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Synergistic Antioxidant and Antimicrobial Effects of the Thymoquinone and Eugenol Combination

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ABSTRACT

Background: The present study aimed to evaluate the *in-vitro* antioxidant and antimicrobial activity of Thymoquinone (TQ), Eugenol (EUG) and their synergistic effects in combination.

Materials: The minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), fractional inhibitory concentration (FIC), and disc diffusion tests were used to determine the antibacterial activity of TQ and EUG. Moreover, Reducing Power Assay and DPPH (2.2-diphenyl-2-picrylhydrazyl) tests were used to indicate the antioxidant ability of TQ and EUG. The combination index (CI) of these compounds was calculated to evaluate their interaction type.

Results: Bacillus cereus, Listeria monocytogenes, and Shewanella SP were the most sensitive bacteria than other studied pathogens to TQ and EUG, with MIC 0.019, 0.07, and 0.07 mg/ml for TQ and 0.6, 0.6, and 0.6 mg/ml for EUG, respectively. The mixture of two ingredients showed a good antimicrobial and synergistic effects against all tested bacteria. The DPPH scavenging activity of the combination of TQ+ EUG were antagonistic in all combined concentrations except for TQ (5 mg/ml) + EUG (1.25 mg/ml) and TQ (2.5 mg/ml) + EUG (0.3 mg/ml) treatments.

Conclusion: The obtained results provided a new combination of antimicrobial and antioxidant agents in drug delivery, especially in food preservation systems, to enhance food shelf life in the food industry.

1. Introduction

Food-borne diseases affect many people annually and have become a major public health issue worldwide [1]. Another area of global concern is the microbial deterioration of food. Nowadays, the tendency to extend food shelf life and prevent oxidation and microbial spoilage has been developed in all food industry sectors [2]. Research on causative agents of food-borne diseases has high priority. For instance, bacteria, viruses, parasites, toxins, and chemicals are causative agents of food-borne infection or intoxication. A large number of illnesses are caused by food-borne pathogens that affect human health and the economy. Bacteria are the most common cause of food-borne diseases [3]. The most common reported food-borne pathogenic bacteria in outbreaks are Shiga toxin-producing *Escherichia coli*, *Escherichia coli* O157:H7, *Staphylococcus aureus*, *Salmonella Enteritidis*, *Salmonella Typhimurium*, *Bacillus cereus*, and *Listeria monocytogenes* [3]. The presence and growth of pathogenic bacteria in food may lead to poisons, spoilage, and food waste [4]. Oxidation is another common cause of food spoilage in the presence of oxygen that reduces nutritional value and produces undesirable flavors in the food due to the reaction between food components such as polyunsaturated fatty acids and atmospheric oxygen [5]. Using preservatives to prevent food contamination and

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spoilage has been considered by food industries. Several reports show adverse effects of synthetic preservatives (antibiotics or synthetic antioxidants) on human health and side effects such as resistance to antibiotics and carcinogenicity [6]. Therefore, the demand for using natural compounds as a promising alternative to antibiotics and chemical preservatives has increased [7]. Natural bioactive substances are available from various sources, including plants, bacteria, animals, fungi, and algae [8]. Herbal sources with numerous bioactive compounds are of particular importance. Lately, medicinal plants have been used dramatically for several ailments due to their easy accessibility, low cost, and fewer side effects than synthetic medicines [9]. Extracted essential oils (EOs) from different plants have been popularized as a safe substitute for synthetic preservatives to control pathogens and oxidation. Phytochemical analyses showed that EOs composed of compounds such as terpenes and other oxygenated components with antimicrobial and antioxidant properties [10]. Thymoquinone (TQ or 2-isopropyl-5-methyl-1, 4-benzoquinone) is the EO's main and most bioactive compound extracted from *Nigella sativa* [11]. This monoterpenoid hydrocarbon is also found in the EOs of other herbs such as caraway (*Carum carvi*), black cumin (*Bunium bulbocastanum*), nutmeg (*Myristica fragrans*), and coriander (*Coriandrum sativum*) [12]. Medical and pharmacologic features of this compound have been reported, including anti-cancerous, anti-diabetic, antimicrobial, anti-inflammatory, antioxidant, antifungal, neuroprotective, anti-ulcerative, and immunomodulatory properties [11,13]. Eugenol (4-allyl-2-methoxyphenol) is one of the main phenolic ingredients in some plants such as clove (*Eugenia caryophyllata*), cinnamon (*Cinnamomum zeylanicum*), nutmeg (*Myristica fragrans*) and basil (*Ocimum basilicum*), soybean (*Glycine max*), beans, coffee, banana, etc. [14]. This compound is a safe substance, and its non-carcinogenicity and non-mutagenicity features have been proven by the Food and Drug Administration [15]. The pharmacological and biological activities of Eugenol, such as antioxidant, antitumor, and anti-inflammatory effects, have been reported in several studies [14, 16]. The EOs′ composition has limited their use in food systems due to their hydrophobic properties and undesirable effects on sensory attributes of food products [17]. Therefore, the food industry has expanded using their recognized biological compounds, such as TQ and EUG. Since using natural preservatives in the food industry has increased, and there have been a few studies about the antimicrobial and antioxidant effects of TQ and its combination with *Eugenol*, the present study aimed to evaluate the in-vitro antioxidant and antimicrobial effects of TQ, Eugenol, and their synergistic effects in combination.

2. Materials and Methods

2.1 Chemicals

TQ , EUG, and 2.2-diphenyl-2-picrylhydrazyl (DPPH) were provided from Sigma-Aldrich (St. Louis, MO, USA). All culture media were purchased from Pronadisa Madrid, Spain. All other chemicals were purchased from Merck (Darmstadt, Germany).

2.2 Microbial strains

The cocktails of lyophilized cultures of *Bacillus cereus*, *Escherichia coli* O157H7, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Shewanella* SP, *Listeria monocytogenes*, and *Salmonella enteritidis* were obtained from the culture collection of the Iranian Research Organization for Science and Technology (IROST), Tehran, Iran (Table 1). The lyophilized bacterial strains were transferred to a broth medium according to the IROST manual and were instantly used to make the desired suspension for the experiments.

Table 1: The cocktails of microorganisms

Lyophilized genus	Strains
Bacillus cereus	ATCC 11778, PTCC1665, PTCC 1857
Escherichia coli O157H7	ATCC 70728
Staphylococcus aureus	ATCC 29213, ATCC 25923, ATCC 9144
Escherichia coli	ATCC 10536, ATCC 15224, ATCC 25922
Pseudomonas aeruginosa	ATCC 15442, ATCC 25619, ATCC 27853
Shewanella SP	ATCC 1711
Listeria monocytogenes	ATCC 19115, ATCC 13932, ATCC 19114
Salmonella enterica subsp. *enterica*	ATCC 14028, ATCC 13076

2.3 Preparation of TQ and EUG Stock Solutions

A stock solution of TQ (20 mg/ml) was prepared in DMSO. Serial dilutions in 10, 5, 2.5, 1.25, 0.62, 0.31, and 0.15 mg/ml in DMSO were prepared for TQ and EUG solutions. All prepared solutions were filtered through 0.22-μm filters [18].

2.4 Preparation of bacterial suspension

In order to prepare the cocktails of microorganisms, bacterial suspensions were prepared in sterile saline solution (0.85% NaCl) from a fresh overnight culture grown in BHI agar (Darmstadt, Germany). All strains were grown separately in BHI broth at 37 °C for 18–20 h. Cell suspensions resuspend with sterile saline solution to provide cell counts of approximately 10^8 CFU/mL with an OD measurement of 0.1 at 625 nm (OD=625 nm) using a spectrophotometer (Milton Roy Company, Warminster, PA). Then the cocktails of strains were obtained using a ratio of 1:1:1 for each strain of *Bacillus cereus, S. aureus, E. coli, Pseudomonas aeruginosa,* and *L. monocytogenes* and a ratio of 1:1 for *Salmonella enterica* strains [19].

2.5 Antimicrobial activity evaluation

MIC, MBC, FIC, and disc diffusion tests were used to

determine the antibacterial activity of TQ and EUG.

2.5.1 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MIC was determined based on the broth microdilution method described by Hassanzadazar et al. (2019). Briefly, in two 96-well microplates, 160 µl of Brain Heart Infusion (BHI) broth and 20 µl of each overnight bacterial suspension (5×10^6 cfu/mL set by 0.5 McFarland standard tubes) were added to each well, respectively. Then, dilutions ranging from 1.2-0.009 mg/ml of TQ and EUG were added to each microplate′ s wells. The wells containing 20 µl inoculum of each bacterium with 180 µL BHI broth and wells containing 180 µL BHI broth and 20 µl filtered TQ or EUG were mentioned as positive and negative controls, respectively. The microplates were incubated for 24 h at 37 °C. The lowest concentrations of TQ and EUG that inhibited the growth of the bacteria were mentioned as the MIC. The wells of microplates without bacterial growth (pre-determined 99 % reduction of the bacterial population) were considered the MBC of TQ or EUG [20].

2.5.2 Fractional inhibitory concentration Index (FICI)

The FICI of the TQ and EUG was determined based on the described method by Sharma et al. (2017) [21]. Briefly, the mixtures of TQ (0.036 mg/ml to 0.009 mg/ml) and EUG (0.6 mg/ml to 0.15 mg/ml) were prepared in DMSO. These concentrations were selected for the combination based on the obtained results of MIC and MBC determination conducted for TQ and EUG separately. FICI of 9 prepared mixtures (0.036TQ+0.6EUG, 0.036TQ+0.3EUG, 0.036TQ+0.15EUG, 0.018TQ+0.6EUG, 0.018TQ+0.3EUG, 0.018TQ+0.15EUG, 0.009TQ+0.6EUG, 0.009TQ+0.3EUG, 0.009TQ+0.15EUG) were evaluated. In each well of a 96-well microplate, 160 µl of BHI broth, 20 µl of the prepared mixtures of TQ and EUG, and 20 µl of bacterial suspension (1×10^6 cfu/ml) were added and incubated at 37 °C for 24 h. FICI was calculated for TQ and EUG concentrations according to the following instructions. The combination of two compounds is total synergistic if Σ FICI ≤0.5, partial synergistic if 0.5<FICI≤0.75, is additive or indifferent if FICI between 0.75 and 2 ($0.75 \leq FICI \leq 2$), and is antagonistic if FICI > 2 (Sharma et al., 2020) [22].

$$FIC_{A=} \frac{\text{MIC (A) in combination}}{\text{MIC (A) alone}}$$

$$FIC_{B=} \frac{\text{MIC (B) in combination}}{\text{MIC (B) alone}}$$

$$\Sigma \text{ FIC index (FICI)} = FIC_A + FIC_B$$

2.5.3 Disc diffusion method

The Agar diffusion test was used to show the effectiveness of TQ and EUG on food-borne bacteria. The susceptibility of each bacterium was determined by measuring the growth inhibition zone (GIZ). For this purpose, 100 µl of each bacterial suspension with a 106 CFU/ml density was spread on the Mueller-Hinton agar plates. Sterile paper discs were placed on the two agar plates, and 10 µl of different concentrations of filtered TQ and EUG solutions (0.62-20 mg/ml) were transferred onto the blank discs separately. The agar plates were incubated at 37 °C. After 24 h, the diameter of the inhibition zone was measured [20].

2.6 Antioxidant activity evaluation

Reducing Power Assay (RPA) and DPPH (2.2-diphenyl-2-picrylhydrazyl) tests were used to determine the antioxidant ability of TQ and EUG. The combination index (CI) of these compounds was calculated to evaluate their interaction type.

2.6.1 Reducing Power Assay (RPA)

The RP test assessed the ability of TQ and EUG solutions to restore Fe3+ ions. For this purpose, TQ and EUG solutions with a concentration of 0.15-10 mg/ml were prepared. 400µl of each concentration of TQ or EUG solutions was mixed with 1 ml of phosphate buffer (0.2 M, pH 6.6) plus 1 ml of potassium ferricyanide (K3Fe (CN) 1%). The mixtures were incubated at 50 °C for 30 min. Following incubation, 1 ml of trichloroacetic acid (10%) was added, and the mixtures were centrifuged (Sigma 3-30K, Germany) at 2500 g for 10 min. Finally, 1 ml of the supernatant solution of each mixture was mixed with 1 ml of distilled water plus 200µl of $FeCl_3$ (0.1%), and the absorbance was measured at 700 nm. Increasing the absorption in the reaction mixture will mean an increase in the RP [23].

2.6.2 2.2-diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity

Radical Scavenging Activity of TQ and EUG was determined based on the scavenging ability of DPPH radicals described by Fathollahi et al. (2019). 50 µl of prepared concentrations of TQ and EUG (0.15, 0.3, 0.6, 1.2, 2.5, 5, 10 mg/ml) and butylated hydroxytoluene (BHT) as a reference antioxidant and control sample mixed with 2 ml of prepared methanolic solution of DPPH (24 µg/ml). The absorbance of the mixtures was measured at 517 nm after shaking and incubation in a dark room for 60 min with a spectrophotometer (LKB, Novaspec, Sweden). Color changing from purple to yellow corresponds inversely to the scavenging activity of the bioactive compounds measured after incubation time. The methanolic DPPH solution and synthetic antioxidant BHT were used as blank and standard solutions, respectively. DPPH-scavenging capacity can be determined with the following equation:

$$SC\% = \frac{A0 - A1}{A0} \times 100\%$$

SC is the scavenging capacity of the bioactive components. A0 was the absorbance of the methanolic DPPH solution without sample solution, and A1 was the absorbance of the solution containing bioactive components [19].

2.6.3 Combination Index (CI)

The combination index (CI) was used to determine the interaction effect between TQ and EUG. The synergistic, additive or antagonistic effects of two phenolic compounds was evaluated based on the DPPH scavenging capacity of TQ and EUG and according to the described method by Mercado-Mercado et al. (2020) [24]. For this purpose, the combination of three concentrations was evaluated: 1.25TQ:0.3EUG mg/ml, 1.25TQ:0.6EUG mg/ml, 1.25TQ:1.25EU mg/ml, 2.5TQ:0.3EUG mg/ml, 2.5TQ:0.6EUG mg/ml, 2.5TQ:1.25EUG mg/ml, 5TQ:0.3EUG mg/ml, 5TQ:0.6EUG mg/ml and 5TQ:1.25EUG mg/ml, respectively. The effects of the interaction of the TQ and EUG or combination index (CI) were determined according to the following formula:

$$CI = \frac{DPPH\%\ (\frac{ab}{a})}{DPPH\%\ (a)} + \frac{DPPH\%\ (\frac{ab}{b})}{DPPH\%\ (b)}$$

- DPPH ab shows the DPPH scavenging activity of the combinations of TQ and EUG, DPPH (a), and DPPH (b), including the result of TQ and EUG alone, respectively. Depending on the type of interactions of the antioxidant compounds, the CI was reported as follows: CI as 0.3-0.7 (synergetic), 0.7-0.85 (moderate synergetic), 0.85–0.90 (slight synergetic), 0.90–1.10 (nearly additive), 1.10–1.20 (slight antagonistic), 1.20-1.45 (moderate antagonistic), and 1.45-3.3 (antagonistic) [25].

2.7 Statistical analysis

The results presented as mean±standard deviation. All assessments were performed in triplicate. Statistical data analysis was performed by the analysis of variance (ANOVA), the Tukey test with a 95% confidence level using SPSS software (Ver. 24.0, Chicago, USA).

3. Results and Discussion

3.1 Antibacterial activity

The results of MIC and MBC values are shown in Table 1. In this method, TQ is more potent than EUG. The most sensitive bacteria were *B. cereus*, *L. monocytogenes, shewanella* SP than other studied pathogens for TQ and EUG with MIC 0.019, 0.07, and 0.07 mg/ml for TQ and 0.6, 0.6, and 0.6 mg/ml for EUG, respectively. The most resistant strains were the *E. coli* with MIC 0.62 and 5 mg/ml for TQ and EUG, respectively (Table 2). The MIC and FIC of the TQ+EUG combination against the studied bacteria are shown in Table 3. The mixture of two ingredients showed a significant antimicrobial and synergistic effect against all bacteria. The best synergistic effect of the combination of TQ+EUG, which indicates the higher effects of two-compound, was related to *E. coli* bacteria with the lowest amount of FICI (0.09). Several researchers reported the antibacterial effects of TQ and EUG, which is consistent with our results. Kouidhi et al. (2011) reported that the MIC values of TQ for *B. cereus, E. coli, S. enterica, S. aureus, L. monocytogenes, P. aeruginosa* are 8, 512, 256, 8, 32, >512 (μg/ml) respectively which are consistent to the results of this study [26]. However, Halawani (2009) reported that MIC values of TQ for *E. coli*, *S. enteritidis*, *P. aeruginosa,* and *S. aureus* bacteria are 800, 400, 400, 3 (μg/ml), respectively, which is not in line with the results of the present study [27]. Furthurmore, Kokoska et al. (2018) reported that the MIC values of TQ for *S. aureus*, *B. cereus*, *E.coli,* and *P. aeruginosa* are 8, 8, 512, and >512 (μg/ml), respectively, which among these bacteria, the susceptibility of *E. coli* was close to the results of the present study [28]. Further, Goel et al. (2018) reported that MIC values of TQ for *P. aeruginosa, E. coli, S. aureus, B. subtilis* bacteria are 1.56, 100, 3.125, 3.125 (μg/ml), respectively which differ from our results [29]. Several studies also reported MIC for EUG against our studied bacteria. El Atki et al. (2019) reported that MIC values of EUG for *E. coli, S. aureus, P. aeruginosa* bacteria are 5.625, 5.625, and >6 (mg/ml), respectively, which reported MIC for *E. coli* is in accordance with the result of the present study [30]. Additionally, Tippayatum et al. (2007) reported that the MIC values of EUG for *L. monocytogenes*, *S. aureus*, *B. cereus*, *E. coli* bacteria are 11, 8, 9, and 8 (mg/ml), respectively. Catherine et al. (2012) reported the MIC values of EUG for *S. aureus*, *B. cereus*, *E. coli* bacteria are 0.20, 0.15, and 0.20 (%). These findings contrast with the results of the present study [4, 31]. There are no studies about the antimicrobial effect of the combinations of TQ+EUG. FICI is commonly used to test the antimicrobial ability of drugs in medicine or bioactive compounds in food microbiology [13]. In this study, the synergistic effects of this combination were seen in the all-prepared mixtures against food-borne pathogens, even at the lowest concentrations except for *B. cereus* (Table 3). As shown in Table 2, the combination of two phenolic compounds had synergistic effects against all the studied bacteria except *B. cereus* with a synergistic partial impact. Their highest synergistic effect was against *E. coli* with FIC=0.09, which indicates the lowest MICs than the MIC of each biomaterial. The results of the disc diffusion method are presented in Table 4. *B. cereus* and other Gram-positive bacteria had the highest GIZ at different concentrations of TQ and EUG. Among the Gram-negative strains, *E. coli* O157H7 and Shewanella were more sensitive than others. The chloramphenicol disc was used as a positive control. A dose-dependent reaction was seen for all studied bacteria in front of the TQ and EUG (Table 3). The TQ showed a better antimicrobial effect than the EUG. The highest and lowest GIZ of studied bacteria in front of the TQ was for *B.cereus* and *S.entritidis* bacteria, respectively, and EUG were related to *B.cereus* and *E.coli* bacteria, respectively. The antimicrobial effect of EUG has been reported in several research [4, 31]; however, at present, there are no antimicrobial reports for TQ based on the disc diffusion test. As expected, the resistance of Gram-negative bacteria to the tested phenolic compounds was more than Gram-positive bacteria due to their outer membrane structure [30]. It should be considered that the mechanisms of action by the aromatic phenolic compounds can be different against various strains of microorganisms based on their functional groups. It was

Table 2: Minimum inhibitory Concentration (MIC) and minimum bactericidal Concentration (MBC) of TQ and EUG (mg/ml)

		Shewanella SP	*P.aeruginosa*	*E.coli*	*B.cereus*	*E.Coli* O157:H7	*S.enterica*	*L.monocytogenes*	*S.aureus*
TQ	MIC	0.07	0.31	0.62	0.019	0.15	0.3	0.07	0.15
	MBC	0.31	1.25	1.25	0.07	0.62	0.62	0.31	0.62
EUG	MIC	0.6	2.5	5	0.6	1.2	2.5	0.6	1.2
	MBC	1.25	5	10	0.6	2.5	5	1.25	2.5

Table 3: Minimum Inhibitory Concentration of combination TQ+EUG (mg/ml)

	Compound	MIC in Combination(mg/m)	FIc	FICI	Result
E.coli	TQ	0.019	0.03	0.09	Synergic
	EUG	0.3	0.06		
P.aeruginosa	TQ	0.019	0.06	0.3	Synergic
	EUG	0.6	0.24		
Shewanella SP	TQ	0.009	0.12	0.37	Synergic
	EUG	0.15	0.25		
B.cereus	TQ	0.009	0.47	0.72	Partial Synergic
	EUG	0.15	0.25		
E.Coli O157:H7	TQ	0.009	0.06	0.31	Synergic
	EUG	0.3	0.25		
S.enteritidis	TQ	0.03	0.09	0.21	Synergic
	EUG	0.3	0.12		
L.monocytogenes	TQ	0.009	0.12	0.37	Synergic
	EUG	0.15	0.25		
S.aureus	TQ	0.009	0.06	0.31	Synergic
	EUG	0.3	0.25		

reported that the growth inhibition of pathogens by TQ is due to the generation of Reactive Oxygen Species (ROS) that results in oxidative stress in bacteria and cell death [29]. According to previous studies, EUG penetrates into the phospholipid bilayer and damages the structure and permeability of the bacterial membrane [30]. In combination with two antimicrobial agents against a bacterial population, the antimicrobial response will likely occur in one of the synergistic, additive, or antagonistic effects (decreasing effect) in comparison with the impact of each one of them [21]. It can be related to the various mechanisms of action of the phenolic compounds, which confirmed each other and targeted the organism at multiple sites [21, 32, 30]. There are no studies on the antimicrobial effects of the combined concentrations of EUG and TQ. Still, several reports about various antimicrobial effects of combining these biomaterials with other bioactive substances [10, 21, 32].

3.2 Antioxidant Activity

The antioxidant potential of the TQ and EUG was evaluated using DPPH and RP methods. Table 5 shows the results of DPPH Radical Scavenging Activity of both compounds compared to the same concentrations of BHT. Dose-response behavior was seen in both biomaterials. The IC_{50} values of TQ and EUG were 1.25 and 0.3 mg/ml, respectively. Obtained results show the higher radical scavenging ability of EUG than TQ. DPPH radical scavenging assay is used to evaluate the hydrogen donating ability of natural compounds as an antioxidant [33]. Hydrogen donating ability is directly related to the number of hydroxyl groups in the molecular structure of TQ and EUG (Figure 1) [34].

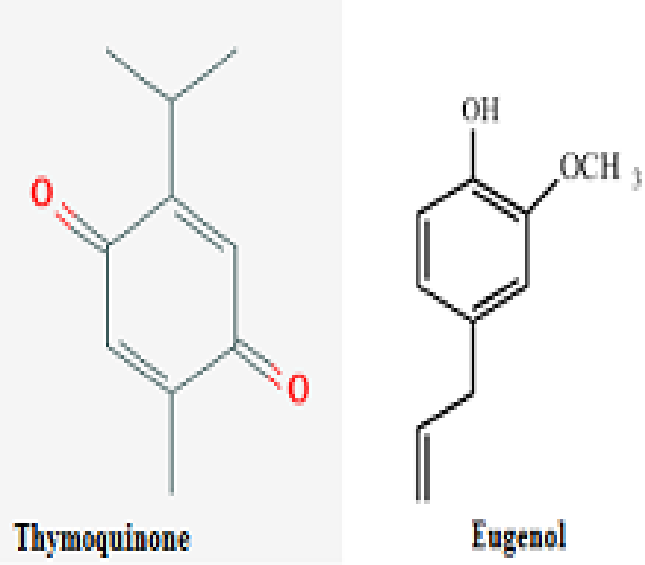

Figure 1: Molecular structure of TQ and EUG

Our results are consistent with the results of previous studies. Zhang et al. (2017) reported that DPPH scavenges activity of EUG is 22.1 ± 3.5 (μg/ml) [15]. The IC50 value of EUG was reported at (0.258±0.26 mg/ml) by Barhouchi et al. (2014) [35]. Khither et al. (2018) reported that the IC_{50} value of TQ is 125.65 ± 0.76 μg/ml [36]. Butt et al. (2019) reported that the IC_{50} for TQ in DPPH assay is 146.8μg/ml [37]. Dose-response results were seen for the RPs of TQ and EUG (Table 5). In this test, both compounds, particularly EUG showed remarkable and strong reducing ability than BHT at similar concentrations.

Table 4: Growth Inhibition Zone (mm) of TQ (mg/ml) and EUG (mg/ml) against Foodborne Pathogens Based on Disk-diffusion Method (Mean ± SD)

TQ	LM	SE	SH	SA	EC	PA	BC	EC O157H7
10	25.16 ± 0.76[a]	18.33 ± 0.57[a]	25.43 ± 0.4[a]	24.68 ± 0.38[a]	26 ± 1[a]	24.66 ± 1.15[a]	32.66 ± 1.52[a]	24.33 ± 0.57[a]
5	18.03 ± 0.45[b]	15.33 ± 0.57[b]	18.5 ± 1.32[b]	17.5 ± 0.5[b]	20 ± 1[b]	17.83 ± 0.76[b]	27.5 ± 0.5[b]	18.5 ± 0.5[b]
2.5	15.01 ± 0.22[c]	13.16 ± 0.28[c]	14.26 ± 0.46[c]	14.8 ± 0.26[c]	15 ± 1[c]	13.93± 0.11[c]	23 ± 1[c]	15.5 ± 0.5[c]
1.25	13.93 ± 0.75[d]	11.83 ± 0.28[d]	13.03 ± 0.55[c]	13.08 ± 0.14[d]	12.66 ± 0.57[d]	12.28 ± 0.3[d]	15.33 ± 0.57[d]	12.83 ± 0.28[d]
0.62	12.56 ± 0.49[e]	9.83 ± 0.28[e]	12.16 ± 0.28[d]	12.25 ± 0.25[d]	10.83 ± 0.76[e]	11.5 ± 0.5[e]	12.33 ± 0.57[e]	11.66 ± 0.57[e]
EUG								
20	15.6±0.17[a]	12.5±0.5[a]	14.5±0.5[a]	15.5±0.5[a]	11.5±0.5[a]	13.53±0.50[a]	18.16±0.76[a]	15.58±0.38[a]
10	13.41±0.52[b]	10.33±0.57[b]	12.43±0.40[b]	13.86±0.23[b]	9.75±0.66[b]	12.25±0.25[a]	15.5±0.5[b]	13.5±0.5[b]
5	10.58±0.38[c]	8.08±0.14[c]	9.16±0.28[c]	10.86±0.23[c]	8.2±0.34[b]	8.33±0.57[b]	12.66±0.57[c]	9.33±0.57[c]
2.5	8.33±0.57[d]	7.08±0.14[c]	7.16±0.28[d]	8.58±0.38[d]	0[c]	7.04±0.27[c]	9.08±0.14[d]	7.83±0.28[d]
Control	20 ± 1[e]	26 ± 1[e]	26.16 ± 0.76[e]	17.33 ± 0.57[e]	26.66 ± 1.15[d]	25.66 ± 0.57[f]	27 ± 1[f]	26 ± 1[e]

*Different small letters in each column show significant difference ($P \leq 0.05$).
**LM: *Listeria monocytogenes*, SE: *Salmonella entritidis*, SH: *Shewanella* SP, SA: *Staphylococcus aureus*, EC: *Escherichia coli*, PA: *Pseudomonas aeruginosa*, BC: *Bacillus cereus*, EC O157H7: *Escherichia coli* O157H7, C: Concentration

Table 5: DPPH radical-scavenging activity and Reducing power (RP) of TQ and EUG (Mean ± SD)

C (mg/ml)	10	5	2.5	1.25	0.6	0.3	0.15
				DPPH			
TQ	97.57±0.86	85.77±1.51	76.54±1.21	65.36±0.66	47.34±1.66	25.70±1.23	12.05±1
EUG	97.68±0.47	95.1±0.70	94.18±0.65	90.11±0.34	82.67±0.76	72.16±0.54	45.94±0.69
BHT	97.19±0.62	96.06±0.93	94.64±0.40	90.64±0.65	83.23±0.47	61.38±0.84	43.46±0.77
				RP			
TQ	1.61±0.01	1.53±0.03	1.40±0	1.31±0.62	1.17±0.01	0.82±0	0.61±0.01
EUG	2.09±0.1	1.81±0	1.72±0	1.63±0.02	1.51±0	1.30±0	1.06±0.04
BHT	1.24±0.04	1.16±0.02	1.11±0.01	1.03±0	0.96±0.02	0.80±0.03	0.72±0.01

Table 6: DPPH and Reducing power (RP) of the combination of TQ+ EUG (mg/ml)

Combination (mg/ml)	DPPH	CI	Effect based on CI	RP
TQ(5) + EUG(1.25)	96.64±0.73	1.09	Nearly additive	1.8±0.01
TQ(5) + EUG(0.6)	94.03±0.74	1.11	Slight antagonistic	1.62±0.02
TQ(5) + EUG(0.3)	93.37±0.45	1.19	Slight antagonistic	1.58±0.01
TQ(2.5) + EUG(1.25)	95.76±0.20	1.15	Slight antagonistic	1.78±0.01
TQ(2.5) + EUG(0.6)	91±0.71	1.14	Slight antagonistic	1.69±0.004
TQ(2.5) + EUG(0.3)	78.75±0.43	1.06	Nearly additive	1.51±0.005
TQ(1.25) + EUG(1.25)	92.39±0.56	1.21	Moderate antagonistic	1.71±0.01
TQ(1.25) + EUG(0.6)	85.76±0.32	1.17	Slight antagonistic	1.61±0.006
TQ(1.25) + EUG(0.3)	80.44±0.55	1.17	Slight antagonistic	1.42±0.007

*Data shows mean value of three independent repeats ± standard deviation
** CI: Combination Index

RPA evaluates reductive ability and the electron-donating capacity of an antioxidant. The reducing ability is generally associated with the presence of reductones, which have been shown to exhibit antioxidant action by breaking the chain reactions and donating a hydrogen atom. The transformation of Fe3+ evaluates it to Fe^{2+} in the presence of hydrogen donator molecules [23]. In this assay, the yellow color of the test solution changes to shades of green and blue depending on the RP of each compound [13]. Mahapatra and Roy (2014) reported that RP of EUG at (100µg/ml) and (80µg/ml) concentrations was 1.2 and 1.1, respectively, which is almost similar to the results of the present study [38]. A study conducted by Barhouchi et al. (2014) showed that RP of EUG, in the 1 and 0.8 mg/ml concentrations was 3.9 and 3.7, respectively. The results are not in line with the results of our study [35]. Gulcin (2011) reported that the RP of EUG in 15 µg/ml concentration was 1.180, which is consistent with our results [13]. In another study, Khither et al. (2018) revealed that the RP of TQ was 0.7 in 2000 µg/ml concentration, which is significantly less than our findings [36]. The results of DPPH radical scavenging activity and RP of the combined phase of TQ and EUG (mg/ml) in 9 concentrations are shown in Table 6. According to the obtained results, the DPPH scavenging activity of the combination of TQ+ EUG were antagonistic in all combined concentrations except for two concentrations TQ (5 mg/ml) + EUG (1.25 mg/ml) and TQ (2.5 mg/ml) + EUG (0.3 mg/ml) which showed additive effects. However, the combination of the two substances did not strongly affect their free form. The results for RP indicated a small increase in the measured values compared to their free form. Further, their additive effect on each other was marginal. Sometimes the antioxidant activity of an antioxidant can be markedly reduced in its mixture with other antioxidants. Several hypotheses interpret the antagonistic mechanism of antioxidants in complex mixtures: Reduction of weaker antioxidants by the stronger ones. Complex formation between antioxidants. Antioxidants polymerization reduces their antioxidant properties. The eventual disappearance of free radicals (No reaction between antioxidants and neutralized radicals). Unknown interactions between antioxidants [39]. The additive efficacy demonstrates the lack of interplay among the antioxidants in a combination mixture due to the independent action of each antioxidant and no interference with another antioxidant′s activity [39]. However, more research is needed to understand better the mechanisms involved in these interactions between TQ and EUG in vitro and as an anti-inflammatory agent or in food matrix to design new functional foods in the future.

4. Conclusion

Our results showed that TQ and EUG have good antimicrobial and antioxidant properties in free forms. The TQ had better antimicrobial effects than the EUG. It depends on the mechanisms of action of these biomaterials against microorganisms. The EUG showed better antioxidant activity than the TQ, depending on their molecular structure. The combination of TQ and EUG showed synergistic antimicrobial effects against studied food-borne pathogens. Based on the CI of DPPH scavenging capacity, the antioxidant activity of the combined phase of TQ+EUG showed antagonistic interactions except for two combinations illustrating additive interactions. It can be concluded that TQ and EUG in free or combination forms can be used as antimicrobial or antioxidant agent in drug delivery systems and for food preservation and enhancement of the food shelf life in the food industry as an ingredient of food or food packaging.

Authors' Contributions

Samira Yousefizadeh : methodology; investigation; resources; data curation; project administration. Majid Aminzare : writing–review; editing; supervision. Hassan Hassanzadazar : Conceptualization; methodology; Formal analysis; resources; data curation; writing–review; editing; visualization; supervision.

Conflicts of Interest

There are no competing interests to declare.

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