

# Antibacterial mechanism and activities of black pepper chloroform extract

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**Abstract** Black pepper extracts reportedly inhibit food spoilage and food pathogenic bacteria. This study explored the antimicrobial activity of black pepper chloroform extract (BPCE) against *Escherichia coli* and *Staphylococcus aureus*. The antibacterial mechanism of BPCE was elucidated by analyzing the cell morphology, respiratory metabolism, pyruvic acid content, and ATP levels of the target bacteria. Scanning electron micrographs showed that the bacterial cells were destroyed and that plasmolysis was induced. BPCE inhibited the tricarboxylic acid pathway of the bacteria. The extract significantly increased pyruvic acid concentration in bacterial solutions and reduced ATP level in bacterial cells. BPCE destroyed the permeability of the cell membrane, which consequently caused metabolic dysfunction, inhibited energy synthesis, and triggered cell death.

**Keywords** Black pepper · Chloroform extract · Respiration metabolism · Antibacterial mechanism · ATP

## Introduction

Pepper (*Piper nigrum* L.) is a medicinal and aromatic liana that originated in India, and was first introduced to China in 1947. At present, this vine is widely planted in Hainan. The cultivation acreage and yield of pepper rank sixth and fifth

worldwide, respectively, with an output of 35,000 tons (Ravindran and Kallapurackal 2012). Pepper is not only used as a spice, but also as a medicinal material. Pepper extracts contain alkaloid (e.g., piperine), terpenes, flavones and volatile oils (e.g., piperidine) that exhibit sedating, detoxification, hypotensive, and anticancer activities (Butt et al. 2012; Meghwal and Goswami 2013; Yoon et al. 2015). Pepper is also used as a preservative and flavor, enhancer in meat and meat-based products (Thiel et al. 2014).

Consumers nowadays demand natural and nontoxic products. Thus, food products should be protected against bacteria during storage to avoid contamination. Chemical preservatives are currently widely utilized, but repeated use of such preservatives may trigger resistance development in pathogenic bacteria, which might lead to serious health problems (Kito et al. 2003). Plants are rich in bioactive substances, such as terpenes, alkaloids, flavonoids, steroids, phenolic, unique amino acid, and polyose. Several substances have been investigated for their biological and antibacterial activities (Pan et al. 2009). Many studies have proven that pepper ethanol extracts and volatile oils can inhibit food spoilage and food pathogens (Sumathykutty and Rao 1988; Nalini et al. 1998; Dorman and Deans 2000; Chaudhry and Tariq 2006; Thiel et al. 2014). Pradhan (Pradhan et al. 1999) demonstrated that phenolic compounds in fresh pepper can inhibit the growth of *Salmonella typhimurium*, *Bacillus*, *Escherichia coli*, and *Staphylococcus aureus*. However, the antimicrobial mechanism of pepper remains unclear to date. This fact has driven food researchers to develop preservation techniques involving natural antiseptics to improve microbial quality with minimal or no toxicity.

Previous studies (Cox et al. 2000; Mader et al. 2007; He et al. 2010; Li et al. 2010) showed that antiseptics kill microbes by changing the structure and function of cellular substances through destruction of the cell wall and membrane;

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inhibition of protein, carbohydrate, and ATP syntheses; and suppression of the activities of key metabolic enzymes.

The present study investigated the antimicrobial mechanism of pepper by evaluating effects of black pepper chloroform extract (BPCE) on the respiration, pyruvic acid concentration, ATP level, and cellular morphology of *E. coli* and *S. aureus*.

## Materials and methods

### Chemicals

Black pepper was purchased from Nanguo Supermarket (Haikou, China). Iodine acetic acid was purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Sodium phosphate was purchased from Guangzhou Chemical Regent Factory (Guangzhou, China). Malonic acid, pyruvic acid, and 2, 4-dinitrophenylhydrazine were purchased from Aladdin Industrial Corporation. An ATP assay kit was purchased from Nanjing JianCheng Bioengineering Institute (Nanjing, China).

### Preparation of BPCE

Air-dried black peppers (100 g) were crushed in a grinder, extracted by stirring with 2 L of 80 % ethanol at 50 °C for 12 h three times, and then filtered. The solvent of combined extracts was evaporated under reduced pressure using a rotary vacuum evaporator at 50 °C, and the remaining water was evaporated at 50 °C by thermostat-controlled water-bath. Turbidity suspensions were prepared by adding 100 mL distilled water to the obtained dried extract. 100 mL of petroleum ether were added into the suspension in the separatory funnel for three times. The lower turbidity suspensions were transferred into another separatory funnels, 100 mL of chloroform were added for three times, agitating and placement after stratification. BPCE was concentrated under vacuum at 30 °C, and then stored at 4 °C for further analysis.

### Microorganisms and growth conditions

*E. coli* (ATCC8739) and *S. aureus* (ATCC6538) were provided by our laboratory. Both strains grew well in nutrient agar medium at 37 °C. *E. coli* and *S. aureus* were routinely cultured overnight at 37 °C for 24 h. The bacteria were washed twice with 0.9 % sterile NaCl, and then resuspended in NaCl. The bacterial concentration was adjusted to  $1-2 \times 10^7$  colony-forming units /mL through Maxwell turbidimetry (Heng et al. 2014). Then, 1.0 mL of *E. coli* or *S. aureus* suspensions was directly added into sterile Erlenmeyer flasks, containing 48 mL of nutrient broth medium. The black pepper chloroform extract (BPCE) was first dissolved in ethanol and then

added in the treated groups to yield the final concentration of minimum inhibitory concentrations (MICs). The control groups had the same volume ethanol but without the extract. Both groups were agitated at 130 rpm in an environmental incubator shaker at 37 °C.

### Electron microscopic observation

The cells were collected through centrifugation at 3000 rpm for 10 min. They were washed thrice with phosphate-buffered saline (PBS, 0.1 mol/L, pH 7.2) and then dehydrated in different concentrations of alcohol. After pre-freezing at -40 °C, the samples were placed in a vacuum freeze drier. The dried samples were observed and photographed under an S-3000N scanning electron microscope (Japan).

### Respiration metabolism of bacteria

The respiratory rate was measured by using a JPB-607A dissolved oxygen detector (Camper and Mcfeters 1979). At the exponential phase, the bacteria were harvested through centrifugation at 3000 rpm for 10 min, washed thrice with NaCl to obtain the final concentration of 10 mg/mL and then stored at 4 °C. PBS (30 mL 0.1 mol/L, pH 7.2), the suspension (10 mL), and glucose substrate (4 mL, 1 %) were transferred to a chamber, and then allowed to react for 5 min in air. Then, a pocket-type dissolved oxygen detector was used to measure the consumed oxygen before the system was hermetically sealed. Typical inhibitors, including malonic acid, iodine acetic acid, sodium phosphate, and BPCE were added. The blank control did not contain any reagent. On the basis of the respiratory rate ( $\mu\text{mol/g min}$ ) of the bacteria before and after extract addition, the respiratory inhibition of bacteria was evaluated as

$$I_R(\%) = \frac{R_0 - R_1}{R_0} \times 100\%$$

where  $I_R$  is the respiratory inhibition of the bacteria after extract addition, and  $R_0$  and  $R_1$  represent the respiratory rates of the bacteria before and after extract addition, respectively.

The respiratory superposing inhibition of the bacteria was expressed as

$$R_R(\%) = \frac{R_1 - R_1'}{R_1} \times 100\%$$

where  $R_R$  is the respiratory superposing inhibition,  $R_1$  represents the respiratory rate after extract addition, and  $R_1'$  stands for the respiratory rate after typical inhibitor addition. The inhibited pathway of respiratory metabolism was determined on the basis of the superposing inhibition.

## Pyruvic acid content

The content of pyruvic acid in the supernatants was measured. The treated and control groups were centrifuged at 6000 rpm for 15 min, and the supernatants were collected and stored in a refrigerator at 4 °C until measurement. The pyruvic acid content was measured using the 2,4-dinitrophenylhydrazine method (Spoel and Dong 2008). The supernatants (0.1 mL) were prepared as described, and 2, 4-dinitrophenylhydrazine (1 mL) was added to test tubes containing 8 % trichloroacetic acid (0.3 mL) by rapid mixing. The mixture was placed in a hot bath at 37 °C for 10 min, added with 10 mL of sodium hydroxide (0.4 mol/L), and then mixed. The absorbance at 520 nm was recorded. The content of pyruvic acid was calculated on a pyruvic acid calibration curve.

## ATP levels

Cells were collected by centrifugation at 5000 rpm for 5 min at 4 °C, washed thrice, and then resuspended in 5 mL of phosphate buffer saline (PBS, pH7.2). Cell suspensions were broken with ultrasound in an ice bath for 5 min (550 W, working for 3 at 3 s intervals,) and then centrifuged at 12,000 rpm for

20 min at 4 °C. The supernatants were collected and stored at a low temperature. The ATP levels were assayed through ATP assay kit (Pang et al. 2013) (Nanjing JianCheng Bioengineering Institute, China). The results were analyzed by UV/Visible Spectrophotometer (UV–VIS, Thermo Fisher Scientific, USA).

## Statistical analysis

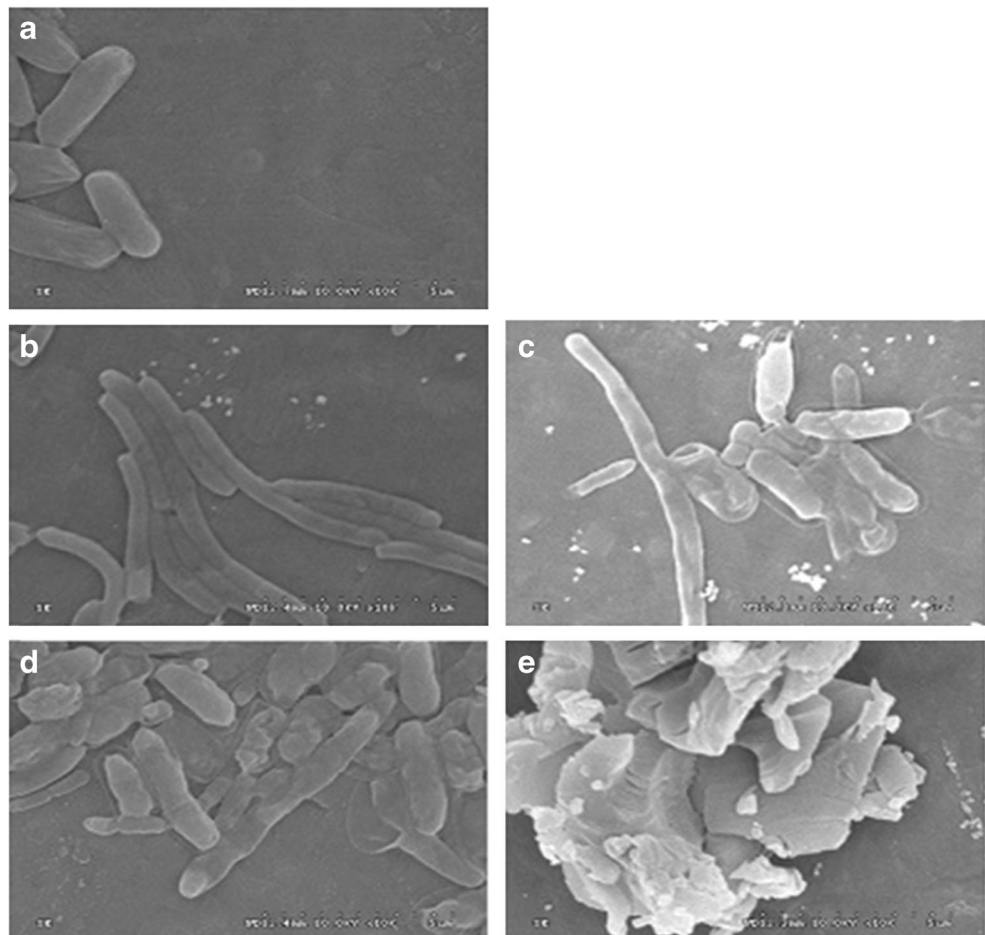
All experiments were performed in triplicate. Data are presented as mean ± SD. The data were analyzed using SPSS 16.0. Statistical significance was assessed using Duncan's one-way multiple comparison. Differences between groups were considered significant at  $p \leq 0.05$ .

## Results and discussion

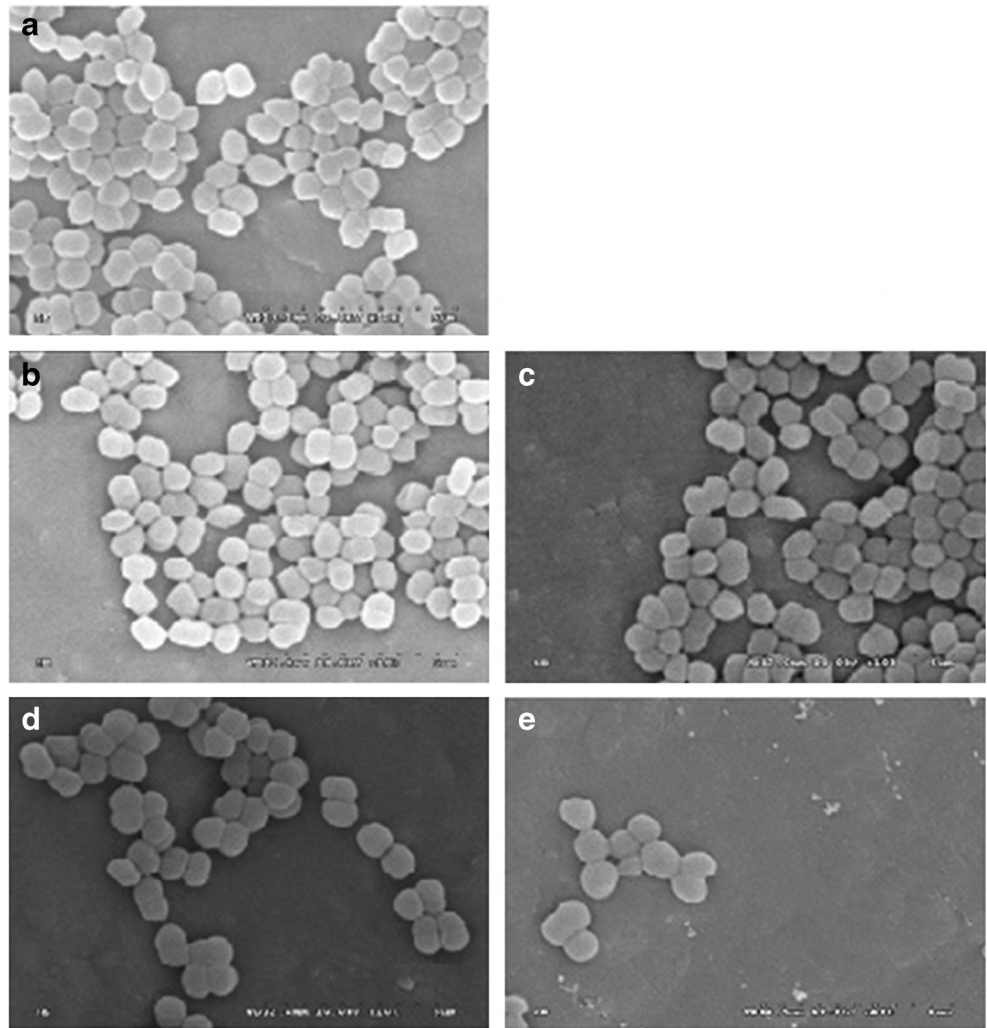
### Electron microscopy observation

The cell membrane plays an important role in maintaining normal bacterial life. This organelle maintains material and energy balance. The effect of BPCE on *E. coli* and *S. aureus*

**Fig. 1** Effect of black pepper chloroform extract on the cell morphology of *E. coli* (control (a); ethanol (b: 8 h, c: 24 h); BPCE (d: 8 h, e: 24 h))



**Fig. 2** Effect of black pepper chloroform extract on the cell morphology of *S. aureus* (control (a); ethanol (b: 8 h, c: 24 h); BPCE (d: 8 h, e: 24 h))



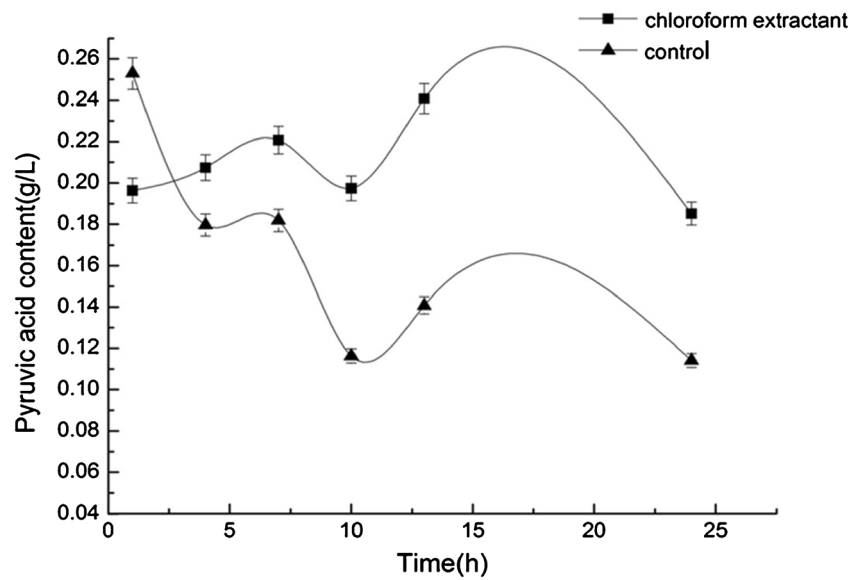
morphology was visualized via scanning electron microscopy (SEM). Figures. 1 and 2 show the SEM images of the physiological morphology of *E. coli* and *S. aureus*. The difference in structural integrity between the control and treatment groups can be clearly observed in the electron micrographs. Normal *E. coli* cells were rod-shaped (Fig. 1a), The bacteria treated with BPCE exhibited more damage to cell membrane (Fig. 1d and e) than those treated with ethanol (Fig. 1b and c). The electron micrographs showed that most of the outmost layer of the *E. coli* cells disappeared at 24th hour of BPCE treatments. Thus, the extract caused minimal leakage of

cellular cytoplasmic contents. After 8 h of treatment with BPCE, the cells began to rupture and aggregate. After 24 h, the cell walls were severely impaired, and the cell boundaries were not distinct. The plasma membrane of the cells completely collapsed, and the release of cellular contents was initiated. Figure 2 shows that *S. aureus* cells were deformed compared with the cells from the normal control and ethanol-treated groups. After 24 h, cell adhesion and boundaries were unclear, and the cell membranes were fractured. Severe plasmolysis and depletion of bacterial cell contents were evident.

**Table 1** Inhibition and superposition by BPCE on the respiratory metabolism of *E. coli* and *S. aureus*

Inhibitor	<i>E. coli</i>		<i>S. aureus</i>	
	Inhibition (%)	Superposition (%)	Inhibition (%)	Superposition (%)
BPCE	17.9	–	13.5	–
Malonic acid	31.3	7.4	32.2	10.3
Sodium phosphate	10.1	21.1	11.7	27.1
Iodine acetic acid	19.3	15.3	17.4	17.9

**Fig. 3** Effect of black pepper chloroform extract on *E. coli* pyruvic acid content



BPCE affects both *E. coli* and *S. aureus*. BPCE alters the permeability of the cellular membrane, induces the leakage of low-molar-mass metabolites and other constituents, and ultimately triggers cell death (Hao et al. 2009; Yao et al. 2012). Several investigators (Rhayour et al. 2003; Lv et al. 2011) found that treatment with essential oils can damage *B. subtilis* or *E. coli* cells by increasing cell permeabilization and disrupting membrane integrity. Their findings agree with the present results.

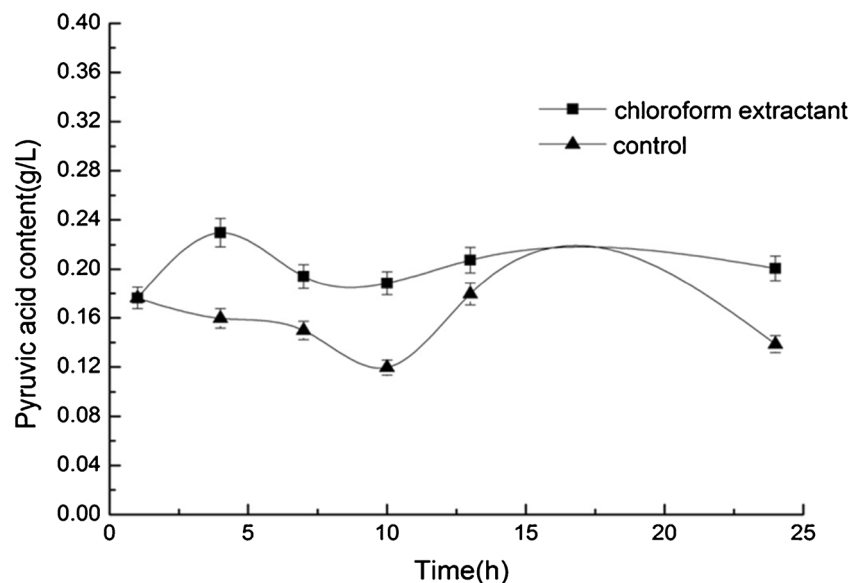
### Bacterial respiratory metabolism

The main purpose of this experiment was to identify the bacterial metabolic pathway inhibited by BPCE. Cells obtain energy by respiratory metabolism. Obstruction of this metabolism

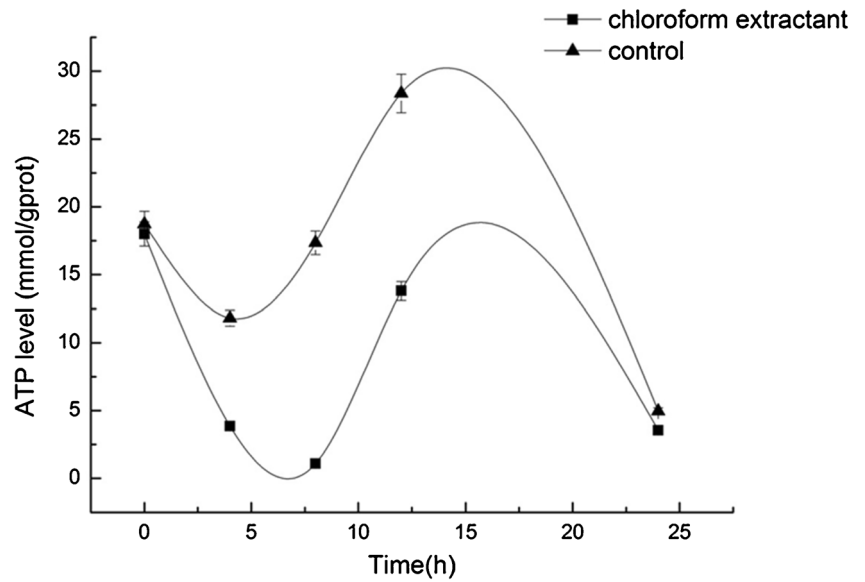
results in the death of bacteria, because the key metabolites and energy cannot be normally and rapidly synthesized. Malonic acid, iodine acetic acid and sodium phosphate inhibit the tricarboxylic cycle (TCA), the Embden–Meyerhof–Parnas pathway (EMP), and the hexosephosphate shunt pathway (HMP) of bacteria. The weaker the synergic inhibition of BPCE and inhibitors, the lower the superposing inhibition of the bacteria; this phenomenon implies that BPCE and the inhibitor have the same pathway of respiratory metabolism (Tong et al. 2005).

As shown in Table 1, the inhibition rates of respiratory metabolism for *E. coli* and *S. aureus* by BPCE were 17.9 and 13.5 %, respectively. The superposing inhibitory rates after adding malonic acid, sodium phosphate, and iodine acetic acid were 7.4, 21.1, and 15.3 % for *E. coli*, respectively,

**Fig. 4** Effect of black pepper chloroform extract on *S. aureus* pyruvic acid content



**Fig. 5** Effect of black pepper chloroform extract on *E. coli* ATP level



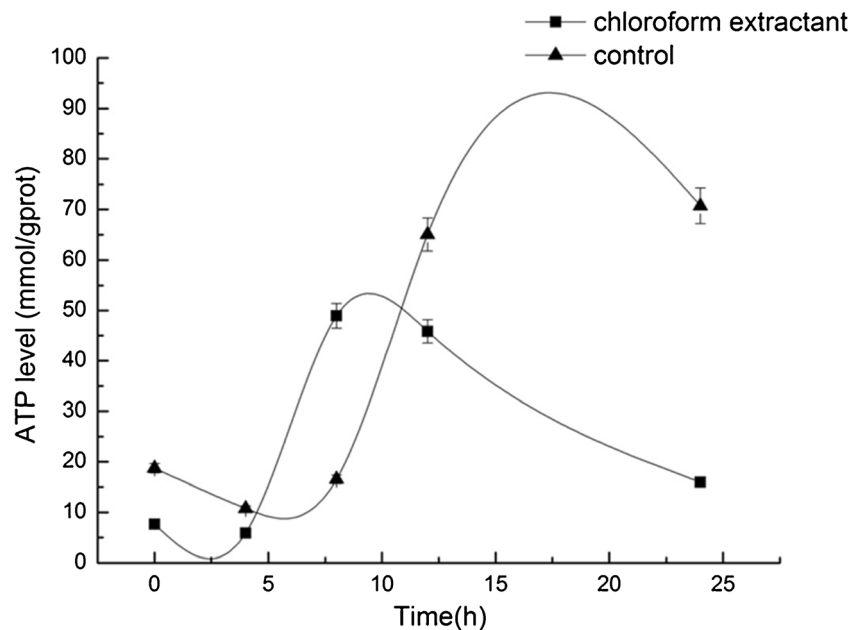
and 10.3, 27.1 and 17.9 % for *S. aureus*, respectively. The results showed that the effects of malonic acid on the superposing inhibition of BPCE for both bacteria were the weakest, while the effects of sodium phosphate were the strongest. These indicate that malonic acid and BPCE inhibited the same pathway of respiratory metabolism, which means BPCE inhibited the TCA pathway. TCA is a highly important metabolic pathway in organisms. This pathway not only provides energy for cells but also provides materials for synthesizing biomacromolecules. Obstruction of this pathway leads to the depletion of succinate dehydrogenase; the reduction of ATP, NADH, and metabolites; and the induction of cell death (Ma et al. 2010). Dong et al. (2010) showed that pomegranate peel inhibited the HMP pathway. While, Zhong et al. (2001) found

that protamine had little impact on respiratory metabolism of bacteria, which is different with our work. Although different bacteriostatic agents have different influence on respiratory metabolism of bacteria, they can still lead to apoptosis of bacteria through other pathway.

**Pyruvic acid content**

Pyruvic acid is an extremely important intermediate. Pyruvic acid connects EMP and TCA to HMP. Pyruvic acid accumulation inhibits the normal physiological metabolism, especially the TCA pathway of bacteria. The TCA pathway is crucial in catabolism and anabolism; blockage of this pathway

**Fig. 6** Effect of black pepper chloroform extract on *S. aureus* ATP level



hinders carbohydrate synthesis (Tomar et al. 2003; Causey et al. 2004; Wang et al. 2015).

In the entire incubation process of *E. coli*, the pyruvic acid content in the control groups slightly increased within 4–7 and 10–12 h. However, the total pyruvic acid content decreased from 0.254 g/L to the final concentration of 0.114 g/L (Fig. 3). Upon the addition of BPCE, the concentration of pyruvic acid significantly increased ( $p \leq 0.05$ ) during the first 7 h. The final value was 0.185 g/L. The values for *S. aureus*, increased within 10–18 h, and then sharply decreased for the control, whereas the values for the treatment group increased except within 4–8 h (Fig. 4). After 24 h, the pyruvic acid concentration in the treatment group was 0.201 g/L, whereas that in the control group was 0.139 g/L. These results indicate the accumulation and leakage of pyruvic acid, which may be attributed to the disrupted selective permeability of the cell membranes in *E. coli* and *S. aureus*. Since pyruvic acid was very important in cells' metabolic, the accumulation of it will lead to abnormal metabolism (Tomar et al. 2003). Besides, the result is consistent with the conclusions of SEM observations and bacterial respiratory metabolism.

#### ATP levels

Cellular ATP concentration indicates viability, and ATP is rapidly degraded when cells die; thus, ATP loss forces the cell to maintain a proton-motive force across the membrane at the expense of the newly synthesized ATP (Palmer et al. 2007). The ATP levels of *E. coli* and *S. aureus* are shown in Figs. 5 and 6, respectively. In the control groups, the ATP levels of both bacteria were increased from 4th hour, and then drastically declined from 12th hour. In the treatment groups, the ATP levels decreased in the first 8 and 4 h for *E. coli* and *S. aureus*, respectively, which marked growth and reproduction. Other variations were similar between the treatment and control groups except *S. aureus*, the ATP level of it decreased after 8th hour. Although the controls and treatment groups had the same variation, the ATP levels of the treatment groups were significantly lower than those of the control groups. The decreased ATP level is probably due to excessive cell apoptosis and excessive ATP consumption, because apoptosis is an ATP-dependent process (Zamaraeva et al. 2005). This phenomenon indicates that BPCE inhibited the respiratory metabolism of both bacteria, which led to obstructed hydrogen acceptance and delivery. Moreover, ATP synthesis was impaired because of the proton-motive force (Carvajal-Arroyo et al. 2014) or the decrease in membrane potential (Cui et al. 2012). Thus, ATP production was inhibited. In other words, BPCE weakened the production capacity of the cells. A previous study (Koshlukova et al. 1999) established a correlation between ATP release and cell killing. Their results showed that intracellular ATP drastically reduced while extracellular ATP accumulated.

In summary, black pepper has exhibited excellent antimicrobial activity to the both bacteria. Piperine, terpenes and flavones are the main chemicals in black pepper. There was one recent study shows monoterpenes in *Crithmum maritimum* L. and *Inula crithmoides* L. essential oils have good antimicrobial activity to *E. coli* and *S. aureus* (Jallali et al. 2014). Piperine also had antimicrobial activity (Salie et al. 1996). Moreover, Dorman and Deans (2000) found the volatile oils exhibited considerable inhibitory effects against Gram-positive and Gram-negative bacteria. Thus, there is a very complex relationship between chemical structures in black pepper and antimicrobial activities, which needs further study.

#### Conclusion

This research elucidated the mechanism by which BPCE inhibits the growth of *E. coli* and *S. aureus* growth by assessing cell morphology, respiration, pyruvic acid content, and ATP level of these bacterial. SEM results showed that the bacterial cells exhibited adhesion, fracture, and accumulation. The antibacterial components of pepper restrained cellular respiration by disrupting the TCA pathway. The accumulation of pyruvic acid and the reduction of ATP proved that BPCE can change cell membrane permeability, destroy bacterial respiratory metabolism and ultimately lead to pyknosis and death. The experimental results provide an approach to exploit safe and healthy natural antimicrobial agents with applications in the food industry. This study is of great significance to food security.

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