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Research Article

PROSOPIS JULIFLORA LEAF EXTRACT DISRUPTS CELL WALL AND CELL MEMBRANE INTEGRITY of *E. COLI* ISOLATED FROM BOVINE UTERINE INFECTIONS TO EXERT BACTERICIDAL EFFECTS

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ABSTRACT: The medicinal properties of *Prosopis juliflora* is reported in traditional medicines, albeit, its exact mechanism(s) of antibacterial action is largely unknown. Herein, we delineated the cellular mechanism of antibacterial activity of *P. juliflora* leaf (PJ) extract against pathogenic *E. coli*. isolated from uterine infections of bovines. The antibacterial activity of PJ extract was found to be initiated at 6 h post-exposure while the complete bactericidal action was exhibited at 12 h post-exposure. Further, relatively lower MIC values (0.39 mg/ml) against the clinical isolate of *E. coli* also support its prominent antibacterial action. Mechanistically, PJ extract produced substantial damage to the bacterial cell wall and interrupted cell membrane integrity leading to release of the intra-cytoplasmic contents and formation of vacuole resulting in cell death. Thus, PJ extract has the potential to be used as an alternative to synthetic antimicrobials to treat uterine infection. Further, it may facilitate the entry of other antimicrobials inside the bacterial cells, however, further research to determine its *in vivo* efficacy is warranted.

Keywords: *Prosopis juliflora*, Bacterial cell wall, Antibacterial mechanism, Uterine infections, Bovines.

INTRODUCTION

Uterine infections in large animals typically encountered following entry of the causative organisms in the uterus during coitus, insemination, parturition and/or after parturition [1]. Additionally, retention of foetal membrane, abortion, dystocia, mounting by infected bulls, unhygienic practices at insemination, hypocalcaemia, season and inadequate nutrition largely contribute to development of uterine infections and inflammation to the innermost lining of the uterus (endometrium) [2, 3]. Uterine infections and endometritis adversely affect the reproductive performances of livestock by altering the ovarian functions, prolongation of the luteal phase, delaying of postpartum estrus as well as reduction in conception rates [4, 5]. Bacterial strains like *Escherichia coli* and *Trueperella pyogenes* along with anaerobic bacteria such as *Fusobacterium necrophorum* and *Prevotella melaninogenica* are commonly associated with clinical and subclinical endometritis in bovines [6, 7, 8]. The incidence rate of clinical endometritis in cattle varies between 18 to 37 % while subclinical endometritis affects 12 to 94 % cattle populations [9, 10].

Uterine infections and clinical endometritis in livestock is frequently treated with intra-uterine and/or systemic administration of synthetic antimicrobials. However, emergence of microbial resistance, lower recovery rates, high treatment costs, and residue levels in animal-derived foods raise a great public health concerns now-a-days [11]. Hence, use of natural drugs and phyto-therapy emerges as an effective alternative to synthetic antimicrobials in treatment of microbial infections due to the presence of diverse groups of active principles and secondary metabolites.

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Thus, using these natural compounds to treat endometritis, either alone or in combination with other antimicrobials, always opens up an efficient alternative and complementary therapeutic approach to combat resistance and infections.

Prosopis juliflora is a member of the Mimosoideae subfamily of the Leguminosae (Fabaceae) family [12]. Several bioactive compounds and secondary metabolites belonging to tannins, phenolics, flavonoids, alkaloids, terpenes and steroids were isolated from different parts of this plants which are reported to have promising pharmacological activities including anticancer, antidiabetic, anti-inflammatory, antimicrobial, antioxidant effects etc. [13, 14, 15]. Moreover, its use in traditional medicine has also been reported widely [16, 17, 18]. most of the studies reported the efficacy of the several compounds extracted from this plant against food-borne pathogens with no such report against microbes of animal origins. However, recently, we have reported the antibiofilm property of *Prosopis juliflora* leaf extract against *Escherichia coli* isolated from clinical endometritis [19]. Here in the present study, we aimed to delineate the additional cellular mechanism of its antibacterial action against pathogenic *E. coli* isolated from clinical cases of uterine infections from large animals.

MATERIALS AND METHODS

Isolation of bacterial strain

Uterine discharges were collected from cows (23 nos.) and buffaloes (19 nos.) that were presented to Veterinary Clinical Complex, CoV.Sc. & A.H., DUVASU, Mathura, during Sept. 2021 - Dec. 2021, with the history of uterine infection and clinical endometritis. To avoid the vaginal contamination, the samples were collected by the double-guarded method and further incubated overnight in buffered peptone media. These pre-enriched samples were then inoculated in MacConkey agar (HiMedia, India) plates in duplicate. For identification of *E. coli*, the lactose fermenting pink colonies were differentiated from non-lactose fermenter (colourless), and the pink colonies were then sub-cultured on MacConkey agar (HiMedia, India) and Eosin Methylene Blue (EMB) agar (HiMedia, India) plates. The colonies were picked and stored as glycerol stock at -20 ºC for further use. All the selected isolates were further characterized by a panel of biochemical tests, *viz*, catalase, oxidase, ONPG, indole, methyl red, Voges-Prausker, citrate test, and triple sugar fermentation test [29]. Reference strain of *E. coli* (ATCC 25922; HiMedia, India) was also used.

All the suspected isolates were further tested for the presence of *Pap, csgA and csgD* genes for genotyping characterization of *E. coli*. For this, bacterial DNA was isolated by snap chill method. Briefly, a loopful of each isolate was mixed thoroughly with the nuclease-free water in microcentrifuge tube and the suspended isolates were denatured at 100 °C in boiling water for 10 minutes. After heat treatment the boiled cell lysate in microcentrifuge tubes were placed immediately on ice for 10 minutes followed by centrifugation at 12,000 rpm for 2 min. The supernatant (2 µl) was used as DNA template. The primer sequences used in the present experiment are summarized in Table 1.

Preparation of extract

The leaves of *Prosopis juliflora* were collected from the Veterinary College campus during the month of June-July and taxonomical identification of the plant was confirmed from CSRI-Central Institute of Medical and Aromatic Plants (CSRI-CIMAP), Lucknow, with Accession No. 8360. The ethanolic crude extract of the leaf was prepared with the help of soxhlet apparatus and the solvent was evaporated using rotatory vacuum evaporator (Cole-Parmer, USA) under reduced pressure and low temperature (<40°C). The yield of the extract was calculated to be 41.42% (w/w). The extract (thereafter mentioned as PJ extract) was stored in air-tight containers at 4°C for further studies.

Estimation of total phenolic acid

Total phenolic content (TPC) in PJ extract was quantified by Folin–Ciocalteu colorimetric method as described earlier [22]. Different concentrations of the extracts (500, 1000 and 2000 µg/ml) were prepared in ethanol. To each concentration, 10% Folin–Ciocalteu reagent (5 ml) and 7% Na_2CO_3 (4 ml) were added, and the final volume was adjusted to 10 ml. The mixture (blue in colour) was then shaken well and incubated for 30 min at 40°C in water bath. The absorbance was measured at 760 nm against blank using multimode plate reader (Molecular Devices, USA). Gallic acid (25, 50, 75, 100, 250 µg/ml) was used to prepare the standard curve and the intrapolated value for the extract was expressed as gallic acid equivalent (GAE) phenolic content. All the experiments were carried out in triplicates.

Estimation of total flavonoid

Total flavonoid content in PJ extract were estimated by aluminium chloride colorimetric assay [23]. Quercetin was used as standard and to 1 ml of each concentration of quercetin (0.25, 0.5, 0.75, 1.0 and 1.5

mg/ml), 4 ml of distilled water was added. At the same time, 0.3 ml of 5% $NaNO_2$ and 0.3 ml of 10% $AlCl_3$ after 5 min was added to the test tube. After 6 min, 1 M NaOH (2 ml) was added to the mixture and the final volume of the mixture was made to 10 ml with distilled water. The absorbance was measured at 510 nm using multimode plate reader (Molecular devices, USA). Similarly, different concentrations of the extracts (2500, 3000 and 4000 µg/ml) were prepared in ethanol and the reaction was carried out as described for quercetin standard. The absorbance for each concentration of the extracts was recorded and quercetin equivalent (QE) flavonoid content was derived from the standard curve.

Evaluation of *in vitro* antibacterial activity

The *in vitro* antibacterial activity of PJ extract was evaluated by agar well diffusion method. Briefly, bacterial suspensions (equivalent to 0.5 McFarland standard) of clinical isolate (S4) and/or ATCC reference strain were inoculated on sterile Mueller-Hinton agar (MHA; HiMedia, India) plates and wells (9 mm diameter) were punched with the help of well puncture device. The freshly reconstituted (20 mg/ml and 25 mg/ml) PJ extract (100 µl) was used to fill the well and the agar plates were incubated at 37ºC for 18 h. Ethanol was used as vehicle control. Further, broth microdilution method was used to determine the minimum inhibitory concentration (MIC) of PJ extract against clinical isolate (S4) and reference strain [19]. The lowest concentration of PJ extract failed to produce any visible bacterial growth was considered as MIC for the test extract. Cefotaxime was used as positive control.

Time kill assay

Time kill assay was performed to assess the effect of PJ extract on bacterial growth curve [24]. Briefly, the freshly grown bacterial isolate was adjusted to visually equivalent turbidity of 0.5 McFarland standard and diluted with MHB to achieve 1:100 dilution. From this dilution, 1 ml each was transferred to two 50 ml sterile tubes containing 10 ml MHB. To one of the tubes, PJ extract (equivalent to 2 MIC) was added, and the other tube was taken as growth control. Immediately after mixing the bacterial suspension in both the tubes, 200 µl was aliquoted from each tube and 100 µl was serially diluted (10-fold) for 6 dilutions. One hundred microliter from first three dilutions (10^{-2}, 10^{-3}, 10^{-4}) from the treatment and control groups were then platted on sterile MHA plates and the process was repeated after 2, 4, 6, 8, 12, 16, 18 and 24 h. For 12, 16, 18 and 24 h lower dilutions (10^{-5}, 10^{-6}, 10^{-7}) were taken for ease in colony counting. The plates were incubated for 18 h and the colonies were counted using colony counter. The experiment was carried out in triplicate and in at least two independent experiments was averaged. The results were expressed as logarithms with corresponding standard errors (mean ± SEM).

Bacterial cell membrane integrity test

The uptake of fluorescent dye by the bacterial cell was evaluated to test the bacterial cell membrane integrity in the absence and presence of PJ extract. Briefly, to 100 µl of bacterial suspension (equivalent to 0.5 McFarland) in Mueller-Hinton broth (MHB; HiMedia, India), PJ extract (equivalent to 2 MIC) was added and incubated at 37 °C for 12 h. The bacterial pellet was then washed twice in PBS by centrifuging at lower speed (3000 rpm for 5 min). The final pellet was dissolved in PBS and to this 1.5 µl CFDA-AM (1 µM) per 200 µl bacterial pellet was added and incubated at 37°C for 15 min. The bacterial suspension was further incubated with 3 µl PI (5 µM) per 200 µl of bacterial pellet and incubated at 37°C for 5 min. Following washing, the supernatant was discarded, and the pellet was resuspended in 200 µl PBS. From this suspension, a thin smear was prepared and visualised under fluorescent microscope. Cefotaxime was used as positive reference control.

Transmission electron microscopy

To elucidate the target site and possible mechanism of action of the extract, transmission electron microscopy (TEM) was performed. The bacterial suspension (equivalent to 0.5 McFarland standard) was exposed to either 2MIC or 4 MIC concentration of PJ extract for 12 h at 37ºC. Cefotaxime was used as positive reference control. The samples were then centrifuged at 3000 rpm for 10 min and the bacterial pellets were then further washed thrice with PBS (pH 7.4) by centrifugation at lower speed. The washed pellet was further suspended in fixative (2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M PBS) and processed for electron microscopy from AIIMS, New Delhi, India.

Data analysis

Results are expressed as mean ± SEM. 'n' denotes the number of replicates in each experimental protocol. Mean values from two groups were analyzed by student's t test using Graph Pad Prism V.4.00 (San Diego, California) and difference in values was considered statistically significant at $p<0.05$.

RESULTS AND DISCUSSION

Isolation and identification of *E. coli* from clinical samples

Twenty-three isolates from 42 clinical samples were found to exhibit 'metallic sheen' on EMB agar and a characteristic pink coloured colony on MacConkey's agar, thus indicative of lactose fermenter. Following microscopic examination, these organisms were found to be Gram-negative rod-shaped. Biochemically, these isolates (9 out of 23 isolates) showed negative for the VP and citrate utilization tests but positive for the indole, methyl red, catalase, ONPG, and sugar fermentation tests. Genotypic characterization of these nine isolates was carried out by amplifying the virulence genes (*viz. Pap, csgA* and *csgD*) from their genomic DNA. ATCC 25922 was used as reference strain for *E. coli*. *Pep1* (336 bp), *csgA* (408 bp), and *csgD* (601 bp) PCR products were amplified only from the genomic DNA of the clinical isolate of S4. Thus, subsequent study was carried out using S4 clinical isolates.

Phytochemical analysis of the extract

The average gallic acid equivalent (GAE) phenolic contents in PJ extract was calculated to be 120.68 ± 1.36 mg/g extract while the average quercetin equivalent (QE) flavonoid contents in PJ extract was calculated to be 148.48 ± 3.27 mg/g extract.

In vitro antibacterial activity of the extract

Using agar well diffusion assay, the antibacterial activity of PJ extract was assessed against the reference strain (ATCC 25922) and clinical isolate (S4) of *E. coli*. In comparison to clinical isolate (S4), PJ extract at both the concentrations (20 mg/ml and 25 mg/ml) exhibited relatively larger zone of inhibition against the reference strain (Table 2). Nonetheless, a marked zone of inhibition was also noted against S4 clinical isolate. Interestingly, PJ extract did not show any concentration-dependent increase in *in vitro* antibacterial activity against the tested organism.

The broth microdilution assay was used to determine the MIC values of PJ extract against the reference strain and clinical isolate of *E. coli.* The MIC values against both S4 clinical isolate and reference strain were calculated to be 0.39 mg/ml.

The growth kinetic of S4 isolate at different time intervals (*viz*. 0, 2, 4, 6, 8, 12, 16, 18 and 24 h) in the absence and presence of PJ extract and/or cefotaxime is illustrated in Fig. 1. The antimicrobial action of PJ extract was revealed from the notion that the growth of S4 isolate was significantly ($p<0.05$) attenuated from 6h post-exposure onwards and complete bactericidal action was exhibited at 12 h post-exposure. Cefotaxime produced its bactericidal effect at 6 h post-exposure.

Effect on cell membrane integrity

As depicted in Fig. 2, the highest level of green fluorescence was visible in negative control slides for both S4 isolate and reference strain indicating the presence of live bacteria with intact cell membrane.

Table 1. Description of primers.

Gene	Sequence (5'-3')	Amplicon Size (bp)	Annealing Temp. (°C)	References
Pap	F-GCAACAGCAACGCTGGTTGCATCAT R-AGAGAGAGCCACTCTTATACGGACA	336	63	[20]
csgA	F-CAGCAATCGTATTCTCCGGTA R-CGTTGTTACCAAAGCCCAACC	408	58	[21]
cgsD	F-TTATCGCCTGAGGTTATCGTTT R-TAAATCTTCTTTGCAGGCGACA	601	58	[21]

Table 2. Comparative zones of inhibition (mm) produced by PJ extract against clinical isolate of *E. coli* (S4) and ATCC reference strain.

Organism tested	Zone of inhibition (mm)	
	PJ extract	
	20 mg/ml	25 mg/ml\
S4 isolate (n=7)	$13.78^{a} \pm 0.55$	$15.11^{b} \pm 0.61$
ATCC 25922 (n=5)	$20.00^{b} \pm 1.54$	$17.5^{b} \pm 0.93$

* Data are presented as Mean ± SEM. Data were analysed by student's t-test. Mean values with different superscripts within a column are statistically significant ($p<0.05$).

However, in the presence of PJ extract, greater number of cells emitted red fluorescence, thus indicating that PJ extract impaired the integrity of the cell membrane for both the strains. Exposure to cefotaxime also showed presence of greater number of PI-positive cells with compromised membrane integrity. Thus, PJ extract exhibited comparable effect on bacterial cells as that

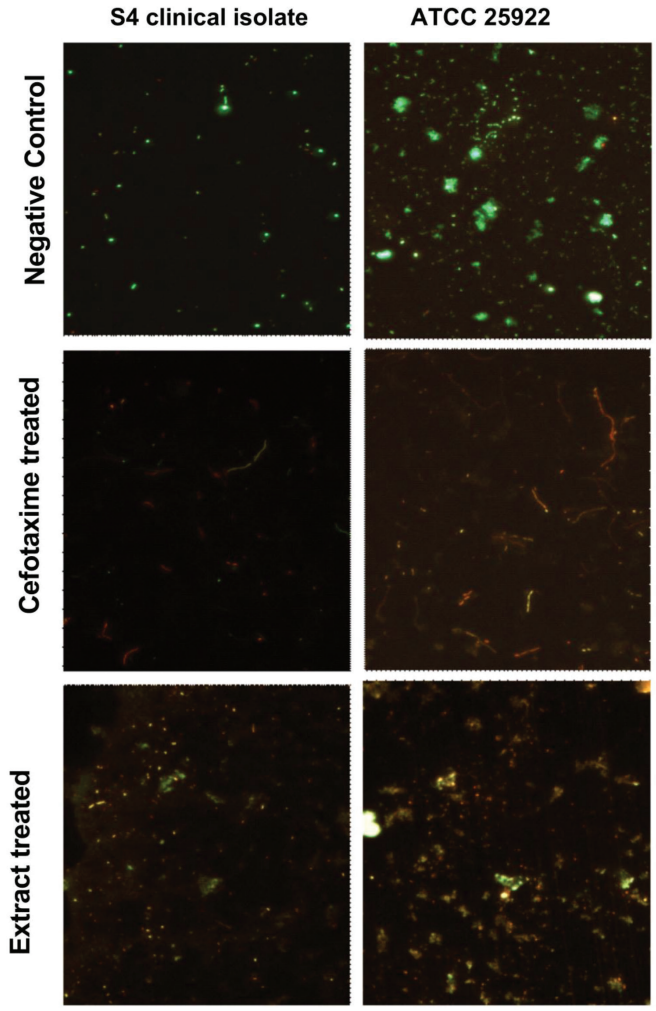

Fig. 2. Effect of *P. juliflora* leave (PJ) extract on cell membrane integrity of clinical isolate (S4) as well as reference strain (ATCC 25922) of *E. coli* as detected by fluorescent microscopy.

observed with cefotaxime.

Effect on bacterial cellular ultrastructure

Fig. 3-6 summarizes representative TEM slides from different groups. Gr-I showed normal bacterial structure with intact cell wall, cell membrane and cytopalsmic contents (Fig. 3a-d). During the binary division the bacteria cells from Gr-I exhibited intact cell membranes (Fig. 3a & b) and uniform distribution of cytoplasmic contents (Fig. 3c & d). Exposure to cefotaxime (Gr-II; positive control) resulted in aberrant bacterial morphology with ruptured cell wall and a loss of cell membrane integrity (Fig. 4a-d). Non-uniform distribution of cytoplasmic contents during binary fusion (Fig. 4b), vacuolation of the cells (Fig. 4c) and leakage of cytoplasmic contents resulting from loss of membrane integrity (Fig. 4a & d) were also observed in the S4 isolate following treatment with cefotaxime. Exposure to lower dose (Gr-III; 2 MIC) of PJ extract also resulted in aberrant bacterial morphology, including ruptured cell wall and lack of cell membrane integrity (Fig. 5a). There was shrinkage of cytoplasmic contents and translucent cytoplasm (Fig. 5b & c) along with detachment of cell membrane (Fig. 5c) and misshapen cells with cytoplasmic vacuolation (Fig. 5d) were noticeable. Exposure to relatively higher concentration of plant extract (Gr-IV; 4 MIC) produced more detrimental effect as evidenced by appearance of abnormal bacterial morphology with intensely disrupted cell wall and loss of integrity of cell membrane (Fig. 6a-d).

The ethanolic PJ extract showed encouraging antibacterial activity against both reference strain and the clinical isolate of *E. coli* (S4) in the current investigation. However, when the extract concentration was raised from 20 mg/ml to 25 mg/ml, the zone of inhibition against these bacterial strains did not increase noticeably. The promising antibacterial activity was further substantiated by its relatively lower MIC value (0.39 mg/ml) against both the reference and clinical strains of *E. coli.* Additionally, the antibacterial effect of PJ extract against S4 isolate was observed to initiate six hours post-exposure, and the complete bactericidal action was achieved at twelve hours later. These findings clearly imply that the antibacterial activity of PJ extract increases with duration of exposure. In agreement to our observations, MIC values against most common Gram-positive and Gram-negative bacteria are reported to varies from 25 µg/ml to 100 µg/ml and the alkaloid fractions of *P. juliflora* leaf extract believed to have the most antibacterial activity when compared to other plant components [25]. The antibacterial efficacy of an ethanolic extract of *P. julifora* leaf, pods, and flowers against a variety of bacterial strains was also reported [26] which is in line with our findings. Recently, we have also reported the antibacterial efficacy of PJ extract against *E.coli* by reducing the biofilm producing ability of the pathogen [19]. The antibacterial effectiveness of *P. juliflora* leaf extract against food spoiling isolates of *S. aureus* and *E. coli* at a concentration of 50 mg/ml was recently reported [27]. Furthermore, according to a report [28], the aqueous

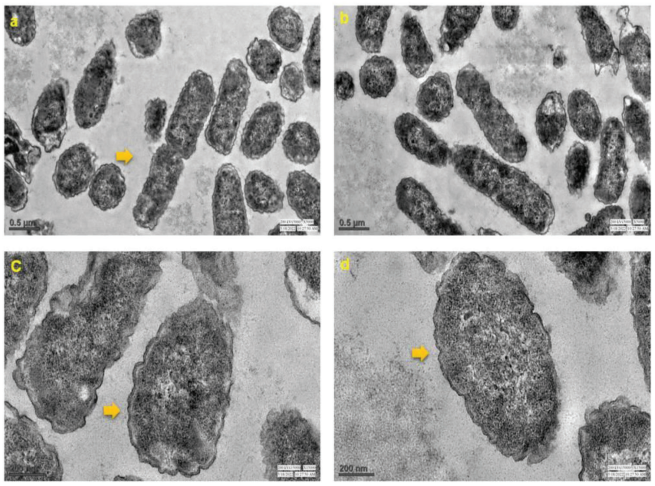

Fig. 3. Representative electron microscopy (TEM) slides from Gr-I showing normal bacterial structure with intact cell wall, cell membrane and cytoplasmic contents (a,b,c,d). [The bacteria undergo normal binary fusion with intact cell membrane (a,b) and uniform distribution of cytoplasmic contents (c, d)].

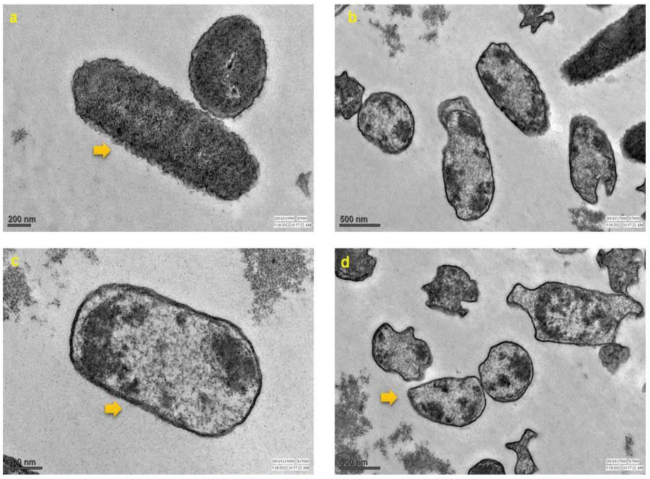

Fig. 5. Representative electron microscopy (TEM) slides from Gr-III showing abnormal bacterial morphology with disrupted cell wall and loss of integrity of cell membrane. [(a). There was shrinking of cytoplasmic contents and translucent cytoplasm (b,c) along with detachment of cell membrane (c). Appearance of misshapen cell (d) with cytoplasmic vacuoles was also prominent following exposure of bacterial cells to *P. juliflora* leave extract (2 MIC)].

extract of *P. julifora* leaf exhibits antibacterial activity against *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Staphylococcus aureus,* and *Enterococcus faecalis*.

Numerous bioactive compounds including terpenes, phenolics, alkaloids, tannins, and flavonoids are reported

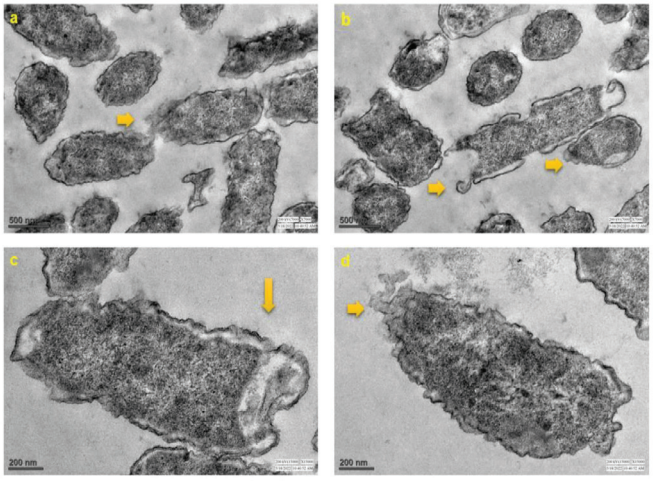

Fig. 4. Representative electron microscopy (TEM) slides from Gr-II showing abnormal bacterial morphology with disrupted cell wall and loss of integrity of cell membrane (a,b,c,d). [Damage of the cell membrane integrity caused leakage of the cytoplasmic contents (a,d). The cell wall was also damaged during binary fusion with non-uniform distribution of cytoplasmic contents (b). Appearance of vacuolation along with detached cell membrane (c) was seen following cefotaxime treatment].

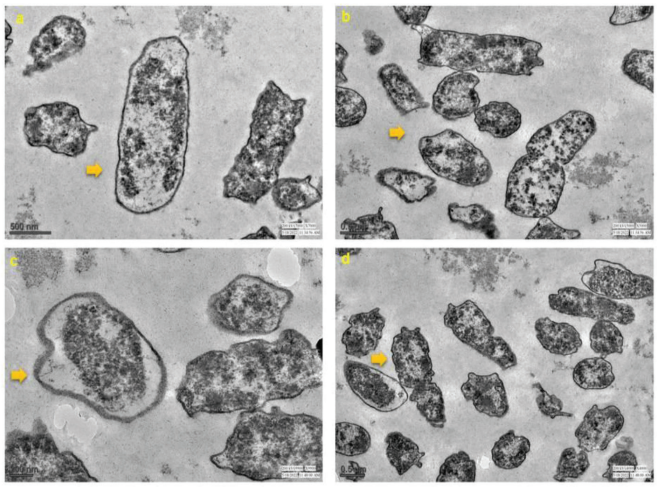

Fig. 6. Representative electron microscopy (TEM) slides from Gr-IV showing abnormal bacterial morphology with intensely disrupted cell wall and loss of integrity of cell membrane (a,b,c,d). [There was clumping of cytoplasmic contents with vacuole formation (a,b) and loos of cell wall integrity (c). Detachment of cell membrane with misshapen cells (b,c) along with condensation and aggregation of chromatin (c,d). Improper distribution of cytoplasmic contents during binary fusion along with loss of cell wall structure (b,d) following exposure of bacterial cells to *P. juliflora* leave extract (4 MIC)].

to be present in *P. juliflora* that are responsible for its antibacterial activity [14, 15]. In the present study we also observed a substantially higher level of flavonoids (148.48 ± 3.27 mg QE/g extract) and total phenolic acid (120.68 ± 1.36 mg GAE/g extract) in PJ extract which are in agreement with the previous findings [29]. Furthermore, this plant contains a number of chemical components that belong to the phytosterol (β-sitosterol), terpenoid diketone (prosopidione), and alkaloid bioactive molecules (secojuliprosopinal, 3'oxojuliprosopine, and juliprosopine) [30].

To better understand the mechanism of antibacterial action of PJ extract, the cellular morphology of bacterial cells treated with the extract was examined. As demonstrated by transmission electron microscopy and fluorescence microscopy, PJ extract mainly destroyed the bacterial cell wall and/or cell membrane in order to produce its bactericidal effect. During bacterial division, it also altered the distribution of the cytoplasmic components. Therefore, by changing the membrane permeability of exposed bacterial populations, PJ extract may facilitate the entry of other conventional antimicrobials inside the bacterial cells and thereby may enhance their effectiveness. Additionally, changes in the cytoskeletal matrix in the *E.coli* were possibly responsible for the appearance of intracytoplasmic vacuole formation after exposure to PJ extract in the current investigation. Furthermore, PJ extract altered the size and form of the exposed bacterial population. The bioactive compounds in the crude extract may alter the protein structure and thereby the fluidity of cell membranes, resulting in membrane permeabilizing actions as reported in other instances [31, 32]. Thus, isolation and identification of the major bioactive phytoconstitutents responsible for this promising antibacterial action may aid to discover novel therapeutic strategy against the most common pathogens of uterine infections in livestock.

CONCLUSION

Taken together, the promising antibacterial action of *P. Juliflora* leaf extract against clinical isolate of *E. coli* from uterine infections in bovines is mediated by disruption of the cell membrane and cell wall integrity resulting in formation of intra-cytoplasmic vacuole and release of cytoplasmic contents. Thus, bioactive compounds in *P. juliflora* leaf extract may be a potential source of antibacterial agent to treat uterine infections in livestock. However, further investigation regarding the *in vivo* validation and safety profiling of the bioactive compounds are warranted.

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