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ORIGINAL PAPER

Antibacterial, antibiofilm, and anti-adhesion activities of *Piper betle* leaf extract against Avian pathogenic *Escherichia coli*

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Abstract

Piper betle leaves have traditionally been used to treat many diseases, including bacterial infections. The present study aimed to investigate the antibacterial, antibiofilm, and anti-adhesion activities of *P. betle* extract against avian pathogenic *Escherichia coli* (APEC). The ethanol extract of *P. betle* leaves demonstrated strong antibacterial activity against clinical isolates of APEC with MIC and MBC values ranging from 0.5 to 1.0 mg/mL as compared with 1% DMSO, a negative control. Disruption and breakdown of the bacterial cells were detected when the cells were challenged with the extract at 2 × MIC. Bacterial cells treated with the extract demonstrated longer cells without a septum, compared to the control. The extract at 1/8, 1/4, and 1/2 × MIC significantly inhibited the formation of the bacterial biofilm of all the tested isolates except the isolate CH10 ($P < 0.05$) without inhibiting growth. At 1/2 × MIC, 55% of the biofilm inhibition was detected in APEC CH09, a strong biofilm producer. At 32 × MIC, 88% of the inhibition of viable cells embedded in the mature biofilm was detected in APEC CH09. Reduction in the bacterial adhesion to surfaces was shown when APEC were treated with sub-MICs of the extract as observed by SEM. Hydroxychavicol was found to be the major compound presented in the leaf extract as detected by GC–MS analysis. The information suggested potential medicinal benefits of *P. betle* extract to inhibit the growth, biofilm, and adhesion of avian pathogenic *E. coli*.

Keywords *Piper betle* · Avian pathogenic *Escherichia coli* · Antibacterial activity · Antibiofilm activity

Introduction

Escherichia coli (*E. coli*) is a Gram-negative commensal bacterium that can be found in the intestinal tract in humans and various species of animals. Although most strains are harmless, some strains can cause fatal diseases (Mare et al. 2021). In addition, the bacterium is a versatile microorganism that contains many pathotypes that cause a variety of diseases. The pathogen is the cause of enteric diseases, such as diarrhea or dysentery (Gomes et al. 2016), referred to diarrheagenic *E. coli* (DEC) strains. Conversely, extra-intestinal pathogenic *E. coli* (ExPEC) represents an emerging pandemic strain that plays roles in infections outside

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the intestinal tract, such as the urinary tract infections (UTIs), bacteremia, and meningitis (Cunha et al. 2017). ExPEC pathotypes are classified into uropathogenic *E. coli* (UPEC), septicemia-associated *E. coli* (SEPEC), neonatal meningitis causing *E. coli* (NMEC), and Avian pathogenic *E. coli* (APEC) that lead to UTIs, septicemia, neonatal meningitis, and colibacillosis in poultry, respectively (Meena et al. 2021). Avian pathogenic *E. coli* (APEC), a causative agent of colibacillosis, is an important pathogen that causes economic losses in poultry production worldwide. Several studies have reported that APEC is highly similar to UPEC and NMEC in humans (Cunha et al. 2017; Jørgensen et al. 2019; Zhuge et al. 2021). Moreover, APEC was considered zoonotic and serves as an external source for human extra-intestinal infections (Kathayat et al. 2021; Stromberg et al. 2017). Hence, poultry can act as a cause of APEC infections in humans. Furthermore, APEC becomes an important pathogen for the poultry industry and public health worldwide.

APEC has a broad selection of virulence factors that promote adhesion and biofilm formation in pathogenesis. Adhesion is the first step in the progress of bacterial infection, followed by colonization to host cells (Ali et al. 2020). The process occurs in matrices that resemble bacteria attached to the pedestal, enabling the formation of biofilms (Rodrigues et al. 2019). Biofilm formation is one of the hallmark strategies used by bacteria to survive in harsh environments (Gu et al. 2019). Due to the development of biofilms, APEC becomes aggregated and difficult to eradicate (Sharma et al. 2016). This mechanism enables pathogenic bacteria to tolerate higher concentrations of antibiotics, leading to chronic infection and the emergence of antibiotic-resistant strains (Gu et al. 2019; Hall et al. 2017; Khairy et al. 2020). Furthermore, the treatment of APEC infections is becoming difficult due to the increasing antibiotic resistance of the pathogenic strains (Duan et al. 2020; Saha et al. 2020).

To overcome the antibiotic-resistant APEC, alternative therapeutics based on natural products have been investigated. In literature, medicinal plants have been reported for antimicrobial activity against *E. coli*, such as seeds of *Annona senegalensis* (Tamfu et al. 2020), and *Rosmarinus officinalis* (Lagha et al. 2019). The present study focused on *Piper betle* (*P. betle*), known as a betel vine. The plant species belongs to the Piperaceae family, a plant native to South and Southeast Asia. Leaves of this plant species have been used in herbal medicine to treat several infectious diseases (Pecková et al. 2018). *P. betle* has antioxidant, antibacterial, and antifungal activities due to its phytochemicals and essential oil (Salehi et al. 2019). The antibacterial activity of the ethanol extract of *P. betle* leaf against *E. coli* has been reported as a bactericidal agent (Nayaka et al. 2021). Furthermore, silver nanoparticles synthesized by *P. betle* extract exhibited antibacterial and antibiofilm activities against several Gram-positive bacteria (Srinivasan et al. 2018).

To the best of our knowledge, no study on this local plant against Avian pathogenic *E. coli* has been scientifically reported. Therefore, the aims of this study were to investigate the antimicrobial activity and the antibiofilm formation ability of *Piper betle* leaf against Avian pathogenic *E. coli*. The antibacterial activity was investigated using disk diffusion assay, broth microdilution assay, and time kill kinetic study. Subsequently, the effects of the extract on the biofilm formation, established biofilm, and adhesion of the pathogens were determined. Finally, the morphology of APEC treated with the extract was observed by scanning electron microscope.

Materials and methods

Bacterial strains and growth condition

Ten clinical isolates of Avian pathogenic *Escherichia coli* including CH01, CH02, CH03, CH04, CH05, CH06, CH07, CH08, CH09, and CH10 isolated from chicken were used and classified as APEC by our research team (Ethical approval with Ref. No. WU-AEC-016-62) as described (Thomrongsuwannakij et al. 2020). The bacteria were isolated from broilers and broiler breeders with colibacillosis in Thailand. Then, five virulence-associated genes including *iroN*, *ompT*, *hlyF*, *iss* and *iutA* were detected using pentaplex PCR as previously described (Thomrongsuwannakij et al. 2020). *E. coli* ATCC25922 (American Type Culture Collection, Manassas) was used as a reference strain. The bacteria were grown on Tryptic Soy Agar (TSA) (Difco, Claix, France) and incubated at 37 °C for 24 h. The pathogens were then inoculated in Tryptic Soy Broth (TSB) (Himedia, Nashik, India) at 37 °C for 18–24 h, stored in TSB containing 25% glycerol at − 80 °C until use.

Preparation of plant extracts

50 g of dry powder of *P. betle* leaves was extracted in 200 mL of 95% ethanol for 7 days at room temperature as described by (Pieczykolan et al. 2019; Mitsuwan et al. 2020a, b). The sample was filtered and then evaporated under reduced pressure. The extract was air-dried at room temperature to eliminate the rest of the solvent, and to maintain the weight of the extract. Then, the extract was balanced daily until the weight stable. The extract was dissolved in 100% dimethyl sulfoxide (DMSO) and stored at 4 °C.

Disk diffusion assay

The disk diffusion method was used to screen the antibacterial activity of *P. betle* extract against clinical isolates of APEC as previously described (Jaber et al. 2021). Briefly,

12.5 µL (200 mg/mL) of plant extract was applied to sterile filter paper disks (6 mm diameter) to give a final concentration of 2.5 mg/disk. Cultures of the bacteria were adjusted to the McFarland No. 0.5 standard and spread on Mueller–Hinton agar (MHA) (Difco, Claix, France) plates. The disks were placed onto the bacterial cultured plates and incubated at 37 °C for 18 h. *E. coli* ATCC25922 was included as the reference strain. Ampicillin (Oxoid, Hampshire, UK) and gentamicin (Oxoid, Hampshire, UK) were used as positive controls. Meanwhile, a disk loaded with 1% DMSO was used as a negative control. The antibacterial activity was evaluated by measuring the diameter of the inhibition zones (mm). The experiment was performed in triplicate. The data were presented as mean ± SD.

Determination of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

The antibacterial activity of the extract against the clinical isolates was determined using the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) as previously described (Jaber et al. 2021). Briefly, the *P. betle* extract was diluted in a 96-well microtitre plate to the final concentration ranging from 2.0 to 0.125 mg/mL in Mueller–Hinton broth (MHB) (Difco, Claix, France). The bacterial suspension (100 µL, 1×10^6 CFU/mL) was inoculated to each well and incubated at 37 °C for 18 h. *E. coli* ATCC25922 was included as the reference strain. Ceftriaxone and 1% DMSO were used as the positive and negative control, respectively. Then, 0.03% of resazurin (Thermo Fisher Scientific, Lancashire, UK) was used to determine the MIC values. The MIC was defined as the lowest concentration that completely inhibited the bacterial growth that present a blue color (Pereira et al. 2021). The MBC was performed with the extract that gave significant MIC values, by streaking the culture on TSA plates. Experiments were assessed in triplicate.

Time-kill study

Time-kill kinetic study of the extract against a representative strain of Avian pathogenic *E. coli* CH09 was investigated. An inoculum (1×10^6 CFU/mL) of the bacterial cultures was grown in the tested medium supplemented with the plant extract at 1, 2, and 4 times of MIC and incubated at 37 °C. A final concentration of DMSO presented in the extract was 1%. Hence, one percent DMSO was used as a negative control. Samples were collected at 0, 2, 4, 6, 8, 12, 18, and 24 h. At each time point, 100 µL of the sample was serially diluted in sterile phosphate-buffered saline (PBS). Viable counts were determined by a drop plate method in TSA. The plates were incubated at 37 °C for 24 h. The experiment was carried out in triplicate and the results were presented as mean log numbers of organisms ± standard deviation.

Biofilm forming ability of Avian pathogenic *E. coli*

The biofilm forming ability of the isolates of Avian pathogenic *E. coli* was determined by crystal violet assay. All clinical isolates of *E. coli* and the reference strain were grown in TSB supplemented with 1% glucose and incubated at 37 °C for 18–24 h. An inoculum (1×10^6 CFU/mL) of the cultures was then grown in the tested medium in 96-well plates and incubated at 37 °C for 24 h. The wells containing biofilm were gently washed with PBS and air-dried. The biofilm was stained with 0.1% (w/v) crystal violet solution (200 µL) for 30 min. Wells were rinsed three times with distilled water to remove excess dye. The biofilms were dissolved in ethanol and measured at an optical density (OD) 570 nm. The biofilm produced by the pathogen was evaluated as described (Gajewska et al. 2020),

OD isolate ≤ OD control = Non biofilm forming ability.

OD control < OD isolate ≤ 2OD control = Weak biofilm forming ability.

OD control < OD isolate ≤ 4OD control = Moderate biofilm forming ability.

4OD control ≤ OD isolate = Strong biofilm forming ability.

Effects of *P. betle* extract on biofilm formation of Avian pathogenic *E. coli*

The effects of *P. betle* extract on biofilm formation of Avian pathogenic *E. coli* were investigated using the crystal violet assay as described (Mitsuwan et al. 2020a, b). Briefly, an inoculum (1×10^6 CFU/mL) of the overnight cultures of the bacteria was then grown in the medium tested in 96-well plates supplemented with *P. betle* extract at 1/2, 1/4, and 1/8 × MIC. One percent DMSO was used as a negative control. The plates were incubated at 37 °C for 24 h. The activity of the extract in the biofilm formation was investigated by crystal violet assay as described above. The inhibitory activity was investigated by the quantification of biofilm formation at the optical density of 570 nm. The relative percentage of biofilm formation was defined as: ($OD_{570\,nm}$ with extract/$OD_{570\,nm}$ without extract) × 100.

Effects of *P. betle* extract on established biofilm of Avian pathogenic *E. coli*

The effects of *Piper betle* extract on the established biofilm of Avian pathogenic *E. coli* were assessed in 96-well plates, as described (Mitsuwan et al. 2020a, b) with modification. Briefly, the overnight culture of *E. coli* (1×10^6 CFU/mL) was added to 96-well microtiter plate and incubated at 37 °C

for 2 days to develop young established biofilm and 5 days to develop mature established biofilm. Each well was replaced with new medium every 2 days. Then, the extract at concentrations of 8–32 times of MIC was added to eradicate the established biofilms, incubated at 37 °C for 24 h. The final concentration of DMSO was 1%, and this concentration of DMSO was used as a negative control. To assess the effect of the extract on established biofilms, the medium was removed and 200 µL of PBS with 10 µL of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, MTT, 5 mg/ml, (Invitrogen, Oregon, USA) was replaced, incubated at 37 °C for 2 h. The MTT assay was used to determine the living bacterial cells. Formazan, the purple insoluble crystals, were dissolved by adding DMSO into each well. Then, the samples were measured at 570 nm by a microplate reader to determine the relative percentage of viability of the biofilm. The relative percentage of the viability was defined as: ($OD_{570\,nm}$ with extract/$OD_{570\,nm}$ without extract) × 100.

Effects of *P. betle* extract on adhesion of Avian pathogenic *E. coli*

The effects of *P. betle* extract on adhesion of APEC were observed by scanning electron microscope (SEM), as described (Mitsuwan et al. 2020a, b) with modification. Briefly, the bacterial cells were cultured in TSB supplemented with 1% glucose, incubated at 37 ℃ for 18 h. The bacterial cells (1×10^6 CFU/mL) were then attached to a sterile glass coverslip (0.5 cm × 0.5 cm) in a 24-well plate containing the extract in concentrations 1/2 and 1/4 × MIC. One percent DMSO was used as a negative control. The samples were incubated at 37 °C for 24 h. Then, the samples were rinsed twice with PBS. Subsequently, glutaraldehyde fixation (2.5% in PBS) was carried out for 2 h. The samples were washed with PBS and dehydrated in a series of graded ethanol (20–100%). The samples were dried in a critical point dryer and coated with gold particles. The adhesion of the bacteria was observed under SEM (Zeiss, Munich, Germany) at The Center for Scientific and Technological Equipment, Walailak University.

Morphology of Avian pathogenic *E. coli* after treatment with *P. betle*

The effects of *P. betle* extract on morphology of the APEC were investigated by SEM as previously reported (Mitsuwan et al. 2020a, b) with some modification. Briefly, the bacterial cells were cultured in MHB, incubated at 37 ℃ for 18 h. The bacterial cells (1×10^6 CFU/mL) were then grown in a centrifuge tube containing the extract at 2 × MIC and 4 × MIC, incubated at 37 ℃ for 24 h. The final concentration of DMSO was 1%, and this concentration of DMSO was used as a negative control. The samples were then centrifuged at 5000 rpm for 5 min. The bacterial pellet was dropped on a sterile glass coverslip (0.5 cm × 0.5 cm), and air-dried. Specimens were fixed in 2.5% glutaraldehyde for 2 h. Then, the samples were dehydrated a series of graded ethanol (20–100%) for 30 min of each step. The specimens were then mounted on aluminum stubs and allowed to dry using a critical point dryer. The samples were then coated with gold particles. Finally, the morphology of the bacteria after treatment with the extract was observed by SEM (SEM-Zeiss, Munich, Germany) at The Center for Scientific and Technological Equipment, Walailak University.

Gas chromatography–mass spectrometry (GC–MS) analysis

To identify the phytochemicals in *P. betle* leaf extract, GC–MS analysis was determined using an Agilent Technologies 7890 B (GC) equipped with 5977A Mass Selective Detector (MS). Briefly, a VF-WAXms capillary column of dimensions 30 m × 250 × 0.25 µm was used with helium gas as the carrier at a flow rate of 1 mL/min. The column temperature was programmed initially at 60 °C, which was increased to 160 °C at 10 °C min^{-1}. Then, the temperature was further increased to 250 °C at 2.5 °C min^{-1}, held time for 15 min. The mass spectrometer was carried out in the electron ionization mode at 70 eV with a source temperature of 230 °C, with continuous scanning from 35 to 500 m/z. The phytochemicals in *P. betle* leaf extract were identified by comparing their mass spectral data with those from the Wiley library.

Statistical analysis

The experiments were performed in triplicate. The results were recorded and entered using the statistical package version 19 (SPSS Inc.Chicago, IL, USA). The results were expressed as mean ± SD. Comparisons between means were carried out according to Duncan's test. In addition, the two-tailed unpaired Student's *t* test was also used. Differences were considered significant at $P < 0.05$.

Results

Preliminary screening of antibacterial activity of *P. betle* extract against Avian pathogenic *E. coli*

The preliminary screening of antimicrobial activity of the extract against the isolates was investigated at a concentration of 2.5 mg/disk. *P. betle* extract had antibacterial activity against clinical isolates of APEC (Table 1 and Fig. 1). The inhibition zone of the extract against the clinical isolates ranged from 18.67 ± 1.53 to 21.33 ± 0.58 mm.

Table 1 Antibacterial activity of the *Piper betle* leaf extract on Avian pathogenic *E. coli* isolated from chicken by disk diffusion assay

Isolates	Inhibition zone (mm)	Antibiotic susceptibility	
		Ampicillin	Gentamicin
CH01	21.00 ± 1.00*	R	S
CH02	20.67 ± 0.58*	R	S
CH03	19.33 ± 1.15*	R	R
CH04	19.00 ± 1.00*	R	S
CH05	20.00 ± 1.00*	R	R
CH06	21.33 ± 0.58*	R	S
CH07	18.67 ± 1.53*	R	S
CH08	19.33 ± 0.58*	R	S
CH09	20.00 ± 1.00*	R	S
CH10	21.33 ± 0.58*	R	S
E. coli ATCC25922	20.00 ± 0.00*	S	S

*Significant difference, $P < 0.05$

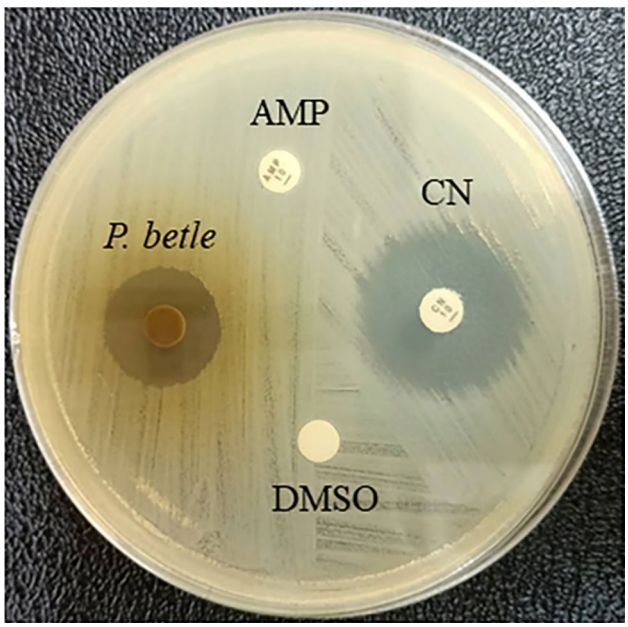

Fig. 1 Activity of *Piper betle* extract against a clinical isolate APEC CH09 as measured by disk diffusion assay. Ampicillin (AMP) and gentamicin (CN) were used as positive control, while DMSO was included as a negative control. Inhibitory activity was presented as zones of inhibition

The inhibition zone of the extract against the reference strain was in the same range as the tested clinical isolates. Ampicillin and gentamicin were included as positive control. All the clinical isolates were found to be resistant to ampicillin. However, 8 isolates (80%) were sensitive to gentamicin.

Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of *P. betle* against Avian pathogenic *E. coli*

The antibacterial activity of *P. betle* extract against ten clinical isolates of Avian pathogenic *E. coli* was determined by a broth microdilution assay. *P. betle* extract showed strong antibacterial activity against the clinical isolates with the MIC values ranging from 0.5 to 1.0 mg/mL (Table 2). In addition, the MBC value of the extract against the isolates was 1.0 mg/mL. It was found that the MIC and MBC values of *P. betle* extract against *E. coli* ATCC25922 were in the same range as those of the tested clinical isolates.

Time-kill study

The time kill curve was determined to confirm the antibacterial effectiveness of *P. betle* extract against Avian pathogenic *E. coli*. A clinical isolate CH09 was used as a representative due to its strong biofilm forming ability. The results demonstrated that the antibacterial activity of *P. betle* extract was dependent on the concentration, resulting in the reduction of the colony-forming unit of the pathogen (Fig. 2). Interestingly, the killing activity of the extract at 4 × MIC against the pathogen was observed within 4 h (3 log inhibition). Furthermore, the viability of the bacteria after treatment with the extract at 2 × MIC decreased by 3 logs in 12 h. The extract at 1 × MIC exhibited bacteriostatic effects against bacteria in 8 h. However, regrowth was observed in the clinical isolate treated with the extract at 1 × MIC.

Table 2 Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of *Piper betle* leaf extract on Avian pathogenic *E. coli* isolated from chicken

Isolates	Antibacterial activity (mg/mL)			
	Piper betle extract		Ceftriaxone	
	MIC	MBC	MIC	MBC
CH1	0.5	1.0	0.000125	0.00025
CH2	1.0	1.0	0.0005	0.0005
CH3	1.0	1.0	0.00025	0.00025
CH4	1.0	1.0	0.0005	0.0005
CH5	1.0	1.0	0.00025	0.00025
CH6	0.5	1.0	0.00025	0.0005
CH7	0.5	1.0	0.0005	0.0005
CH8	0.5	1.0	0.0005	0.0005
CH9	0.5	1.0	0.00025	0.00025
CH10	0.5	1.0	0.00025	0.00025
ATCC25922	0.5	1.0	0.00025	0.00025

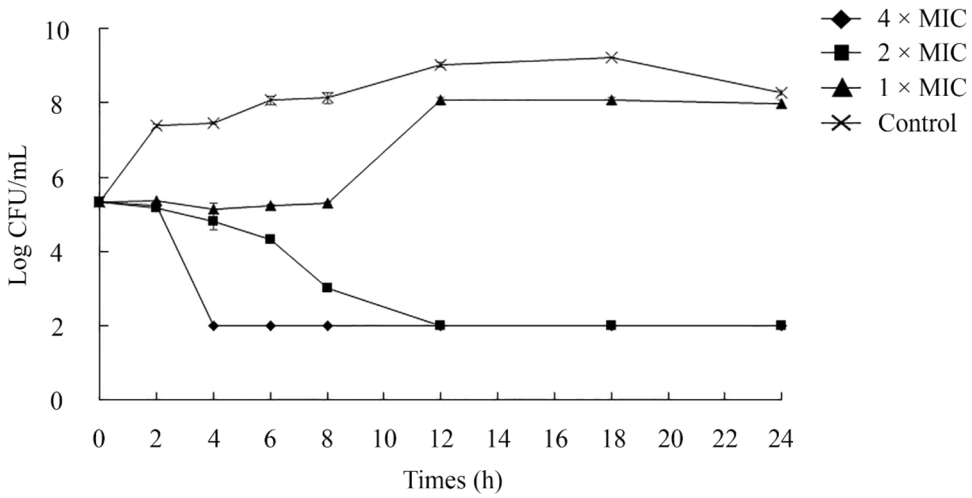

Fig. 2 Time-kill curves of Avian pathogenic *E. coli* CH09 after treatment with *P. betle* extract. The bacterium was treated with the extract at 4 × MIC, 2 × MIC, 1 × MIC. One percent DMSO was used as a negative control. Each symbol indicates the mean ± SD

Morphylogy of Avian pathogenic *E. coli* after treatment with *P. betle* extract

The morphology of APEC cells treated with *P. betle* extract was observed by SEM. Rod shape and smooth surface of the bacterial cells were observed in the control groups of the isolate (Fig. 3A–C) and the reference strain (Fig. 3J–L). It was found that both of the isolate (Fig. 3D–I) and the reference strain (Fig. 3M–R) treated with the extract showed an abnormal shape when compared to the control. Long cell formation and dried shape of the cells were observed when the pathogens were treated with the extract in 2 × MIC and 4 × MIC. Furthermore, disruption and breakdown of the bacterial cells were detected when the cells were challenged with the extract. It was noted that the bacterial cells treated with the extract demonstrated longer cells without septum when compared to the control. However, the normal size of the cells was observed.

P. betle reduced biofilm formation in Avian pathogenic *E. coli*

To investigate the activity of *P. betle* extract on the biofilm formation of APEC, the biofilm forming ability of the clinical isolates was evaluated. It was found that 3 isolates (30%) including CH6, CH9, and CH10 were strong biofilm formers. Whereas, 7 isolates (70%) including CH1, CH2, CH3, CH4, CH5, CH7, and CH8 produced weak biofilm formation. Hence, all the strong biofilm forming and 2 weak biofilm forming isolates were used as representative APEC to test the effects of the extract on the biofilm formation.

The activity of *P. betle* extract as inhibitors against the biofilm formation of APEC was determined using the crystal violet assay. The results demonstrated that *P. betle* extract at 1/8 × MIC, 1/4 × MIC, and 1/2 × MIC significantly inhibited the bacterial biofilm formation of the clinical APEC isolates (Fig. 4B) ($P < 0.05$) without growth inhibition (Fig. 4A). In 1/2 × MIC of the extract, 55% of the biofilm inhibition was detected in APEC CH09, a strong biofilm producer. It was observed that the extract reduced the biofilm formation in the reference strain. However, the growth of the reference strain was significantly inhibited by the extract in 1/8 × MIC, 1/4 and 1/2 × MIC.

Activity of *P. betle* on established biofilm of Avian pathogenic *E. coli*

The activity of the ethanolic extract of *P. betle* leaves on the established biofilm of APEC was assessed by the MTT assay. As shown in Fig. 5, a significant reduction in the viability of young (2-day-old biofilm) and mature (5-day-old biofilm) biofilm-grown cells was observed after the treatment with the extract at 8 × MIC when compared with the control ($P < 0.05$). In 32 × MIC of *P. betle* extract, a 94% of the inhibition of viable cells embedded in the young established biofilm was detected in APEC CH09, a strong biofilm former (Fig. 5A). In addition, the 88% reduction in the viability of the isolate in the mature biofilm was detected following the treatment with the *P. betle* extract at 32 × MIC, compared to the control (Fig. 5B).

Inhibition of adhesion of Avian pathogenic *E. coli*

The present study showed that *P. betle* reduced biofilm formation and eliminated the established biofilm of APEC. It is recognized that adhesion is the first step of biofilm formation. Hence, the activity of *P. betle* extract on adhesion of the pathogen to glass surfaces was investigated using SEM. The study was performed on representative bacteria named CH09, a strong biofilm producer and the reference strain. In the control groups, the bacteria, including the clinical isolate (Fig. 6A–C) and the reference strain (Fig. 6J–L) could

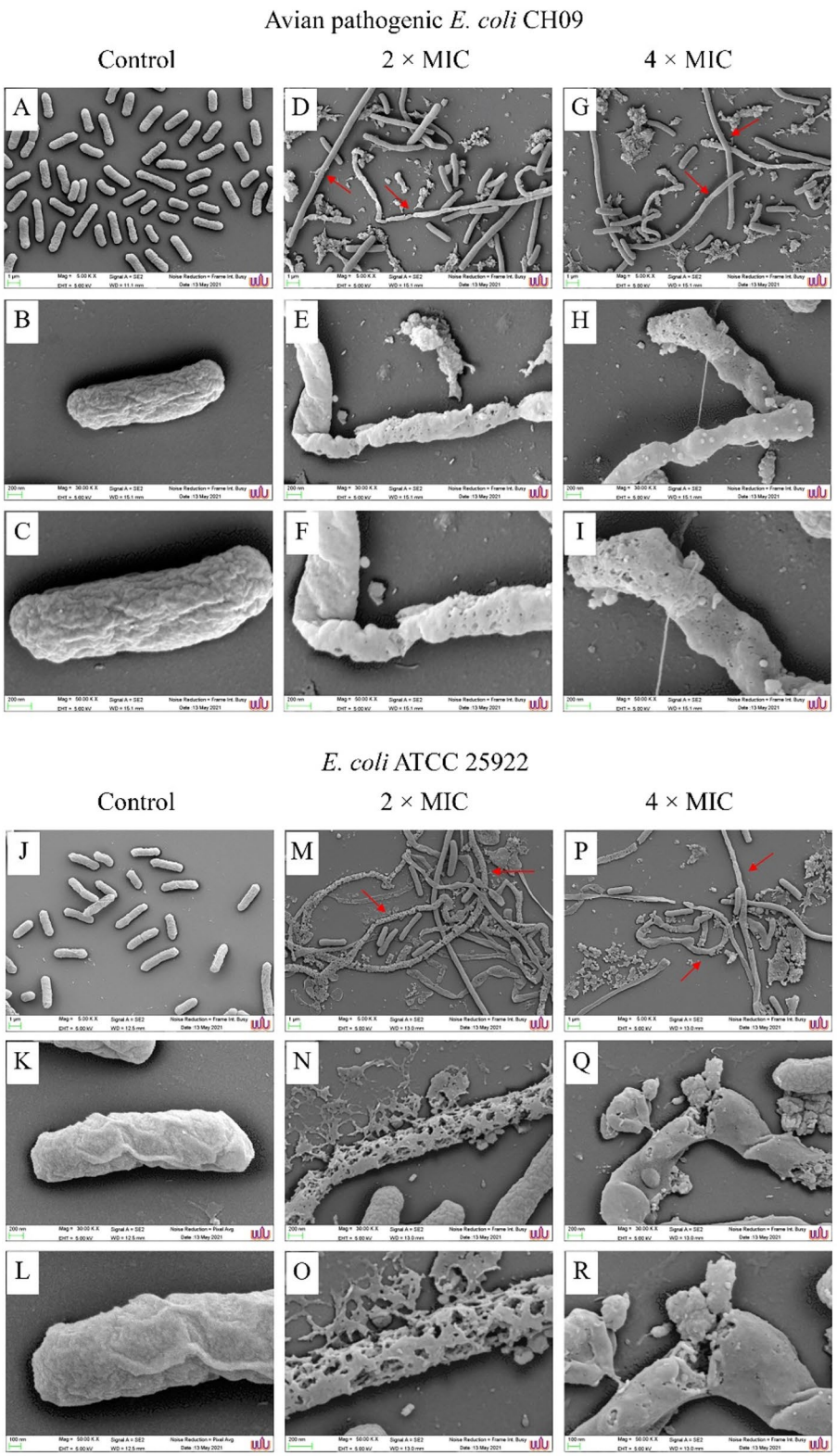

Fig. 3 Morphylogy of Avian pathogenic *E. coli* CH09 (**A**–**I**) and ATCC25922 (**J**–**R**) after treatment with *P. betle* extract observed by SEM. The bacterial cells were treated with the extracts at 2×MIC and 4×MIC. One percent DMSO was used as a negative control. Magnifications were revealed as: **A**, **D**, **G**, **J**, **M**, **P**= 5000X; **B**, **E**, **H**, **K**, **N**, **Q**= 30,000X; **C**, **F**, **I**, **L**, **O**, **R**= 50,000X

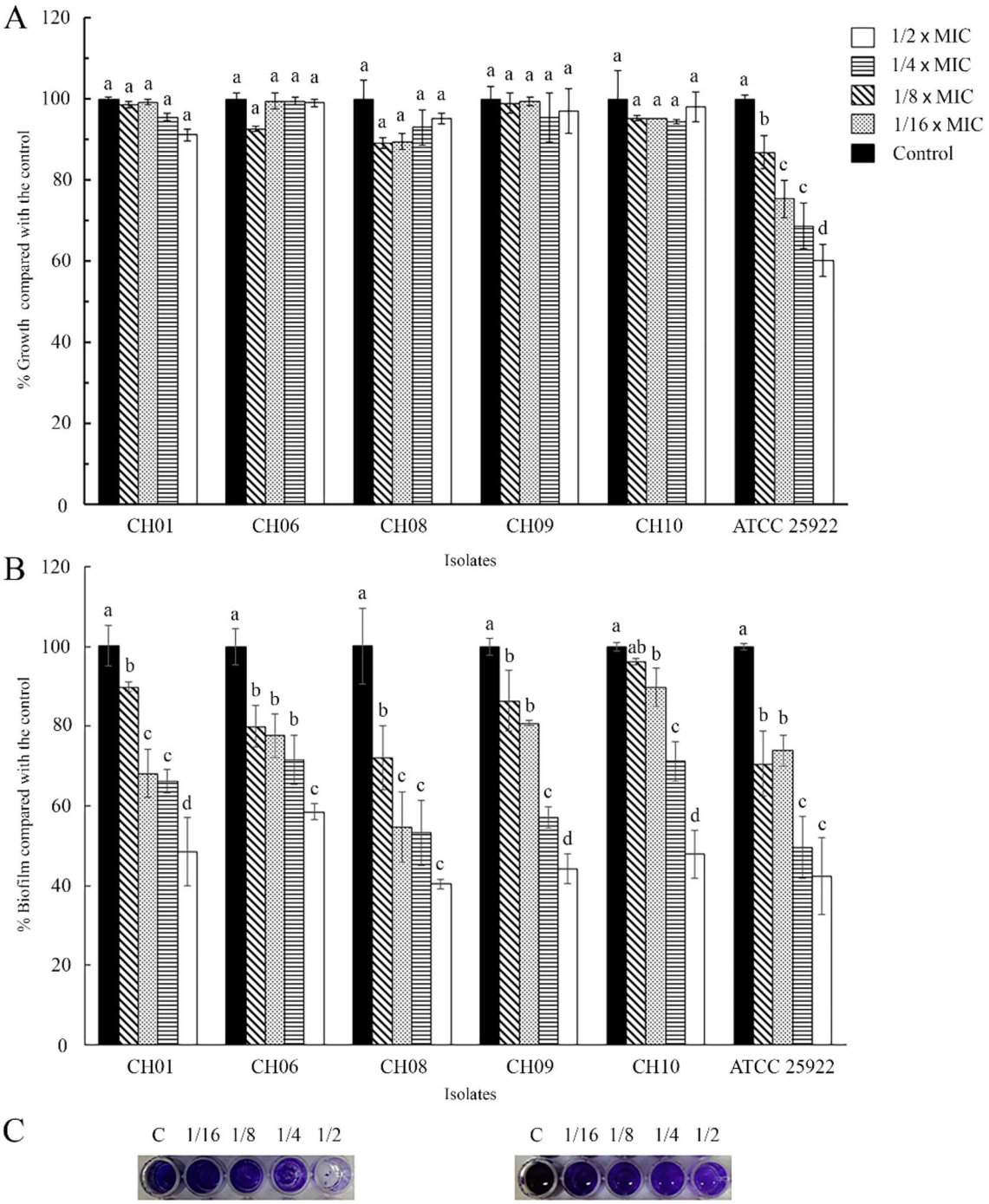

Fig. 4 Effects of *P. betle* extract on the growth (**A**) and biofilm formation (**B**) of Avian pathogenic *E. coli*. The pathogens were grown in the medium supplemented with *P. betle* extract at sub-MICs. The biofilms were stained with crystal violet. Photos of stained biofilm cells (**C**) attached to microtiter wells before (left) and after adding of DMSO to dissolve the biofilm (right). One percent DMSO was used as a negative control. Comparisons between means were carried out according to Duncan's test. Differences were considered significant at $P < 0.05$

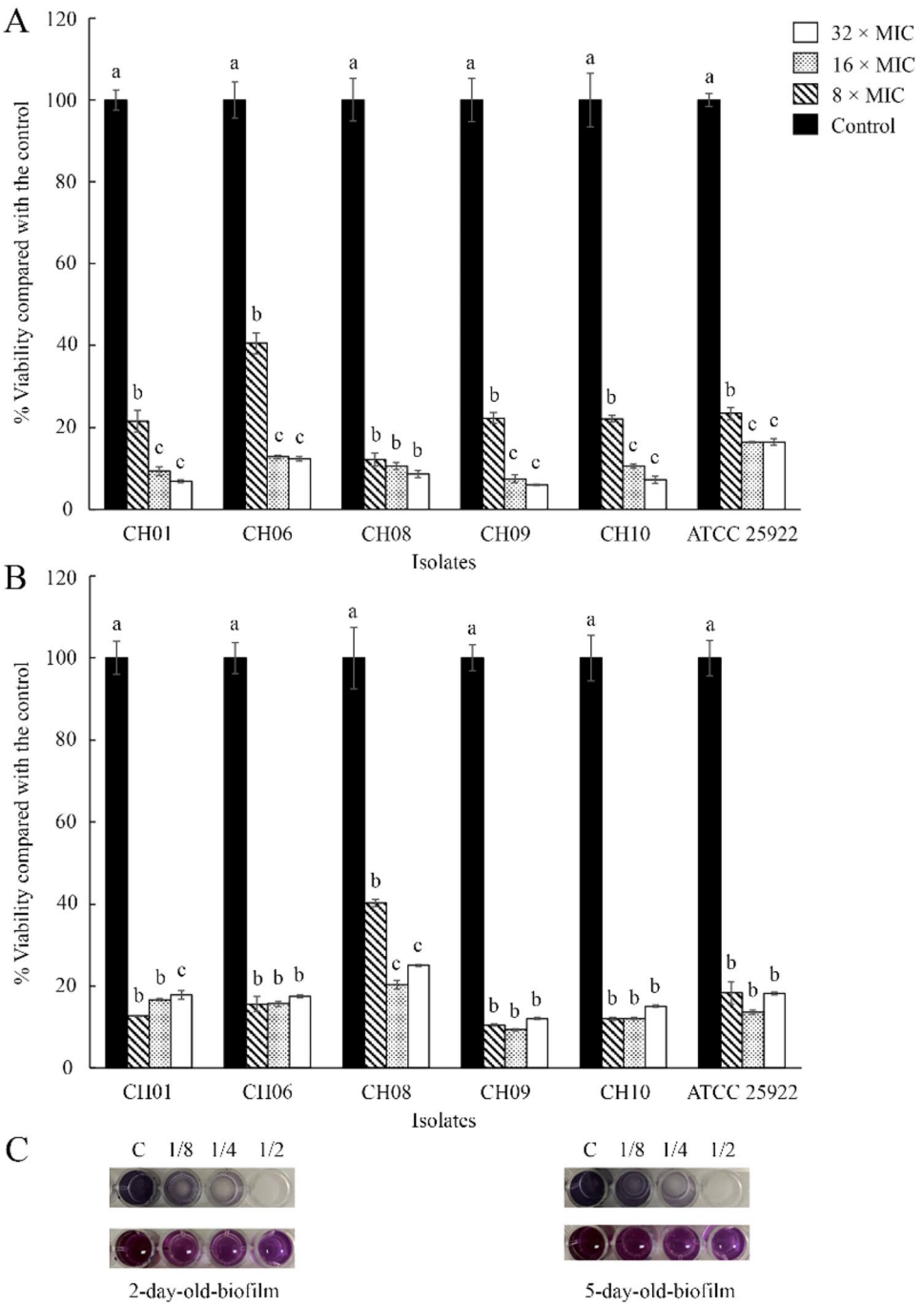

Fig. 5 Effects of *P. betle* extract on established biofilm of Avian pathogenic *E. coli*. The bacteria were grown in the medium supplemented to produce young, 2 days (**A**) and mature, 5 days (**B**) biofilms. The biofilm was treated with *P. betle* extract at different concentrations. One percent DMSO was used as a negative control. The viable biofilm cells were stained with MTT. Photos of stained biofilm cells (**C**) attached to microtiter wells before (upper) and after adding of DMSO to dissolve the biofilm (below). Comparisons between means were carried out according to Duncan's test. Differences were considered significant at $P < 0.05$

regularly bind to the surface. The reduction in the bacterial adhesion to surfaces was observed when APEC were treated with sub-MICs of *P. betle* extract when compared with the control in 24 h (Fig. 6D–I, M–R).

Identification of the phytochemicals in *P. betle* leaf extract using GC–MS analysis

To identify the phytochemicals in *P. betle* leaf extract, GC–MS analysis was determined. A total of 53 compounds were detected from *P. betle* leaf extract as observed by GC–MS analysis (Table 3). Hydroxychavicol was found to be the major compound presented in the leaf extract, followed by phenol, 2-methoxy-4-(2-propenyl), and chavicol. It was observed that 54.61% total peak area of hydroxychavicol was detected in the leaf extract.

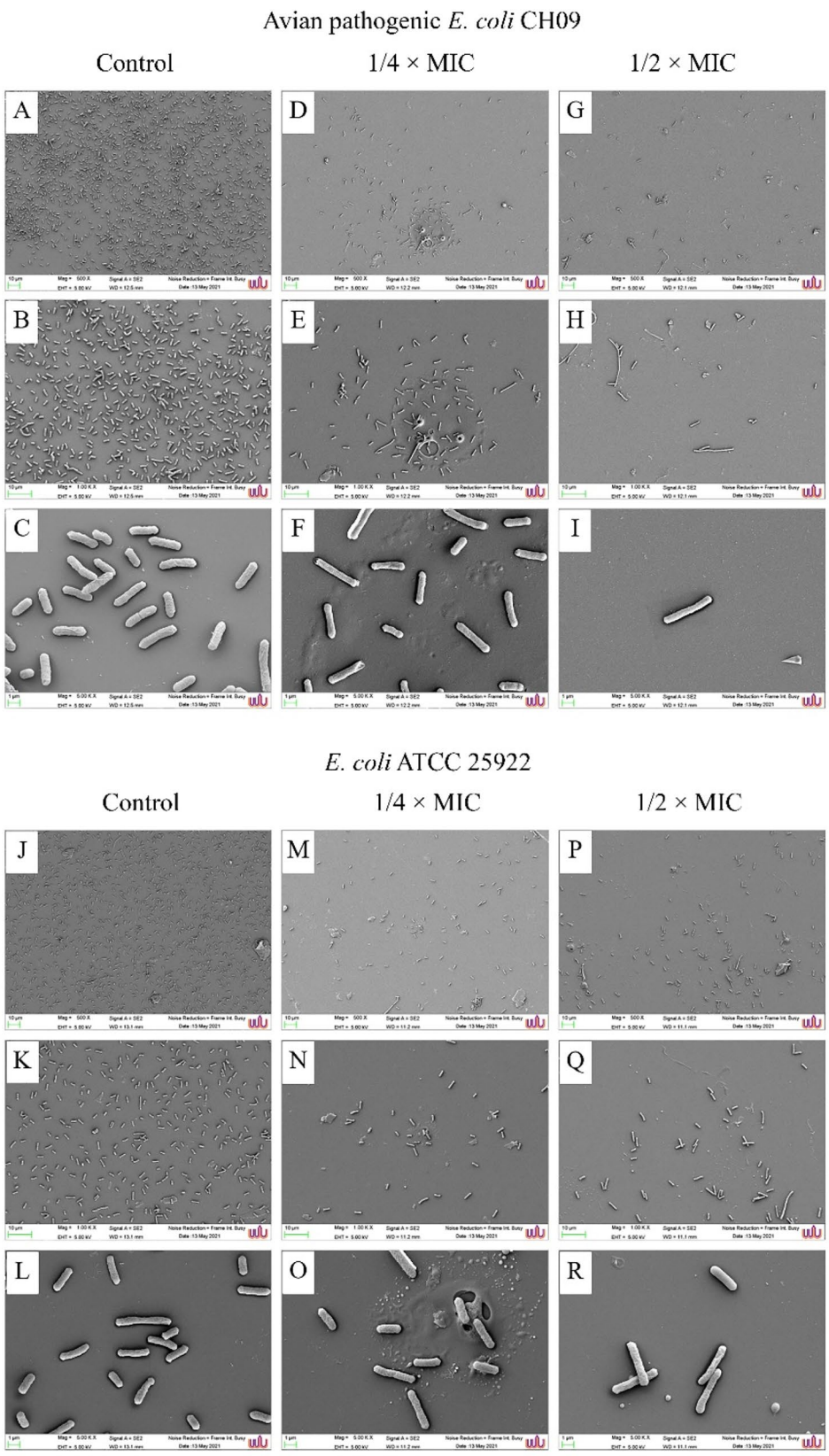

Fig. 6 Activity of *P. betle* extract on the adhesion of Avian pathogenic *E. coli* CH09 (**A**–**I**) and ATCC25922 (**J**–**R**) observed by SEM. The bacterial cells were cultured in the medium supplemented with the extracts at 1/4 × MIC and 1/2 × MIC. One percent DMSO was used as a negative control. Magnifications were revealed as: **A**, **D**, **G**, **J**, **M**, **P** = 500X; **B**, **E**, **H**, **K**, **N**, **Q** = 1000X; **C**, **F**, **I**, **L**, **O**, **R** = 5000X

Table 3 Chemical composition of *Piper betle* leaf extract

No	RT min	% (Total)	Compounds
1	9.5880	0.05	Eucalyptol
2	13.2911	1.28	Acetic acid
3	13.4922	0.04	1-(1-ethyl-2,3-dimethyl-cyclopent-2-enyl)-ethanone
4	13.9525	0.12	Copaene
5	14.2935	0.04	(−)-beta-bourbonene
6	14.5066	0.08	Linalool
7	14.8874	0.13	cis-alpha-bergamotene
8	14.9471	0.09	Tricyclo[2.2.1.0(2,6)]heptane, 1,7-dimethyl-7-(4-methyl-3-pentenyl)-, (−)-
9	15.0976	0.10	cis-alpha-bergamotene
10	15.2340	0.02	(1R,2S,6S,7S,8S)-8-Isopropyl-1-methyl-3-methylenetricyclo[4.4.0.02,7]decane-rel-
11	15.2909	0.31	Caryophyllene
12	15.4386	0.02	1H-Cycloprop[e]azulene, decahydro-1,1,7-trimethyl-4-methylene-, (1aR,4aR,7R,7aR,7bS)-(+)-
13	15.8847	0.02	Bicyclo[2.2.1]heptane, 2-methyl-3-methylene-2-(4-methyl-3-pentenyl)-, (1S-endo)-
14	16.0069	0.15	cis-beta-farnesene
15	16.0950	0.04	Anethole
16	16.1717	0.11	alpha-humulene
17	16.2911	0.04	(1S,5S)-4-methylene-1-((R)-6-methylhept-5-en-2-yl)bicyclo[3.1.0]hexane
18	16.3649	0.54	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-, (1.alpha.,4a.beta.,8a.alpha.)-
19	16.6235	0.01	1H-cyclopropa[a]naphthalene, 1a,2,3,5,6,7,7a,7b-octahydro-1,1,7,7a-tetramethyl-, [1aR-(1a.alpha.,7.alpha.,7a.alpha.,7b.alpha.)]-
20	16.6832	0.06	1,3-cyclohexadiene, 5-(1,5-dimethyl-4-hexenyl)-2-methyl-, [S-(R*,S*)]-
21	16.7656	0.51	1H-benzocycloheptene, 2,4a,5,6,7,8,9,9a-octahydro-3,5,5-trimethyl-9-methylene-, (4aS-cis)-
22	17.1293	0.61	delta-Cadinene
23	17.2799	0.21	Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-
24	17.4447	0.05	Naphthalene, 1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)-
25	18.6552	0.06	2,4-dimethyl-quinoline
26	18.8683	0.07	2-octen-1-ol, 3,7-dimethyl-
27	18.9876	0.02	2-Isopropenyl-4a,8-dimethyl-1,2,3,4,4a,5,6,8a-octahydronaphthalene
28	19.1439	0.05	Glutaric acid, 2-ethylhexyl farnesyl ester
29	19.6383	0.03	(−)-5-Oxatricyclo[8.2.0.0(4,6)]dodecane,,12-trimethyl-9-methylene-, [1R-(1R*,4R*,6R*,10S*)]-
30	19.7406	0.21	methyleugenol
31	20.4709	0.06	1,3-dihydroxyacetone dimer
32	20.9255	0.05	1H-cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1ar (1a.alpha,4a.alpha,7.beta,7a.beta,7b.alpha.)]-
33	21.1756	0.07	3-cyclohexen-1-ol, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-
34	21.2949	0.16	Phenol, 2-methoxy-3-(2-propenyl)-
35	21.5961	29.01	Phenol, 2-methoxy-4-(2-propenyl)-
36	21.8348	0.06	tau-cadinol
37	22.3804	0.18	Hexadecanoic acid, ethyl ester
38	22.8236	0.07	(−)-5-oxatricyclo[8.2.0.0(4,6)]dodecane,,12-trimethyl-9-methylene-, [1R-(1R*,4R*,6R*,10S*)]-
39	22.9998	0.14	Gossonorol
40	23.4308	5.50	Chavicol
41	24.3381	0.07	(2E)-3-(2-hydroxyphenyl)-2-propenoic acid
42	25.8612	0.04	Octadecanoic acid, ethyl ester
43	26.2590	0.18	(E)-9-octadecenoic acid ethyl ester
44	26.9722	0.12	Phytol, acetate
45	27.2080	0.22	Linoleic acid ethyl ester
46	28.5066	0.32	9,12,15-octadecatrienoic acid, ethyl ester, (Z,Z,Z)-
47	28.8078	1.97	Phytol

Table 3 (continued)

No	RT min	% (Total)	Compounds
48	30.7769	0.07	(3S,3AR,%S8AR,9AR)-3-({[2-diethylnmino)ethyl]amino}methyl)-5,8adimethyl-3A,5,6,7,8,8A,9,9A octahydronaphtho[2,3-B]furan-2(3H)-one
49	33.8855	0.40	n-Hexadecanoic acid
50	35.8603	54.61	Hydroxychavicol
51	37.5027	0.12	9-octadecenoic acid (Z)-, tetradecyl ester
52	38.2869	0.25	9,12-octadecadienoic acid (Z,Z)-
53	39.4292	0.52	9,12,15-octadecatrienoic acid, (Z,Z,Z)-
Total	99.26		

Discussion

Avian pathogenic *E. coli* is a crucial pathogen that causes economic losses in poultry production across the globe. Furthermore, the pathogen has been considered zoonotic and serve as an external source for human extra-intestinal infections. According to its strong virulence factor, including biofilms, the eradication of the pathogen by the host's immune system and antibiotic is failed. Furthermore, the treatment of bacterial infections is becoming difficult due to the increased antibiotic resistance of bacteria (Duan et al. 2020; Saha et al. 2020). In the present study, we focused on natural products that have antibacterial and anti-virulence activities as a therapeutic strategy for an efficient method for the control of APEC.

The results exhibited that the ethanolic extract of *P. betle* leaves had strong antibacterial activity against APEC, as revealed by both disk diffusion assays, followed by MIC and MBC determination, as well as Time-kill kinetic study. The antibacterial activity of *P. betle* extract against those pathogens acquired in hospitals, including methicillin-resistant *Staphylococcus aureus*, ESβL *Klebsiella pneumoniae*, ESβL *Pseudomonas aeruginosa*, and ESβL *E. coli* have been reported (Valle et al. 2015). The values of the inhibition zone, MIC, and MBC of *P. betle* extract against APEC were in the same range as those of the previous reports (Valle et al. 2015). The leaf extract of *P. betle* has formerly been reported as an effective antibacterial agent. According to the results of MIC and MBC values, the MBC/MIC ratio was less than 2 times that was considered a bactericidal agent (Nayaka et al. 2021). However, the bacteriostatic effect of the extract of *P. betle* has been documented (Taukoorah et al. 2016). Nevertheless, the present study revealed bactericidal effects of the ethanolic extract of *P. betle* against Avian pathogenic *E. coli*. Hence, the present study supports strong antibacterial activity of *P. betle* extract exhibiting low value of the MIC and MBC obtained from the results.

Interestingly, leaves of *P. betle* contain various phytochemical constituents, such as phenol, flavonoid, and tannin (Taukoorah et al. 2016), contribute to its therapeutic potential. The main bioactive compounds in the leaf extracts of the plant species and their essential oil have been reported to consist of eugenol, phytol, 4-chromanol, hydroxychavicol, carvacrol, chavicol, chavibetol, and allylpyrocatechols 1 (Nayaka et al. 2021). Eugenol, a pure compound present in the plant, had pronounced antibacterial activity against *E. coli* with very low MIC values (Jeyakumar et al. 2021). It has been suggested that eugenol altered membrane permeability and disrupted the cells of *E. coli*, resulting in leakage of intracellular contents (Jeyakumar et al. 2021). Similarly, the disruption and breakdown of the APEC cells were detected when the cells were challenged with *P. betle* extract. It was noted that the bacterial cells treated with the extract demonstrated longer cells without septum when compared to the control. The results revealed that hydroxychavicol was found to be the major compound presented in the leaf extract of *P. betle*. It has been reported that hydroxychavicol, a key ingredient in *P. betle*, induces bacterial cell death by DNA damage, ROS generation, and inhibition of the cell division (Singh et al. 2018). Furthermore, hydroxychavicol suppressed the expression of SulA, a protein under the control of SOS response (Singh et al. 2018), resulting in an inhibition of the cell division by inhibiting the formation of the FtsZ ring in response to genotoxic stress (Mukherjee et al. 1998). It has been documented that FtsZ mutant *E. coli* exhibited long cell shape without FtsZ ring formation. Therefore, the inhibition of FtsZ may be one of the possible effects of *P. betle* extract against the bacteria.

It was accepted that bacterial biofilms play a crucial role in the pathogenesis of the infection. In addition, the mechanism enables pathogens to tolerate higher concentrations of antibiotics, leading to chronic infections and the emergence of antibiotic-resistant strains (Gu et al. 2019; Hall et al. 2017; Khairy et al. 2020). The present study demonstrated that *P. betle* extract acted as an inhibitor of biofilm formation of the APEC. The mode of action of phytochemicals in inhibiting biofilms may involve in the inhibition of quorum sensing system and killing viable cells embedded in the biofilm (Reen et al. 2018). It understood that biofilm formation is regulated by the quorum sensing system, a cell-to-cell

communication process (Wu et al. 2021). The ethanol extract of *P. betle* leaves inhibited the quorum sensing in *Pseudomonas aeruginosa*, resulting in an inhibition of virulence factors, including biofilms (Datta et al. 2016). In addition, eugenol, a key ingredient in *P. betle*, reduced biofilms in *E. coli* O157: H7 by suppressing important genes that are required for the formation of the biofilm and the attachment and effacement phenotype (Kim et al. 2016).

Adhesion is the first step to progress in the formation of the biofilm. The present study demonstrated that *P. betle* extract reduced the adhesion of the APEC cells to the surfaces observed by SEM. Similarly, the anti-adhesion activity of *P. betle* extract against *Streptococcus mitis* on saliva-coated glass surfaces has been reported (Razak et al. 2003). In addition, eugenol, a bioactive compound in *P. betle* leaves, reduced the production of fimbria in *E. coli* O157: H7, resulting in inhibition of the adhesion and biofilms (Kim et al. 2016). Our results may indicate that *P. betle* extract reduced the adhesion of APEC cells to the surface resulting in the inhibition of the biofilms. The further investigation, isolation of pure compounds from *P. betle* extract should be investigated. In addition, antibacterial mechanisms of the extract and the pure compounds against the pathogens should be studied. The further study should be performed in vivo to provide insight on the mechanism of *P. betle* extract against the APEC in chickens. In addition, the activity of the extract on anti-inflammatory activity of white blood cells isolated from chicken should be investigated.

Conclusion

The present study reveals a bactericidal activity of *P. betle* extract against the APEC cells. The disruption and breakdown of the bacterial cells were detected when the cells were challenged with the extract at 2 × MIC. It was noted that the bacterial cells treated with the extract at 2 × MIC demonstrated longer cells without a septum when compared to the control. The extract at concentration 1/16 × MIC and 8 × MIC inhibited the biofilm formation and eradicated the established biofilm in the APEC, respectively. In addition, the reduction of bacterial adhesion to the surfaces was observed when APEC cells were treated with 1/4 × MIC of *P. betle* extract when compared to the control. Hydroxychavicol was found to be the major compound presented in the leaf extract, followed by Phenol, 2-methoxy-4-(2-propenyl), and chavicol. It was observed that 54.61% total peak area of hydroxychavicol was detected in the leaf extract. The information suggested potential medicinal benefits of *P. betle* extract to inhibit the growth, biofilm, and adhesion of Avian pathogenic *E. coli*.

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Author contributions WM, SS, PK, and VN conceived and designed the experiments. WM, PK, JC, NM and CR performed the experiments. PK, JC, TT and WM analyzed and interpreted the data. WK and WM analyzed statistical analysis. TT, PW, VN, and MLP contributed reagents, materials, analysis tools or data. WM, PK, MLP, and TT wrote the paper.

Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors declare no conflict of interest.

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