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Original Research Article

EFFECTS OF EXTRACTS OF *GONGRONEMA LATIFOLIUM*, *ZINGIBER OFFICINALE*, AND *ALLIUM SATIVUM* ON FAECAL SHEDDING OF BETA-LACTAM-RESISTANT *ESCHERICHIA COLI* IN BROILERS

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ABSTRACT

Antimicrobial resistance (AMR) poses a global public health challenge, with beta-lactam–resistant *Escherichia coli* (*E. coli*) posing significant risks to poultry production and food safety. This study evaluated the antibacterial effects of aqueous extracts of *Gongrenema latifolium* (*G. latifolium*), *Zingiber officinale* (*Z. officinale*), and *Allium sativum* (*A. sativum*) on faecal shedding of beta-lactam–resistant *E. coli* in broilers raised in Abia State, Nigeria. A total of 144 broiler chicks were randomly assigned into four groups (A–D): control, *G. latifolium*-treated, *Z. officinale*-treated, and *A. sativum*-treated, with each group subdivided into three dosage levels (low, medium, and high). Birds were orally challenged with beta-lactam–resistant *E. coli* (10^6 CFU/ml) at days 7 and 21 and treated with the respective plant extracts for three weeks. Faecal samples were collected weekly and cultured on Eosin Methylene Blue agar for coliform enumeration. Data were analyzed using one-way ANOVA at a 95% confidence level. All plant extracts significantly ($P \leq 0.05$) reduced the faecal shedding of beta-lactam–resistant *E. coli* compared to the control group. The highest reduction in colony-forming units (CFUs) was observed in broilers treated with high-dose *G. latifolium* extracts, followed by medium doses of *A. sativum* and *Z. officinale* extracts. The antibacterial effects varied with dose, with higher extract concentrations yielding greater reductions in CFU counts. The findings demonstrate that aqueous extracts of *G. latifolium*, *A. sativum* and *Z. officinale* possess significant antibacterial activities against beta-lactam–resistant *E. coli* in broilers, suggesting their potential as natural alternatives to synthetic beta-lactam antibiotics in poultry production. Further studies are recommended to determine optimal dosages, toxicity profiles, and solvent-specific extract efficacy.

Keywords: *Allium sativum*, beta- lactam resistance, broilers, *Escherichia coli*, *Gongronema latifolium*, herbal antimicrobials, *Zingiber officinale*

INTRODUCTION

Antibiotic resistance is a serious public health problem that threatens human, animal and environmental health globally (Islam *et al.*, 2021). Of particular concern is resistance to beta-lactam antibiotics, which remain among the most widely used antimicrobial agents in poultry production for disease prevention and treatment. The increasing prevalence of beta-lactam–resistant bacteria has compromised treatment efficacy, increased production costs, and heightened the risk of resistant pathogens entering the food chain.

Escherichia coli (*E. coli*) is a common pathogen, a major cause of colibacillosis, a disease that causes acute fatal

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septicemia and other severe conditions in birds, leading to severe mortality rates in broiler flocks and consequently, high economic losses. The emergence of beta-lactam–resistant *E. coli*, including strains capable of producing extended-spectrum beta-lactamases (ESBLs), complicates treatment and poses a risk to both animal and human health due to the potential for transmission through the food chain (Poirel *et al.*, 2018). These resistant strains reduce the therapeutic value of commonly used beta-lactam antibiotics and contribute to the persistence of infection in poultry environments. In response to rising resistance, there is increasing interest in alternative antimicrobial strategies that reduce reliance on conventional antibiotics.

The use of herbs and spices in poultry production has gained significant attention as a viable alternative to antibiotics, which has been widely used as growth promoters and in disease prevention (Nadir *et al.*, 2014). Herbs and spices rich in bioactive compounds offer a range of benefits that can enhance the health, welfare and productivity of broiler chickens (Abd *et al*., 2022). They have been shown to exhibit broad-spectrum antimicrobial activities, including antibacterial, antifungal, antiparasitic and antiviral effects (Okoye, 2018) that make them useful in treating various infections and diseases in animals.

Furthermore, *G. latifolium* (called *utazi* in Igbo language) leaves and *Z. officinale* (ginger) extracts derived from the roots of *Z. officinale*, have been documented to possess anti-inflammatory, antioxidant, antiulcer properties, and protect against oxidative stress (Ozkur *et al*., 2022, Mbaeyi-Nwaoha *et al*., 2023, Saravanan, 2024). Garlic extract, derived from *Allium sativum* is a rich source of bioactive compounds, particularly allicin, which is responsible for its medicinal properties (El-Saber *et al*., 2020). The sulphur compounds in *A. sativum* are known for their antimicrobial, antiviral and antifungal properties, making it a valuable herb for promoting overall health (Hayat *et al.*, 2022). These properties make such plants promising candidates for mitigating infections caused by beta-lactam–resistant pathogens in poultry systems.

Given the increasing prevalence of beta-lactam resistance among poultry-associated *E. coli* in Nigeria and the need for sustainable antimicrobial alternatives, this study was therefore, designed to evaluate the effects of the aqueous extracts of *G. latifolium*, *Z. officinale* and *A. sativum* extracts on the faecal shedding of beta-lactam–resistant *Escherichia coli* in broiler chickens raised in Abia state, Nigeria

MATERIALS AND METHODS

STUDY LOCATION

The study was conducted at the poultry farm of the College of Veterinary Medicine, Michael Okpara University of Agriculture, Umudike, Abia State. Umudike is located in Ikwuano Local Government Area, at approximately 5.49° N latitude and 7.54° E longitude**,** within the humid tropical rainforest zone of Nigeria. A map showing the location of the study area within Abia State and Nigeria is presented in Figure I.

Figure I: Map of Abia State showing Ikwuano Local Government Area

SOURCING OF THE HERBS AND SPICES

The *G. latifolium* leaves, *Z. officinale* and *A. sativum* rhizomes were sourced from the local Orie-Ugba market and were properly identified in the Department of Crop Science, Michael Okpara University of Agriculture, Umudike, Nigeria, with batch numbers CVM/VPP/24/30, CVM/VPP/24/32 and CVM/VPP/24/31 respectively.

PREPARATION OF THE LEAVES AND SPICE FOR EXTRACTION

The *G. latifolium* leaves, *Z. officinale* and *A. sativum* spices were thoroughly washed, cut into smaller sizes and air-dried

under room temperature. The dried herbs and spices were thereafter ground to powder using a dry blender.

Aqueous extracts of these herb and spices were made and stored at 4℃ until use according to the method described by Azwanida, (2015).

SOURCING OF TEST ORGANISMS

Test organisms (a beta-lactam–resistant *E. coli*) used in this study were sourced from the Veterinary Microbiology laboratory, Michael Okpara University of Agriculture, Umudike, Nigeria. They were isolates obtained from broilers in a separate experimentally study.

ISOLATION OF THE TEST ORGANISM

The isolates were sub-cultured on MacConkey Agar and incubated aerobically at 37℃ for 24-48 hours. The suggestive colonies (lactose fermenting colonies on MacConkey agar) were sub-cultured again on Eosin Methylene Blue (EMB) Agar and incubated aerobically for 24-48 hours.

PHENOTYPIC IDENTIFICATION OF THE ISOLATES

The isolates that produced typical colonies with a green-metallic sheen were identified using standard biochemical procedures such as Gram staining, citrate test, indole test, and triple sugar iron test according to Bergey's manual of systemic bacteriology (Garrity & Holt, 2001; Whitman, 2015).

ANTIMICROBIAL SUSCEPTIBILITY TEST

Antibiotic sensitivity test was carried out using a modified Kirby Bauer disc diffusion method (Syal *et al*., 2017). It was performed using commercially available antibiotic discs on Mueller-Hinton Agar (MHA). Fresh18-24hours bacterial colonies from solid media was transferred into peptone water and adjusted to McFarland standards. A lawn culture of the standardized suspension was then made on the surface of MHA and the plates were allowed to stand for 30 minutes to permit absorption of excess surface moisture before antibiotic disc application. The antibiotic discs were dispensed onto the surfaces of inoculated plates. The plates were incubated aerobically between 18-24 hours at 37℃. After incubation, the diameters of the zones of inhibition were measured for each antimicrobial agent and compared with the CLSI guidelines (CLSI, 2020). The following beta-lactam antibiotics were used; Augumentin (10µg/20µg) Cefotaxime (30µg) Ceftazidime (30µg) Aztreonam (30µg) Meropenem (10µg)

EXPERIMENTAL BIRDS

One hundred and forty-four broilers were used for this study. They were kept under deep litter system and fed *ad-libitum* with feed and water; adequate biosecurity measures were maintained. Sample size was calculated according to formula by Thrushfield (2005).

EXPERIMENTAL INFECTION AND TREATMENT

At 7 days of age, birds in the infected groups were orally challenged with 1 ml *E. coli* suspension **(**10^6 CFU/mL**),** with a booster challenge on day 21. Treatment with plant extracts commenced one week post-challenge and continued daily for three consecutive weeks. Birds were given Gumboro vaccine on day 10 and Newcastle disease vaccine Lasota (NDV-L) at 21 days. All vaccines used were procured from the National Veterinary Research Institute (NVRI), Vom, Plateau state, Nigeria. The birds were randomly allocated into four different groups labelled A-D, which were also divided into 3 sub-groups (I-III); with 36 birds per group, and 12 within each subunit or subgroup. The Experimental design is presented in Table I.

ETHICAL APPROVAL

Ethical Approval was received from the College of Veterinary Medicine, Michael Okpara University of Agriculture, Umudike with Ethical Approval Number MOUAU/CVM/REC/202516.

PREPARATION OF INNOCULUM

The identified cultured isolates of *E. coli* were cultured overnight in peptone water and standardized to a suspension of 10^6 CFU/ml and administered to the all birds across treatment and control groups at day 7 in drinking water and repeated at day 21.

TABLE I: EXPERIMENTAL DESIGN

Group	Subgroup	Infection status	Treatment Administered
A(Control)	A1	Not infected	Water ONLY
	A2	Infected	Antibiotic
	A3	Infected	NONE
B(Utazi)	B1	Infected	Utazi 100mg/kg
	B2	Infected	Utazi 200mg/kg
	B3	Infected	Utazi 300mg/kg
C(Ginger)	C1	Infected	Ginger 100mg/kg
	C2	Infected	Ginger 200mg/kg
	C3	Infected	Ginger 300mg/kg
D(Garlic)	D1	Infected	Garlic 100mg/kg
	D2	Infected	Garlic 200mg/kg
	D3	Infected	Garlic 300mg/kg

FAECAL COLIFORM ANALYSIS

About 2-3 samples of freshly voided faeces were collected weekly from the poultry pens into a sterile petri-dish from each of the subunits in each group and taken immediately to the Veterinary Microbiology Laboratory, Michael Okpara University of Agriculture, Umudike for coliform investigation.

For determination of coliform counts, 1g of the freshly collected voided faecal sample was homogenized in 9ml of sterile distilled water in a test tube and a 10-fold serial dilution was done. Serial dilutions up to 1: 10^6 were prepared. From these mixtures, 100µL from 10^4 – 10^6 dilutions were dropped on a dried surface of Eosin Methylene Blue (EMB) agar plate and spread homogenously on the agar. This was repeated in triplicates. The plates were allowed to stand for about 10 minutes and thereafter incubated aerobically at 37°C for 24 hours.

Viable colonies formed after incubation was counted systematically using a colony counter (J-2 2024-02). The colony forming unit (CFU) from each of the counted plates was determined using the formula:

CFU/ml = Number of colonies X dilution factor /Volume of culture used

Where: dilution factor = 10^6; volume of culture used = 1ml (100µl)

STATISTICAL ANALYSIS

Data generated from this study were analysed using Statistical package for Social Sciences (SPSS) version 23.0. One-way analysis of variance (ANOVA) was used to compare the means. The statistical confidence was set at 95 % ($P \leq 0.05$).

RESULTS

EFFECT OF AQUEOUS EXTRACTS OF *G. LATIFOLIUM (UTAZI*) ON FAECAL SHEDDING OF B-LACTAM-RESISTANT *E. COLI*

Treatment with aqueous *G. latifolium* leaf extract resulted in a reduction in faecal shedding of beta-lactam-resistant *Escherichia coli* in treated birds (Table II). While reductions in CFU counts were not consistently significant at earlier sampling points, a statistically significant effect was observed by Day 28 post-infection. At day 28, all extract-treated groups exhibited significantly lower CFU counts compared with the infected untreated and antibiotic-treated controls, with the greatest reduction recorded in birds receiving the high-dose extract. These findings suggest a time- and dose-dependent antimicrobial effect of *G. latifolium*, especially with prolonged administration.

TABLE II: EFFECT OF *GONGRENEMA LATIFOLIUM* LEAF AQUEOUS EXTRACTS ON THE COLIFORM FORMING UNIT OF B-LACTAM-RESISTANT *E. COLI*

Groups	Day 7	Day14	Day 21	Day 28
A1	ND	ND	ND	ND
A2	1.68 ± 3.33^{a}	1.97 ± 5.70^{a}	1.90 ± 4.12^{b}	2.43 ± 1.55^{b}
A3	1.68 ± 3.33^{a}	1.98 ± 0.00^{a}	2.21 ± 1.53^{b}	18.36 ± 1.06^{a}
B1	1.67 ± 6.66^{a}	1.96 ± 3.30^{a}	3.35 ± 0.34^{a}	1.63 ± 1.58^{c}
B2	1.68 ± 3.33^{a}	1.97 ± 6.07^{a}	2.21 ± 1.84^{b}	1.74 ± 0.37^{c}
B3	1.67 ± 6.67^{a}	1.96 ± 3.33^{a}	1.72 ± 0.63^{b}	0.13 ± 2.33^{d}

Values are expressed as mean ± standard deviation (SD). Superscripts (a–d) indicate significant differences within the same column**;** means with different superscripts differ significantly at $P \leq 0.05$. KEY: ND**:** Not detected; A1: Uninfected and untreated; A2: Infected + treated with antibiotics; A3: Infected but untreated; B1: Infected and treated with 100mg/kg of extract; B2: Infected and treated with 200mg/kg of extract; B3: infected and treated with 300mg/kg of extract

EFFECT OF *Z. OFFICINALE (GINGER*) EXTRACT ON FAECAL SHEDDING OF B-LACTAM-RESISTANT *E. COLI*

Administration of *Z. officinale* extract significantly reduced faecal shedding of β-lactam–resistant *E. coli* in infected birds, with the effect most pronounced by day 28. The medium-dose group showed the greatest reduction in CFU counts, followed by the low- and high-dose groups, indicating a dose-related but non-linear response. All *Z. officinale*-treated groups had significantly lower faecal bacterial loads compared with the infected untreated control ($P < 0.05$), highlighting the potential of *Z. officinale* phytochemicals to suppress intestinal colonization by resistant *E. coli*.

TABLE III: EFFECTS OF *Z. OFFICINALE (GINGER)* EXTRACT ON THE COLIFORM FORMING UNIT (CFU) OF B-LACTAM-RESISTANT *E. COLI*

Groups	Day 7	Day 14	Day 21	Day 28
A1	ND	ND	ND	ND
A2	1.68 ± 3.33^{a}	1.97 ± 5.70^{a}	1.90 ± 4.12^{b}	2.43 ± 1.55^{b}
A3	1.68 ± 3.33^{a}	1.98 ± 0.00^{a}	2.21 ± 1.53^{b}	18.36 ± 1.06^{a}
C1	1.68 ± 3.30^{a}	1.96 ± 8.81^{a}	2.59 ± 2.30^{a}	0.22 ± 0.66^{c}*
C2	1.67 ± 5.77^{a}	1.96 ± 5.76^{a}	2.21 ± 4.73^{ab}	0.19 ± 0.20^{c}*
C3	1.68 ± 0.00^{a}	1.97 ± 6.66^{a}	1.42 ± 1.01^{b}	1.65 ± 1.12^{b}*

Values are expressed as mean ± standard deviation. Superscripts (a–d) indicate significant differences within the same column; means with different superscripts differ significantly. *****: Significantly different from the infected untreated control group (A3) at $P < 0.05$ KEY: ND = Not detected. A1: Uninfected and untreated; A2: Infected + treated with antibiotics; A3: Infected but untreated); C1: Infected and treated with 100mg/kg of extract; C2: Infected and treated with 200mg/kg of extract; C3: Infected and treated with 300mg/kg of extract.

EFFECT OF *A. SATIVUM* (*GERLIC*) EXTRACT ON FAECAL SHEDDING OF B-LACTAM RESISTANT *E. COLI*

Administration of *A. sativum* extract significantly reduced faecal shedding of β-lactam–resistant *E. coli* in infected birds, particularly from day 21 post-infection. The medium-dose (200mg/kg) group demonstrated the greatest reduction in CFU counts, followed by the high-dose group, while the low dose (100mg/kg) group showed no significant effect compared with the infected untreated control. Overall, medium- and high-dose *A. sativum* treatments effectively lowered faecal bacteria loads relative to both antibiotic-treated and untreated controls ($P \leq 0.05$), highlighting its potential as a non-antibiotic intervention against β-lactam–resistant bacteria.

TABLE IV: EFFECT OF *A. SATIVUM* (*GARLIC*) EXTRACT ON THE COLIFORM FORMING UNIT (CFU) OF B-LACTAM-RESISTANT *E. COLI*

Groups	Day 7	Day 14	Day 21	Day 28
A1	ND	ND	ND	ND
A2	1.68 ± 3.33^{a}	1.97 ± 5.70^{a}	1.90 ± 4.12^{b}	2.43 ± 1.55^{b}
A3	1.68 ± 3.33^{a}	1.98 ± 0.00^{a}	2.21 ± 1.53^{b}	18.36 ± 1.06^{a}
D1	1.67 ± 5.80^{a}	1.96 ± 3.33^{a}	1.92 ± 1.78^{b}	18.46 ± 2.62^{a}
D2	1.67 ± 3.33^{a}	1.97 ± 1.15^{a}	1.51 ± 8.37^{bc}	1.15 ± 4.86^{c}*
D3	1.67 ± 3.33^{a}	1.97 ± 6.60^{a}	2.39^{bc}	$\pm 4.32^{b}$*

Values are expressed as mean ± SD. Superscripts (a, b, c) indicate significant differences within the same column ($P < 0.05$). *= significantly different from infected untreated control (A3). KEY: A1: Uninfected and untreated; A2: Infected + treated with antibiotics; A3: Infected but untreated); D1: Infected and treated with 100mg/kg of extract; D2: Infected and treated with 200mg/kg of extract; D3: Infected and treated with 300mg/kg of extract

DISCUSSION

Herbs and spices play a significant role in poultry production, offering numerous benefits which include antimicrobial effects, antioxidant effects, immune system support and growth promotion (Rafeeq *et al*., 2022).

In this study, aqueous extracts of *G. latifolium, Z. officinale*, and *A. sativum* administered at graded doses (100mg/kg, 200mg/kg and 300mg/kg) significantly reduced the faecal shedding of beta-lactam–resistant *Escherichia coli* in broiler chickens compared with the control groups. This finding supports the growing interest in plant-based antimicrobials as alternatives to conventional antibiotics in poultry production. The observed reduction in faecal shedding across the treatment groups, particularly at day 28, indicates sustained antibacterial activity of the plant extracts against beta-lactam–resistant *E. coli*. The significant differences recorded between the treated groups and the untreated control groups confirm the inhibitory effects of these extracts on intestinal colonization by resistant *E. coli* strains. These effects may be attributed to the presence of bioactive phytochemicals capable of disrupting bacterial cell walls, inhibiting enzyme activity, and impairing metabolic functions, mechanisms that are particularly relevant for organisms resistant to beta-lactam antibiotics.

G. latifolium extract demonstrated a pronounced dose-dependent reduction in faecal shedding of β-lactam–resistant *E. coli*, particularly at higher concentrations. This finding supports earlier reports that both aqueous and ethanolic extracts of *G. latifolium* possess antibacterial activity (Ojiako *et al*., 2020). Although limited information exists on its effect on faecal shedding of β-lactam–resistant *E. coli* in broilers, the antibacterial activity observed in this study may be linked to phytochemicals such as alkaloids, tannins, and saponins that could exert inhibitory effects on bacterial growth (Ejembi *et al*., 2022).

Similarly, *Zingiber officinale* extract significantly reduced faecal shedding of beta-lactam-resistant *E. coli*, corroborating findings by Saba *et al*. (2023), who observed a reduction in the faecal microbial load in broiler chickens infected with experimentally induced *E. coli* infection. This also aligns with studies by Gull *et al.* (2012), who demonstrated notable antimicrobial activity *by Z. officinale*. The antimicrobial activity of *Z. officinale* is attributed to the presence of phytochemical compounds (such as gingerol, oleoresins), phenolic compounds (such as eugenol, shogaols, zingerone, gingerdiols, gingerols) and their synergistic interactions with other compounds like β-sesquiphellandren and zingiberen that exert synergistic antibacterial effects (Singh & Singh, 2018; Mara *et al*., 2020). This finding highlights the potential role of *Z. officinale* as a complementary non-antibiotic strategy for mitigating the burden of β-lactam–resistant bacteria in poultry production, with implications for antimicrobial resistance containment and food safety. By day 21, there were differences between treatment groups, with *Z. officinale*-treated birds showing lower faecal CFU counts compared with the infected untreated control.

This suggests the onset of suppression of intestinal colonization by resistant *E. coli*. The effect was most pronounced at day 28 post-treatment, when all *Z. officinale*-treated groups exhibited significantly reduced faecal shedding relative to the infected untreated group ($P < 0.05$), confirming the sustained antimicrobial influence of *Z. officinale* against β-lactam–resistant strains.

A. sativum extract also demonstrated notable antibacterial effects. Higher doses consistently produced greater effects than lower doses, suggesting a concentration-dependent response. This response is consistent with earlier studies reporting reduced intestinal *E. coli* populations in broiler chickens treated with *A. sativum* preparations (Sarica *et al*., 2005; Mahmoud *et al.,* 2006). This may be as a result of the presence of organosulphate compounds together with other antibacterial sulphur compounds such as allicin and allinase known for their strong antibacterial activities against coliform bacteria (Norman *et al.,* 2023).

The results demonstrate that extract significantly reduced faecal shedding of β-lactam–resistant *Escherichia coli* in experimentally infected birds, with the effect becoming pronounced after prolonged treatment. During the early phase of the experiment (days 7 and 14), no significant differences were observed among the infected groups, indicating that the extracts did not exert an immediate bactericidal effect

The persistence of high CFU counts in the low-dose *A. sativum* group at day 28 suggests that sub-therapeutic concentrations may be insufficient to suppress β-lactam–resistant *E. coli*. Similar observations have been reported in previous studies, where the effectiveness of herbal extracts was influenced by factors such as plant chemical composition, extraction method, dosage, storage conditions, and environmental factors (Malhotra, 2017). This highlights the importance of optimizing dosage and treatment duration when using herbal extracts for antimicrobial purposes.

The dose-dependent reduction in coliform counts observed across treatment groups suggests that higher concentrations of the plant extracts enhanced antibacterial efficacy. This pattern is consistent with previous reports that associate increased concentrations of phytochemicals with improved antimicrobial activity. In particular, the superior performance of *G. latifolium* at higher doses may be attributed to its rich phytochemical profile, while the antibacterial effect of *A. sativum* is likely linked to allicin and related sulphur compounds known to disrupt bacterial cell membranes. The observed reduction in faecal shedding of beta-lactam–resistant *E. coli* has important implications for poultry health and food safety. Persistent faecal shedding of resistant bacteria contributes to environmental contamination and facilitates transmission along the food chain.

By reducing bacterial load, these herbal extracts may help limit the spread of beta-lactam–resistant *E. coli* within poultry farms and beyond.

The findings of this study show that aqueous extracts of *G. latifolium*, *Z. officinale*, and *A. sativum* significantly reduced faecal shedding of beta-lactam–resistant *Escherichia coli* in broiler chickens; supporting growing evidence that plant-derived bioactive compounds possess antibacterial activity against antibiotic-resistant bacteria and may serve as alternatives to conventional antimicrobials in poultry production systems.

CONCLUSION

This study demonstrates that aqueous extracts of *G. latifolium*, *Z. officinale*, and *A. sativum* significantly reduce faecal shedding of beta-lactam–resistant *Escherichia coli* in broiler chickens. These findings underscore the potential of locally available medicinal plants as sustainable alternatives to synthetic beta-lactam antibiotics in poultry production, particularly in low- and middle-income settings where antimicrobial resistance poses a growing threat due to gross antibiotic misuse and poor regulatory enforcement.

This study focused specifically on beta-lactam–resistant *E. coli*. However, its focus on a single antibiotic class and the use of aqueous extracts only was limiting. Further studies incorporating additional antibiotic classes, extraction solvents, toxicity profiles of *A. sativum*, *Z. officinale* and *G. latifolium* and their mechanistic investigations are recommended.

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CONFLICT OF INTEREST

Authors declare that there is no conflict of interest.

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