infectious microbes has limited the lifespan of these new antimicrobial agents [1]. Clinical microbiologists are applying various strategies to combat drug-resistant microbes, including the search for novel antimicrobial agents from plants. Because from the recent past, plants have been an integral part of traditional medicine system and indigenous people of the world rely on plant-based medicines to cure various diseases. Besides, plant-derived antimicrobial agents are gaining popularity because of easy availability and less side effects [2]. Furthermore, antimicrobial drugs derived from plants will be new to the microbes as antimicrobial drugs of present times are mostly bacterial, or fungal origin [2] and therefore, microbes are less likely to develop resistance against plant-derived antimicrobial drugs.

Plants contain a variety of secondary metabolites such as phenols, quinones, flavonoids, alkaloids, tannins, terpenoids that have been proven to possess antimicrobial activity [3-6]. In most cases, these secondary metabolites are synthesized by plants in response to plant pathogen attack. The mechanism of plant antimicrobial activity is poorly understood, although there are some reports on the probable mechanism of action of phytochemicals such as flavonoids have been reported to inhibit DNA synthesis in *Proteus vulgaris* [7], alkaloids like berberine have been proposed to be potent inhibitors of various enzymes like lactate and malate dehydrogenase [8]. Phenolics were thought to exert their antimicrobial effect by inhibition of enzymes through reacting with sulfhydryl groups containing compounds [9]. Quinone, a class of phytochemicals is known to provide a source of stable free radicals as well as bind to proteins irreversibly inhibiting bacterial growth [10].

Investigation of the mechanism of bacterial killing by the novel antimicrobial agents may provide a better understanding of resistance mechanisms for the advancement of future drugs. Bactericidal antibiotics have been reported to kill bacteria by a common mechanism of cell death involving the induction of ROS, which subsequently results in DNA damage, protein oxidation and lipid peroxidation [11]. Antibiotic like quinolone, a gyrase inhibitor is known to interfere with the iron regulatory pathway resulting information of ROS which eventually cause cell death [12]. Plants are known to produce activated oxygen species to fight against pathogens [13]. The main objective of this study was to find out whether ROS generated oxidative stress is responsible for bacterial killing by *L. salicifolia* leaf extract. Furthermore, morphological changes in bacterial cells due to plant extract treatment were also demonstrated.

L. salicifolia (Roxb. ex Nees) Hook. f. commonly known as 'Dighloti' has been a part of traditional medicine systems in North East India from ancient time. The Apatani people of Ziro valley in Arunachal Pradesh are known to use the fruit of *L. salicifolia* in the treatment of bone fracture and stomach disorder [14]. The leaves of these plants are used in dysentery and pneumonia traditionally in Assam. *L. salicifolia* is cultivated for feeding Muga silk worms in North East India.

Rastogi and Borthakur [15] isolated two alkaloids dicentrinone and nordicentrine from leaves of *L. salicifolia*. There are reports on the antibacterial activity of these two alkaloids [16]. Phytochemical screening [17] of *L. salicifolia* leaf extract showed the presence of flavonoids, alkaloids, terpenoids, and peptides. There are few reports on the antibacterial activity of *Litsea* sp. [18, 19].

There is the previous report on the insecticidal activity of *L. salicifolia* [20]. This is the first report on antibacterial activity and mechanism of action of *L. salicifolia* leaf extract.

MATERIALS AND METHODS

Chemicals and reagents

Solvents (analytical grade) were obtained from Merck (Mumbai, Maharashtra). Chemicals including nitroblue tetrazolium chloride (NBT) and chloramphenicol were obtained from HiMedia Laboratories Pvt. Ltd. Dimethyl sulfoxide was obtained from HiMedia Laboratories Pvt. Ltd.

Preparation of extracts

Fresh leaves of *L. salicifolia* were collected locally from the foothills of Guwahati, Assam and the plant was identified at Botany Department, Gauhati University, Assam (voucher no. GUBH <17869>). The collected leaves were shade dried, and the dried leaves were ground to a fine powder using an electronic grinder. The powdered leaves were extracted with a series of solvents, PE, CHF and MT in the sequence of increasing polarity at room temperature using soxhlet apparatus and water extract was prepared by pouring double distilled water onto the fine powder and kept for 72 h at room temperature. The solvent extract and the filtrate of water extract were evaporated to dryness under reduced pressure using rotary vacuum evaporator [17].

Bacterial strain and culture condition

Two human pathogenic bacteria, one Gram positive (*S. aureus* MTCC 96) and one Gram-negative (*E. coli* MTCC 443) were obtained from MTCC and Gene Bank (MTCC). Strains were maintained at 4 °C on nutrient agar with periodical subculturing every month. The active culture required for bioassay was prepared by inoculation of a loopful of each strain into 10 ml of nutrient broth and incubated in a shaker at 37 °C for 18 h.

Preliminary antibacterial assay

The preliminary antibacterial assay was carried out using disc diffusion method [21]. With the help of a glass spreader standardized inoculums of approximately 1× 10^5CFU/ml (0.5 McFarland standard) were evenly spread over the surface of the plate containing Muller Hinton Agar media (MHA). Discs of 6 mm diameter prepared from Whatman filter paper No. 1, loaded with plant sample were placed on the surface of the medium and kept for 30 min at room temperature under laminar flow for compound diffusion. Chloramphenicol (10 µg per disc) was used as positive control and blank discs with dimethyl sulfoxide (DMSO) were used as negative control. The plates were incubated for 18 h at 37 °. Each experiment was performed thrice and zone of inhibition was recorded in millimeters.

Determination of MIC and MBC

For determination of MIC and MBC macro-broth dilution method was used [22]. The bacteria (10^5CFUs/ml) were grown in tubes containing a different concentration of plant extracts for 18h. MIC was considered to be the lowest concentration of the extract at which there was no visible turbidity. MBC was taken as the lowest concentration at which there was no visible bacterial growth of cultures after re inoculation in fresh media.

Assay of oxidative stress on bacterial cells

Oxidative stress on the bacterial cell was determined using the NBT method [23]. To the bacterial suspensions (10^5 CFU/ml) 0.1 ml of Hanks' balanced salt solution (HBSS) was added and incubated with plant extract for 5, 30, 60, 90 and 120 min time intervals, respectively at 37 ˚C. To each of the solutions, 0.5 ml of NBT at a concentration of 1 mg/ml was added and incubated at 37 ˚C for 30 min. 0.1 M HCL was added to each of the solutions to stop the reaction, and bacterial pellet was obtained by centrifugation. The reduced NBT is extracted out with DMSO and the pellet was diluted with HBSS. Formation of Formazan blue was measured at an absorption spectrum of 575 nm using a spectrophotometer.

Preparation of cells for TEM analysis

Changes in morphology and cell envelope were studied using a high-resolution TEM (JEM 2100; Jeol) with an accelerating voltage of 200 KeV. Bacterial culture of cell density mentioned above was treated with the plant extract for 1 h at 37 ˚C. Untreated control cells were cultured in nutrient broth in the absence of extract. Cell pellets obtained from the treated and control cell suspension were diluted with 0.1M sodium cacodylate buffer and mixed thoroughly with an equal volume of 2% glutaraldehyde and then left for 15 min at 4 ˚C.

The pellet obtained after centrifugation (10000 rpm/10 min) was refixed with 2% glutaraldehyde for 1 h at 4 ˚C. The pellet was then rinsed with 0.1M sodium cacodylate buffer, and post fixation was carried out with 2% osmium tetroxide (OsO_4). The samples were dehydrated with graded acetone series and embedding in the pure embedding medium using Beem capsules. Ultrathin sections were prepared in copper grids and allowed to dry. The sections on the grid were stained using a double staining technique involving uranyl acetate and lead citrate staining.

DNA isolation

Plasmid DNA was isolated [24] from the control and extract treated S. aureus and E. coli cells in order to understand the effect of the extract on bacterial DNA. The bacterial cells were treated for 30 min, 60 min and 90 min. The isolated DNA was analyzed using gel electrophoresis.

Statistical analysis

Student's t-Test was used to study the level of significance (p value<0.05 was considered significant). Each experiment was carried out in triplicate (mean±SD).

RESULTS

Antibacterial activity of *L. salicifolia* leaf extract

In the preliminary screening, all the four extract of *L. salicifolia* leaf showed antibacterial activity against *S. aureus* with CHF extract showing a zone of inhibition equal to that of the positive control (chloramphenicol). *E. coli* appeared to be sensitive only against chloroform extract (table 1).

The MIC and MBC values were depicted in table 2 and table 3 respectively. The MIC values of CHF extract were 0.076±0.02 mg/ml for *S. aureus* and 0.096±0.041 mg/ml for *E. coli*. AQ extract exhibited highest MIC value for *S. aureus*. Although all the four extracts appeared to be inhibitory against *S. aureus*, CHF extract was found to be most effective showing lowest MIC and MBC values.

Table 1: Growth inhibition of *E. coli* and *S. aureus* by PE, CHF, MT and AQ extract of *L. salicifolia*

Plant name	Pathogens	Inhibition zone (mm)					
		PE	CHF	MT	AQ	Cont*	Cont**
Litsea salicifolia (Roxb. ex Nees) Hook. f.	*E. coli*	NI	9.83±0.23	NI	NI	16.17±0.23	NI
	Staphylococcus aureus	15.5±0.4	17.67±0.47	15.33±0.9	8.67±0.9	17.67±0.4	NI

NI: no inhibition; Cont*: positive control; cont**: Negative control, each experiment was carried out in triplicate (mean±SD)

Table 2: MIC of PE, CHF, MT and AQ extract against *S. aureus* and MIC of CHF extract against *E. coli*

Extract	MIC (mg/ml)	
	S. aureus	*E. coli*
PE	0.25±0.05	NI
CHF	0.076±0.02	0.096±0.04
MT	0.38±0.076	NI
AQ	1.33±0.57	NI

NI: No inhibition, each experiment was carried out in triplicate (mean±SD)

Table 3: MBC of PE, CHF, MT and AQ extract against *S. aureus* and MBC of CHF extract against *E. coli*

Extract	MBC (mg/ml)	
	S. aureus	*E. coli*
PE	0.92±0.09	NI
CHF	0.4±0.08	0.65±0.05
MT	2.3±0.5	NI
AQ	4±0.5	NI

NI: No inhibition, each experiment was carried out in triplicate (mean±SD)

Effect of plant extracts on oxidative stress

To investigate the role of oxidative stress on the antibacterial action of the CHF extract we studied the possible involvement of ROS pathway in the present system by performing NBT assay. The results of NBT assay revealed that there is an increase in ROS production with increasing time and reaches maximum in 90 min, after which there was no further increase in the case of *S. aureus* (fig. 1a).

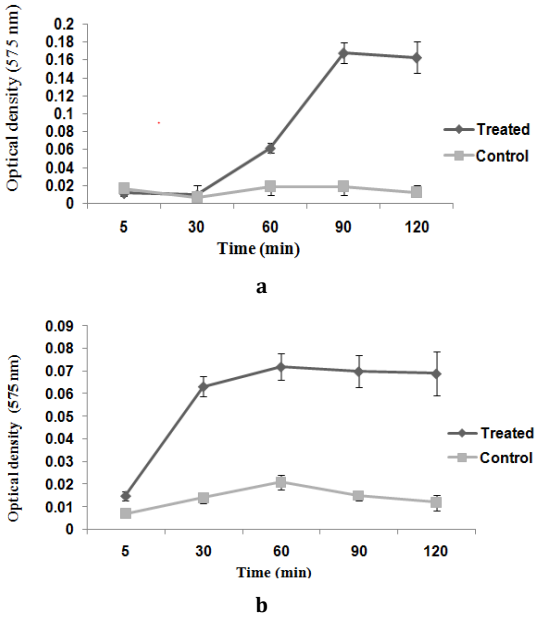

Fig. 1: Effect of chloroform extract on the intracellular ROS concentration of: (a) *S. aureus* (b) *E. coli*

each bar represents mean±SD (n=3), Means of treated are significant (p<0.05) compared to control

In case of *E. coli* also there was an increase in the formation of Formazan blue confirming enhanced ROS production with time, showing the maximum optical density (OD) in 60 min. The control cells showed a negligible increase in oxidative stress in both *S. aureus* and *E. coli* (fig. 1b). Means of treated bacterial cells are significant compared to untreated control, as analyzed by Student's t-test (p<0.05).

Changes in morphology in *S. aureus* and *E. coli* cells treated with *L. salicifolia* CHF extract

Analysis of the external morphological features of the two treated bacterial strains showed significant morphological changes. Untreated *E. coli* cells appeared to be normal in shape with a uniform inner membrane, an undamaged, intact outer membrane and thin periplasmic space (fig. 2a). The treated *E. coli* cells incubated with CHF extract showed swollen periplasmic space and some electron dense material got accumulated in the periplasmic space from the cytosol (fig. 2b and fig. 2c). There was leakage of the cytoplasmic content due to damage of the outer and inner membrane and finally complete disintegration of both the membranes (fig. 2d).

The untreated *S. aureus* cells showed normal coccal morphology under TEM micrographs with intact cell envelope (fig. 3a). The treated *S. aureus* cells showed damaged cell wall and membranes releasing electron dense particles in the surrounding environment (fig. 3b) and some cells appeared to undergo complete lysis (fig. 3c). Some cells were seen without a cell wall (fig. 3d and fig. 3e).

DNA analysis

The plasmid DNA isolated from the control *S. aureus* cells showed two bands on the gel, one band for supercoiled circular DNA and the other for nicked DNA and no DNA could be seen in the loading wells.

However, there was the formation of single-stranded breaks in the plasmid DNA isolated from the treated *S. aureus* and there are negligible, or no supercoiled DNA band could be seen in the gel (fig. 4). DNA could be seen on the loading well in case of treated cells.

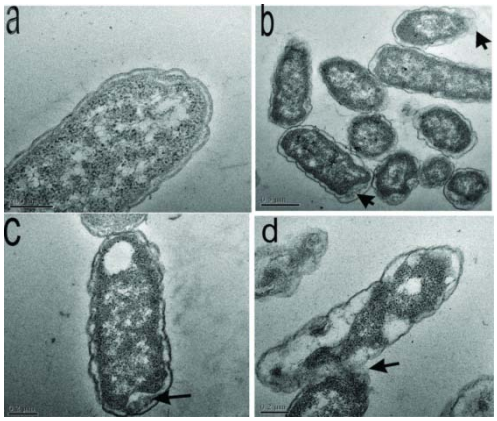

Fig. 2: TEM micrographs of *E. coli*:

(a) Untreated bacteria. (b) and (c) after treatment with CHF extract there is a separation of inner and outer membrane and periplasmic space is swollen, accumulation of some electron dense particle in the periplasmic space (Arrow indicate swollen periplasmic space containing electron dense materials). (d) Leakage of cytoplasmic content due to membrane damage (indicated by an arrow) after treatment

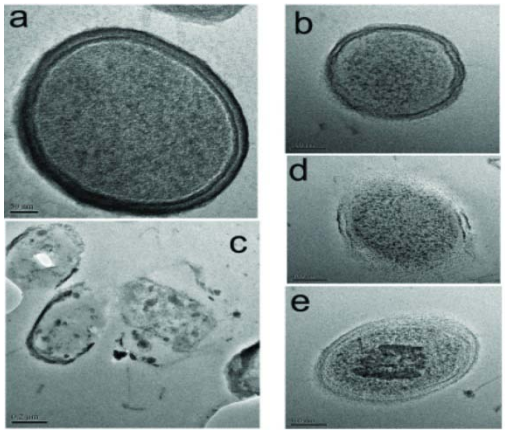

Fig. 3: TEM micrographs of *S. aureus.*

(a) Untreated bacteria. (b) After treatment with CHF extract, there is the disintegration of cell wall and cell membrane, leakage of cytoplasmic content. (c) Complete lysis of some cells after treatment. (d) and (e) some cells are lacking cell wall after treatment

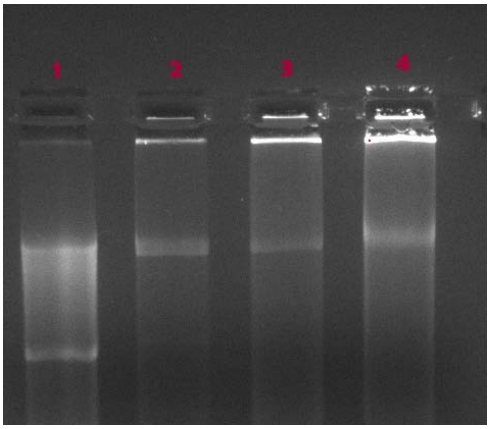

Fig. 4: Plasmid DNA bands analyzed by gel electrophoresis. Lane 1: Control DNA isolated from *S. aureus*, Lane 2: DNA isolated from *S. aureus* cells treated with *L. salicifolia* extracts for 30 min, Lane 3: DNA isolated from *S. aureus* cells treated with extracts for 60 min and Lane 4: DNA isolated from *S. aureus* cells treated with extracts for 90 min

DISCUSSION

Although various plants have been studied for their antibacterial activity [25], their mechanism of action on bacteria is not clearly understood in most cases. In the present work, it is evident from the results that the *L. salicifolia* leaf extracts are more effective against *S. aureus* than *E. coli*. This is due to structural differences in the cell envelope of these two bacteria [26]. Gram-positive bacteria lack the additional outer membrane present in Gram-negative bacteria which is selectively permeable and does not allow some drugs and antibiotics to penetrate the cell. CHF extract was found to be effective against both *E. coli* and *S. aureus*. CHF extracts from plants have been previously reported to provide good antibacterial activity [27-29]. The broad spectrum activity of CHF extract is probably because of its ability to extract phytochemicals active against both Gram-positive and Gram-negative strains.

To detect the level of ROS production in the treated bacterial cells, NBT dye was used. Production of ROS in aerobic cells is a natural process that involves various intracellular and extracellular sources and cells have their own defense mechanism to handle free radical-induced oxidative stress. If the level of oxidative stress exceeds the normal, it can pose threat to the cell. Most of the bactericidal antibiotic such as ciprofloxacin [30] kill bacteria by stimulation of ROS-induced oxidative damage [11]. In the present work, extract treated *S. aureus* and *E. coli* showed a significant increase in ROS production compared to the untreated bacterial cells. The crude plant extract contains a variety of chemical constituents that act synergistically on bacterial cells, leading to the formation of free radicals. Phytochemicals like flavonoids, quinones and peptides are known to form complex with bacterial cell walls and create pores [2]. Possibly the formation of pores in the bacterial cell wall allowed various other chemical constituents in the extracts to penetrate the cell that in turn triggers various biochemical pathways resulting in the production of excessive ROS in the bacterial cytoplasm leading to cell death.

TEM micrographs showed considerable changes in morphology of treated cells. The extract treated *E. coli* cells showed separation of inner membrane from the cell wall and there was disruption of the outer and inner membrane. Some cells were with swollen periplasmic space containing cytoplasmic materials. The innate immune system of plants produces antimicrobial peptides as a first line of defense in response to pathogen attack that is known to interact with membrane lipids [31]. The interaction of the chemical constituents of the crude extract with the outer membrane of Gram-negative bacteria might have caused disruption and thereby destabilizing the outer surface which eventually cause splitting of the inner membrane. Probably disruption of the membranes led to the accumulation of cytoplasmic content in the periplasmic space. Treated *S. aureus* cells also showed cell wall damage. Some cells completely lost their cell wall. The probable reason could be the ability of some plant constituents to interfere with the process of bacterial cell wall formation, thereby causing disruption of cell wall [2]. Similar phenomena were also observed in *Magnolia officinalis* extract treated *S. aureus* cells [32]. *The Aquilaria crassna* leaf extract also reported exerting its antibacterial effect against *S. epidermidis* by disruption of cell wall [33]. There is the previous report that oxidative stress due to ROS production causes alteration of expression of bacterial membrane transporters [34]. Therefore, structural changes and cell membrane damage caused due to the interaction between plant extract and bacterial cells could be the possible cause or consequence of cell death in the present system. The possible reason for the increase in nicked DNA formation and loss of supercoiled DNA in case of CHF extract treated *S. aureus* cells could be due to binding of plant constituents to bacterial DNA or enzymes involved in DNA replication, which resulted in bacterial DNA breakage forming relaxed circular DNA. Plant constituents might have bound to DNA, thereby interfering movement in the gel and thus forming faint bands. There are previous reports on flavonoids and alkaloids showing antibacterial action by binding to DNA [7]. In case of *E. coli* also, there were significant changes in the banding pattern of control and treated cells in the gel.

CONCLUSION

The present work, for the first time provides information for *L. salicifolia* antibacterial activity and its mechanism of action on Gram positive and Gram negative bacteria. *L. salicifolia* possesses potent antibacterial activity and is able to kill bacteria involving generation of ROS induced oxidative stress. In the present study, we performed the antibacterial activity of PE, CHF, MT and AQ extract of *L. salicifolia* leaf. CHF extract appeared to have broad spectrum antibacterial activity. Further experiments to isolate specific compounds from the plant responsible for antibacterial activity are in progress. Cytotoxicity studies of the extracts will be required to provide information on the effect of the extract on the human body.

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CONFLICTS OF INTERESTS

Authors have no conflicts of interest to declare

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