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ARTICLE

EVALUATION OF HEATED CHICKEN EGGSHELL POWDER AS ANTIBACTERIAL AGAINST ESBL (EXTENDED SPECTRUM BETA LACTAMASE) PRODUCING *Escherichia coli*: *IN-VITRO* AND *IN-SILICO* STUDIES

[*Evaluasi Serbuk Cangkang Telur Ayam yang Dipanaskan sebagai Antibakteri terhadap Escherichia coli Penghasil ESBL (Extended Spectrum Beta Lactamase): Studi In-Vitro dan In-Silico*]

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ABSRACT

Antimicrobial resistance (AMR) has become a critical global health concern, particularly with the increasing prevalence of *Extended-Spectrum β-Lactamase* (ESBL)-producing *Escherichia coli*. This study aimed to evaluate the antibacterial activity of heated chicken eggshell powder (HCEP) against ESBL-producing *E. coli* through both *in-vitro* and *in-silico* approaches. The *In-silico* molecular docking studies revealed that calcium diglyceroxide exhibited the strongest binding affinity toward dihydrofolate reductase – 5.9 kcal/mol, suggesting disruption of folate metabolism as a potential antibacterial mechanism. Meanwhile, *in-vitro* results demonstrated measurable antibacterial activity, with inhibition zones ranging from 6.90 to 12.36 mm and minimum inhibitory concentration (MIC) values of 1.421 µg/ml for non-ESBL and 2.842 µg/ml for producing-ESBL *E. coli*. The antibacterial effect was attributed to calcium oxide content, which generates reactive oxygen species (ROS) and induces a highly alkaline microenvironment. The correlation analysis showed a strong positive relationship between sample concentration and inhibition zone as antibacterial against *E. coli* producing-ESBL (r = 0.987). These suggest that increasing the concentration of the HCEP sample results in a larger inhibition zone. Overall, these findings highlight HCEP as a potential alternative antibacterial agent, particularly in topical applications, while also offering a sustainable approach to eggshell waste utilization.

Keywords: Chicken eggshell, antibacterial, *in-vitro, in-silico*

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ABSTRAK

Resistensi antimikroba telah menjadi masalah kesehatan global yang sangat krusial terutama dengan meningkatnya prevalensi Escherichia coli penghasil Extended-Spectrum β-Lactamase (ESBL). Penelitian ini bertujuan untuk mengevaluasi aktivitas antibakteri dari serbuk cangkang telur ayam yang dipanaskan terhadap Escherichia coli penghasil ESBL melalui pendekatan in-vitro dan in-silico. Hasil uji in-silico melalui docking molekuler menunjukkan bahwa senyawa kalsium digliseroksida memiliki afinitas ikatan terkuat terhadap enzim dihidrofolat reduktase, dengan nilai energi ikat sebesar –5,9 kcal/mol, yang mengindikasikan potensi gangguan terhadap metabolisme folat sebagai salah satu mekanisme kerja antibakteri. Sedangkan, hasil uji in-vitro menunjukkan adanya aktivitas antibakteri diukur dengan zona hambat berkisar antara 6,90 hingga 12,36 mm, serta nilai Konsentrasi Hambat Minimum (KHM) sebesar 1,421 µg/ml untuk non-ESBL dan 2,842 µg/ml penghasil-ESBL E. coli. Efek antibakteri ini diduga berasal dari kandungan kalsium oksida yang menghasilkan reactive oxygen species (ROS) dan menciptakan lingkungan basa kuat. Analisis korelasi menunjukkan adanya hubungan positif yang kuat antara konsentrasi sampel dan zona hambat sebagai antibakteri melawan E. coli penghasil ESBL (r = 0,987). Hasil ini menunjukkan bahwa peningkatan konsentrasi sampel HCEP menghasilkan zona hambat yang lebih besar. Secara keseluruhan temuan ini menyoroti HCEP sebagai agen antibakteri alternatif yang potensial khususnya untuk aplikasi topikal, serta memberikan pendekatan berkelanjutan dalam pemanfaatan limbah cangkang telur.

***Kata kunci**: Cangkang telur ayam, antibakteri, in-vitro, in-silico*

INTRODUCTION

Antibiotic Microbial Resistance (AMR) has emerged as one of the major challenges in global health today (Safitri *et al.*, 2024). The World Health Organization (WHO) has stated that bacterial resistance is one of the ten global health threats to humanity, with an estimated death toll of around 5.2 million people in East and Southeast Asia over the next decade (Cristina, 2023). The *Enterobacterales* group has been identified by the World Health Organization (WHO) as its top priority in combating antibiotic resistance due to the emergence of *Extended-Spectrum β-Lactamase* (ESBL) resistance. Among this group, *Escherichia coli* is the bacterium with the highest resistance rate (Noster *et al*., 2021). *E. coli* has also been reported as a significant cause of bloodstream infections (BSI), contributing to both community and hospital-acquired cases (Sunarno *et al.*, 2023).

In addressing the growing challenge of antimicrobial resistance, the exploration of alternative sources with antibacterial potential has become increasingly important. One such source is eggshell waste, which is abundantly produced as a byproduct of the food industry and household consumption. According to an estimation by the United States Department of Agriculture (USDA), global egg consumption is projected to reach approximately 8,917 million dozen by 2028, highlighting the enormous amount of eggshell waste that will inevitably be generated (Das *et al*., 2022). Several studies have indicated that eggshells possess notable antimicrobial properties, making them a promising candidate for alternative or complementary strategies in combating bacterial infections. Therefore, the utilization of eggshell waste not only offers potential biomedical applications but also provides an environmentally sustainable solution to reduce organic waste accumulation.

Chicken eggshells consist of the main compound calcium carbonate ($CaCO_3$) by 90.9%. $CaCO_3$ heated by high-temperature calcination will produce calcium oxide (CaO) compounds that have antibacterial activites (Chadijah *et al*. 2016). Al-Azzawi *et al*. (2023) reported that heated chicken eggshell powder (HCEP) has antibacterial ability with a diameter inhibition zone of 22 mm against *E. coli*. Meanwhile, research by Ohshima *et al.* (2015) reported that chicken eggshell powder has a minimum inhibitory concentration (MIC) against *E. coli* bacteria of 0.43 mg/ml. Heated chicken eggshells exhibit two primary antibacterial mechanisms. The first involves the generation of reactive oxygen species (ROS) which induce oxidative stress leading to bacterial DNA damage and subsequent cell death (Nath *et al.*, 2021). The second mechanism is associated with their highly alkaline nature with a pH of approximately 12.96, creating a strongly base environment in which bacteria are unable to survive (Farah *et al.*, 2019; Razak *et al.*, 2023).

In-vitro and *in-silico* studies of the antibacterial activity of chicken eggshell powder against ESBL-producing *E. coli* have never been conducted before. Antibacterial activity testing through *in-vitro* methods can demonstrate the potential of heated eggshell powder as an alternative antibiotic.

Meanwhile, *in-silico* studies investigate the interactions of compounds contained in heated eggshell powder, namely CaO, $CaCO_3$, $Ca(OH)_2$, SO_3, Mn_2O_3, Fe_2O_3, and $C_6H_{14}CaO_6$, to determine which compounds may contribute to the antibacterial mechanism (de Oliveira *et al.*, 2020; Das *et al*., 2022). It is expected that these *in-vitro* and *in-silico* studies will serve as scientific evidence that heated eggshell powder can be utilized as a new antibacterial alternative to combat resistant bacteria.

MATERIALS AND METHODS

Materials

In-vitro Studies

Meropenem antibiotic were obtained from local drug store, distilled water, sterile disc paper (Himedia®), ethanol 70%, Mueller Hinton Agar (Himedia®), Mueller Hinton Broth (Himedia®), sodium chloride (NaCl 0.9%), glycerin, chicken eggshell powder, incubator (memmerth®), analytical balance (Shimadzu AUY-220®), McFarland Suspension 0,5 (Himedia®), autoclave (Hiclave HVE-50®), Biosafety Cabinet (Thermo fisher scientific®),(Iwaki/pyrex®), microscope, sterile cutton swab, micropipette (Endo®), micropipette tip (Biologix®) and 96-well microplate (Biologix®).

In-silico Studies

Notebook computer (Acer®), Biova Discovery Studio Visualizer, autodocktools v1.5.7, pymol v2.5.3, pyrx v0.8, ligplot$^+$ v2.3.1 and IBM SPSS Statistics 26.

Bacterial Strain

Escherichia coli ATCC 25922 as non-ESBL bacteria and clinical isolate *E. coli* as Producing-ESBL bacteria were obtained from Tanjungpura University Pontianak and Indonesia University.

Methods

Molecular Docking

The *in-silico* analysis in this study was carried out to examine the interactions between five receptors of *E. coli* that represent the main mechanisms of antibiotic action (PDB IDs: 2EX6, 1DYJ, 4DUH, 2O7O, and 5IGJ). The binding energy of the test compounds was compared with a positive control, which in this study was meropenem. The first step involved obtaining the test compounds from heated chicken eggshell powder (HCEP) via PubChem (https://pubchem.ncbi.nlm.nih.gov/). The compounds contained in HCEP include CaO, $CaCO_3$, $Ca(OH)_2$, SO_3, Mn_2O_3, Fe_2O_3, and $C_6H_{14}CaO_6$ (de Oliveira *et al*., 2020; Das *et al*., 2022). Ligand and receptor preparation was performed using AutoDock Tools v1.5.7. Validation was carried out with PyMol v2.5.3, while docking simulations were conducted with PyRx v0.8. Visualization of ligand–receptor interactions was carried out using LigPlot+ v2.3.1.

Preparation of Heated Chicken Eggshells Powder

Chicken eggshells were collected from the Food Merchant around Pontianak City, West Kalimantan. Separate the membrane from the eggshell using tweezers. The membrane of chicken eggshells possesses antibacterial activity (Aprilisna *et al.*, 2015). Therefore, separating the membrane from the eggshell is necessary to obtain unbiased data. Then clean the eggshell by soaking it in water. Put the eggshell into the oven at 105^{o}C for 90 minutes. Furthermore, chicken eggshells were pulverized with a blender and then sieved using a 120 mesh sieve. The finely ground eggshell powder was stored in a dry container (Chadijah *et al.*, 2016; Farid *et al*., 2021).

The calcination process using a furnace was carried out at the Laboratorium Terpadu of Universitas Tanjungpura. The eggshell powder was put into the furnace and then run at 900^{o}C for 3 hours. After calcination, the heated chicken eggshell powder (HCEP) was stored in a desiccator and weighed (Ohshima *et al.*, 2015; Nath *et al.*, 2021).

Organoleptic and Moisture Content Test

The organoleptic test was carried out through physical observation using the five senses to describe the color, shape, and odor. Meanwhile, the moisture content test was conducted by weighing 1 gram of chicken eggshell powder on a pre-calibrated moisture balance tray, then covering it and initiating the drying process until the percentage of moisture content was displayed. The determination of moisture content was performed in triplicate (Utami *et al.*, 2017).

Test Solution Preparation

Bacteria Subculture was carried out by taking bacteria from pure stock using 1 sterile ose needle and then streaked on MHA media. After that, incubate the MHA media for 24 hours at 37ºC (LRH Dima and Astuty Lolo, 2016). Preparation of bacterial suspensions is done by taking the subculture bacteria using 1 sterile ose needle and then inserted in a test tube containing 10 ml of 0.9% NaCl. Homogenize the bacterial suspension using a vortex. After that, the bacterial suspension was compared with McFarland 0.5 (Himedia®) standard solution, which is equivalent to 1.5 x 10^8 CFU/ml (Aviany and Pujiyanto, 2020).

Disc Diffusion Test

Solutions series were made at concentrations of 5%, 10%, 20%, and 30% (µg/ml) by weighing 0.25; 0.5; 1; and 1.5 grams of HCEP, respectively and then dissolved in 5 ml of glycerin in a vial. Bacterial suspension was pipetted as much as 25 µL and then dripped on MHA (Himedia®) media and then spread using a spreader on the entire surface of the agar (Makalew *et al*., 2016). Take a sterile disc paper and soak it in each concentration of HCEP for 5 minutes. After that, place the disc paper containing HCEP on the previously marked MHA (Himedia®) media. The negative control used was glycerin, while the positive control used was meropenem antibiotic (10 µg/disc). Petri dishes were incubated for 24 hours at 37ºC. The results of the incubation showed the inhibition zone formed around the disk was then measured using a caliper with mm units. Three measurements were taken and then averaged (Mulqie *et al.*, 2022). Disc diffusion testing was done as many as 3 replications.

Microdilution Test

Microdilution Test of HCEP were counducted to determining minimum inhibitory concentration (MIC) using a sterile microplate 96 well. A total of 100 µl of MHB (Himedia®) media was inserted in column wells 1-12 in rows A-D. Next, take the test sample with a concentration of 22.727 µg/ml as much as 100 µl into column 1 and homogenized. After that, take 100 µl in column 1 and then transfer to column 2 and homogenize. The dilution procedure was repeated until column 10 and then 100 µl of the final column was taken and discarded. The total volume of columns 1-10 becomes 100 µl (first dilution). Add 10 µl of bacterial suspension into the wells of columns 1-11 in rows A-C. The total volume after being given the bacterial suspension becomes 110 µl (second dilution). Column 11 was used as the growth control (GC), containing 110 µl MHB (Himedia®) media and bacterial suspension, while column 12 was used as the negative control (NC) containing only 100 µl MHB (Himedia®) media. Row 12 was not given a bacterial suspension in order to serve as a control comparison (CC) when observing the difference in turbidity between wells that were given a bacterial suspension and those that were not. The microplate was incubated at 37ºC for 24 hours. Then, bacterial growth was observed by looking at the turbidity and the formation of bacterial colonies in each well. The smallest concentration of the column that showed clarity was determined as the minimum inhibitory concentration value (Makalew *et al*., 2016; Mulqie *et al.*, 2022).

Statistical Analysis

The bacterial diameter inhibition zone data were analyzed for significant differences using IBM SPSS Statistics 26, with a significance at *p-value* < 0.05. The minimum inhibitory concentration (MIC) was determined from the lowest concentration in the dilution series that showed complete clarity. Subsequently, the results of the sample concentration and Inhibition zone tests were compared using Pearson's correlation test in IBM SPSS Statistics 26.

RESULTS

Molecular Docking

Table 1. Docking Validation of *Escherichia coli* Receptors (*Validasi Proses Docking pada Reseptor Escherichia coli*).

No.	Receptors (*Reseptor*)	Protein PDB ID	RMSD (Å)	Binding Energy (Kcal/mol) (*Energi Ikatan (Kkal/mol)*)
1.	Dihydrofolate Reductase	1DYJ	0.862	-8.9
2.	DNA gyrase subunit B	4DUH	0.484	-8.5
3.	Penicillin Binding Protein-4	2EX6	1.536	-6.4
4.	TetR Ribosom 30S	2O70	0.545	-9.4
5.	Macrolide 2'-phosphotransferase type I Ribosom 50S	5IGJ	0.382	-9.0

Table 2. Molecular Docking of HCEP Compounds Againts *Escherichia coli* receptors (*Pendockingan Molekuler Senyawa HCEP terhadap Reseptor Escherichia coli*).

No.	Ligand (*Ligand*)	CID	Binding Energy (kcal/mol) (*Energi Ikatan (Kkal/mol)*)				
			1DYJ	4DUH	2EX6	2O7O	5IGJ
1.	Calcium oxide[a]	14778	-2.5	-2.8	-2.5	-2.5	-2.5
2.	Calcium carbonate[a]	10112	-3.9	-3.9	-3.9	-3.2	-3.8
3.	Calcium hydroxide[a]	6093208	-3.1	-3	-3.2	-2.8	-3.3
4.	Calcium diglyceroxide[a]	32162225	-5.9	-5.2	-5	-4.6	-5.4
5.	Sulfur trioxide[a]	1099	-3.3	-3.3	-3.5	-2.9	-3.5
6.	Manganese(II)oxide[a]	160959	-3.7	-4.1	-4.4	-4	-4.6
7.	Iron(III)oxide[a]	518696	-4.2	-4	-4.1	-4.5	-4.2
8.	Meropenem[b]	441130	-7.2	-6.3	-5.8	-7.1	-6.4
9.	Native Ligand[c]	-	-8.9	-8.5	-6.4	-9.4	-9.0

a = HCEP compounds, b = antibiotics control, c = native ligand of each *Escherichia coli* receptors *(a = senyawa HCEP, b = kontrol antibiotik, c = ligan native dari setiap reseptor Escherichia coli)*

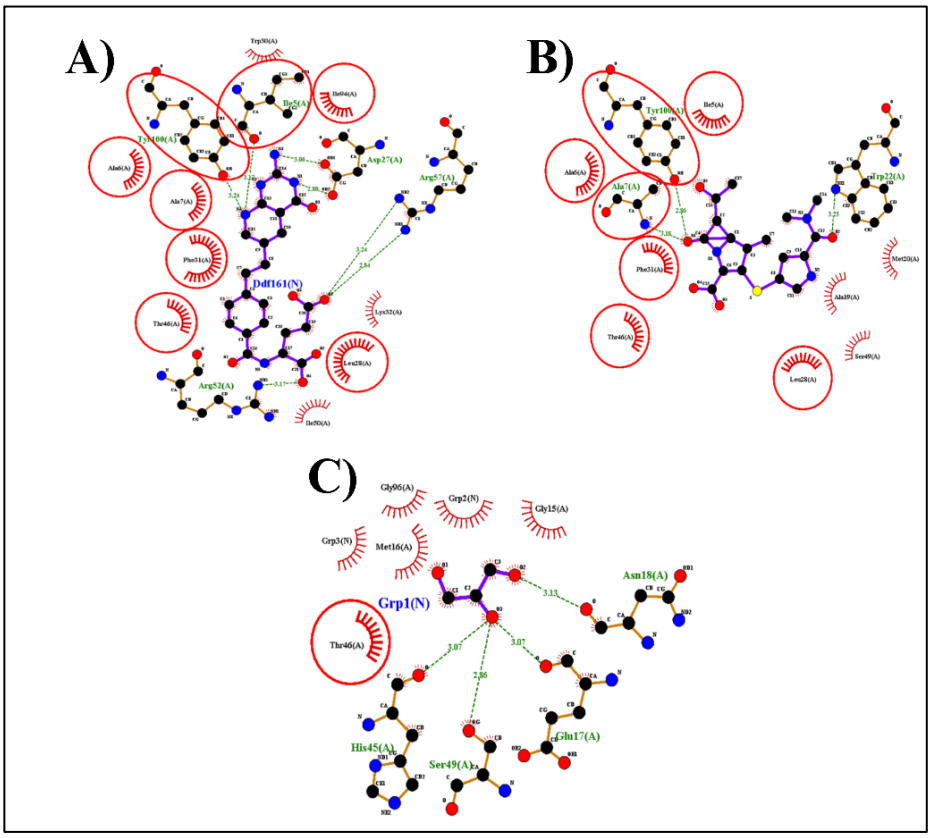


Figure 1. Interaction Between Ligand, Calcium diglyceroxide and Meropenem againts Dihydrofolate Reductase of *Escherichia coli*: Ligand Native (a), Meropenem (b) and Calcium diglyceroxide (c) (*Interaksi antara Ligand, Kalsium digliseroksida, dan Meropenem terhadap Dihidrofolat Reduktase dari Escherichia coli: Ligand Native (a), Meropenem (b), dan Kalsium Digliseroksida (c)*).

Organoleptic and Moisture Content Properties of HCEP

(a) (b)

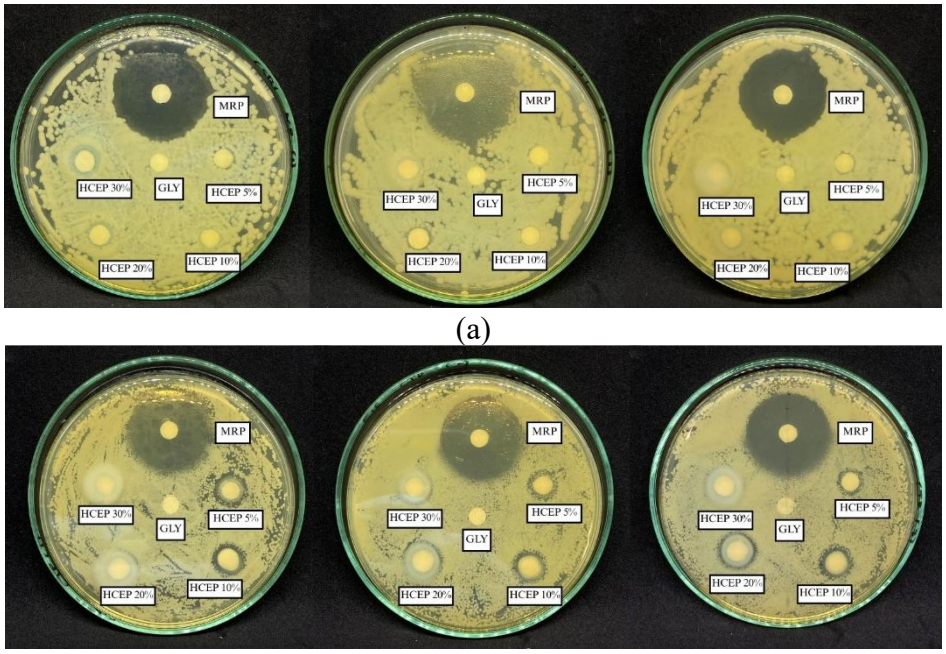
Figure 2. Characteristics of (a) Non-Heated Chicken Eggshell Powder (NCEP) and (b) Heated Chicken Eggshell Powder (HCEP) (*Karakteristik (a) Serbuk Cangkang Telur Ayam yang Tidak Dipanaskan dan (b) Serbuk Cangkang Telur Ayam yang Dipanaskan*).

Table 3. Result of Organoleptic and Moisture Content of HCEP (*Hasil Uji Organoleptik dan Kadar Air dari Serbuk Cangkang Telur Ayam yang Dipanaskan*).

Result (*Hasil*)	**Before Calcination** (*Sebelum Kalsinasi*)	**After Calcination** (*Setelah Kalsinasi*)
Organoleptic *(Organoleptik)*	Color: brownish-white (*Warna: putih kecoklatan)* Shapes: fine powder (*Bentuk: serbuk halus*) Smell: signature (*Bau: khas cangkang telur*)	Color: white (*Warna: putih*) Shapes: fine powder (*Bentuk: serbuk halus*) Smell: odorless (*Bau: tidak berbau*)
Moisture Content (%) *(Kadar Air (%))*	1.58 ± 0.076	0.68 ± 0.072*

*result of the average of three repetitions *(*hasil rata-rata dari tiga kali pengulangan)*

Antibacterial Activity of HCEP



(a)

(b)

Figure 3. Inhibition Zone of HCEP Againts (a) *Escherichia coli* non-ESBL and (b) *Escherichia coli* Producing-ESBL in three replicate *(Zona Hambat Serbuk Cangkang Telur yang Dipanaskan terhadap (a) Escherichia coli non-ESBL dan (b) Escherichia coli penghasil ESBL dalam tiga ulangan)*

Table 4. Result of Diameter Inhibiton Zone HCEP Againts *Escherichia coli* non-ESBL and *Escherichia coli* Producing-ESBL (*Hasil Diameter Zona Hambat HCEP terhadap Escherichia coli non-ESBL dan Escherichia coli penghasil ESBL*).

Bacteria (*Bakteri*)	**Group Test** (*Test Grup*)	**Diameter of Inhibition Zone (mm) n=3** (*Diameter Zona Hambat*)
Escherichia coli non-ESBL	Glycerin (Control Negative) *(Gliserin (Kontrol Negatif))*	0^{a}
	Meropenem (Control Positive) *(Meropenem (Kontrol Positif))*	$29.26^{b} \pm 0.58$
	5%	$10.11^{ab} \pm 0.69$
	10%	$10.40^{ab} \pm 0.73$
	20%	$12.36^{ab} \pm 0.66$
	30%	$11.78^{ab} \pm 0.66$
Escherichia coli Producing-ESBL	Glycerin (Control Negative) *(Gliserin (Kontrol Negatif))*	0^{a}
	Meropenem (Control Positive) *(Meropenem (Kontrol Positif))*	$33.10^{b} \pm 0.48$
	5%	$6.90^{ab} \pm 0.17$
	10%	$7.68^{ab} \pm 0.19$
	20%	$8.63^{ab} \pm 0.17$
	30%	$9.30^{ab} \pm 0.13$

a = significant difference to the meropenem ($p<0.05$), b = significant difference to the gylcerin ($p<0.05$) *(a = berbeda signifikan dengan meropenem ($p<0,05$), b = berbeda signifikan dengan gliserin ($p<0,05$))*

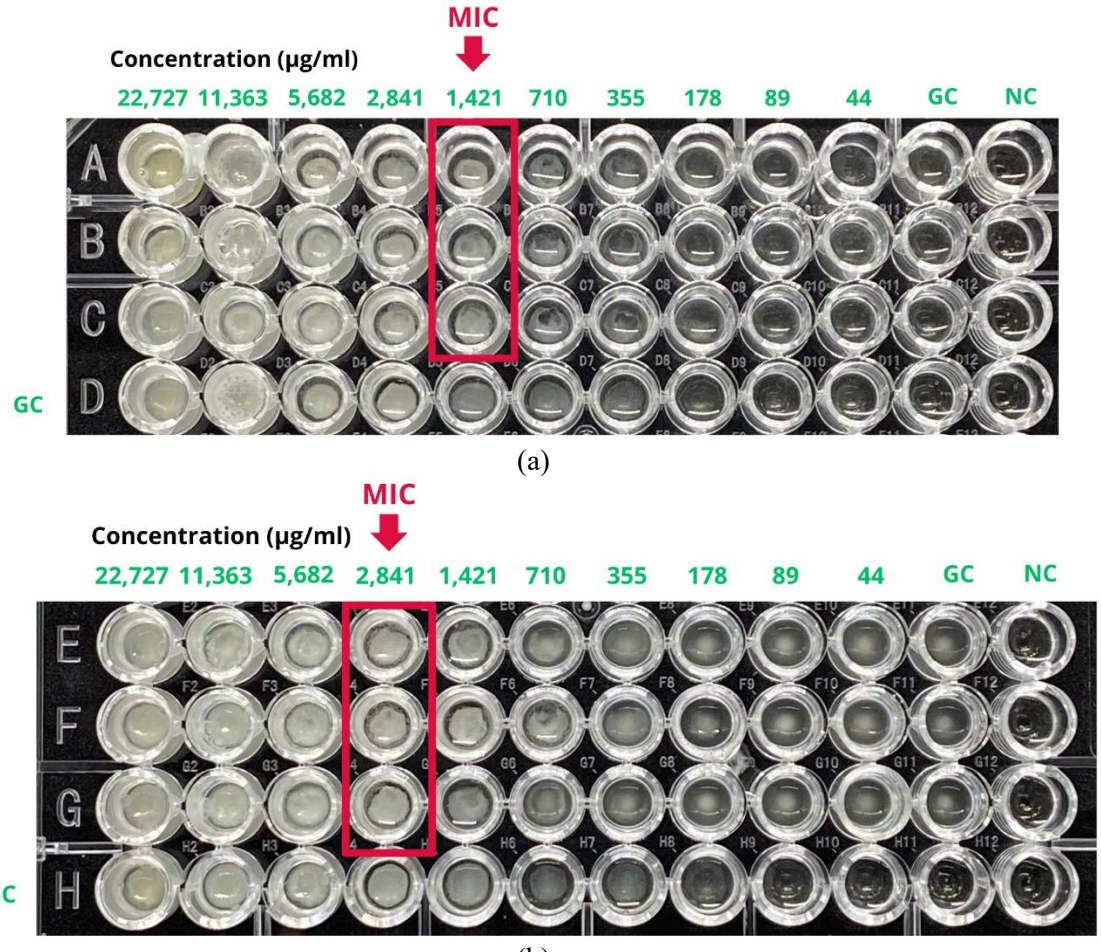


Figure 4. Minimum Inhibitory Concentration of HCEP against (a) *Escherichia coli* non-ESBL and (b) *Escherichia coli* Producing ESBL (*Konsentrasi Hambat Minimum HCEP terhadap (a) Escherichia coli non-ESBL dan (b) Escherichia coli penghasil ESBL*).

Table 5. Minimum Inhibitory Concentration (MIC) Observations of HCEP against (a) *Escherichia coli* non-ESBL and (b) *Escherichia coli* producing ESBL *(Pengamatan Konsentrasi Hambat Minimum dari HCEP terhadap (a) Eschericia coli non-ESBL dan (b) Eschericia coli penghasil-ESBL).*

Bacteria (*Bakteri*)	**Minimum Inhibitory Concentration (µg/ml)** (*Konsentrasi Hambat Minimum(µg/ml)*)
Escherichia coli non-ESBL	1.421*
Escherichia coli Producing-ESBL	2.842*

*result of the average of three repetitions *(*hasil rata-rata dari tiga kali pengulangan)*

Correlation Analysis Between Sample Concentration and Inhibition Zone

Table 6. Correlation Analysis Between Sample Concentration and Inhibition Zone Againts *Escherichia coli* non-ESBL and *Escherichia coli* Producing ESBL *(Analisis Korelasi Antara Konsentrasi Sampel dan Zona Inhibisi Terhadap Escherichia coli non-ESBL dan Escherichia coli penghasil ESBL*).

Correlation (*Korelasi*)	**Sample Concentration** (*Konsentrasi Sampel*)	**Inhibition Zone *Escherichia coli* non-ESBL** (*Zona Inhibisi Escherichia coli non-ESBL*)	**Inhibition Zone *Escherichia coli* Producing-ESBL** (*Zona Inhibisi Escherichia coli penghasil ESBL*)
Sample Concentration *(Konsentrasi Sampel)*	1	0.866	0.987**
Inhibition Zone *Escherichia coli* non-ESBL *(Zona Inhibisi Escherichia coli non-ESBL)*	0.866	1	0.866
Inhibition Zone *Escherichia coli* Producing-ESBL *(Zona Inhibisi Escherichia coli penghasil ESBL)*	0.987**	0.866	1

**Correlation is significant at the ($p<0.05$), level *(Korelasi signifikan pada tingkat ($p<0.05$))*

DISCUSSION

Molecular Docking

Docking validation revealed RMSD values below 2 Å across all protein targets, specifically ranging from 0.382 to 1.536 Å. Such low RMSD values are widely regarded in the literature as a benchmark for accurate pose prediction in molecular docking studies. Wang *et al.* (2016) affirm that an RMSD below 2.0 Å between the docked and crystal ligand conformations indicates a successful reproduction of the native binding mode, reflecting robust sampling capability of the docking algorithm (Wang *et al.*, 2016). Another comprehensive assessment underscores that mean RMSD values under 2.0 Å reliably demonstrate that the docking program has effectively recapitulated the experimentally observed molecular interactions (Rao *et al.*, 2007). The result of docking validation of *Escherichia coli* receptors can be seen in Table 1.

The *in-silico* results demonstrated that the inorganic compounds contained in heated chicken eggshell powder (HCEP) exhibited varying binding energy toward essential protein targets of ESBL-producing *E. coli*. Overall, the binding energies of HCEP compounds were higher compared to the antibiotics control and native ligands, suggesting that these inorganic molecules form relatively weaker interactions. Nevertheless, certain compounds displayed more prominent interactions than others. The result of molecular docking of HCEP compounds against *E. coli* receptors can be seen in Table 2.

The *in-silico* docking analysis highlighted that the strongest interaction among HCEP compounds was observed with the folic acid reductase (1DYJ) target. Specifically, calcium diglyceroxide ($C_6H_{14}CaO_6$) demonstrated the lowest binding energy compared to other HCEP constituents, suggesting that this compound may interfere with the folate biosynthesis pathway in *E. coli*. Since folic acid metabolism is essential for nucleotide synthesis, inhibition at this stage can significantly disrupt bacterial DNA replication and cell survival. This finding aligns with previous studies reporting that inhibitors of dihydrofolate reductase exert potent antibacterial activity by impairing the production of tetrahydrofolate, a crucial cofactor in nucleic acid synthesis (He *et al.*, 2020). The result of ligand-receptor interactions is shown in Figure 1.

The visualization ligand-receptors interaction demonstrated that compounds from HCEP, particularly calcium diglyceroxide ($C_6H_{14}CaO_6$), interacted with the active pocket of dihydrofolate reductase (DHFR) (PDB_ID : 1DYJ). The interaction revealed that these ligands established several hydrogen bonds with polar residues and hydrophobic contacts with residues typically involved in the folate-binding site, such as Asp27, Phe31, Leu28, Ile94, and Arg98. These residues are crucial for substrate stabilization and catalytic function of DHFR, suggesting that occupancy of this binding pocket may interfere with folate metabolism in *E. coli*. As folate reduction is essential for nucleotide biosynthesis, such interactions could disrupt DNA synthesis and impair bacterial proliferation, even if the binding energy of HCEP compounds were moderate compared to standard antibiotics.

In addition to these molecular interactions, the antibacterial activity of HCEP is reinforced by its physicochemical properties. Heat treatment of eggshells produces calcium oxide (CaO), which generates an alkaline microenvironment (high pH) and promotes the formation of reactive oxygen species (ROS) in aqueous media. Previous studies have shown that this combination of alkalinity and oxidative stress exerts broad bactericidal effects by damaging bacterial membranes, proteins, and nucleic acids (Ohshima *et al.*, 2015; Ismael *et al.*, 2024). Therefore, while the docking results suggest moderate binding energy of HCEP constituents to DHFR, the observed antibacterial effect *in-vitro* may result from a synergistic mechanism: partial inhibition of the folate pathway through DHFR binding, combined with non-specific but potent bactericidal effects mediated by ROS generation and elevated pH.

Organoleptic and Moisture Content Properties of HCEP

The preparation of chicken eggshell powder was carried out by weighing 320.2 grams of powder and calcined at 900^{o}C for 3 hours. The results of the heated chicken eggshell powder (HCEP) obtained a weight of 174.83 grams with a yield of 54.6%. The organoleptic test results of HCEP produced white color, fine powder and odorless Figure 2. These characteristics are in accordance with the calcium oxide (CaO) compound. The results of the moisture content test obtained in HCEP have met the requirements of <10% (Kemenkes RI, 2020).

The mass reduction of eggshell powder after calcination is caused by the release of CO2 gas and the decomposition of organic compounds. The decomposed organic compound components of eggshells are derived from proteins such as mucopolysaccharides consisting of chondroitin sulfate A and B, glucosamine, galactosamine, galactose, mannose, and sialic acid (Gago and Dala Ngapa, 2021). There is a difference between the color and odor of chicken eggshell powder before heating. This is because chicken eggshells have protoporphyrin IX pigment, which gives a brown color, and hydrogen sulfide compounds that cause a typical egg odor (Ghosh *et al.*, 2018; Wang *et al.*, 2023). The results of the organoleptic and moisture content test of HCEP can be seen in Table 3.

Disc Diffusion Assay

The inhibition zone values of HCEP against *E. coli* non-ESBL and *E. coli* producing-ESBL bacteria were determined using the disc diffusion method. The results of the diameter of inhibition zone showed that HCEP has antibacterial activity against *E. coli* non-ESBL and *E. coli* producing-ESBL, can be seen in Table 4. When compared to ESBL-producing bacteria, there is a reduction in the inhibition zone diameter between *E. coli* non-ESBL and *E. coli* producing-ESBL, as seen in Figure 3. This occurs not because ESBL enzymes are able to hydrolyze the compounds contained in

HCEP powder, but is instead suspected to be due to other resistance mechanisms, such as efflux pump activity. Efflux pumps are proteins located in the bacterial cell wall that transport substrates such as antibiotics from inside the bacterium to the outside. This mechanism prevents HCEP powder that has just entered the bacterial cell from causing damage, as it is immediately expelled by the efflux pump (Sharma *et al*., 2019).

The compound content that is thought to have antibacterial activity from HCEP is calcium oxide (CaO). CaO can form free radical reactive oxygen species (ROS), which will produce oxidative stress, resulting in damage to bacterial DNA which leads to cell death. The radical compound produced by HCEP is superoxide O_2^-. These ROS can kill bacteria by oxidizing thiol groups, damaging proteins and DNA (Dryden, 2018). In response to ROS, bacteria will secrete enzymes that can bind to radical compounds to reduce the damage that occurs. Enzymes produced by bacteria in fighting ROS are glutathione, nitric oxidase, and peroxiredoxins which work as antioxidants (Mishra and Mishra, 2015). However, the large amount of ROS that exceeds the antioxidants produced results in ROS still being able to kill bacteria with all the defenses produced (Li *et al.*, 2021).

Another mechanism that is thought to have antibacterial ability is the alkaline condition of HCEP. Calcium oxide contained in HCEP has a pH value of 12.8, classified as a strong base (Alam *et al*., 2022). A pH value that is maintained between pH 11-12 during the incubation process can have an antibacterial effect on bacteria (Farah *et al.*, 2019). The higher the pH of the test compound, the higher the antibacterial activity. The mutation ability of bacteria is only able to control acidic pH compared to alkaline pH (Kincses *et al.*, 2021). An increase in pH can cause the cellular respiration process of bacteria to become dysfunctional, causing bacteria to be unable to survive (Khan *et al.*, 2023).

The diameter of the inhibition zone obtained by HCEP against *E. coli* non-ESBL was 12.36 mm, while HCEP against *E. coli* producing-ESBL 9.30 mm. These results are in accordance with the research Al-azzawi *et al*. 2023 produced chicken eggshell powder has an diameter of the inhibition zone of 8-13 mm against *E. coli*. The positive control serves as a comparison of the results of the inhibition zone between the antibiotic and the test sample, while the negative control is used to determine whether there is an antibacterial effect on the provision of glycerin as a solvent for the test sample (Sari *et al.*, 2019; Alda *et al.*, 2022).

Microdilution Assay

The minimum inhibitory concentration (MIC) of HCEP against *E. coli* non-ESBL and *E. coli* Producing-ESBL bacteria were performed using the broth microdilution method. The MIC result of HCEP against *E. coli* non-ESBL and *E. coli* producing-ESBL in Figure 4 and Table 5. The results of the MIC observation of HCEP powder on *E. coli* non-ESBL obtained the first well that gave clarity at the 5th well, so that the MIC value was obtained at 1.421 µg/ml. However, HCEP powder on *E. coli* producing-ESBL obtained the first well that gave clarity at the 4th well, so that the MIC value was obtained at 2.842 µg/ml. The MIC value exceeds the range of MIC ability category > 1.000 µg/ml. The high MIC value will affect the pharmacokinetics/pharmacodynamics of the drug dose to be administered. MIC that is too high allows the administration of drugs with very high doses. This can result in drug overdose, causing poisoning/toxicity (Magréault *et al.*, 2022). This finding concludes that HCEP cannot be developed as an oral medication, but it can be formulated into topical dosage forms such as ointments and gels.

Correlation Analysis Between Sample Concentration and Inhibition Zone

The Pearson correlation analysis between sample concentration and inhibition zone diameter demonstrated a strong positive relationship against *E. coli* non-ESBL ($r = 0.866$) and *E. coli* producing-ESBL ($r = 0.987$). Statistically significant correlation ($p < 0.05$) was observed for the inhibition zone of *E. coli* producing-ESBL, whereas no significant correlation ($p > 0.05$) was found for the inhibition zone of *E. coli* non-ESBL. These findings indicate that increasing the concentration of the HCEP sample results in a larger inhibition zone against *E. coli* producing-ESBL.

CONCLUSION

Based on the results, heated chicken eggshell powder (HCEP) exhibited notable antibacterial activity against ESBL-producing *Escherichia coli* as demonstrated by both *in-vitro* and *in-silico* analyses. The *in-silico* molecular docking revealed that calcium diglyceroxide exhibited the strongest binding affinity toward dihydrofolate reductase (-5.9 kcal/mol), suggesting inhibition of folate metabolism as a possible antibacterial mechanism. Meanwhile, *in-vitro* test showed inhibition zones ranging from 6.90 to 12.36 mm, with MIC values of 1.421 µg/ml for non-ESBL strains and 2.842 µg/ml for ESBL strains. The correlation analysis showed a strong positive relationship between sample concentration and inhibition zone as antibacterial activity against *E. coli* producing-ESBL (r = 0.987). These sugest that increasing the concentration of the HCEP sample results in a larger inhibition zone. In conclusion, HCEP shows significant potential as an antibacterial agent, particularly for topical applications, while also serving as a sustainable approach to eggshell waste management.

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AUTHOR CONTRIBUTIONS

FN: creating the research concept and performing the final revision of the manuscript; HI: developing the research design, writing the initial manuscript draft, and reviewing data interpretation; HK: assisting in formulating the research design, conducting the literature review, and revising the manuscript draft; SNN: contributing to data analysis, preparing figures and tables, and assisting in manuscript revision; IF: conducting methodological validation, compiling results, and editing the manuscript for clarity and consistency; AP: collecting research data and drafting the research article.

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