Acta Pharm. Sci. Vol 60:(4), 2022
DOI: 10.23893/1307-2080.APS6027

Does eugenol have potential as an anti-campylobacter and antioxidant compound in the food industry and clinical settings?

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ABSTRACT

Though there exist high food safety standards and legislations in developed countries, *Campylobacter* infections have remained a major public health problem. *Campylobacter jejuni* and *Campylobacter coli* are the principal causes of bacterial food-borne gastroenteritis in humans. *Campylobacter* infections are closely related to the consumption of contaminated chicken. *Campylobacter* species develop resistance to existing antibiotics thanks to the genomic plasticity. There is therefore a higher need to develop effective approaches for bacterial control in modern food industry and clinical settings. Presented study has evaluated the anti-campylobacter activity and antioxidant capacity of eugenol, the primary phenolic component of clove oil. Two *C. jejuni* strains (ATCC 33560 and one chicken isolate) and two *C. coli* strains (NCTC 12525 and one chicken isolate) were used in the study. The anti-campylobacter activity of eugenol was analyzed by microbroth dilution method. Minimum inhibitory and minimum bactericidal concentration (MIC and MBC) were in the range of 0.64-1.28 mg mL^{-1}. Besides, our MTT assay findings showed that eugenol has strong scavenging ability. These outcomes have supported both the anti-campylobacter and antioxidant activity of eugenol. Eugenol is generally recognized as safe and is a promising antimicrobial compound against the genus *Campylobacter*.

Keywords: *Campylobacter jejuni*, *Campylobacter coli*, Eugenol, Clove oil, Antibacterial Activity

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(Received 29 Jul 2022, Accepted 22 Aug 2022)

INTRODUCTION

Campylobacter species, specially *C. jejuni* and *C. coli*, are the primary cause of bacterial food-borne gastroenteritis in humans [1]. The World Health Organisation (WHO) has suggested that food-borne *Campylobacter* infections affected more than 95 million people around the earth in 2010 [2]. Human Campylobacteriosis has currently been recorded as the mostly reported infection in the European Union. It is notable that *Campylobacter* infections represent 50% of the total cases of gastrointestinal infections [3]. Apart from acute morbidity, chronic several complications such as reactive arthritis (RA), inflammatory bowel disease (IBD) and Guillain Barre Syndrome (GBS) have also been reported following *Campylobacter* infection in humans [4]. Moreover, *Campylobacter* infections are responsible for 31% of GBS cases globally [5].

Campylobacter species are common in the digestive tract of livestock and domestic animals. Essentially the thermophilic *Campylobacter* species are a commensal microorganism in broiler flocks with up to 10^{10} colony forming units (CFU) per gram of faeces [1]. Modern poultry slaughter relies on automated equipment posing a challenge to processors to avoid bacterial contamination in the slaughter line. As a result, contamination of poultry carcasses during slaughter is often inevitable [6]. The most *Campylobacter* infections in humans are caused by the consumption of contaminated foods of animal origin, such as under-cooked poultry and un-pasteurized milk [7,8]. However, it has been reported that the main cause of human campylobacteriosis is eating contaminated chicken in developed countries [3]. Chicken consumption is responsible for 29.2%, 65-69% and 56.5% of human campylobacteriosis in USA, Canada and UK, respectively [9]. Thus, ensuring microbiological control at all stages of the food chain will reduce the risk of human *Campylobacter* infections.

Campylobacter infections are typically self-limiting and not require medical treatment. The focus of medical interventions in the most cases are hydration and electrolyte repletion. However, antibiotics are used in high-risk patients such as the immunocompromised and the elderly. The genus *Campylobacter* creates a variety of mechanisms for resistance to clinically important antibiotics thanks to the genomic plasticity [8]. Antibiotic-resistant *Campylobacter* is listed as one of the underlying pathogens on the WHO list for creating new treatment strategies [10]. Concerns about antibiotic-resistant pathogens and food safety have increased the interest in developing alternative approaches to combat pathogens. Natural compounds from roots, leaves and flower buds of medicinal plants may have a prominent medicinal potential without developing resistance in several pathogens [11]. Besides, the inclusion of natural compounds in combination therapies for medical treatment is also of great interest.

Clove has been used by civilizations for many years due to its fragrance and flavor. This desirable characteristics make it worth for culinary and therapeutic uses. Eugenol is the major phenolic component of clove essential oil, derived from the flower buds and leaves of *Syzygium aromaticum* (L.) Merr. et L.M.Perry (syn. *Eugenia caryophyllus* (Spreng.) Bullock et S.G.Harrison). It is a yellow colored liquid with the molecular weight of 164.2 g mol^{-1}. The antioxidant property of eugenol, which has been proven by various studies, has recently became the focus of attention of researchers [12,13]. Current studies have also suggested the analgesic, antiproliferative and anti-inflammatory properties of eugenol [14,15]. Further, there is extensive literature on the antimicrobial activity of eugenol against wide variety of human pathogens such as *Salmonella* Typhmurium, *Candida albicans* and *Escherichia coli* [16-18]. However, there are limited studies reporting the *in-vitro* antimicrobial activity of eugenol against *Campylobacter* species. The present study therefore assessed the *in-vitro* antimicrobial activity of eugenol against *C. jejuni* and *C. coli* strains. We have also aimed to present the *in-vitro* antioxidant activity of eugenol as ascorbic acid equivalent.

METHODOLOGY

Chemicals

Dimethyl sulfoxide (DMSO; Sigma-Aldrich, Chemie GmbH, Germany), Mueller Hinton Broth (MHB; Sharlau Microbiology, Belgium), Modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA; CM0739, OXOID), Mueller Hinton Agar (MHA; M1084-500G, HIMEDIA), Eugenol (Sigma-Aldrich, Chemie GmbH, Germany), Peptone Water (PW; LAB104, LABM) and CampyGen 3.5 L sachet (CN0035A, OXOID) were used in the present study.

Bacterial strains and preparation of bacterial suspension

In the present study, *C. jejuni* (one chicken isolate and ATCC 33560) and *C. coli* (one chicken isolate and NCTC 12525) strains were used. Isolated and reference strains were obtained from chicken meat purchased from a local market and Refik Saydam National Type Culture Collection Laboratory (Ankara, Turkey), respectively. All bacterial strains were preserved in a growth medium containing 20% glycerol at -80 °C. Initially, each *Campylobacter* strain was cultivated on separate mCCDA plate and incubated at 37 °C for 48 hours under the microaerobic condition created with CampyGen 3.5 L sachet. The cells were then adjusted to 0.5-McFarland opacity (1-2 x 10^8 CFU mL^{-1}) with 10 mL of PW [19]. Each prepared bacterial suspension was homogenized by vortexing for 1 minute prior to the antibacterial activity test.

Preparation of eugenol solution

Eugenol (99% reagent plus) was purchased from Sigma-Aldrich (Chemie GmbH, Germany). The stock solution was prepared by dissolving 0.96 mL of eugenol in sterilised distilled water (50 mL) containing 1% DMSO. The stock solution (20.480 mg mL^{-1}) is then 2-fold-diluted with sterile MHB to yield 10.240 mg, 5.120 mg, 2.560 mg, 1.280 mg, 0.64 mg, 0.32 mg, 0.16 mg, 0.08 mg and 0.04 mg mL^{-1} solutions.

Analyze of bacterial growth inhibiton and determination of minimum inhibitory concentration

The MIC was determined using the microbroth dilution method recommended by Clinical and Laboratory Standards Institute [20]. Briefly, 100 µL of the serial dilution of eugenol (0.04-10.240 mg mL^{-1}) was transferred to 96-well microplates. Subsequently, bacterial suspension (1-2 x 10^8 CFU mL^{-1}) were prepared for each *Campylobacter* strain. 10 µL of each bacterial suspension was inoculated to the wells of separate microplates in six replicates. The control wells were prepared with broth media (sterilized product testing), eugenol (for negative control), and bacterial suspension (for positive control). Optical density (OD) was determined for each well by a spectrophotometer (Byonoy GmbH Absorbance 96, Germany) at an absorbance of 600 nm prior to incubation, T_0. Then, microplates were incubated in the jar for 24 hours at 37 °C under microaerobic condition created with CampyGen. The OD of the wells was measured again by the same spectrophotometer at an absorbance of 600 nm after the incubation, T_{24}. The OD for each well at T_0 was subtracted from the OD for each well at T_{24} and the average of the differences for six replicates was ultimately calculated.

The average OD of the positive control well was assigned a value of 100% bacterial growth. Afterwards, a standard curve was generated using the known percentage of bacterial growth by linear regression analysis. Accordingly, the percentage of bacterial growth was determined for each dilution of eugenol in the test wells. The growth inhibition data were obtained using the following equation. Percentage of inhibition = 100 – Percentage of growth. The MIC value was considered as the minimum eugenol concentration achieved growth inhibition above 95% at the absorbance of 600 nm.

Determination of minimum bactericidal concentration

The MBC values were determined after obtaining the MIC values. Briefly, the dilutions corresponding to ½MIC, MIC and 2MIC were cultured on MHA and incubated under microaerobic conditions created with CampyGen at 37°C for

48 hours. The MBC was regarded as the lowest eugenol concentration that inactivated more than 99.99% of bacteria present. Six replicates were performed for each *Campylobacter* strain and eugenol dilution.

Determination of antioxidant activity

The antioxidant activity of eugenol was determined by the MTT [3- (4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] method previously defined by Liu & Nair (2010) [21]. The MTT (1 mg mL^{-1}) was dissolved in sterilised distilled water. A stock solution of vitamin C (L-ascorbic acid, 600 µmol, 0.105 mg mL^{-1}) and eugenol (6.5 µmol, 1.067 g/mL) was two-fold diluted with distilled water. 190 µL of MTT solution and 10 µL of each eugenol dilution were mixed in an eppendorf tube and incubated at 37 °C for 4 hours. After incubation, 200 µL of DMSO was added to each tube and vortexed again to dissolve the blue formazan salt. Then 100 µL of each mixture was transferred to 96-well microplates in four replicates. The results were evaluated by spectrophotometer (Byonoy GmbH Absorbance 96, Germany) at an absorbance of 570 nm. A standard curve was generated with the OD values of two fold dilution of Vitamin C (y=bx+a) by linear regression analysis. Vitamin C equivalence of eugenol was determined according to the standard curve.

Statistical analysis

The results are stated as mean and standard deviation ($\bar{X} \pm SD$). Antimicrobial and antioxidant activity tests were performed in 6 replicates and 4 replicates, respectively. Data were analyzed using standard curves created with Microsoft Excel graphical charts.

RESULTS and DISCUSSION

Anti-campylobacter activity of eugenol

The antimicrobial activity of essential oils has long been described by Martindale (1910) [22]. In the literature to date, significant results on the antimicrobial activity of eugenol have accumulated. Despite limited research reporting the activity of eugenol against the genus *Campylobacter*, our findings have supported that eugenol has significant antimicrobial activity as noted in the literature. The antimicrobial activity of eugenol has been demonstrated against a wide range of pathogens such as parasites, fungi, gram positive and negative bacteria [23,24]. The antimicrobial potential is generally attributed to the free –OH groups in the structure of eugenol [12]. Previous studies have reported that eugenol targets microorganism viability by increasing cell membrane permeability, inhibiting membrane-associated ATPase activity, and arresting cellular

respiration [25,26]. Recent studies have reported the effects of eugenol on targeting microorganism viability as well as modulating various aspects of virulence [27]. However, *Campylobacter* has a natural ability to develop antimicrobial resistance thanks to its genomic plasticity [28].

Research on antimicrobial activity against *Campylobacter* species has mainly focused on essential oils of thyme and oregano [29,30]. A very few studies have been conducted on the anti-campilobacter activity of eugenol obtained from clove essential oil. Friedman et al. (2002) previously reported that *C. jejuni* strains are highly sensitive to eugenol [31]. Similarly, our findings revealed the activity of eugenol against *C. jejuni* and *C. coli* strains. The limited research has showed that the MIC value of eugenol against thermophilic *Campylobacter* species ranges from 0.05 to 1.280 mg mL^{-1}. According to Kuete (2010), antimicrobial activity is classified as weak (MIC ≥ 0.625 mg mL^{-1}), moderate (0.1 < MIC < 0.625 mg mL^{-1}) and significant (MIC < 0.1 mg mL^{-1}) [32]. In our study, MIC values of eugenol against the tested *Campylobacter* strains ranged from 0.64 to 1.280 mg mL^{-1} (Table 1).

Table 1. Minimum inhibitory and minimum bactericidal concentration of eugenol against *Campylobacter* strains.

Bacterial strain	MIC[#] (mg mL^{-1})	MBC[¥] (mg mL^{-1})	MBC/MIC
C.jejuni (chicken isolate)	0.64	1.28	2
C. jejuni ATCC 33560	1.28	1.28	1
C. coli (chicken isolate)	0.64	1.28	2
C. coli NCTC 12525	0.64	0.64	1

[#] *minimum inhibitory concentration*

[¥] *minimum bactericidal concentration*

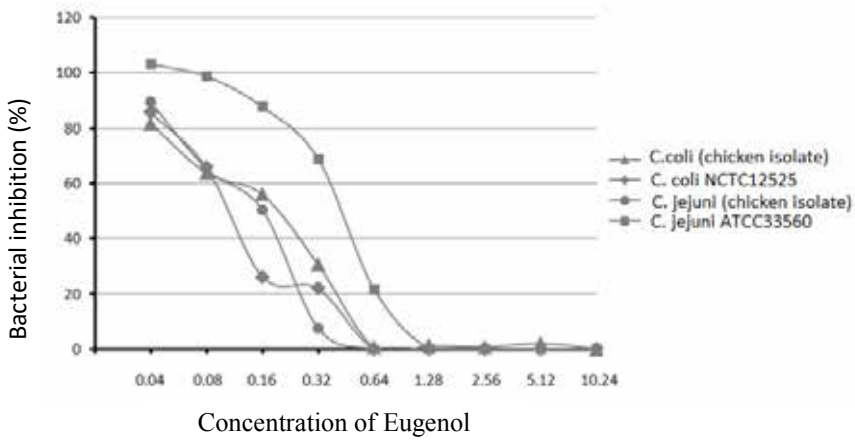

Figure 1. Assesing the efficiency of eugenol on bacterial growth inhibition at different concentrations. The studied concentrations were 0.04, 0.08, 0.16, 0.32, 0.64, 1.280, 2.560, 5.120 and 10.240 mg/mL, respectively.

The percent growth inhibition of tested eugenol concentrations against *C. jejuni* and *C. coli* strains is shown in Figure 1. Understanding the antimicrobial activity of essential oils is possible by determining the MIC value, which is the starting point of the research. However, different MIC findings reported in the literature have revealed some contradictions. These conflicts may arise from factors such as bacterial subtype, laboratory method, solvent and emulsifier used in research [33]. We determined the MIC of eugenol (1% DMSO) as 0.64 and 1.280 mg mL^{-1} against isolated and reference strains of *C. jejuni*, respectively. Similarly, Grilli et al. (2013) has reported that the MIC of eugenol (with ≤ 5% ethanol) is the 1.280 mg mL^{-1} for the both strains using the microbroth dilution method [34]. Although the solvent types were different, the MIC value in the study by Grilli et al. (2013) is in line with our findings. Also, we have observed the MIC of eugenol as 0.64 mg mL^{-1} against both isolated and reference strains of *C. coli*. Similarly, the MIC of eugenol was found to be 0.5 mg mL^{-1} against *C. coli* strains by Hassan et al. (2019) using the agar dilution method [35]. The MIC of eugenol against *Campylobacter* have mostly been determined using the microbroth dilution method. The lowest MIC values (0.05 mg mL^{-1} and 0.01 mg mL^{-1}, respectively) of eugenol against *C. jejuni* and *C. coli* strains by microbroth dilution method were recently recorded by Gahamanyi et al (2020) [36].

Some authors have reported synergistic interaction between eugenol and certain antibiotics as a possible strategy. Palaniappan et al. (2010) have suggested that eugenol (0.1 mg mL^{-1}) can reduce the MIC of penicillin and tetracycline against antibiotic-resistant *S.* Typhimurium, *E. coli* and *S. aureus* [37]. Addition-

ally, synergistic interactions between eugenol and streptomycin against *S.* Typhimurium and *L. monocytogenes* have been reported [38]. Remarkably, more than 8,000 *C. jejuni* sequence types have been reported to be registered by 2020 [39]. In the last decade, ciprofloxacin- and tetracycline- resistant *Campylobacte*r has been gradually increasing in the European population [40]. Therefore, the combination of antimicrobial compounds against *Campylobacter* strains are considered as a new alternative strategy [41]. To our knowledge, the anti-campylobacter activity of eugenol in combination with antibiotics or essential oils has not been evaluated to date. Further studies evaluating the anti-campylobacter activity of eugenol in combination with various antimicrobial agents should be warranted.

The increasing prevalence of antibiotic-resistant bacteria remains one of the major threats to public health worldwide. Knowledge of the pharmacodynamic characteristic of antimicrobial compounds can provide a reasonable approach to determine the optimal concentration against pathogens, improving public health. Therefore, it may be clinically necessary to determine the minimum bactericidal concentration (MBC) in order to understand the pharmacodynamic characteristics of antimicrobial molecules. Antimicrobial activity can be subdivided into two main categories as bacteriostatic and bactericidal activity, depending on the pharmacodynamic characteristics. In several studies, the activity of antimicrobial compounds is considered as bactericidal if the MBC/MIC ratio is ≤ 4. Some researchers have therefore suggested the bactericidal potential of eugenol against *Campylobacter* species [36]. Similarly, our findings demonstrated that the MBC/MIC ratio of eugenol against both *C. jejuni* and *C. coli* strains was < 4, resulting in bactericidal activity (Table 1). However, bactericidal activity of eugenol should be confirmed by further studies using time-kill tests.

Antioxidant activity of eugenol

Free radicals are responsible for a variety of adverse effects on health promotion and food stability. Scavenging free radicals and reducing the formation of free radicals is important for maintaining human health and food quality [42,43]. Free radical scavenging is a known reaction in which antioxidant molecules inhibit oxidation. These reactions are used in antioxidant activity studies and provide rapid detection of the scavenging activity of antioxidant compounds. The ability of antioxidant molecules to scavenge free radicals is often attributed to the bond-dissociation energy of the hydroxyl (–OH) group [44]. There is no widely used reasonable method for the determination of antioxidant activity. The 2,2, diphenyl-picrylhydrazil (DPPH) method and the trolox equiva-

lent antioxidant capacity (TEAC) method are commonly used [18]. In the present study, the scavenging ability of each antioxidant compound was determined by a simple and inexpensive MTT method [21].

As seen in Table 2, our findings showed that eugenol and vitamin C (ascorbic acid) dose-dependently reduced the MTT.

Table 2. Optical density of antioxidant compounds at 570 nm and vitamin C equivalence of eugenol

Concentration of Vitamin C (µmol/mL)	X ± SD[#] (nm)	Concentration of Eugenol (µmol/mL)	X ± SD[¥] (nm)	Vitamin C Equivalent (µmol/mL)
600	0.224±0.014	6.500	1.075±0.068	3478.222
300	0.106±0.028	3.250	0.999±0.057	3224.519
150	0.068±0.010	1.625	0.585±0.023	1844.519
75	0.060±0.014	0.812	0.253±0.019	739.333
37.5	0.051±0.012	0.406	0.105±0.003	246.000
18.75	0.039±0.004	0.203	0.063±0.002	106.370

[#] *optical density values of two fold dilution of Vitamin C*

[¥] *optical density values of two fold dilution of Eugenol*

Vitamin C is known as a broad spectrum antioxidant that can react with a range of detrimental radicals such as reactive oxygen species (ROS) and organic radicals [44]. In this study, we have demonstrated that eugenol has greater scavenging and reduced ability compared to vitamin C. Similarly, there are a wide range of *in-vivo* and *in-vitro* studies proving the potent antioxidant activity of eugenol [12,13]. Eugenol has been generally recognized as safe (GRAS) food additive by the US Food and Drug Administration. Further, it has significant potential to prevent oxidative stress-related diseases [13]. Therefore, eugenol has promising bioactivity for the prevention of chronic diseases as well as for use as a natural preservative in the food industry. However, the pro-oxidative and harmful effect of high concentrations have been highlighted. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has proven that the maximum allowable daily intake of eugenol for humans is 2.5 mg kg^{-1} body weight [45].

Campylobacter infections are the most common bacterial cause of food-borne illness around the earth. Consumption of contaminated chicken is mainly responsible for *Campylobacter* infections in developed countries. In the last decade, antibiotic-resistant *Campylobacte*r has been gradually increasing in

the European population. The researchers therefore focus on alternative treatment methods using natural antimicrobial agents. The antimicrobial activity of eugenol is one of the most popular research areas in recent years. Additionally, the antioxidant capacity of eugenol is remarkable for maintaining human health and food stability. Therefore, the goal of the presented research was to assess the anti-campylobacter as well as antioxidant action of eugenol. Our findings have highlighted the importance of eugenol for *Campylobacter* control. Eugenol may be useful to control *Campylobacter* in the modern food industry, where food preservation technologies are becoming increasingly important. However, it is necessary to comprehend the action mechanism and synergistic action of eugenol before it is used in clinical settings.

ACKNOWLEDGMENT

This study was supported by Marmara University Research Council (BAPKO, TDK-2021-10289)

AUTHOR CONTRIBUTIONS

Murat Gürbüz: Conceptualization, Methodology, Investigation, Software, Data curation, Writing – original draft, Writing - review & editing.

Burcu İrem Omurtag Korkmaz: Conceptualization, Methodology; Investigation, Writing - review & editing.

Serol Korkmaz: Conceptualization, Methodology, Investigation, Software, Writing - review & editing.

DECLARATION OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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