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Metabolic perturbations and key pathways associated with the bacteriostatic activity of *Clitoria ternatea* flower anthocyanin fraction against *Escherichia coli*

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Abstract

Clitoria ternatea flowers are rich in anthocyanins and possess various biological activities. Specifically, the antibacterial mechanism of action of *C. ternatea* anthocyanins remains unknown and was investigated in *Escherichia coli*. A time–kill assay was used to assess the antibacterial activity and the metabolic perturbations in *E. coli* were investigated utilizing liquid chromatography–mass spectrometry (LC-MS)-based metabolomics. Pathway analyses were carried out for metabolites showing ≥2-fold changes. The anthocyanin fraction remarkably reduced the growth of *E. coli* at 4 h by 95.8 and 99.9% at minimum inhibitory concentration (MIC) and 2× MIC, respectively. The anthocyanin fraction (MIC) had a bacteriostatic effect and was shown to have perturbed glycerophospholipids (1-acyl-sn-glycero-3-phosphoethanolamine, phosphatidylglycerol, diacylglycerol and cardiolipin), amino acids (valine, tyrosine and isoleucine) and energy (ubiquinone and NAD) metabolites at 1 and 4 h. This study demonstrated significant metabolic perturbations of the glycerophospholipid, amino acid and energy metabolism, with these being the key pathways involved in the bacteriostatic activity of anthocyanins from *C. ternatea*, which may have promise as bacteriostatic agents for *E. coli*-related infections.

DATA SUMMARY

All data is available at https://doi.org/10.26180/21687398.v1[1].

INTRODUCTION

Although *Escherichia coli*, Gram-negative bacteria of the family *Enterobacteriaceae*, comprise a common, normal part of the flora in the human gut, they have become a concern because of their pathogenic strains, which are known to cause severe infections, along with the increase in the emergence of multidrug resistance globally [2]. *E. coli* have been identified as significant causative pathogens in bloodstream and urinary tract infections worldwide in community and healthcare settings [3]. They have displayed high rates of resistance to antibiotics such as aminoglycosides, aminopenicillins, fluoroquinolones and third-generation cephalosporins, further complicating and limiting treatment options [3].

The rise in antimicrobial resistance means there is a need for new antimicrobial agents or alternative therapeutic methods/strategies to target infectious diseases. Plants are a rich source of compounds with diverse functional groups in which many are yet to be explored for their therapeutic potential [4]. The *Clitoria ternatea* ('blue pea' or 'butterfly pea') plant belongs to the family *Fabaceae* and is known to grow in various countries in Asia and South and Central America [5, 6]. The leaves, flowers and roots of this plant have been used since ancient times to treat a variety of ailments and health conditions besides reports on their various biological activities (e.g. antioxidant, antidiabetic, anti-inflammatory, antifungal and antiproliferative/anticancer) [5, 6].

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Abbreviations: ADM, agar dilution method; ATCC, American Type Culture Collection; ATP, adenosine triphosphate; BHI, brain heart infusion; c.f.u., colony-forming units; CL, cardiolipin; DG, diacylglycerol; ECMDB, *E. coli* Metabolome Database; FAD, flavin adenine dinucleotide; FC, fold change; KEGG, Kyoto Encyclopedia of Genes and Genomes; LC-MS, liquid chromatography–mass spectrometry; MIC, minimum inhibitory concentration; NAD, nicotinamide adenine dinucleotide; PC, phosphatidylcholine; PCA, principal component analysis; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine; TCA, tricarboxcylic acid; TRIS, 2-amino-2-(hydroxymethyl)propane-1,3-diol.

C. ternatea flowers have ornamental value and are also used as a food colouring agent due to the presence of the compounds known as anthocyanins [7]. The crude extract of the flower has been reported to show antibacterial activity against various bacterial strains (e.g. *E. coli*, *Klebsiella pneumoniae*, *Bacillus subtilis* and *Staphylococcus aureus*), which has mainly been attributed to its major compounds, flavonols and anthocyanins [8, 9].

Metabolomics is a comprehensive analysis investigating large-scale metabolite levels in biological systems. It can be further categorized as targeted (identifies and quantifies selected metabolites of interest, e.g. enzymes, substrates or compounds of a particular pathway) or untargeted (involves measuring all metabolites in a desired biological experimental system, which usually generates a new hypothesis based on the outcome) approach, depending on the nature of the study [10, 11]. Metabolomics is increasingly being used to elucidate the mechanism of action of drugs in a biological system for drug discovery and development [12]. Our previous study [13] obtained an anthocyanin fraction from this flower to determine the antibacterial activity against various pathogenic bacterial strains. Antibacterial activity was observed against *B. subtilis*, *E. coli* and *Bacillus cereus*. As *E. coli* is a pathogen of importance, the present study aimed to determine the antibacterial activity and the mechanism of action of the anthocyanin fraction against *E. coli* through a metabolomics study.

METHODS

Preparation and extraction of samples

Clitoria ternatea cv. Double Blue flowers (freshly harvested) were procured from a horticultural nursery in Subang Jaya, Malaysia. *C. ternatea* flower petals were plucked carefully for use in the study, while the remaining parts were discarded. Before extraction, the petals were cut into smaller pieces. Ethanol extracts of the fresh flower material were prepared by immersing the flowers in 50% ethanol with a solid to liquid ratio of 1:20 (g ml^{-1}) for 3 h at room temperature (25 °C) with constant shaking. Extracts were then vacuum-filtered, and a rotary evaporator was used to concentrate the solution under vacuum at 45 °C. The concentrated solution was freeze-dried and stored at −80 °C until further analysis.

Semi-purification of crude extract by Amberlite XAD-16 column chromatography

The anthocyanin fraction was obtained according to the method in a previous study [14]. Distilled water (100 ml) was used to dissolve the freeze-dried extract of *C. ternatea* flowers (5 g), adjusted to pH 2 using 1M HCl followed by partitioning with ethyl acetate (100 ml) to facilitate the removal of flavonols. The aqueous fraction (containing anthocyanins) was collected and again partitioned two more times with ethyl acetate. The aqueous fraction (containing anthocyanins) was concentrated to a volume of 10 ml under vacuum at 37 °C in a rotary evaporator. The aqueous fraction (containing anthocyanins) was subjected to further purification using Amberlite XAD-16 column chromatography. A chromatography column [S24/29, 25(D)×300 mm (l)] with a sintered glass disc (porosity 0) and a stopcock with a PTFE key were used for the open column chromatography. The Amberlite XAD-16 adsorbent was immersed in methanol overnight prior to loading to ¾ of the column height. Briefly, 1 l of purified water was used to rinse the column followed by activation with 0.5 l of aqueous sodium hydroxide (2%) solution. Acidified water (1 l) was used to wash the column to condition it to pH 3. The concentrated aqueous fraction (10 ml) of the anthocyanin fraction was loaded onto the column, which was then rinsed with acidified water (0.3 l at pH 3) to remove phenolic acids at a flow rate of 10 ml min^{-1}. Anthocyanins were then eluted with acidified methanol [95:5, methanol : acidified water (pH 2), v/v]. The methanol (contains anthocyanins) fraction was freeze-dried and stored at −80 °C until further analysis. The extraction yield of the anthocyanin fraction using Amberlite XAD-16 column chromatography was 18.2%, as reported in our previous study [13].

Time–kill assay of *E. coli* ATCC 25922

The culture of *E. coli* ATCC 25922 was prepared from frozen stock (−80 °C) on tryptic soy agar plates and incubated at 37 °C for 18 h. A single colony was obtained and inoculated into brain heart infusion (BHI) broth (10 ml), incubated at 37 °C for 18 h in a shaking water bath (180 r.p.m.). The overnight culture diluted with BHI broth (1:100) was grown to obtain an OD_{600nm} of 0.50 to achieve an early logarithmic growth phase (i.e. ~10^8 c.f.u. ml^{-1}), followed by dilution (1:100) in 20 ml of BHI broth alone (control group) and BHI broth containing *C. ternatea* anthocyanin fraction at ½ MIC (5 mg ml^{-1}), MIC (10 mg ml^{-1}) and 2× MIC (20 mg ml^{-1}) for 1, 4 and 24 h. One millilitre of sample was taken at 0, 1, 4 and 24 h and serial dilutions were performed according to the pour plate method as described below to determine bacterial growth (c.f.u. ml^{-1}). The formula below was used to count the percentage reduction in the total viable count of c.f.u.:

$$\text{Percentage reduction} = \frac{\text{Count of untreated group at } x \text{ interval} - \text{count of treated group at } x \text{ interval}}{\text{Count of untreated group at } x \text{ interval}} \times 100$$

Growth curve (pour plate method)

The effect of the anthocyanin fraction at ½ MIC (5 mg ml^{-1}), MIC (10 mg ml^{-1}) and 2× MIC (20 mg ml^{-1}) for 1, 4 and 24 h on *E. coli* ATCC 25922 growth was evaluated using the standard pour plate method [15]. Serial dilutions (1:10) at 10^3 to 10^6 of treated bacteria culture were performed and 100 µl of individual diluted bacteria culture was dispensed onto the bottom of Petri dishes. Then 18 ml of molten Mueller–Hinton agar (45 °C) was poured and mixed with the sample by gently swirling the plate. The plates

were incubated at 37 °C for 24 h after the agar solidified. The number of *E. coli* ATCC 25922 colonies was recorded from plates with 30–300 colonies as c.f.u. The microbial data are presented as log c.f.u. ml^{-1} [16].

Statistical analysis

The experiments above were performed in independent triplicates and the results were expressed as the mean value±standard deviation. The data were analysed using one-way analysis of variance (ANOVA) followed by post-hoc Tukey's test to analyse the statistical difference exhibited by *E. coli* ATCC 25922 in terms of growth in untreated (control) and treated groups [½ MIC (5 mg ml^{-1}), MIC (10 mg ml^{-1}) and 2× MIC (20 mg ml^{-1})]. The significance was set at $P<0.05$ using SPSS 23 software (IBM, New York, USA).

Bacterial culture preparation for metabolome analysis

Bacterial isolates of *E. coli* ATCC 25922 were sub-cultured from −80 °C stocks onto tryptic soy agar. A single colony was obtained and inoculated in BHI broth (10 ml). It was incubated overnight at 37 °C with constant shaking (180 r.p.m.). The overnight culture was diluted by 1:100 (2.5 ml was inoculated into 500 ml) of BHI broth and grown to obtain an OD_{600} of 0.5 (mid-exponential growth phase) at 37 °C with constant shaking (180 r.p.m.). Subsequently, 20 ml of mid-exponential culture ($OD_{600}=0.5$) was collected into a 50 ml Falcon tube (this was used as 0 h). For each sample, 50 ml of the mid-exponential culture was treated with the anthocyanin fraction of *C. ternatea* (AF) resuspended in BHI broth at MIC (10 mg ml^{-1}), while the untreated group served as a control sample. Three biological replicates of each group were prepared. The bacterial cultures (20 ml) of treated and untreated groups were collected at 1 and 4 h.

Extraction and liquid chromatography–mass spectrometry (LC-MS) analysis of cellular metabolites

Cellular metabolites were extracted using a previously reported method [17, 18]. The bacterial cultures (20 ml) of treated and untreated groups at 0, 1 and 4 h were immediately transferred into ice-cold 50 ml Falcon tubes. To stop the metabolic processes, the samples were quenched rapidly in a dry ice/ethanol bath for 30 s. The culture was then normalized to 0.5 at OD_{600} (cell counts were at 10^8 c.f.u. ml^{-1}) using fresh BHI broth. The normalized culture (10 ml) was centrifuged (3220 ***g*** at 4 °C for 10 min) and the cell pellet was obtained. Ice-cold 0.9% sodium chloride (2 ml) was used to wash the cell pellet twice followed by centrifugation (3220 ***g*** at 4 °C for 5 min). The residue after centrifugation was removed. After discarding the supernatant, 0.5 ml of dry-ice cold chloroform : methanol : water (1:2:0.8, v/v) extraction solvent containing internal standard at 1 µM of 2-amino-2-(hydroxymethyl)propane-1,3-diol (TRIS) was added to resuspend the cells and facilitate the extraction of the intracellular metabolites. Liquid nitrogen was used to freeze the samples and allowed to thaw on ice to promote the release of intracellular metabolites. The freeze–thaw process was performed thrice and followed by centrifugation (3220 ***g*** at 4 °C for 10 min). The supernatant was obtained and transferred to 1.5 ml centrifuge tubes and centrifuged (14400 ***g*** at 4 °C for 10 min). The samples were stored at −80 °C prior to LC-MS analysis. The samples (3 µl) were analysed on a 1290 Infinity LC system coupled to a 6520 Accurate-Mass Q-TOF mass spectrometer with a dual ESI source (Agilent, Santa Clara, CA, USA) coupled to a HILIC-Z column (2.7 µm, polymeric, 100×2.1 mm; Infinitylab Poroshell, Agilent). The run was performed in positive and negative ion modes at a resolution of 35 000. The detection range was 85 to 1275 *m/z*. The mobile phases were 20 mM ammonium acetate (A) and acetonitrile (B) at a flow rate of 0.3 ml min^{-1}. The applied condition was as follows: 20% of solvent A and 80% B to 50% B over 15 min, and to 5% B at 18 min. The system was washed with 95% A and 5% B for 3 min followed by 20% A and 80% B for 8 min to re-equilibrate the column. Nitrogen was used as the desolvation gas, at 300 °C and a flow rate of 60 l h^{-1}, and He gas was used as the damping gas, with a declustering potential of 40 eV, a collision energy of 5 eV and a collision cell entrance potential of 10 eV. The samples were run in a single batch of LC-MS to avoid batch-to-batch variability [19].

Data processing, bioinformatics and statistical analysis

MZmine 3 was used to analyse raw LC-MS data for chromatographic peak detection. Metabolites were putatively identified by comparing the *m/z* values and molecular formula with the online databases *E. coli* Metabolome Database (ECMDB: https://ecmdb.ca/), Kyoto Encyclopedia of Genes and Genomes (KEGG, https://www.genome.jp/kegg/) and LIPID MAPS (https://www.lipidmaps.org/) [20–22]. The peak height was used to quantify the amount of each metabolite and was normalized by the median and log-transformed utilizing MetaboAnalyst 5.0. Principal component analysis (PCA) was applied to the treated and untreated groups. Univariate statistical analysis was performed using Welch's *t*-test ($P<0.05$) to identify significant changes between groups. Metabolites that were significant and had ≥2-fold change (FC) (i.e. $\log_2$ FC ≥1 or ≤1) were further analysed for metabolic changes between treated and untreated groups. Pathway analysis of significantly perturbed metabolites was carried out using ECMDB, KEGG and LIPID MAPS [20–22].

RESULTS AND DISCUSSION

Antibacterial activity of the *C. ternatea* anthocyanin fraction against *E. coli* ATCC 25922

The antimicrobial activity of *C. ternatea* anthocyanin fraction against a laboratory strain *E. coli* ATCC 25922 utilizing the agar dilution method (ADM) showed the MIC value to be 10 mg ml^{-1} at 24 h in our previous study. The MIC is defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a micro-organism after overnight incubation [13]. The concentration of the fraction was found not to affect the viability of *E. coli* at ≤5 mg ml^{-1} (viability >95%). A time–kill assay was performed with *E. coli* treated at ½ MIC (5 mg ml^{-1}), MIC (10 mg ml^{-1}) and 2× MIC values (20 mg ml^{-1}) of *C. ternatea* anthocyanin fraction (Fig. 1). The time–kill assay showed that the fraction at ½ MIC value did not affect the bacterial growth over a period of 24 h. The reduction of bacterial growth (c.f.u. ml^{-1}) by treatment groups was determined at respective time points compared to the untreated group. At 1 h, the bacterial growth (c.f.u. ml^{-1}) was 11.2×10^5, 6.0×10^5 and 7.4×10^5 at ½ MIC, MIC and 2× MIC, respectively, compared to the untreated control, which was 17.6×10^5 c.f.u. ml^{-1}. While at 4 h, the bacterial growth (c.f.u. ml^{-1}) was 17.4×10^6, 8.0×10^5 and 2.0×10^3 at ½ MIC, MIC and 2× MIC, respectively, compared to the untreated control, which was 19.0×10^6 c.f.u. ml^{-1}. The highest reduction of bacterial growth (c.f.u. ml^{-1}) by the treatment group was observed at 4 h, with 95.8 and 99.9% reduction at MIC and 2× MIC, respectively (Fig. 1).

Our previous study and another group of researchers found that the crude flower extract (up to 50 mg ml^{-1}) did not have antibacterial activity against *E. coli* [8, 13]. However, it was different to that reported by a study that showed the crude extract to have an MIC value of 1.25–5.0 mg ml^{-1} (broth dilution method) against *E. coli*, while another study reported an activity at 100 mg ml^{-1} in a disc diffusion assay [9, 23].

The impact of regional differences of the compound composition of the *C. ternatea* crude flower extract is unknown, as the composition was not reported in these studies. The crude flower extract is known to have a much lower content of anthocyanins, suggesting that the high content of anthocyanins (composed mainly of ternatin B2/B3, ternatin D1 and ternatin D2) in the fraction used in this study is responsible for the antibacterial activity. This can also be supported by the findings observed in *B. cereus* and *B. subtilis* strains, where the MIC of the anthocyanin extract was 16 times lower than the MIC of the crude extract [13].

The anthocyanin composition in the fraction is based on our previous study, which determined the composition of anthocyanins (%) via LC-MS analysis in the anthocyanin fraction obtained from Amberlite XAD-16 open column chromatography [13]. Ternatin B2, ternatin D1 and ternatin D2 were the most abundant anthocyanins, with a peak area of 23.9, 20.4 and 20.0%. Other anthocyanins, such as ternatin B3, ternatin B4, ternatin C1, ternatin C2 and ternatin D3 were also detected and reported by other studies [13, 24].

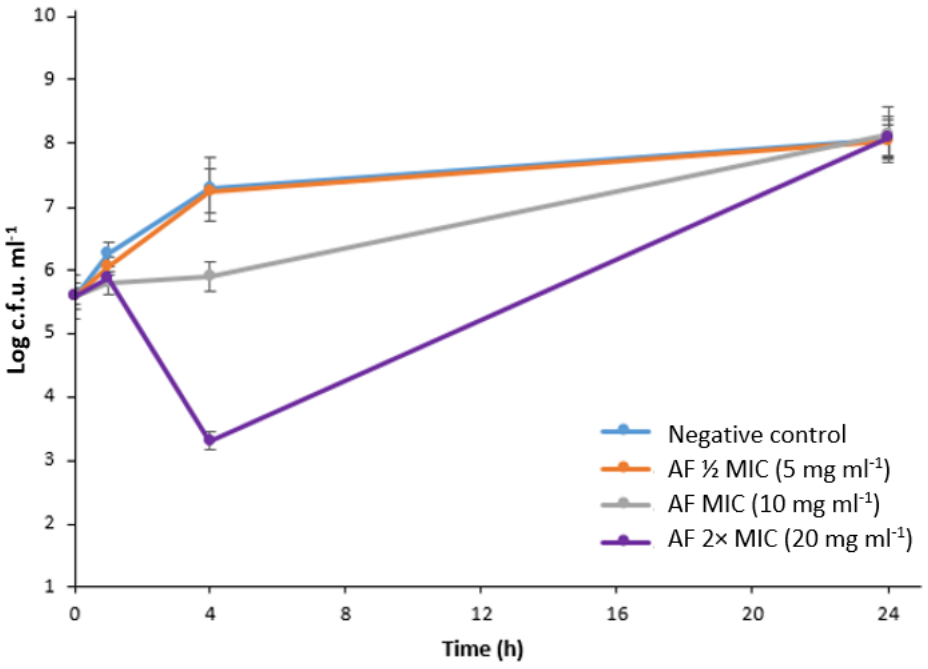

Fig. 1. Time–kill assay. Log c.f.u. ml^{-1} of *E. coli* ATCC 25922 for 1, 4 and 24 h treated with *C. ternatea* anthocyanin fraction (AF) at ½ MIC (5 mg ml^{-1}), MIC (10 mg ml^{-1}) and 2× MIC (20 mg ml^{-1}). The standard deviations of log c.f.u. ml^{-1} of three biological replicates are represented by the error bars (P<0.05).

The MIC (10 mg ml^{-1}) of the *C. ternatea* anthocyanin fraction was determined in our previous study using the agar dilution method [13]. However, in this current study, the time–kill study was performed using the suspension method (in a flask with large volume as the metabolomics study requires a larger amount of culture to proceed with study analysis) with constant shaking, which may further accelerate the growth of bacterial culture, contributing to the effect observed at 24 h in the current study.

In this current study, the *C. ternatea* anthocyanin fraction at MIC and 2×MIC value at 4 h displayed a bacteriostatic effect, as the reduction of log c.f.u. ml^{-1} of *E. coli* ATCC 25922 (Fig. 1) at 4 h for MIC or 2× MIC was not greater than a 3 $\log_{10}$-fold decrease in c.f.u. [25], which shows that not all bacterial cells are killed at 4 h and the bacteriostatic effect is not maintained at 24 h. A similar pattern has also been observed in another study with other natural compounds from garlic and guava [26]. It is also suggested that the antibacterial activity exhibited by *C. ternatea* anthocyanin fraction may be contributed by the synergistic activity of the anthocyanins in the fraction.

Bacteriostatic agents can be defined as agents that are able to prevent the growth of bacteria without causing bacterial cell death directly [27, 28]. Drugs with a bacteriostatic effect are more advantageous than those that are bactericidal as they may help to prevent drug resistance [27–29]. Observations have shown bacteriostatic agents to cause a lower incidence of toxic shock and more tolerable side effects clinically [27]. Apart from that, from our previous study [13], although the crude extracts of *C. ternatea* flower displayed toxicity at doses above 156.3 µg ml^{-1} against HEK-293 cells (human embryonic kidney cells), the findings from the acute toxicity study in rats show the extract to be rather safe for consumption.

An acute toxicity study was performed using albino Wistar rats, which were treated orally with 50% ethanol extract of *C. ternatea* flowers at 2000 mg kg^{-1} body weight. The treatment group showed no signs of mortality or abnormality and there was no significant difference in the haematological values compared to the control untreated group, which indicates no acute toxicity of *C. ternatea*

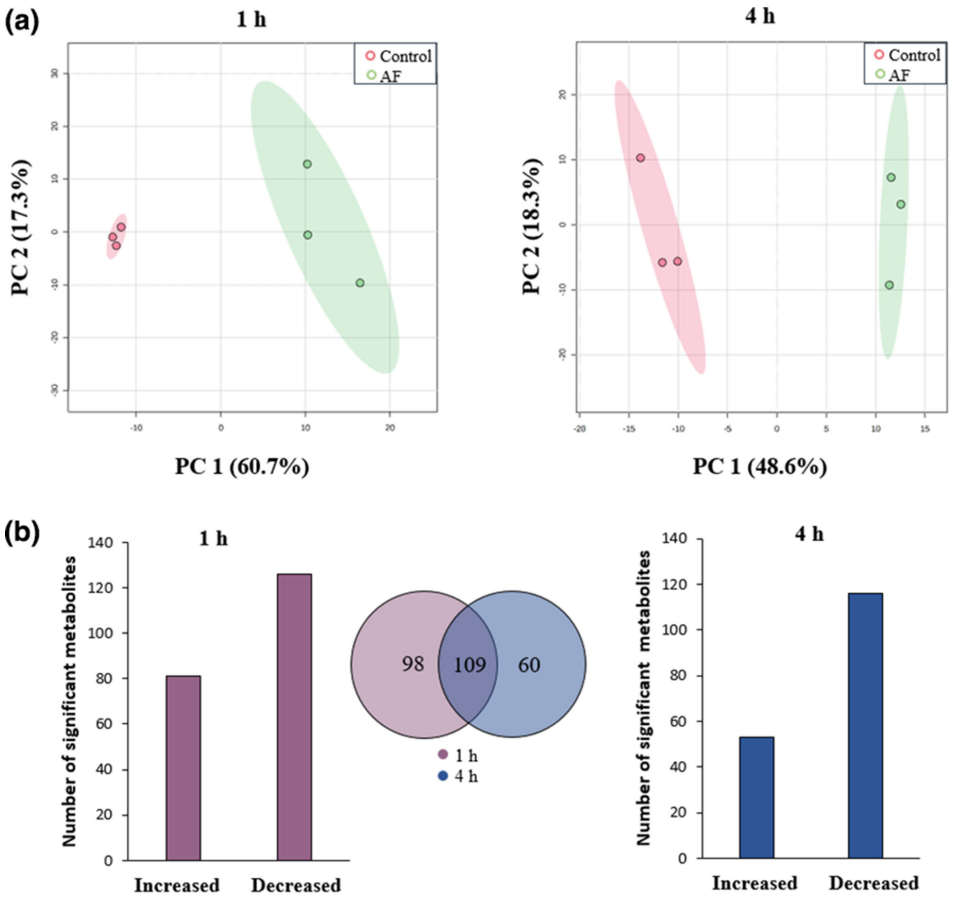

Fig. 2. Metabolomic analyses of *E. coli* ATCC 25922 treated with *C. ternatea* flower anthocyanin fraction (AF) at 1 and 4 h. (a) The metabolic changes in *E. coli* with treatment with *C. ternatea* AF as indicated by principal component analysis (PCA) plots at 1 and 4 h from the control. (b) Bar charts and Venn diagram of significantly perturbed metabolites in *E. coli* treated with AF at 1 (purple) and 4 h (blue). Significantly perturbed metabolites were selected based on $\log_2$ FC ≤−1 or ≥1 and P<0.05.

flower extracts up to 2000 mg kg^{-1} [30]. The anthocyanin fraction could potentially be used to reduce *E. coli*-related infections. However, further studies investigating the toxicity in preclinical models would be essential.

Metabolic profile of *E. coli* ATCC 25922 treated with *C. ternatea* anthocyanin fraction

The mechanism of action of the anthocyanin fraction of *C. ternatea* for its bacteriostatic activity against *E. coli* is not known and was further investigated utilizing untargeted metabolomics analysis to determine key signalling pathways that may be responsible for the antibacterial effect observed. Untargeted metabolomics was used, as it is not known which metabolites are or would be affected upon treatment with *C. ternatea* anthocyanin fraction. LC-MS-based metabolomics was used for analysing polar and nonpolar small molecules [31]. The metabolic perturbations of the *C. ternatea* anthocyanin fraction against *E. coli* were determined at its MIC (10 mg ml^{-1}) at 1 and 4 h treatment (Fig. 2). Approximately 800 metabolites were detected in the control (untreated) and treated groups at 1 and 4 h and were subjected to PCA followed by statistical analysis utilizing the *t*-test. A clear clustering between treatment and control groups was observed for the metabolite profile at 1 (PC1=60.7%) and 4 h (PC1=48.6%) using PCA (Fig. 3a). The $\log_2$ fold change (≤−1 or ≥1) was determined for the metabolites that were significantly perturbed in the treatment group compared to the control. In total, the number of significantly affected metabolites at 1 h was 81/126 (up/down) and 53/116 (up/down) at 4 h. (Fig. 2b). These metabolites were then subjected to identification utilizing the online databases ECMDB, KEGG and LIPID MAPS followed by pathway analysis. The significantly perturbed metabolites were from the metabolism of lipids, amino acids and energy, which are further discussed below.

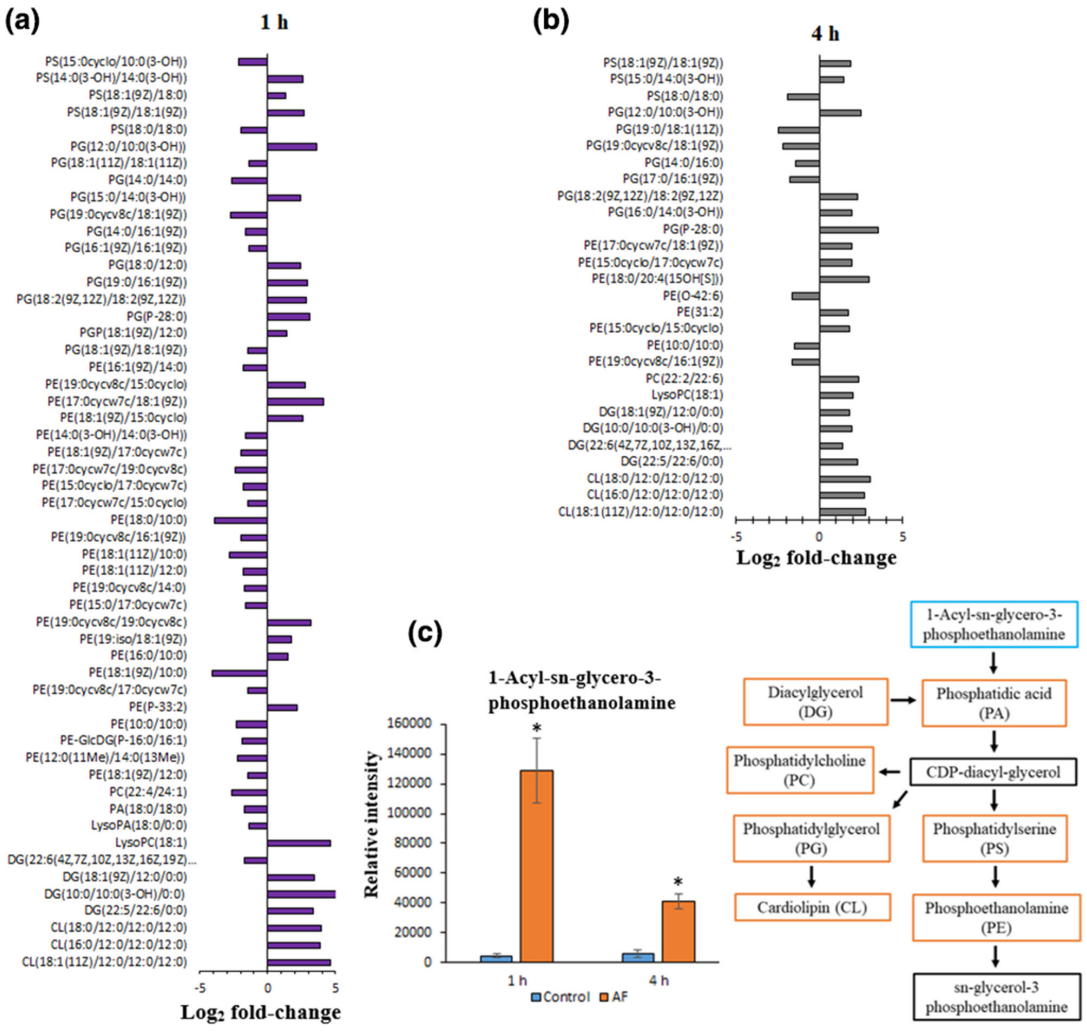

Fig. 3. Metabolomic analysis of *E. coli* ATCC 25922 treated with *C. ternatea* flower anthocyanin fraction (AF) at 1 and 4 h. *E. coli* lipid perturbations upon treatment with AF at 1 (a) and 4 h (b). The schematic diagram and bar charts (c) show the significantly perturbed lipids in *E. coli* following AF treatment at 1 and 4 h of the metabolites in lipid biosynthesis pathways. Blue/orange in the schematic diagram or '*' show significantly perturbed metabolites ($\log_2$ FC ≤1 or ≥1 and P<0.05).

Perturbation of phospholipid and glycerophospholipid metabolism by *C. ternatea* anthocyanin fraction

Glycerophospholipids are important components of the outer membrane (a dual membrane composed of lipopolysaccharide in the outer leaflet and glycerophospholipids in the inner leaflet) of Gram-negative bacteria, which is essential to protect the bacteria from toxic compounds and harsh environmental conditions. The inner membrane (symmetrical bilayer) of the bacteria, by contrast, is known to be composed of phospholipids, which are essential for the maintenance of barrier permeability and in support of its membrane proteins [32].

The glycerophospholipid molecules are mainly phosphatidylethanolamine (PE), followed by phosphatidylglycerol (PG), phosphatidylcholine (PC), phosphatidylserine (PS) and cardiolipin (CL). CL is synthesized in the inner membrane and transported to the outer membrane [33].

The treatment of *C. ternatea* anthocyanin fraction at 10 mg ml^{-1} (MIC) was found to have exerted growth inhibition and significantly perturbed the glycerophospholipid metabolites of *E. coli* at 1 and 4 h (Fig. 3). The levels of glycerophospholipids such as PE (18:0/10:0), PS [15:0cyclo/10:0(3-OH)], PG [19:0cycv8c/18:1(9Z)] and PE [18:1(11Z)/10:0] were decreased ($\log_2$ FC=−3.87 to −2.09), while 1-acyl-sn-glycero-3-phosphoethanolamine, PG [12:0/10:0(3-OH)], diacylglycerol (DG) (22:5/22:6/0:0) and CL (16:0/12:0/12:0/12:0) were increased ($\log_2$ FC=3.37 to 4.88) by *C. ternatea* anthocyanin fraction treatment at 1 h.

At 4 h, PS (18:0/18:0), PG [19:0/18:1(11Z)], PE (10:0/10:0) and PG [17:0/16:1(9Z)] were among those that decreased ($\log_2$ FC=−2.48 to −1.47), while 1-acyl-sn-glycero-3-phosphoethanolamine, CL (18:0/12:0/12:0/12:0), DG (22:5/22:6/0:0) and PE (31:2) were among those that increased ($\log_2$ FC=1.74 to 3.07).

A larger number of glycerophospholipid metabolites were perturbed at 1 h compared to 4 h (Fig. 3). The perturbations observed in the glycerophospholipid metabolites indicate membrane damage induced by *C. ternatea* anthocyanin fraction treatment. This may trigger enhanced synthesis and transport of glycerophospholipids to re-establish the outer membrane barrier of a bacterium [34]. This is also supported by the significant increase in the level of CL metabolites. CL metabolites play an essential role in remodelling of the bacterial outer membrane, as CL is also important for bacterial structural integrity and cell function [35].

E. coli membranes are mainly composed of PE (75%) followed by PG (20%) and CL (5%) [36, 37]. Changes in the environmental conditions or exposure to antibacterial agents can impose stress on bacteria, triggering certain responses to enable their survival and requiring major metabolic reprogramming [36]. The results obtained on the phosphoglycerolipid metabolite levels upon treatment with the anthocyanin fraction showed disruption of the optimum conditions required for membrane integrity, which may have caused alterations in cell physiology, which also compromises the integrity of the cell.

Compromised integrity of the cell membrane upon treatment could have led to a series of different cellular effects, such as cellular envelope structure modification, disruption of energy transduction metabolic pathways and impairment of membrane and macromolecule synthesis [37]. This can be supported by the findings reported in another study for the antibacterial effect of the anthocyanins of blueberries. The anthocyanins elicited bacterial cell membrane damage, resulting in leakage of intracellular materials as well as facilitating entry into the cell. It also further disrupted the tricarboxcylic acid (TCA) cycle and energy metabolism of the bacteria [38]. The results obtained in our study showed a remarkable effect of *C. ternatea* anthocyanin fraction, which triggered the perturbation of various phospholipids to cause disruption of membrane integrity to inhibit the growth of *E. coli*.

Perturbation and inhibition of amino acid and energy metabolism of *C. ternatea* anthocyanin fraction

The disruption of bacterial membrane integrity by *C. ternatea* anthocyanin fraction could have facilitated its entry into the cell, affecting the stability of the intracellular environment and leading to the perturbations of the level of amino acid metabolites. The amino acids leucine ($\log_2$ FC=−1.04 and −1.74), isoleucine ($\log_2$ FC=−1.26 and −2.03), valine ($\log_2$ FC=−1.86 and −2.27), tyrosine ($\log_2$ FC=−1.64 and −1.96) and phenylalanine ($\log_2$ FC=−1.24 and −1.91) were reduced at both 1 and 4 h of treatment, respectively. There was a higher reduction of the metabolite levels of amino acids at 4 h compared to 1 h (Fig. 4a). The effect on the level of amino acid metabolites was higher later at 4 h as compared to the effect on glycerophospholipid metabolites, which was higher at 1 h (Fig. 3). This may be a subsequent effect that occurs after the perturbation of glycerophospholipids upon entry of anthocyanins into the cells.

Amino acids are the basic building blocks of proteins, and are also biomolecules that are essential in the biological functions of micro-organisms, such as metabolism, growth and survival of cells, formation of cell walls, division of cells and bacterial cell communication (quorum sensing). Enzymes, being composed of amino acids and classified as proteins, play a crucial role in facilitating the resistance of microbes to antimicrobial drugs [39]. The glycolytic pathway, the TCA cycle and the pentose phosphate pathway are oxidative pathways to obtain energy. The reduction of amino acid metabolite levels may reflect it being used as a substitute for carbon source due to the bacteriostatic effect of the anthocyanin fraction. This may be an adaptation mechanism of bacteria to thrive in environmental conditions to maximize their chance of survival [40, 41].

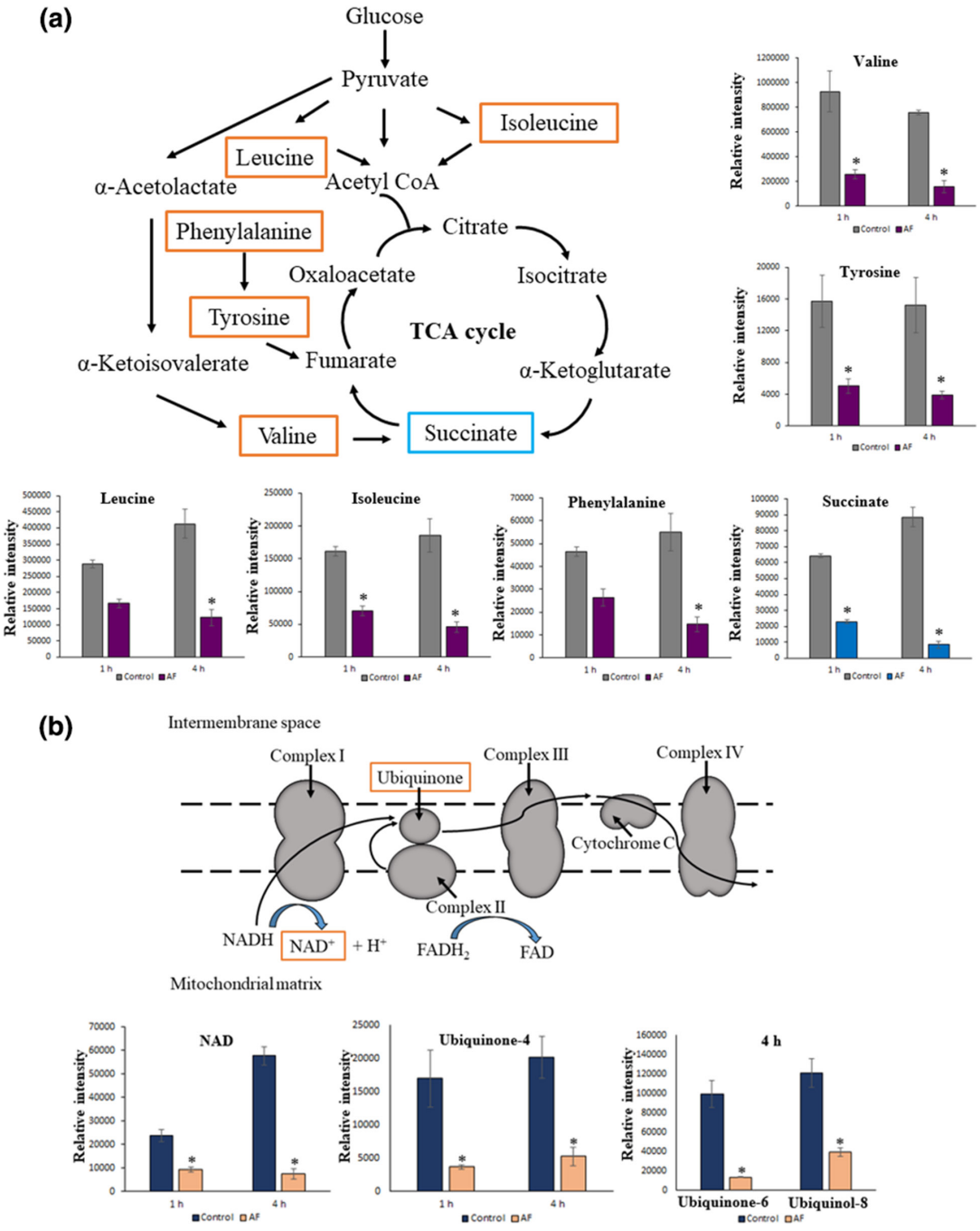

Fig. 4. Metabolomic analyses of *E. coli* ATCC 25922 treated with *C. ternatea* flower anthocyanin fraction (AF) at 1 and 4 h. (a) Significant perturbations of amino acids and (b) energy metabolites in *E. coli* treated with AF at 1 and 4 h are represented in a schematic diagram and/or bar charts; '*' indicates significantly perturbed metabolites (log_2 FC ≤1 or ≥1 and P<0.05).

The TCA cycle is involved in the oxidation of different metabolic intermediates derived from the catabolism of lipids, carbohydrates and certain amino acids. For each turn of the TCA cycle, three molecules of NAD^+ are reduced to NADH and one molecule of FAD is reduced to $FADH_2$. These molecules then transfer their energy to the electron transport chain, which in turn releases energy so that it can be converted to adenosine triphosphate (ATP) as an energy source for bacteria [42]. Disruption of the metabolic activity of the bacterial TCA cycle causes the weakening of cellular respiration, making the energy supply inadequate, which can lead to cell death [43].

At the examined treatment concentration (MIC=10 mg ml^{-1}) (Fig. 4a), *C. ternatea* anthocyanin fraction affected the TCA cycle, where it was found to have reduced the metabolite level of succinate ($\log_2$ FC=−1.28 and −3.8) at both 1 and 4 h, respectively, with a higher reduction at 4 h. The reduction of succinate by the anthocyanin fraction of *C. ternatea* may have disrupted the energy metabolism of the electron transfer chain by reducing the electron transfer to ubiquinone as the rate of the electron transfer from ubiquinone to NAD is limited by the low content of succinate, which serves as the main reductant of ubiquinone [41, 44].

In *E. coli*, ubiquinone is a lipid that plays an important role in the electron transfer chain to generate energy [45]. Upon treatment with the anthocyanin extract, the levels of NAD ($\log_2$ FC=−1.33 and −2.96) and ubiquinone-4 ($\log_2$ FC=−1.58 and −1.94) declined significantly at 1 and 4 h, respectively, with a higher reduction at 4 h (Fig. 4b). The metabolite levels of ubiquinone-6 ($\log_2$ FC=−2.88) and ubiquinol-8 ($\log_2$ FC=−1.61) were decreased at 4 h but not at 1 h. The anthocyanin fraction affected the TCA of *E. coli*, inducing a reduced level of its intermediate (succinate). This might have weakened its cellular respiration, reducing the energy supply, finally leading to cell growth inhibition [43].

The findings can be further supported by the effect of blueberry anthocyanins, which disrupted the bacterial TCA cycle and reduced the level of ATPase, impedeing respiratory metabolism. The blueberry anthocyanins could have acted by increasing the efflux of ATP from the cytoplasm of pathogens [38]. The production rate of formazan (to represent the metabolic activity of pathogens in the TCA cycle) gradually decreased with increasing concentration of blueberry anthocyanins to block the TCA of pathogens. The weakening of cellular respiration and inadequate energy supply may lead to cell death [38]. These findings show that the inhibition of energy production also plays an important role in the growth inhibition or killing of bacteria, as also reported in other studies [34, 46].

In conclusion, the anthocyanin fraction of *C. ternatea* has a potential bacteriostatic effect against *E. coli*. To the best of our knowledge, this is the first study to reveal significant metabolic perturbations of *C. ternatea* anthocyanin fraction against *E. coli*, revealing its mechanism of action. The anthocyanins in the fraction could have acted synergistically in exerting the bacteriostatic effect by perturbing glycerophospholipid metabolism, amino acid and energy metabolism, with these being the major pathways involved in the effect of *C. ternatea* anthocyanin fraction against *E. coli*.

Structure–activity relationship studies would be beneficial to understand the effect of the structural groups responsible for or contributing to the bioactivity. Future studies looking into combination treatment of *C. ternatea* anthocyanin fraction with clinical agents may be beneficial; its potential action as a bacteriostatic agent might enhance the antibacterial effect of clinical agents. Assessment of the impact on bacterial membrane integrity using imaging studies would be highly beneficial. It is also essential to carry out further studies to determine its effect on clinical and multidrug-resistant *E. coli* strains. The anthocyanin fraction could potentially be used to reduce *E. coli*-related infections. However, further studies investigating toxicity in pre-clinical models are also essential. This study provides novel metabolomic information on the mechanism of action of *C. ternatea* anthocyanin fraction to reduce *E. coli*-related infections.

Funding information
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Author contributions
E.J.J. contributed to data curation, formal analysis, investigation and writing – original draft preparation. M.L.H. contributed to supervision and writing – review. J.L. contributed to supervision and writing – review. W.S.C. contributed to conceptualization, funding acquisition, project administration, resources, supervision and writing – review and editing.

Conflicts of interest
The authors declare that there are no conflicts of interest.

References

1. Sim C. Metabolic perturbations and key pathways associated with the Antibacterial activity of *Clitoria Ternatea* flower Anthocyanin fraction against *Escherichia coli*. *Figshare*. 2022. DOI: 10.26180/21687398.v1.
2. Poirel L, Madec JY, Lupo A, Schink AK, Kieffer N, *et al*. Antimicrobial resistance in *Escherichia coli*. *Microbiol Spectr* 2018;6:6–4.
3. De Oliveira DMP, Forde BM, Kidd TJ, Harris PNA, Schembri MA, *et al*. Antimicrobial resistance in ESKAPE pathogens. *Clin Microbiol Rev* 2020;33:e00181-19.
4. Anand U, Jacobo-Herrera N, Altemimi A, Lakhssassi N. A comprehensive review on medicinal plants as antimicrobial therapeutics: potential avenues of biocompatible drug discovery. *Metabolites* 2019;9:258.
5. Jeyaraj EJ, Lim YY, Choo WS. Extraction methods of butterfly pea (*Clitoria ternatea*) flower and biological activities of its phytochemicals. *J Food Sci Technol* 2021;58:2054–2067.
6. Mukherjee PK, Kumar V, Kumar NS, Heinrich M. The ayurvedic medicine *Clitoria ternatea*—from traditional use to scientific assessment. *J Ethnopharmacol* 2008;120:291–301.
7. Vidana Gamage GC, Lim YY, Choo WS. Anthocyanins from *Clitoria ternatea* flower: biosynthesis, extraction, stability, Antioxidant activity, and applications. *Front Plant Sci* 2021;12:792303.

8. Leong C-R, Kamarul Azizi MA, Taher MA, Wahidin S, Lee K-C, *et al*. Anthocyanins from *Clitoria ternatea* attenuate food-borne *Penicillium expansum* and its potential application as food biopreservative. *Nat Prod Sci* 2017;23:125.
9. Uma B, Prabhakar K, Rajendran S. Phytochemical analysis and antimicrobial activity of *Clitorea ternatea* LINN against extended spectrum beta Lactamase producing Enteric and urinary pathogens. *Asian J Pharm Clin Res* 2021;2009:94–96.
10. Lin Y-W, Han M-L, Zhao J, Zhu Y, Rao G, *et al*. Synergistic combination of polymyxin B and enrofloxacin induced metabolic perturbations in extensive drug-resistant *Pseudomonas aeruginosa Front Pharmacol* 2019;10:1146.
11. Zhou B, Xiao JF, Tuli L, Ressom HW. LC-MS-based metabolomics. *Mol Biosyst* 2012;8:470–481.
12. Peng B, Li H, Peng XX. Functional metabolomics: from biomarker discovery to metabolome reprogramming. *Protein Cell* 2015;6:628–637.
13. Jeyaraj EJ, Lim YY, Choo WS. Antioxidant, cytotoxic, and antibacterial activities of *Clitoria ternatea* flower extracts and anthocyanin-rich fraction. *Sci Rep* 2022;12:14890.
14. Chang YJ, Pong LY, Hassan SS, Choo WS. Antiviral activity of betacyanins from red pitahaya (*Hylocereus polyrhizus*) and red spinach (*Amaranthus dubius*) against dengue virus type 2 (GenBank accession no. MH488959). *Access Microbiol* 2020;2:acmi000073.
15. Benson HJ. *Bensons Microbiological Applications: Laboratory Manual in General Microbiology*. Boston: McGraw-Hill Higher Education; 2005.
16. Brown AE. *Benson's Microbiological Applications: Laboratory Manual in General Microbiology*. New York, USA: McGraw-Hill. Inc; 2005.
17. Han M-L, Zhu Y, Creek DJ, Lin Y-W, Gutu AD, *et al*. Comparative metabolomics and transcriptomics reveal multiple pathways associated with polymyxin killing in *Pseudomonas aeruginosa*. *mSystems* 2019;4.
18. Maifiah MHM, Cheah S-E, Johnson MD, Han M-L, Boyce JD, *et al*. Global metabolic analyses identify key differences in metabolite levels between polymyxin-susceptible and polymyxin-resistant *Acinetobacter baumannii*. *Sci Rep* 2016;6:22287.
19. Maifiah MHM, Creek DJ, Nation RL, Forrest A, Tsuji BT, *et al*. Untargeted metabolomics analysis reveals key pathways responsible for the synergistic killing of colistin and doripenem combination against *Acinetobacter baumannii*. *Sci Rep* 2017;7:45527.
20. Sajed T, Marcu A, Ramirez M, Pon A, Guo AC, *et al*. ECMDB 2.0: a richer resource for understanding the biochemistry of *E. coli*. *Nucleic Acids Res* 2016;44:D495–501.
21. Kanehisa M, Goto S. KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Res* 2000;28:27–30.
22. Fahy E, Sud M, Cotter D, Subramaniam S. LIPID MAPS online tools for LIPID research. *Nucleic Acids Res* 2007;35:W606–12.
23. Kamilla L, Mnsor SM, Ramanathan S, Sasidharan S. Antimicrobial activity of *Clitoria Ternatea* (L.) extracts. *Pharmacologyonline* 2009;2009:731–738.
24. Ichiyanagi T, Nashimoto M, Terahara N. Gastrointestinal absorption of ternatins, polyacylated anthocyanins derived from butterfly pea (*Clitoria ternatea* L.) petals in rats. *BPB Reports* 2021;4:136–141.
25. Khader R, Tharmalingam N, Mishra B, Felix L, Ausubel FM, *et al*. Characterization of five novel anti-MRSA compounds identified using a whole-animal *Caenorhabditis elegans*/*Galleria mellonella* sequential-screening approach. *Antibiotics* 2020;9:9.
26. Shetty S, Shetty RM, Rahman B, Reddy MS, Shetty SR, *et al*. Comparison of time-kill assay to evaluate the antimicrobial efficacy of garlic (Allium Sativum). *Contemp Clin Dent* 2021;12.
27. Loree J, Lappin SL. Bacteriostatic antibiotics. In: *StatPearls*. Treasure Island (FL): StatPearls Publishing, 2021.
28. Pankey GA, Sabath LD. Clinical relevance of bacteriostatic versus bactericidal mechanisms of action in the treatment of Gram-positive bacterial infections. *Clin Infect Dis* 2004;38:864–870.
29. Rani R, Sharma D, Chaturvedi M, Parkash Yadav J. Antibacterial activity of twenty different endophytic fungi isolated from *Calotropis procera* and time kill assay. *Clin Microbiol* 2017;06:6.
30. Srichaikul B. Ultrasonication extraction, bioactivity, antioxidant activity, total flavonoid, total phenolic and antioxidant of *Clitoria ternatea* linn flower extract for anti-aging drinks. *Phcog Mag* 2018;14:322.
31. Schrimpe-Rutledge AC, Codreanu SG, Sherrod SD, McLean JA. Untargeted metabolomics strategies-challenges and emerging directions. *J Am Soc Mass Spectrom* 2016;27:1897–1905.
32. Ruiz N, Kahne D, Silhavy TJ. Transport of lipopolysaccharide across the cell envelope: the long road of discovery. *Nat Rev Microbiol* 2009;7:677–683.
33. Douglass MV, Cléon F, Trent MS. Cardiolipin aids in lipopolysaccharide transport to the gram-negative outer membrane. *Proc Natl Acad Sci* 2021;118:118.
34. Aye SM, Galani I, Han M-L, Karaiskos I, Creek DJ, *et al*. Lipid A profiling and metabolomics analysis of paired polymyxin-susceptible and -resistant MDR *Klebsiella pneumoniae* clinical isolates from the same patients before and after colistin treatment. *J Antimicrob Chemother* 2020;75:2852–2863.
35. Dong H, Zhang Z, Tang X, Huang S, Li H, *et al*. Structural insights into cardiolipin transfer from the Inner membrane to the outer membrane by PbgA in Gram-negative bacteria. *Sci Rep* 2016;6:30815.
36. Needham BD, Trent MS. Fortifying the barrier: the impact of lipid A remodelling on bacterial pathogenesis. *Nat Rev Microbiol* 2013;11:467–481.
37. Rowlett VW, Mallampalli VKPS, Karlstaedt A, Dowhan W, Taegtmeyer H, *et al*. Impact of membrane phospholipid alterations in *Escherichia coli* on cellular function and bacterial stress adaptation. *J Bacteriol* 2017;199:e00849-16.
38. Sun X, Zhou T, Wei C, Lan W, Zhao Y, *et al*. Antibacterial effect and mechanism of anthocyanin rich Chinese wild blueberry extract on various foodborne pathogens. *Food Control* 2018;94:155–161.
39. Idrees M, Mohammad AR, Karodia N, Rahman A. Multimodal role of amino acids in microbial control and drug development. *Antibiotics* 2020;9:9.
40. He R, Chen W, Chen H, Zhong Q, Zhang H, *et al*. Antibacterial mechanism of linalool against *L. monocytogenes*, a metabolomic study. *Food Control* 2022;132:108533.
41. Liu M, Feng M, Yang K, Cao Y, Zhang J, *et al*. Transcriptomic and metabolomic analyses reveal antibacterial mechanism of astringent persimmon tannin against Methicillin-resistant *Staphylococcus aureus* isolated from pork. *Food Chem* 2020;309:125692.
42. Jurtshuk P. Bacterial metabolism. *Med Microbiol* 1996;4.
43. Yuan Y, Liu L, Guo L, Wang L, Liu Y. Antibacterial mechanism of rose essential oil against *Pseudomonas putida* isolated from white *Hypsizygus marmoreus* at cellular and metabolic levels. *Ind Crops Prod* 2023;196:116523.
44. Pinegin B, Vorobjeva N, Pashenkov M, Chernyak B. The role of mitochondrial ROS in antibacterial immunity. *J Cell Physiol* 2018;233:3745–3754.
45. Aussel L, Pierrel F, Loiseau L, Lombard M, Fontecave M, *et al*. Biosynthesis and physiology of coenzyme Q in bacteria. *Biochim Biophys Acta* 2014;1837:1004–1011.
46. Zhao J, Han M-L, Zhu Y, Lin Y-W, Wang Y-W, *et al*. Comparative metabolomics reveals key pathways associated with the synergistic activity of polymyxin B and rifampicin combination against multidrug-resistant *Acinetobacter baumannii*. *Biochem Pharmacol* 2021;184:114400.

Peer review history

VERSION 4

Editor recommendation and comments

https://doi.org/10.1099/acmi.0.000535.v4.1

Rubén de Dios; Brunel University London, Life Sciences, UNITED KINGDOM

Date report received: 13 June 2023
Recommendation: Accept

Comments: Thank you very much for your efforts in implementing all the suggestions and corrections to improve your manuscript. This work is now suitable for publication. Congratulations!

Author response to reviewers to Version 3

RESPONSE TO EDITOR'S COMMENTS:

1)L393-394: "Amino acids in the form of enzymes" reads as if amino acids had catalytic activity themselves. Please rephrase to properly convey that enzymes are proteins, and thus are formed by amino acids

Response:

This has now been rephrased (lines 393-394).

2) L394-396: The glycolytic pathway, the TCA cycle and the pentose phosphate pathway are oxidative pathways to obtain energy, reduced coenzymes and metabolic precursors that are streamed to other metabolic branches, such as amino acid anabolic reactions (as illustrated in Figure 4A). However, this reads as if those oxidative pathways were used for making amino acids. Please rephrase this.

Response:

This has now been rephrased (lines 395-396).

3) L467: I would really like to commend the authors for availing their data to the public. As an Open Research Platform, we really value these efforts for transparency. However, some more explanation on the content that is shared would be needed in this "Data summary" section. Additionally, it would be ideal if the content that is shared had any sort of legend that allowed the reader to precisely know what they will find in the different spreadsheets. If this modification cannot be made directly on the shared document, please add the explanation related to the different data sheets in this section as well.

Response:

Modification has been made directly on the shared document. The title of each sheet has been included at the top of each respective sheet which corresponds to the related data. Sheet 0 lists all the titles of the 8 sheets.

VERSION 3

Editor recommendation and comments

https://doi.org/10.1099/acmi.0.000535.v3.1

Rubén de Dios; Brunel University London, Life Sciences, UNITED KINGDOM

Date report received: 28 May 2023
Recommendation: Minor Amendment

Comments: I would like to thank the authors for their efforts in applying the comments and suggestions in their manuscript. However, there are still some minor points to amend. Please find them below: ·L393-394: "Amino acids in the form of enzymes" reads as if amino acids had catalytic activity themselves. Please rephrase to properly convey that enzymes are proteins, and thus are formed by amino acids ·L394-396: The glycolytic pathway, the TCA cycle and the pentose phosphate pathway are oxidative pathways to obtain energy, reduced coenzymes and metabolic precursors that are streamed to other metabolic branches, such as amino acid anabolic reactions (as illustrated in Figure 4A). However, this reads as if those oxidative pathways were used for making amino acids. Please rephrase this. ·L467: I would really like to commend the authors for availing their data to the public. As an Open Research Platform, we really value these efforts for transparency. However, some more explanation on the content that is shared would be needed in this "Data summary" section. Additionally, it would be ideal if the content that is shared had any sort of legend that allowed the reader to precisely know what they will find in the different spreadsheets. If this modification cannot be made directly on the shared document, please add the explanation related to the different data sheets in this section as well.

Author response to reviewers to Version 2

RESPONSE TO EDITOR'S COMMENTS:

1) Although it was suggested by Reviewer 1 and fixed to some extent, a clean-cut definition of the difference between bactericidal and bacteriostatic activity, which happens to be important for the interpretation of the results, is not entirely achieved. Please provide it supported with the appropriate references.

Response:

The reduction of log CFU/mL of *E. coli*ATCC 25922 (Figure 1) at 4 h for MIC or 2 × MIC is not greater than 3 $\log_{10}$-fold decrease in colony forming units (Khader et al., 2020) and therefore the anthocyanin fraction of *C. ternatea*flower is bacteriostatic. A correction has been made and supporting references have now been included (lines 258 - 263).

References

Khader, R., Tharmalingam, N., Mishra, B., Felix, L., Ausubel, F. M., Kelso, M. J., & Mylonakis, E. (2020). Characterization of five novel anti-MRSA compounds identified using a whole-animal Caenorhabditis elegans/Galleria mellonella sequential-screening approach. Antibiotics, 9(8), 449.

Shetty, S., Shetty, R. M., Rahman, B., Reddy, M. S., Shetty, S. R., Vannala, V., ... & Halkai, R. (2021). Comparison of time-kill assay to evaluate the antimicrobial efficacy of garlic (allium sativum) and guava (psidium guajava) extracts on periodontal pathogens. Contemporary Clinical Dentistry, 12(4), 389.

2) In line with the previous comment, as the conclusion is that the flower fraction of study is specifically bactericidal, I strongly recommend to consider a change of the title including this, rather than the generic "antibacterial activity"

Response:

A correction has been made and confirmed the anthocyanin fraction to be bacteriostatic. The title has now been changed and specific to "bacteriostatic":

Metabolic perturbations and key pathways associated with the bacteriostatic activity of Clitoria ternatea flower anthocyanin fraction against Escherichia coli

3) In comment 19 from Reviewer 1, there is a discussion on the toxicity of the anthocyanin fraction on eukaryotic cells and rats. However, this is not included in the manuscript. I strongly suggest introducing this in the manuscript where appropriate, as it will contribute to enrich the discussion.

Response:

This discussion has now been included in the manuscript (lines 270 - 280).

4) Remove brackets from *E. coli*strain name (e.g. L161, L220, L279)

Response:

The brackets have now been removed

5) L230: use "respectively" to match each CFU count to each MIC-based concentration

Response:

"respectively" has now been added to match each CFU count to each MIC-based concentration (lines 231 and 233).

6) L207, L208, L215: include citations for the different databases

Response:

Citations for the different databases have now been included (lines 209 and 216)

7) Statistical analysis (L154-158): state which statistical test is applied to which dataset, as well as the significance threshold (p-value), for example in the respective figure legends

Response:

This information has now been included (lines 156-160). Statistical analysis tests for metabolomics section are on lines 212-215.

8) L381: "Amino acids being the basic building blocks of proteins, are also biomolecules that are essential in the biological functions of microorganisms…" Please correct the grammar in this sentence

Response:

The grammar in the sentence has now been changed (lines 390-394).

9) In the interest of transparency and reproducibility, consider making the whole metabolomics dataset publicly available, either as supplementary material or uploading to the appropriate database and citing it in the manuscript. Please refer to the Microbiology Society Open Data Policy for more information (https://www.microbiologyresearch.org/open-data)

Response:

The availability of the data (https://doi.org/10.26180/21687398.v1) has now been included in the manuscript (line 467).

10) An exhaustive revision of the references is needed, especially with respect to appropriateness and format. Examples of this are:

o L309-314: reference 25 is used for explaining the differences between OM and IM. However, this is explained in the introduction of reference 25, it is not a result of that work. Please use the original refences when possible and appropriate throughout the manuscript.

Response:

The reference has now been changed (line 323).

o L314-317: reference 26 has to do with the trafficking of phospholipids and CL in mitochondria, not in bacteria. Please refer to a more content-appropriate article to support this information.

Response:

The reference has now been changed (line 326).

o L390-394: The TCA cycle is not directly involved in the oxidation of lipids, carbohydrates and amino acids. It is involved in the oxidations of different metabolic intermediates that derive from the catabolism ofthose via multiple branches of the metabolism (e.g. pyruvate, alpha-ketoglutarate). Please correct and be as precise as possible in general statements throughout the manuscript. Furthermore, reference 34, used for supporting this, refers to the TCA cycle in skeletal muscle, whilst there is plenty of information directly related to the TCA cycle in bacteria.

Response:

The sentence has been corrected and the reference has now been changed (lines 400-401 and line 405).

o L394-395: reference 35 is unrelated to the TCA and/or bacterial respiration, and thus to this statement

Response:

The reference has now been changed (lines 406 and 421).

o L414: reference "Sun"

Response:

The reference has now been included (line 425).

VERSION 2

Editor recommendation and comments

https://doi.org/10.1099/acmi.0.000535.v2.1

Rubén de Dios; Brunel University London, Life Sciences, UNITED KINGDOM

Date report received: 22 April 2023
Recommendation: Major Revision

Comments: Dear Authors, Thank you very much for your efforts in implementing the reviewers' suggestions and comments in your revised manuscript. It has undoubtedly gained in quality and methodological soundness. However, there are still some points that need amendment. Please address the following: • Although it was suggested by Reviewer 1 and fixed to some extent, a clean-cut definition of the difference between bactericidal and bacteriostatic activity, which happens to be important for the interpretation of the results, is not entirely achieved. Please provide it supported with the appropriate references. • In line with the previous comment, as the conclusion is that the flower fraction of study is specifically bactericidal, I strongly recommend to consider a change of the title including this, rather than the generic "antibacterial activity" • In comment 19 from Reviewer 1, there is a discussion on the toxicity of the anthocyanin fraction on eukaryotic cells and rats. However, this is not included in the manuscript. I strongly suggest introducing this in the manuscript where appropriate, as it will contribute to enrich the discussion. • Remove brackets from E. coli strain name (e.g. L161, L220, L279) • L230: use "respectively" to match each CFU count to each MIC-based concentration • L207, L208, L215: include citations for the different databases • Statistical analysis (L154-158): state which statistical test is applied to which dataset, as well as the significance threshold (p-value), for example in the respective figure legends • L381: "Amino acids being the basic building blocks of proteins, are also biomolecules that are essential in the biological functions of microorganisms…" Please correct the grammar in this sentence • In the interest of transparency and reproducibility, consider making the whole metabolomics dataset publicly available, either as supplementary material or uploading to the appropriate database and citing it in the manuscript. Please refer to the Microbiology Society Open Data Policy for more information (https://www.microbiologyresearch.org/open-data) • An exhaustive revision of the references is needed, especially with respect to appropriateness and format. Examples of this are: o L309-314: reference 25 is used for explaining the differences between OM and IM. However, this is explained in the introduction of reference 25, it is not a result of that work. Please use the original refences when possible and appropriate throughout the manuscript. o L314-317: reference 26 has to do with the trafficking of phospholipids and CL in mitochondria, not in bacteria. Please refer to a more content-appropriate article to support this information. o L390-394: The TCA cycle is not directly involved in the oxidation of lipids, carbohydrates and amino acids. It is involved in the oxidations of different metabolic intermediates that derive from the catabolism of those via multiple branches of the metabolism (e.g. pyruvate, alpha-ketoglutarate). Please correct and be as precise as possible in general statements throughout the manuscript. Furthermore, reference 34, used for supporting this, refers to the TCA cycle in skeletal muscle, whilst there is plenty of information directly related to the TCA cycle in bacteria. o L394-395: reference 35 is unrelated to the TCA and/or bacterial respiration, and thus to this statement o L414: reference "Sun"

Author response to reviewers to Version 1

RESPONSE TO REVIEWERS

Manuscript number: ACMI-D-22-00204

Title: Metabolic perturbations and key pathways associated with the antibacterial activity of Clitoria ternatea flower anthocyanin fraction against Escherichia coli

Authors: Ethel Jeyaseela Jeyaraj; Mei-Ling Han; Jian Li; Wee Sim Choo

REVIEWER 1:

In this manuscript, the authors present findings of the anthocyanin fractions from Clitoria ternatea flowers as having a bacteriostatic effect on *E. coli*. Through metabolome analysis they show the involvement of disrupted membrane integrity, perturbed glycerophospholipid and energy metabolism as the key pathways involved in bacteriostatic effects of the plant. The manuscript

is concise and well written, and congratulations to the authors for these exciting and interesting findings. While it is clear that exposure to the anthocyanin fractions has an impact of *E. coli*, the precise nature of this impact needs some additional clarity and the focus of the findings of the metabolomic data needs to be sharpened.

1)- The terms bacteriostatic and antibacterial are both used to describe the effects of *C. ternatea*throughout the paper. The authors should clarify the distinction between the two terms and select.

Response:

The antibacterial activity of *C. ternatea*anthocyanin fraction was investigated, the observed effect was found to be bacteriostatic as growth was observed at 24 h treatment time and the metabolomics study was done at the MIC only. The term antibacterial has been now changed to bacteriostatic throughout as determined from the time-kill assay.

2)- It needs to be made clear in the main text to the reader if the strain of E. coli is being tested is of clinical origin or is multidrug resistant. If the strain is not of clinical origin or MDR, do the authors think the extract will be effective against these more recalcitrant strains.

Response:

The *E. coli*strain used in this study was a laboratory strain. This has now been mentioned in the beginning of the "Discussion" section (lines 219-220). An additional statement has now been included in the "Conclusion" section suggesting further studies to be conducted on clinical and MDR strains (lines 431-432).

3)- The authors need to explore the true nature of the effect of this fraction in more detail. The MIC was previously determined as 10mg/ml, however from Figure 1 it is quite clear that at 24 hours, the E. coli have overcome the inhibitory effect of the fraction and have recovered to the same CFU as the negative control. The other issue with this figure is that at 2xMIC there is a clear killing effect as stated in the text the CFU drops from 7.4 x 10^5 to 2 x10^2. This suggests that the fraction may be bactericidal. The other issue with this figure is that in all conditions it appears that there is an increase in bacterial growth from 0-1 hour, how can this be explained given the inhibitory nature of the fraction, you would expect the effect to be immediate. Given this is a fraction however, rather than a pure compound there could be synergistic and antagonistic interactions taking place at once. This section would benefit from a growth curve analysis with readings every 15 minutes.

Response:

The MIC (10 mg/mL) of *C. ternatea*anthocyanin fraction was determined in our previous study (Jeyaraj et al., 2022) using the agar dilution method. However, in this current study, the time-kill study was done using the suspension method (in a flask with large volume as the metabolomics study requires a larger amount of culture to proceed with study analysis) with constant shaking which may further accelerate the growth of bacterial culture thus contributing to the effect seen at 24 h in the current study. In this current study, *C. ternatea*anthocyanin fraction at MIC value at 4 h displays a bacteriostatic effect while at 2 × MIC potentially displays bactericidal effect with a high possibility that not all bacterial cells are killed at 4 h as a bacteriostatic or bactericidal effect is not maintained at 24 h. It is also suggested that the antibacterial activity exhibited by *C. ternatea*anthocyanin fraction may be contributed by the synergistic activity of the anthocyanins in the fraction. It would be beneficial for further studies to be conducted on the effect of the *C. ternatea*anthocyanin fractionon the growth curve analysisat shorter time intervals to further understand its bacteriostatic/bactericidal effect at MIC and 2 × MIC. This paragraph has now been included in the manuscript (lines 254-267).

4)- The authors base a significant component on the MIC that was previously determined in Jeyaraj et al.,2022. However, this MIC was determined in an agar dilution method, and as much of this study is based in broth cultures, it would be very beneficial to conduct an MIC assay in liquid broth. The benefit of this also is that there may be regional or temporal impacts on anthocyanin concentration that would lead to differences in the MIC from batch to batch. Findings should also be put in context with Uma et al., 2009 where the crude flower extract had an MIC of between .25-10 mg/mL.

Response:

The use of broth cultures in this current study as compared to the previous study findings on MIC value using agar dilution method, and the essential need for further studies on growth curve analysis has now been mentioned in main text (lines 254-267). We were not able to determine the MIC values using the broth dilution method due to the strong extract colour which masks the detection of the absorbance value at the higher concentration range tested. Based on our findings from the agar dilution method (Jeyaraj et al., 2022), the growth inhibition effect was not seen for the crude extracts on *E. coli*up to 40 mg/mL which correlates to findings from other studies but was different to that reported by Uma et al., 2009. It is unknown on the impact of regional differences of the compound composition of the *C. ternatea*crude flower extract as it was not reported in their study. This has now been included in the manuscript (lines 235-241).

5)- For the time kill assays - what was the anthocyanin fraction resuspended in? Is a vehicle control needed for the assay?

Response:

The anthocyanin fraction was resuspended in the same bacterial growth media (BHI broth)thus a vehicle control was not needed for the assay. This information has now been included in the manuscript (line 132 and line 168).

6)- The authors need to state their definition of MIC within the text.

Response:

The definition of MIC has now been included in the manuscript (lines 221-223).

7)- Is it known that the concentrations of anthocyanins are the same in each flower? (Dependant on the size/ stage of the flower).

Response:

Mature flowers (bloomed) were used in the current study and is commonly used in other bioactivity studies. Our previous study (Jeyaraj et al., 2021) investigated the phytochemical composition of this flower and was found to have a similar profile as reported by other studies.

Jeyaraj, E. J., Lim, Y. Y., & Choo, W. S. (2021). Effect of organic solvents and water extraction on the phytochemical profile and antioxidant activity of Clitoria ternatea flowers. ACS Food Science & Technology, 1(9), 1567-1577.

8)- Does the geographical location of flower impact the concentration of anthocyanins present?

Response:

Currently it is not known on the direct effect comparison of geographical location of *C. ternatea*flower and the impact on the concentration of anthocyanins. Although the phytochemical composition (%) was found to be similar, the reported TAC (total anthocyanin content) values compared to other studies ranged from 0.33 - 5.6 mg CGE/g which can be affected by the extraction method used (Jeyaraj et al., 2021).

Jeyaraj, E. J., Lim, Y. Y., & Choo, W. S. (2021). Effect of organic solvents and water extraction on the phytochemical profile and antioxidant activity of Clitoria ternatea flowers. ACS Food Science & Technology, 1(9), 1567-1577.

9)- The authors should provide a brief description of the agar dilution method used in this paper, as well as citing their previous paper, to make it easier for the reader.

Response:

The use of agar dilution method to obtain MIC value in the previous study and that used in the current study has now been included in the manuscript (lines 254-267)

10)- Were the treated samples and untreated controls used for metabolomic analysis subjected to the same processes/vehicle as the treatment?

Response:

All samples were processed under the same conditions.

11)- The text states (line 162) that the MIC is used for metabolome analysis - if the bacteria does not grow (bacteriostatic) at this concentration then is there likely to be pleiotropic effects of the bacteria being in different growth phases, particularly at the 4 hour timepoint?

Response:

The MIC (10 mg/mL) of *C. ternatea*anthocyanin fraction was determined in our previous study (Jeyaraj et al., 2022) using the agar dilution method. However, in this current study, the time-kill study was done using the suspension method (in a flask with large volume as the metabolomics study requires a larger amount of culture to proceed with study analysis) with constant shaking which may further accelerate the growth of bacterial culture thus contributing to the effect seen at 1 or 4 h. The metabolic effect is already being seen at 1 h and a further increase in bacterial growth is not seen at 1 h to 4 h suggesting the effect to be taking place at 1 h onwards.

12)- Line 300 the authors state that there is a greater perturbation in glycerophospholipid metabolitesat 1 hour compared to 4 hours. However, based on figure1 the bacteria has continued to grow at the 1 hour time point (although it is not clear if this increase in growth is statistically significant), how does this fit with the glycerophospholipid hypothesis?

Response:

There was no significant difference between the bacterial growth at 1 and 4 h in Figure 1. This suggests that the effect on glycerophospholipid metabolite perturbations to be taking place at 1 h onwards to 4 h.

13)- The study would be greatly strengthened by some validation of the impact on membrane integrity using perhaps live cell imaging or a kit such as LIVE/DEAD BacLight Bacterial viability kit.

Response:

We highly agree it would be beneficial to validate on the impact on membrane integrity using imaging studies. However, we do not have the resources to carry this out. A suggestion for further studies looking at this aspect has now been included in lines 430-431.

14)- Line 354 how does this higher reduction at 4 hours as compared to 1 hour compared with the previous section on the impact on glycerophospholipids?

Response:

This could be possibly due to the subsequent effects that may occur after the perturbation of glycerophospholipids upon entry of anthocyanins into the cells. This has now been included in lines 377-381. The disruption of the bacteria membrane integrity (glycerophospholipids) by *C. ternatea*anthocyanin fraction could have facilitated its entry into the cell which affected the stability of the intracellular environment leading to the perturbations of the level of amino acid metabolites. Thus, the effect on the level of amino acid metabolites was higher later at 4 h as compared to glycerophospholipids (lines 371-373).

15)- Authors should show the ubiquinol-6 and -8 at 1 hr to give a full picture of the results.

Response:

The metabolite levels of ubiquinone-6 and ubiquinol-8 were decreased at 4 h but not at 1 h thus it was not shown in Figure 4. A statement for the effect not seen at 1 h for ubiquinone-6 and ubiquinol-8 has now been included in line 407-408.

16)- Greater weight should be added to the effect on the TCA cycle as this is strengthened by the previous work which the authors have cited by Sun et al., 2018.

Response:

More information has now been included in lines 412-417.

17)- If standards of Ternatins are available to purchase, authors should consider this as a control for their assays.

Response:

Currently standards for ternatins are not available for purchase.

18)- For the bacterial culture preparation for metabolome analysis - why was a different agar (nutrient agar) used to grow the colonies than in the time kill assay?

Response:

This has now been corrected and changed to Tryptic Soy agar in lines 161-162.

19)- The authors state in line 402-404 that the anthocyanin fraction could be used to reduce *E. coli*related infections however this does not align with previous work published by the authors which states that the anthocyanin rich extract displays toxicity at doses above 156.3 µg/mL against HEK-293 cells (Jeyaraj et al.,2022, Figure 3), which is much lower the MIC described in this work. Greater context needs to be added here.

Response:

From the previous study (Jeyaraj et al., 2022), although the crude extracts of *C. ternatea*flower displayed toxicity at doses above 156.3 µg/mL against HEK-293 cells, the findings from the acute toxicity study in rats show the extract to be rather safe for consumption. An acute toxicity study was done using albino Wistar rats which were treated orally with 50% ethanol extract of *C. ternatea*flowers at 2000 mg/kg body weight. The treatment group showed no signs of mortality or abnormality and there was no significant difference in the hematological values compared to the control untreated group which indicates no acute toxicity of *C. ternatea*flower extracts up to 2000 mg/kg (Srichaikul, 2018). "The anthocyanin fraction could be potentially used to reduce *E. coli*-related infections. However further studies investigating the toxicity in pre-clinical models would be essential." This statement has now been included in lines 432-434.

Jeyaraj, E. J., Lim, Y. Y., & Choo, W. S. (2022). Antioxidant, cytotoxic, and antibacterial activities of Clitoria ternatea flower extracts and anthocyanin-rich fraction. Scientific Reports, 12(1), 14890.

Srichaikul, B. (2018). Ultrasonication extraction, bioactivity, antioxidant activity, total flavonoid, total phenolic and antioxidant of Clitoria ternatea linn flower extract for anti-aging drinks. Pharmacognosy Magazine, 14(56).

20)- References needed for the following:

o Line 63, second sentence

o Line 71, second sentence

o Line 246, second sentence

o Line 248, second sentence

o Line 308

Response:

The references have now been included/removed:

o Line 63, second sentence: Line 65

o Line 71, second sentence: Line 73

o Line 246, second sentence: This statement and reference has been removed.

o Line 248, second sentence: This statement and reference has been removed.

o Line 308: Line 337

21) Line 251, reference does not support the statement. Needs to be rewritten to fit the reference or a new reference is needed.

§ Reference paper states "It was demonstrated that the sugars do not contribute to the binding affinity or the DNA cleavage selectivity, although the presence of the sugar enhances the DNA cleavage efficiency by 2-5 times"

Response:

This statement and reference have been removed. “The anthocyanins in the fraction could have acted in synergism for exerting the bacteriostatic effect by perturbing the glycerophospholipid metabolism, amino acid and energy metabolism being the major pathways involved for the effect of *C. ternatea*anthocyanin fraction in *E. coli*. Structure-activity relationship studies would be beneficial to understand the effect of the structural groups responsible or contributing to the bioactivity”. This has now been included in lines 423-427.

22) Lines 223-225, Findings from Gram positive bacteria are compared to Gram negative are not representative and thus cannot support the findings and perhaps more relevant examples could be used.

Response:

This was referring to findings from our previous study which determined the antibacterial activity of the crude extract and anthocyanin-rich fraction of *C. ternatea*against various Gram-positive and Gram-negative bacteria. This statement intended to show the higher antibacterial activity (based on MIC value) of the anthocyanin-rich fraction when compared to the crude extract.

23) Figure 1, Line 237 - Are the replicates technical or biological?

Response:

The replicates are biological and has now been included in line 276.

REVIEWER 2:

The work has some value and significance, although the advantages and disadvantages should be considered:

Advantages:

the work investigates mechanism of action of anthocyanins fraction against E. coli - it is new

Disadvantages:

The phytochemical analysis of the obtained fraction was not presented, hence indeed we don't know what were the active compounds used in the experiment.

The methodology lacks many details, and in general is not precise, strongly needs clarification, also in the section related to bacterial metabolites identification.

In the discussion: it is suggested to rethink the presented observations in regard to the study "Cell Metab. 2019 Aug 6; 30(2): 251-259. doi: 10.1016/j.cmet.2019.06.009: 10.1016/j.cmet.2019.06.009". The authors of the manuscript under evaluation suggest that decreased energy production results in bacterial cell death, however in the earlier paragraphs they stated that observed effect of anthocynanins was bacteriostatic (lines 225-226). It is contradictory. Also, if you analyse the suggested work, you will find

the information that decreased metabolic activity in bacteria is strictly related to bacteriostatic effect of antibiotics. Hence it is mandatory to re-evaluate observations to make correct conclusions.

Response:

The amendments have been made based on the remarks below.

Other remarks:

1)Please make it clear: was the extraction yield 50%? The 10 g of plant material was used to prepare extract, than 5g of the extract was used for semi-purification. Was it possible to obtain 5g of the extract from 10g of plant material?

Response:

The moisture content and yield of crude extract from fresh flowers were determined and reported in our previous study (Jeyaraj et al., 2021). The sentence has now been clarified to provide the ratio of flower material to solvent used to obtain the crude extract "Ethanol extracts of the fresh flower material were prepared by immersing the flowers in 50% ethanol at a solid to liquid ratio of 1:20 (g/mL)" in lines 99-100.

The anthocyanin-rich fraction was prepared from the crude extract of *C. ternatea*flowers as reported in our previous study (Jeyaraj et al., 2022). The extraction yield of the anthocyanin-rich fraction using Amberlite XAD-16 column chromatography was 18.2%. This information has now been included in lines 122-124.

Jeyaraj, E. J., Lim, Y. Y., & Choo, W. S. (2021). Effect of organic solvents and water extraction on the phytochemical profile and antioxidant activity of Clitoria ternatea flowers. ACS Food Science & Technology, 1(9), 1567-1577.

Jeyaraj, E. J., Lim, Y. Y., & Choo, W. S. (2022). Antioxidant, cytotoxic, and antibacterial activities of Clitoria ternatea flower extracts and anthocyanin-rich fraction. Scientific Reports, 12(1), 14890.

2)Line 107: what was used for pH adjustment?

Response:

The pH was adjusted using 1M HCl. This has now been included in line 107.

3)Line 112: what was the size (weight) of the amberlite resin?

Response:

Chromatography column [S24/29, 25(D) × 300mm (L)] with sintered glass disc (porosity 0) and stopcock with PTFE key were used for the open column chromatography. The Amberlite XAD-16 adsorbent was immersed in methanol overnight prior loading to ¾ of column height. Further information on the column type, Amberlite adsorbent used and loading method has now been included in lines 112-115.

4)Line 115: was water used to dissolve sample?

Response:

The aqueous fraction (containing anthocyanins) was concentrated to a volume of 10 mL under vacuum at 37°C in a rotary evaporator. This has now been included in lines 109-111.

5)Line 117: how the flow rate was controlled?

Response:

The number of drops for a total volume of solution was determined first to estimate the volume per drop (determine the number of drops/mL of solution). It was then calculated to adjust the number of drops per minute to achieve 10 mL/min (the column stopcock was adjusted to obtain the number of drops per minute).

6)What was the final yield of the purified anthocyanins fraction?

Response:

The anthocyanin-rich fraction was prepared from the crude extract of *C. ternatea*flowers as reported in our previous study (Jeyaraj et al., 2022). The extraction yield of the anthocyanin-rich fraction using Amberlite XAD-16 column chromatography was 18.2%. This information has now been included in lines 122-124.

Jeyaraj, E. J., Lim, Y. Y., & Choo, W. S. (2022). Antioxidant, cytotoxic, and antibacterial activities of Clitoria ternatea flower extracts and anthocyanin-rich fraction. Scientific Reports, 12(1), 14890.

7)Line 128: What was the volume of cultures treated with anthocyanins?

Response:

The sentence has now been rephrased to "followed by dilution (1:100) in 20 mL of BHI broth alone (control group) and BHI broth containing *C. ternatea*anthocyanin fraction at ½ MIC (5 mg/mL), MIC (10 mg/mL) and 2× MIC (20 mg/mL) for 1, 4, and 24 h in lines 131-133.

8)It is not stated anywhere what solvent was used to dissolve the frozen anthocyanins for the antimicrobial experiments.

Response:

BHI broth was used to dissolve the anthocyanin fraction. This information has now been included in lines 132 and 168.

9)Could you please clarify the volume of the experimental culture: line 159 states 50 ml, while line 167 states 20ml.

Response:

For each sample, 50 mL was prepared for treated and untreated groups with 3 biological replicates. From the 50 mL, 20 mL was collected at 1 h and 20 mL was collected from the remaining source at 4h. This has now been further explained in lines 163-171.

10) Line 170: what was used to normalize cultures?

Response:

Fresh BHI broth was used to normalize the cultures. This has now been included in line 178.

11) Line 179: was the residue after centrifugation removed?

Response:

The residue after centrifugation was removed. This has now been included in line 181.

12) Lines 181-185: the parameters of ion source and detector should be given.

Response:

This has now been included in lines 197-199.

13) Line 193: authors reported that retention time was used for identification, so were authentic standards used? because in databases retention time is rather not reported. It also would be good to specify the allowed mass difference which was applied during identification (delta ppm). Can you please specify how the internal standard was used in the calculations?

Response:

Authentic standards were not used. We used m/z values to assist or identify a particular metabolite which appears across all samples (control and treated groups at 1 and 4 h) and compared to the available online databases (*E. coli*Metabolome Database (ECMDB), Kyoto Encyclopedia of Genes and Genomes (KEGG) and LIPID MAPS) to determine the putative metabolites. The statement on using retention time to identify metabolites in database has now been removed.

14) Line 219: it is hard to believe that you can dissolve 100mg of plant extract in 1 ml! Rather suspension can be obtained.

Response:

Further clarification and explanation have now been included to compare results obtained by other studies in lines 235-239.

15) Line 220-223: Can you support this statement with reference? The other authors report also ternatin A1, A2, A3, B1, B2, B3, B4, preternatin A3 (https://www3.e-kenkyu.com/bpb-reports-online-journal/uploads/manuscript/file/137/4_136.pdf).

Response:

The statement "high content of anthocyanins (composed mainly of Ternatin B2, Ternatin D1 and Ternatin D2) in the fraction used in this study responsible for the antibacterial activity" is based on our previous study (Jeyaraj et al., 2022) which determined the composition of anthocyanins (%) via LC-MS analysis in the anthocyanin-rich fraction obtained from Amberlite XAD-16 open column chromatography. Ternatin B2, ternatin D1 and ternatin D2 were the most abundant anthocyanins with a peak area of 23.93, 20.44, and 20.03 %. Other anthocyanins such as ternatin B3, ternatin B4, ternatin C1, ternatin C2, ternatin D3 were also detected.This information and the suggested reference have now been included in lines 246-252.

Jeyaraj, E. J., Lim, Y. Y., & Choo, W. S. (2022). Antioxidant, cytotoxic, and antibacterial activities of Clitoria ternatea flower extracts and anthocyanin-rich fraction. Scientific Reports, 12(1), 14890.

Ichiyanagi, T., Nashimoto, M., & Terahara, N. (2021). Gastrointestinal absorption of ternatins, polyacylated anthocyanins derived from butterfly pea (Clitoria ternatea L.) petals in rats. BPB Reports, 4(4), 136-141.

16) Lines 241-251: it is not clear why the discussion about the structure of compounds present in used fraction is placed in the section "Metabolic profile of *E. coli*(ATCC 25922) treated with *C. ternatea*anthocyanin fraction".

Response:

This has now been removed.

17) Lines: 394-395: this statement about glycoside residues is not supported by any proof in the evaluated work, firstly, the composition of the used fraction was not determined, secondly, the glucose moiety can be substituted by p-coumaroyl or malonyl residues.

Response:

This statement has been removed. "The anthocyanins in the fraction could have acted in synergism for exerting the bacteriostatic effect by perturbing the glycerophospholipid metabolism, amino acid and energy metabolism being the major pathways involved for the effect of *C. ternatea*anthocyanin fraction in *E. coli*. Structure-activity relationship studies would be beneficial to understand the effect of the structural groups responsible or contributing to the bioactivity". This has now been included in lines 423-427.

VERSION 1

Editor recommendation and comments

https://doi.org/10.1099/acmi.0.000535.v1.5

Rubén de Dios; Brunel University London, Life Sciences, UNITED KINGDOM

Date report received: 09 January 2023
Recommendation: Major Revision

Comments: In this manuscript, the authors describe the consequences at the metabolic level of a flowers extract on E. coli, discussion a possible mechanism of action for its antimicrobial activity. In the current global context, the description of possible new antimicrobials represents a meaningful contribution to multiple fields. The manuscript has been reviewed by two experts in the field and their reviews are enclosed. Both reviewers have a number of major issues that need to be addressed. Most importantly, for the sake of clarity, the methodology section needs substantial improvement. Furthermore, the consistency with previous literature, the coherence between different section of the manuscript and the clarity of a number of concepts used in this work need improvement. Please consider the reviewers' comments thoroughly and address their concerns point by point in a separate document. A revised manuscript should include appropriate revisions.

Reviewer 2 recommendation and comments

https://doi.org/10.1099/acmi.0.000535.v1.3

Elwira Sieniawska; Medical University of Lublin: Uniwersytet Medyczny w Lublinie, POLAND
https://orcid.org/0000-0003-4773-0329

Date report received: 31 December 2022
Recommendation: Major Revision

Comments: The work has some value and significance, although the advantages and disadvantages should be considered: Advantages: the work investigates mechanism of action of anthocyanins fraction against E. coli - it is new Disadvantages: The phytochemical analysis of the obtained fraction was not presented, hence indeed we don't know what were the active compounds used in the experiment. The methodology lacks many details, and in general is not precise, strongly needs clarification, also in the section related to bacterial metabolites identification. In the discussion: it is suggested to rethink the presented observations in regard to the study "Cell Metab. 2019 Aug 6; 30(2): 251-259. doi: 10.1016/j.cmet.2019.06.009: 10.1016/j.cmet.2019.06.009". The authors of the manuscript under evaluation suggest that decreased energy production results in bacterial cell death, however in the earlier paragraphs they stated that observed effect of anthocynanins was bacteriostatic (lines 225-226) . It is contradictory. Also,

if you analyse the suggested work, you will find the information that decreased metabolic activity in bacteria is strictly related to bacteriostatic effect of antibiotics. Hence it is mandatory to re-evaluate observations to make correct conclusions. Other remarks: Please make it clear: was the extraction yield 50%? The 10 g of plant material was used to prepare extract , than 5g of the extract was used for semi-purification. Was it possible to obtain 5g of the extract from 10g of plant material? Line 107: what was used for pH adjustment? Line 112: what was the size (weight) of the amberlite resin? Line 115: was water used to dissolve sample? Line 117: how the flow rate was controlled? What was the final yield of the purified anthocyanins fraction? Line 128: What was the volume of cultures treated with anthocyanins? It is not sated anywhere what solvent was used to dissolve the frozen anthocyanins for the antimicrobial experiments. Could you please clarify the volume of the experimental culture: line 159 states 50 ml, while line 167 states 20ml. Line 170: what was used to normalize cultures? Line 179: was the residue after centrifugation removed? Lines 181-185: the parameters of ion source and detector should be given Line 193: authors reported that retention time was used for identification, so were authentic standards used?, because in databases retention time is rather not reported. It also would be good to specify the allowed mass difference which was applied during identification (delta ppm). Can you please specify how the infernal standard was used in the calculations? Line 219: it is hard to believe that you can dissolve 100mg of plant extract in 1 ml! Rather suspension can be obtained. Line 220-223: Can you support this statement with reference? The other authors report also ternatin A1, A2, A3, B1, B2, B3, B4, preternatin A3 (https://www3.e-kenkyu.com/bpb-reports-online-journal/uploads/manuscript/file/137/4_136.pdf). Lines 241-251: it is not clear why the discussion about the structure of compounds present in used fraction is placed in the section "Metabolic profile of E. coli (ATCC 25922) treated with C. ternatea anthocyanin fraction" Lines: 394-395: this statement about glycoside residues is not supported by any proof in the evaluated work, firstly, the composition of the used fraction was not determined, secondly, the glucose moiety can be substituted by p-coumaroyl or malonyl residues.

Please rate the manuscript for methodological rigour
Poor

Please rate the quality of the presentation and structure of the manuscript
Satisfactory

To what extent are the conclusions supported by the data?
Partially support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?
No

Is there a potential financial or other conflict of interest between yourself and the author(s)?
No

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?
Yes

Reviewer 1 recommendation and comments

https://doi.org/10.1099/acmi.0.000535.v1.4

Anonymous.

Date report received: 22 December 2022
Recommendation: Major Revision

Comments: 1. Methodological rigour, reproducibility and availability of underlying data Good but the MIC section and the effect on growth need to explored in greater detail. 2. Presentation of results Good, particularly for the metabolomics although the data does not appear to have been uploaded to a metabolomics database which would support transparency. 3. How the style and organization of the paper communicates and represents key findings Good 4. Literature analysis or discussion Quite a few sections need to be more appropriately referenced as highlighted above. 5. Any other relevant comments In this manuscript, the authors present findings of the anthocyanin fractions from Clitoria ternatea flowers as having a bacteriostatic effect on E. coli. Through metabolome analysis they show the involvement of disrupted membrane integrity, perturbed glycerophospholipid and energy metabolism as the key pathways involved in bacteriostatic effects of the plant. The manuscript is concise and well written, and congratulations to the authors for these exciting and interesting findings. While it is clear that exposure to the anthocyanin fractions has an impact of E. coli, the precise nature of this impact needs some additional clarity and the focus of the findings of

the metabolomic data needs to be sharpened. Important Revisions - The terms bacteriostatic and antibacterial are both used to describe the effects of C. ternatea throughout the paper. The authors should clarify the distinction between the two terms and select. - It needs to be made clear in the main text to the reader if the strain of E. coli is being tested is of clinical origin or is multidrug resistant. If the strain is not of clinical origin or MDR, do the authors think the extract will be effective against these more recalcitrant strains. -The authors need to explore the true nature of the effect of this fraction in more detail. The MIC was previously determined as 10mg/ml, however from Figure 1 it is quite clear that at 24 hours, the E. coli have overcome the inhibitory effect of the fraction and have recovered to the same CFU as the negative control. The other issue with this figure is that at 2xMIC there is a clear killing effect as stated in the text the CFU drops from 7.4 x 10^5 to 2 x10^2. This suggests that the fraction may be bactericidal. The other issue with this figure is that in all conditions it appears that there is an increase in bacterial growth from 0-1 hour, how can this be explained given the inhibitory nature of the fraction, you would expect the effect to be immediate. Given this is a fraction however, rather than a pure compound there could be synergistic and antagonistic interactions taking place at once. This section would benefit from a growth curve analysis with readings every 15 minutes. - The authors base a significant component on the MIC that was previously determined in Jeyaraj et al.,2022. However this MIC was determined in an agar dilution method, and as much of this study is based in broth cultures, it would be very beneficial to conduct an MIC assay in liquid broth. The benefit of this also is that there may be regional or temporal impacts on anthocyanin concentration that would lead to differences in the MIC from batch to batch. Findings should also be put in context with Uma et al., 2009 where the crude flower extract had an MIC of between .25-10 mg/mL. - For the time kill assays - what was the anthocyanin fraction resuspended in? o Is a vehicle control needed for the assay? - The authors need to state their definition of MIC within the text. - Is it known that the concentrations of anthocyanins are the same in each flower? (Dependant on the size/ stage of the flower) - Does the geographical location of flower impact the concentration of anthocyanins present? - The authors should provide a brief description of the agar dilution method used in this paper, as well as citing their previous paper, to make it easier for the reader. - Were the treated samples and untreated controls used for metabolomic analysis subjected to the same processes/vehicle as the treatment? - The text states (line 162) that the MIC is used for metabolome analysis - if the bacteria does not grow (bacteriostatic) at this concentration then is there likely to be pleiotropic effects of the bacteria being in different growth phases, particularly at the 4 hour timepoint. ? - Line 300 the authors state that there is a greater perturbation in glycerophospholipid metabolites at 1 hour compared to 4 hours. However, based on figure1 the bacteria has continued to grow at the 1 hour time point (although it is not clear if this increase in growth is statistically significant), how does this fit with the glycerophospholipid hypothesis? - The study would be greatly strengthened by some validation of the impact on membrane integrity using perhaps live cell imaging or a kit such as LIVE/DEAD BacLight Bacterial viability kit. - Line 354 how does this higher reduction at 4 hours as compared to 1 hour compared with the previous section on the impact on glycerophospholipids? - Authors should show the ubiquinol-6 and -8 at 1 hr to give a full picture of the results. - Greater weight should be added to the effect on the TCA cycle as this is strengthened by the previous work which the authors have cited by Sun et al., 2018. - If standards of Ternatins are available to purchase, authors should consider this as a control for their assays. - For the bacterial culture preparation for metabolome analysis - why was a different agar (nutrient agar) used to grow the colonies than in the time kill assay? - The authors state in line 402-404 that the anthocyanin fraction could be used to reduce E. coli related infections however this does not align with previous work published by the authors which states that the anthrocyanin rich extract displays toxicity at doses above 156.3 µg/mL against HEK-293 cells (Jeyaraj et al.,2022 , Figure 3), which is much lower the MIC described in this work. Greater context needs to added here. - Minor points - Refences needed for the following: o Line 63, second sentence o Line 71, second sentence o Line 246, second sentence o Line 248, second sentence o Line 308 o Line 251, reference does not support the statement. Needs to be rewritten to fit the reference or a new reference is needed. § Reference paper states "It was demonstrated that the sugars do not contribute to the binding affinity or the DNA cleavage selectivity, although the presence of the sugar enhances the DNA cleavage efficiency by 2-5 times" - Lines 223-225, Findings from Gram positive bacteria are compared to Gram negative are not representative and thus cannot support the findings and perhaps more relevant examples could be used. - Figure 1, Line 237 - Are the replicates technical or biological?

Please rate the manuscript for methodological rigour
Good

Please rate the quality of the presentation and structure of the manuscript
Good

To what extent are the conclusions supported by the data?
Partially support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?
No

Is there a potential financial or other conflict of interest between yourself and the author(s)?
No

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?
Yes

SciScore report

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