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•Research article•

Inhibition of *Escherichia coli* nitroreductase by the constituents in *Syzygium aromaticum*

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[ABSTRACT] Gut bacterial nitroreductases play an important role in reduction of various nitroaromatic compounds to the corresponding *N*-nitroso compounds, hydroxylamines or aromatic amines, most of which are carcinogenic and mutagenic agents. Inhibition of gut nitroreductases has been recognized as an attractive approach for reducing mutagen metabolites in the colon, so as to prevent colon diseases. In this study, the inhibitory effects of 55 herbal medicines against *Escherichia coli* (*E. coli*) nitroreductase (*Ec*NfsA) were examined. Compared with other herbal extracts, *Syzygium aromaticum* extract showed superior inhibitory potency toward *Ec*NfsA mediated nitrofurazone reduction. Then, the inhibitory effects of 22 major constituents in *Syzygium aromaticum* against *Ec*NfsA were evaluted. Compared with other tested natural compounds, ellagic acid, corilagin, betulinic acid, oleanic acid, ursolic acid, urolithin M5 and isorhamnetin were found with strong to moderate inhibitory effect against *Ec*NfsA, with IC_{50} values ranging from 0.67 to 28.98 mol·L^{-1}. Furthermore, the inhibition kinetic analysis and docking simulation demonstrated that ellagic acid and betulinic acid potently inhibited *Ec*NfsA (K_i < 2 μmol·L^{-1}) in a competitively inhibitory manner, which created strong interactions with the catalytic triad of *Ec*NfsA. In summary, our findings provide new scientific basis for explaining the anti-mutagenic activity of *Syzygium aromaticum*, where some newly identified *Ec*NfsA inhibitors can be used for developing novel agents to reduce the toxicity induced by bacterial nitroreductase.

[KEY WORDS] Gut bacterial nitroreductase; *Ec*NfsA inhibitor; *Syzygium aromaticum*; Anti-mutagenic activity
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Introduction

The gastrointestinal tract is a complex ecosystem that harbors the most numerous microbial community, consisting of approximately 10^{13} microbial cells [1, 2]. The gut bacteria can metabolize environmental chemicals with various enzymes and the enzymes are classified into five core enzyme families (azoreductases, nitroreductases, *β*-glucuronidases, sulfatases and *β*-lyases) [3-6]. Compared with other intestinal enzymes, nitroreductases are closely related with the production of carcinogenic and mutagenic metabolites in the colon, due to its reduction of nitroaromatic compounds to nitroso-, *N*-hydroxy-intermediates and aromatic amines through a NAD(P)H-dependent ping-pong bi-bi kinetic mechanism, which can interact with biomolecules such as DNA [5-9]. Researches revealed that nitroreductases from the intestinal microbial communities were the main contributors in stimulating the toxicity of exogenous nitroaromatic chemicals [10-13]. In addition, it has also been found that the activity of fecal nitroreductases from colon cancer patients was significantly higher than that from healthy subjects [8]. Thus, bacterial nitroreductases have become a potential molecular target for reducing the risk of carcinogenic and genetic toxicity [14-16], and developing nitroreductase inhibitors will be helpful to prevent pathological changes in the colon.

However, few nitroreductases inhibitors have been found due to the diversity in both substrates and catalytic activity [17]. As rich natural product resources, herbal medicines and edible plants have been considered good choices for drug candidates with a satisfactory safety profile during a long history

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of clinical practice [18-20]. In the current study, the inhibitory effects of 55 herbal extracts against bacterial nitroreductases were evaluated using *E. coli* nitroreductase (*Ec*NfsA) as a model enzyme, as it is considered the representative oxygen-insensitive nitroreductase [11, 12]. The results showed that *Syzygium aromaticum* extract potently inhibited *Ec*NfsA mediated nitrofurazone reduction. Next, the inhibitory effects of twenty-two main constituents in *Syzygium aromaticum* on *Ec*NfsA were tested. Seven compounds including ellagic acid, corilagin, betulinic acid, oleanic acid, ursolic acid, urolithin M5 and isorhamnetin showed strong to moderate inhibitory effects on *Ec*NfsA in a dose-dependent manner. Furthermore, the kinetic analysis demonstrated that ellagic acid and betulinic acid inhibited *Ec*NfsA induced-nitrofurazone reduction in the competitive mode with K_i values of 0.40 and 0.68 μmol·L^{-1}, respectively. The docking simulation confirmed that both ellagic acid and betulinic acid closely bound to the active sites of *Ec*NfsA through hydrogen bondings and Pi-alkyl interactions. In conclusion, the two newly identified potent *Ec*NfsA inhibitors from *Syzygium aromaticum* can be used as lead compounds for the development of anti-mutagenic medicines induced by bacterial nitroreductases.

Material and Methods

Chemicals and reagents

Fifty-five herbal products were provided by Tianjiang Pharmaceutical Co., Ltd. (Jiangsu, China). The major constituents collected from *Syzygium aromaticum*, including ellagic acid (**1**), corilagin (**2**), methyl eugenol (**3**), eugenol (**4**), protocatechuic acid (**5**), gallic acid (**6**), betulinic acid (**7**), oleanic acid (**8**), ursolic acid (**9**), isorhamnetin (**10**), quercetin (**11**), luteolin (**12**), kaempferol (**13**), quercetin-3-*O*-glucuronide (**14**), hyperoside (**15**), luteolin-7-*O*-*β*-D-glucuronide (**16**), luteoloside (**17**), quinic acid (**18**), malic acid (**19**), citric acid (**20**), maltopentaose (**21**) and urolithin M5 (**22**) (purities > 98%) were obtained from Chengdu Pufei De Biotech Co., Ltd. (Chengdu, China). The chemical structures of the twenty-two compounds from *Syzygium aromaticum* are showed in Fig. 1. Nitrofurazone (NFZ) and NADPH were purchased from MedChemExpress (USA). The stock solutions of NFZ and each compound (100 mmol·L^{-1}) were prepared in DMSO and stored at 4 °C until use. *E. coli* competent cell DH5*α*, NdeI/EcoRI enzyme and DNA Extraction Kit were purchased from Takara Co., Ltd. (Japan). *E. coli* competent cell BL21 (DE3), pET-28a (+) vector and Plasmid MiniPrep Kit were purchased from Transgen BioTech (Beijing, China). *E. coli* K12 was obtained from Shanghai Biology Collection Center (Shanghai, China). The Tris-HCl buffer (20 mmol·L^{-1}, pH 7.4) was prepared using Millipore water and stored at 4 °C for later use. DMSO was of LC grade (Tedia, USA).

Enzyme preparation

E. coli K12 genomic DNA was extracted and purified with the DNA Extraction Kit, and utilized as the template for amplifying *Ec*NfsA. Forward primer: 5′-CGCGCGGCAGC CATATGACGCCAACCATTGAACT-3′ (underline: NdeI); Reverse primer: 5′-RGACGGAGCTCGAATTCTTAGCG-CGTCGCCCAACCCTGTTTGTGCAAATAATCCAG-3′ (underline: EcoRI). After purified and cloned into pET-28a (+) vector, the recombinant plasmid was transformed into *E. coli* Competent Cell DH5*α*. Plasmids with positive clones were identified by sequencing pET-28a (+)-*Ec*NfsA and compared with the *Ec*NfsA sequence. Identified recombinant pET-28a (+)-*Ec*NfsA was transformed into *E. coli* BL21 (DE3) host strain through the method of heat shock. The transformants were inoculated into 100 mL LB liquid medium containing 50 μg kanamycin·mL^{-1} with shaking at 120 r·min^{-1}. When the OD_{600} reached 0.4−0.6, IPTG was added to 0.3 mmol·L^{-1} and the culture was further incubated at 30 °C and 120 r·min^{-1} for 5 h. The cells were harvested by centrifugation at 8000 r·min^{-1} at 4 °C for 10 min. The sediment was re-suspended in PBS (0.1 mol·L^{-1}, pH 6.8) and then sonicated in ice bath. The resultant suspension was centrifuged at 12 000 r·min^{-1} at 4 °C for 10 min, obtaining a crude enzyme solution. Then, the crude enzyme solution was loaded onto a Ni-NTA column equilibrated with PBS. *E. coli* nitroreductase (*Ec*NfsA) was eluted with 100 mmol·L^{-1} imidazole, and subsequently the salt removed by dialysis. According to previous studies [21], the desalted solution was freeze-dried and the concentration of the purified protein was measured. Both the crude protein extract and purified protein were analyzed by SDS-PAGE (Fig. S1). The K_m value of *Ec*NfsA mediated NFZ reduction was determined to be 32.35 ± 3.42 μmol·L^{-1}, while the V_{max} was 1.268 ± 0.035 pmol·min^{-1}·μg^{-1} protein (Fig. S2).

Enzyme inhibition assay

The inhibitory effects of herbal extracts or compounds against *E. coli* nitroreductase (*Ec*NfsA) were investigated using NFZ as a probe substrate. Briefly, the incubation mixture (with a total volume of 200 μL) contained 189 μL Tris-HCl buffer (20 mmol·L^{-1}), 2 μL NADPH solution (0.1 mmol·L^{-1}), 2 μL enzyme solution (2 μg·mL^{-1}), 2 μL sample solution (herbal extracts or natural compounds) or DMSO (a negative control), and 5 μL substrate (20 μmol·L^{-1}). After pre-incubation at 37 °C for 3 min, reduction reaction with or without the tested sample started by the addition of NFZ. Then, the absorbance was determined on a multi-mode microplate reader (BioTek Synergy H1, Hybrid Reader, Vermont, USA) at a wavelength of 400 nm. The residual activities of *Ec*NfsA were calculated using the following formula: the residual activity (%) = (the absorbance intensity in the presence of extracts or compounds)/the absorbance intensity in the negative control (DMSO alone) × 100%.

Inhibition kinetic analysis

The inhibition behaviors of both ellagic acid (**1**) and betulinic acid (**7**) against *E. coli* nitroreductase (*Ec*NfsA) were carefully investigated. The inhibition constant (K_i) values were measured using different concentrations of NFZ in the presence or absence of various concentrations of each in-

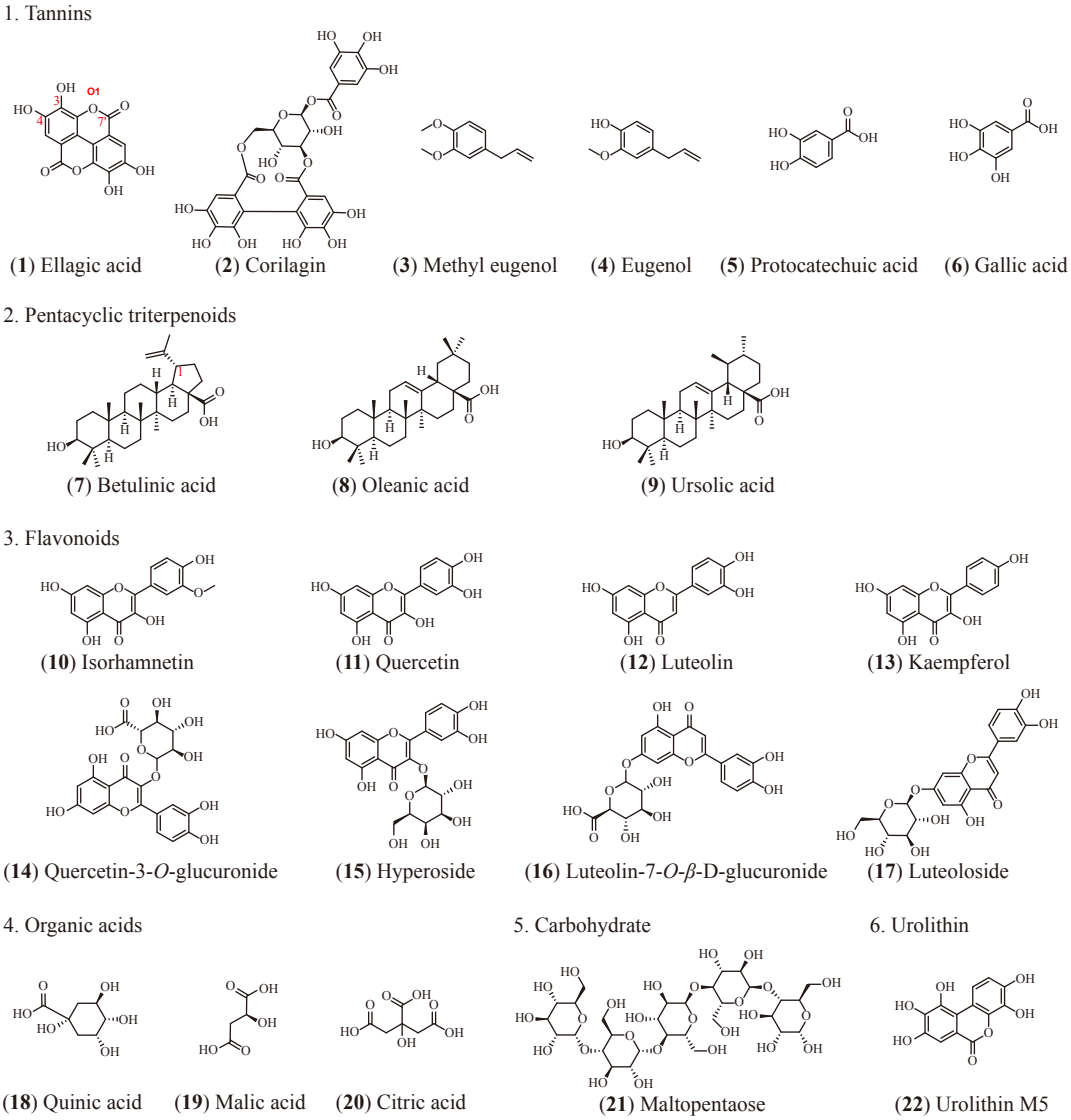

Fig. 1 Structures of major constituents from *Syzygium aromaticum*

hibitor. The K_i values of the inhibitors were calculated using the second plot of the Lineweaver-Burk plots. The calculation equations were previously showed [22]. Moreover, the inhibition kinetic types (competitive, non-competitive and mixed) of ellagic acid (**1**) and betulinic acid (**7**) were determined according to the previous calculation equations.

Molecular docking simulation

The 3D structures of ellagic acid (**1**) and betulinic acid (**7**) were drawn using ChemBioDraw Ultra 14.0 (CambridgeSoFt, Corporation, MA, USA) and energy minimization was performed. AutoDockTools version 4.2.6 (Scripps Research, La Jolla, CA, USA) was used for docking simulation of *Ec*NfsA docked with the inhibitor or the substrate (nitrofurazone). The crystallographic structure of *Ec*NfsA (PDB ID: 1F5V) was obtained from Protein Data Bank. The water of protein crystallization was removed, and hydrogen atoms and Kollman charges were added using AutoDock Tools. As the experimental results were competitive inhibition, an apposite cavity was selected to contain the active site of the protein during docking simulation, while protein molecular docking was performed for the inhibitors. According to Lamarck genetic algorithm, an optimal predicted conformation was obtained. The binding of inhibitors to the 3D models of protein receptors was visualized using PyMol software package (PyMol 2.3.2, Schrodinger, LLC, NY, USA).

Synergistic or antagonistic effects of ellagic acid and betulinic acid against EcNfsA

As previously described [23], the potential synergistic or antagonistic interactions of ellagic acid (**1**) and betulinic acid (**7**) against *Ec*NfsA-mediated NFZ reduction were explored, and the isobolograms were constructed using ellagic acid (**1**) and betulinic acid (**7**) under the concentrations of IC_{30}, IC_{50}, and IC_{70}, respectively. The combination index (CI), and the sum of the reduction index of the two inhibitors, were calculated using the following equation. (CI < 1: synergism, CI = 1: additive effect and CI > 1: antagonism).

$$\text{Combination index (CI)} = [(D)_1/(D_x)_1] + [(D)_2/(D_x)_2]$$

Statistical analysis

All inhibition assays were conducted in triplicate. The IC_{50} values and K_i values were evaluated according to nonlinear regression by GraphPad Software 8.0 (GraphPad Software, Inc., San Diego, CA, USA). Data were shown as mean ± SD.

Result

Inhibitory effects of herbal extracts on EcNfsA

First, the inhibitory potentials of fifty-five kinds of herbal extracts (at a final concentration of 100 μg·mL^{-1}) towards *Ec*NfsA were analyzed using nitrofurazone as the substrate [24]. As shown in Fig. 2A, the results showed that *Syzygium aromaticum* extract was superior to other extracts in inhibiting *Ec*NfsA-mediated reduction of nitrofuzone. Then, the dose-inhibition curve of *Syzygium aromaticum* extract towards *Ec*NfsA was plotted. As shown in Fig. 2C, the IC_{50} value for *Syzygium aromaticum* extract against *Ec*NfsA was evaluated to be 3.39 ± 0.44 μg·mL^{-1}. This finding demonstrated that the standard extract of *Syzygium aromaticum* strongly inhibited the reducing activity of *Ec*NfsA, and further investigation was suggested to explore the main constituents in *Syzygium aromaticum* with strong inhibitory effects against *Ec*NfsA.

Inhibitory effects of the major constituents in Syzygium aromaticum against EcNfsA

The inhibitory activity of twenty-two main constituents in *Syzygium aromaticum* were carefully examined at the concentration of 100 μmol·L^{-1}. As shown in Fig. 3A, the twenty-two compounds exhibited different inhibitory effects, in which seven compounds including ellagic acid (**1**), corilagin (**2**), betulinic acid (**7**), oleanic acid (**8**), ursolic acid (**9**), isorhamnetin (**10**) and urolithin M5 (**22**) showed strong inhibitory potency in *Ec*NfsA-mediated reduction of nitrofurazone *in vitro*. Next, the inhibitory potentials of the seven constituents against *Ec*NfsA were evaluated at three different concentrations (1, 10, and 100 μmol·L^{-1}). As shown in Fig. 3B, ellagic acid (**1**) and betulinic acid (**7**) efficiently blocked the activity of *Ec*NfsA at the concentration of 10 μmol·L^{-1}, with inhibition rates of 81.64% and 97.19%, respectively. Moreover, the dose-inhibition curves of these constituents were plotted. As shown in Figs. 3C−3I and Table 1, the IC_{50} values of ellagic acid (**1**), corilagin (**2**), betulinic acid (**7**), oleanic acid (**8**), ursolic acid (**9**), urolithin M5 (**22**) and isorhamnetin (**10**) were in the range of 0.67−28.98 μmol·L^{-1}, in which ellagic acid (**1**) and betulinic acid (**7**) were found to be the most effective inhibitors towards *Ec*NfsA with IC_{50} values of 0.67 ± 0.04 and 2.67 ± 0.08 μmol·L^{-1}, respectively.

Inhibitory mechanism of ellagic acid (1) and betulinic acid (7) against EcNfsA-meidated nitrofurazone reduction

The two potent inhibitors ellagic acid (**1**) and betulinic acid (**7**) were selected to further investigate the inhibition modes against *Ec*NfsA. The time-dependent inhibition curves revealed that both the compounds were time-independent inhibitors, implying that they were the reversible inhibitors of *Ec*NfsA (Fig. S4). Furthermore, the inhibition modes and

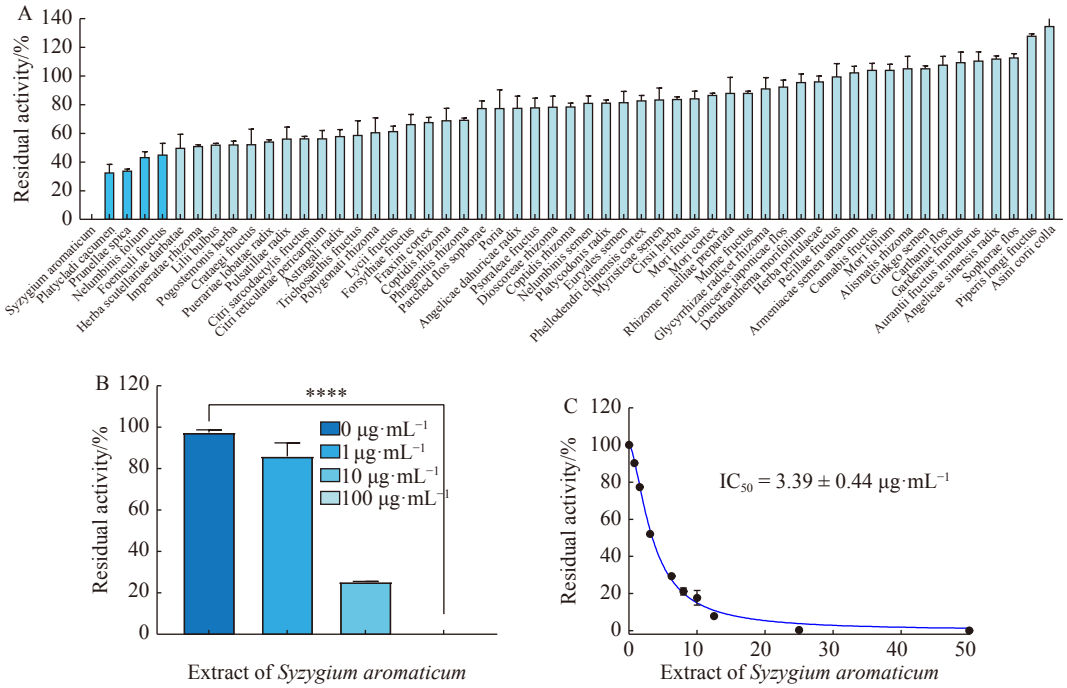

Fig. 2 Inhibitory effects of 55 herbal extracts on *Ec*NfsA at the concentration of 100 μg·mL^{-1} (A), the inhibitory effects of *Syzygium aromaticum* extract against *Ec*NfsA at three final concentrations (1, 10, and 100 μg·mL^{-1}) (B), and a dose-inhibition curve of the extract of *Syzygium aromaticum* on *Ec*NfsA (C). Data are expressed as mean ± SD (n = 3). $^{****}P < 0.0001$ *vs* the control group (without inhibitor)

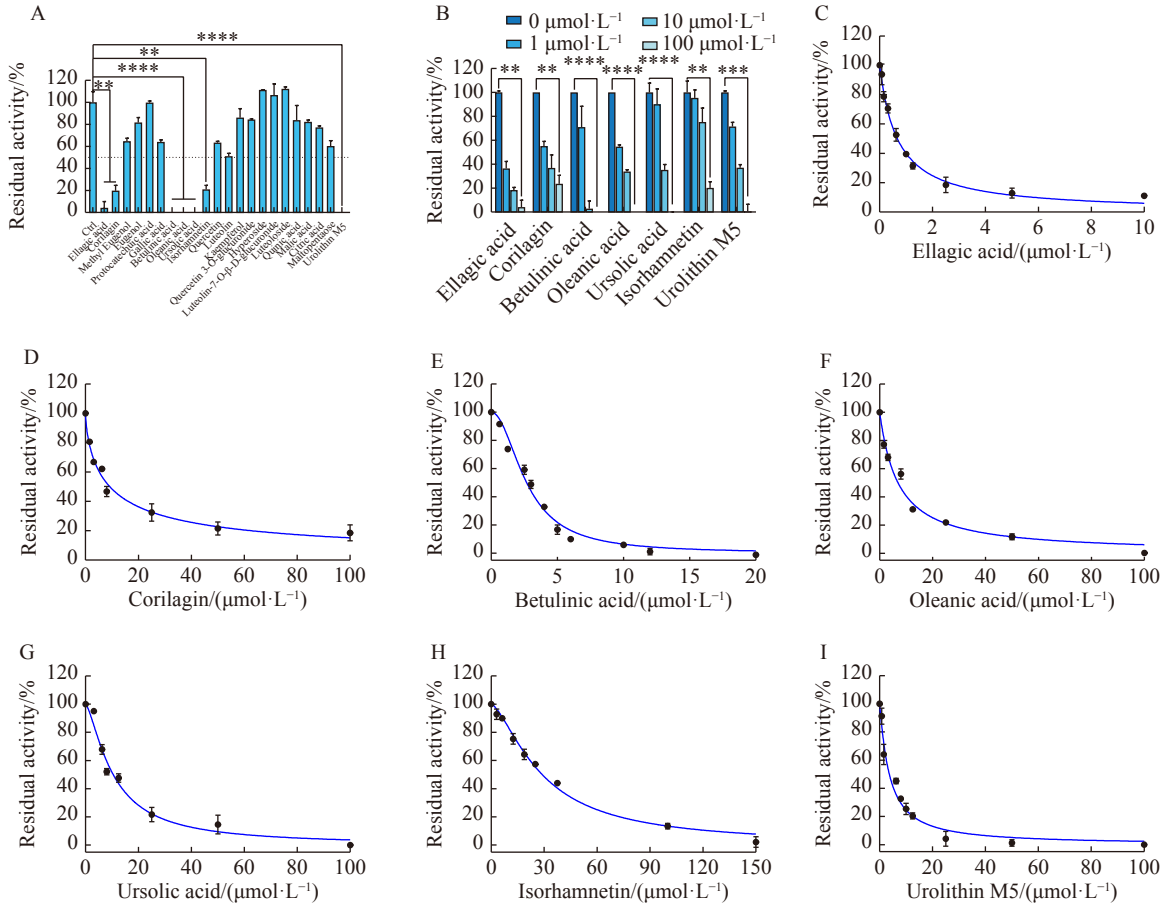

Fig. 3 Inhibitory effects of the major constituents in *Syzygium aromaticum* against *Ec*NfsA at the concentration of 100 μmol·L^{-1} (A). Inhibitory effects of the seven constituents on *Ec*NfsA at three final concentrations (1, 10, and 100 μmol·L^{-1}) (B). The dose-inhibition curves of the seven major constituents in *Syzygium aromaticum* (C−I) on *Ec*NfsA. Data were expressed as mean ± SD (n=3). $^{**}P$< 0.01, $^{***}P$ < 0.001 and $^{****}P$ < 0.0001 *vs* the control group (without inhibitor)

constants were determined according to the Lineweaver-Burk plots. As shown in Fig. 4 and Table 2, both ellagic acid (**1**) and betulinic acid (**7**) were competitive inhibitors with K_i values of 0.40 and 0.68 μmol·L^{-1}, respectively. All these results suggested that both ellagic acid (**1**) and betulinic acid (**7**) may block *Ec*NfsA activity through binding into the catalytic triad of the enzyme.

Molecular docking simulation

To deeply understand the inhibitory mechanisms of the newly identified inhibitors ellagic acid (**1**) and betulinic acid (**7**) against *Ec*NfsA-mediated reduction of nitrofurazone, molecular docking simulation was conducted using a crystal structure of *Ec*NfsA (PDB ID: 1F5V) as previously reported. The binding poses with the lowest binding energies were analyzed. As shown in Figs. 5A−5D and Fig. S5, NFZ and two natural *Ec*NfsA inhibitors (ellagic acid and betulinic acid) strongly interacted with amino acid residues around the catalytic cavity of *Ec*NfsA, which were overlapped with the binding area of nitrofurazone. These findings were in good agreement with the experimental data of competitive inhibition modes of ellagic acid (**1**) and betulinic acid (**7**) against *Ec*NfsA-mediated reduction of NFZ. As depicted in Figs. 5E and 5G, the phenolic groups at the C-3, C-4 sites of ellagic acid (**1**) created very strong hydrogen bonding interactions with Arg-225 (1.9 Å) and Ser-41 (2.4 Å, 3.0 Å), respectively, while the Arg-225 (2.5 Å) made strong hydrogen bonding with the oxygen atoms at the O-1 position. At the same time, the carbonyl group at the C-7′ formed hydrogen bonds with Asn-134 (1.8 Å). These results suggested that hydrogen bonding was the primary contributor to the binding of ellagic acid (**1**) on *Ec*NfsA. In addition, betulinic acid (**7**) interacted with *Ec*NfsA by producing hydrogen bonding with Ser-41 (2.4 Å) and Pi-Alkyl interaction with Tyr-199 and Tyr-200 (Figs. 5F and 5H). It was known that Ser-41, Asn-134 and Arg-225 were the active sites of *Ec*NfsA, which played a crucial role in substrate recognition and catalysis of nitrofurazone [25, 26]. Moreover, the substrate generated strong interactions with Ser-41 (2.7 Å, 2.7 Å) and Arg-225 (2.3 Å, 2.2 Å) of *Ec*NfsA *via* hydrogen bonds (Fig. 6). These results suggested that both ellagic acid (**1**) and betulinic acid (**7**) docked into the active residues with nitrofurazone on *Ec*NfsA, and then functioned as competitive inhibitors against *Ec*NfsA-mediated reduction of NFZ. All findings were consistent with the experimental results, in which ellagic acid (**1**) and betulin-

Table 1 Inhibitory effects (IC_{50} values) of the major constituents in *Syzygium aromaticum* against *Ec*NfsA (mean ± SD, *n* = 3)

Class	No.	Compounds	MW	IC_{50}/(μmol·L^{-1})
Tannins	**1**	Ellagic acid	302.19	0.67 ± 0.04****
	2	Corilagin	634.45	9.4 ± 0.83****
	3	Methyl eugenol	178.23	> 100
	4	Eugenol	164.20	> 100
	5	Protocatechuic acid	154.12	> 100
	6	Gallic acid	170.12	> 100
Pentacyclic triterpenoids	**7**	Betulinic acid	456.7	2.67 ± 0.08****
	8	Oleanic acid	456.70	6.95 ± 0.48****
	9	Ursolic acid	456.70	10.48 ± 0.59****
Flavonoids	**10**	Isorhamnetin	316.26	28.98 ± 1.01****
	11	Quercetin	302.24	> 100
	12	Luteolin	286.24	> 100
	13	Kaempferol	286.24	> 100
	14	Quercetin 3-*O*-glucuronide	478.36	> 100
	15	Hyperoside	464.38	> 100
	16	Luteolin-7-*O*-*β*-D-glucuronide	462.36	> 100
	17	Luteoloside	448.38	> 100
Organic acids	**18**	Quinic acid	192.17	> 100
	19	Malic acid	134.09	> 100
	20	Citric acid	192.12	> 100
Carbohydrate	**21**	Maltopentaose	828.72	> 100
Urolithin	**22**	Urolithin M5	276.2	3.57 ± 0.36****

$^{****}P < 0.0001$ *vs* the control group (without inhibitor)

ic acid (**7**) were first identified as potent competitive inhibitors against *Ec*NfsA-mediated reduction of NFZ.

Synergistic or antagonistic effects of ellagic acid and betulinic acid against EcNfsA

Meanwhile, the potential synergistic or antagonistic drug interactions were evaluated using ellagic acid (**1**) and betulinic acid (**7**), which were the most potent inhibitors in *Syzygium aromaticum*. As showed in Fig. 7, Fig. S6, Tables 3 and 4, all the points were located on the right side of each additive line, with the CI index > 1. These results clearly indicated that the combination of ellagic acid (**1**) and betulinic acid (**7**) exhibited little synergistic effect on *Ec*NfsA-mediated NFZ reduction, which also confirmed the competitive inhibition modes of these two inhibitors on *Ec*NfsA.

Discussion

Nitroaromatic compounds are always xenobiotic chemicals with carcinogenicity and genotoxicity, It has been found that the toxicity of these compounds is highly related with the products formed during reduction of the nitro groups by bacterial nitroreductases [27, 28]. The reduced products from nitroaromatics such as hydroxylamino or *N*-nitroso compounds can interact with DNA, causing toxic and mutagenic effects [15, 29, 30]. Thus, the exploration of nitroreductase inhibitors is an important alternative for relieving nitroaromatics toxicity.

Herbal medicines or edible plants containing natural phytochemical compounds are good choices for screening the inhibitors of gut bacterial enzymes, which are always active in the intestinal lumen rather than in the circulation [31]. With increasing studies on intestinal microbial communities, the regulatory effects of natural constituents on gut bacterial enzymes have attracted lots of attentions [32-34]. In this study, the inhibitory effects of fifty-five herbal extracts on *E. coli* nitroreductase (*Ec*NfsA) mediated nitrofurazone reduction were evaluated. Compared with other plant extracts investigated, *Syzygium aromaticum* extract (100 μg·mL^{-1}) exhibited the strongest inhibitory effect against *Ec*NfsA mediated nitrofurazone reduction (Fig. 2A). *Syzygium aromaticum* has a history of safe use as a Chinese crude drug, which produces a wide variety of bio-active compounds such as tannins, sesquiterpenes and triterpenoids [35-38]. A number of studies

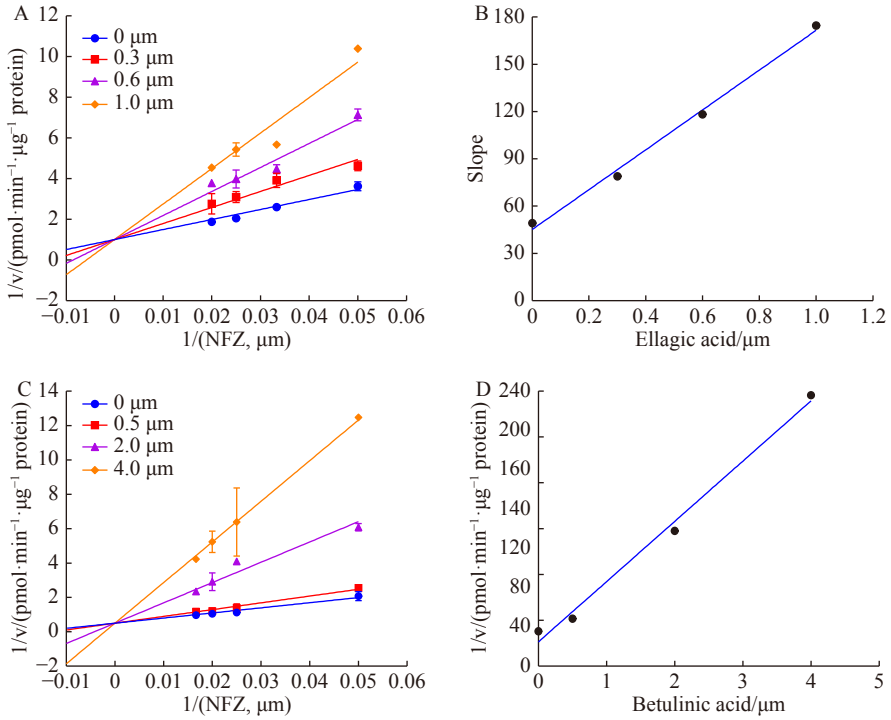

Fig. 4 The inhibitory kinetics of two strong *Ec*NfsA inhibitors in *Syzygium aromaticum*. Left: The Lineweaver-Burk plot of ellagic acid (A) and betulinic acid (C) against *Ec*NfsA-mediated reduction of nitrofurazone. Right: The secondary plot from the Lineweaver-Burk plot for *Ec*NfsA inhibition by ellagic acid (B) and betulinic acid (D). Data are expressed as mean ± SD (n = 3)

Table 2 Inhibition constant (K_i) and inhibition types of compounds against *Ec*NfsA-mediated reduction of nitrofurazone (mean ± SD, n = 3)

Compounds	IC_{50}/(μmol·L^{-1})	K_i/(μmol·L^{-1})	Inhibition mode	Goodness of fit (R^2)	S/(kcal·mol^{-1})
Ellagic acid	0.67 ± 0.04****	0.40	Competitive	0.96	−8.0
Betulinic acid	2.67 ± 0.08****	0.68	Competitive	0.98	−7.6

$^{****}P < 0.0001$ *vs* the control group (without inhibitor)

showed that *Syzygium aromaticum* possessed various pharmacological activity including sedative, antibacterial, anti-inflammatory and antioxidant effects [39-42]. Recently, it has been reported that *Syzygium aromaticum* suppressed mutagenic activity induced by nitroheterocyclic compounds in dietary supplements through inhibiting SOS response [43, 44]. As another important mechanism by which nitroheterocyclic compounds exerted mutagenic effects, gut bacterial nitroreductases reduced them into *N*-nitroso compounds or amines, so as to generate carcinogenicity and toxicity [45, 46]. Based on the primary screening results, *Syzygium aromaticum* extract was found to be the most effective in inhibiting *Ec*NsfA among all tested herbal extracts, which indicated the presence of active components which can provide a new explanation for the anti-mutagenic activity of *Syzygium aromaticum*.

Furthermore, the inhibitory effects of twenty-two main components in *Syzygium aromaticum* were systematically investigated. As shown in Figs. 3C−3I and Table 1, seven components including ellagic acid (**1**), corilagin (**2**), betulinic acid (**7**), oleanic acid (**8**), ursolic acid (**9**), urolithin M5 (**22**) and isorhamnetin (**10**) displayed strong to moderate inhibitory effects against *Ec*NfsA mediated nitrofurazone reduction with IC_{50} values ranging from 0.67 to 28.98 μmol·L^{-1}. It was worthy to note that the total content of the seven compounds were higher than 199.3 mg·g^{-1}, especially the content of the two potent inhibitors ellagic acid (**1**) and betulinic acid (**7**) which were determined as high as 175.7 and 16.9 ± 0.3 mg·g^{-1}, respectively [47, 48]. In addition, urolithin M5, one of the main metabolite of ellagic acid by the gut microbiota [49], also exhibited moderate inhibitory effect with an IC_{50} value of 3.57 ± 0.36 μmol·L^{-1}. All these results indicated that the excellent inhibitory effect of *Syzygium aromaticum* was probably produced by these major components, while ellagic acid (**1**) and betulinic acid (**7**) were the most potent inhibitors against *Ec*NfsA induced nitrofurazone reductions. As the major chemical constituents in *Syzygium aromaticum*, ellagic acid and betulinic acid exerted a variety of pharmacological activity including anti-mutation and anti-cancer effects, such as colorectal cancer, mouth cancer, breast cancer, bladder

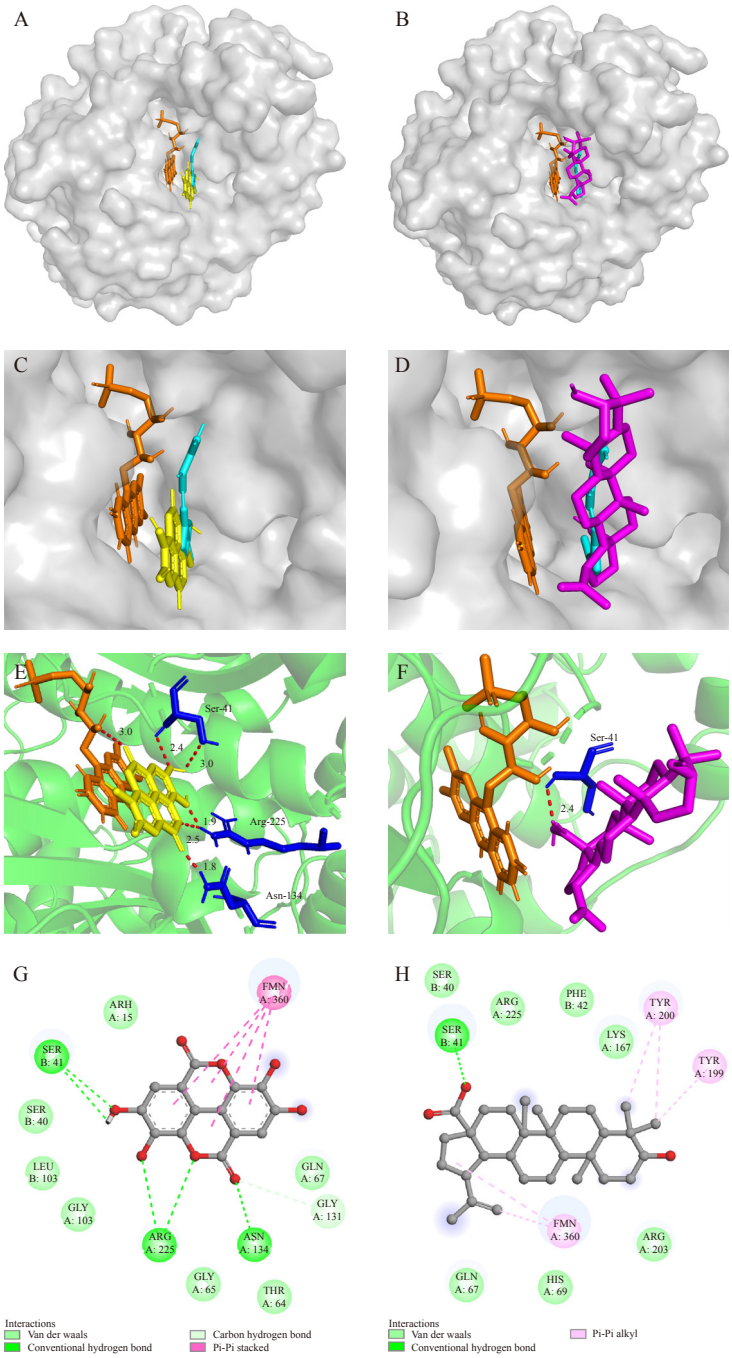

Fig. 5 The stereodiagram of ellagic acid (A) and betulinic acid (B) in *Syzygium aromaticum* combined with the crystal structure of *Ec*NfsA; A stereo view of the crystal structure of *Ec*NfsA (PDB ID: 1F5V) combined with ellagic acid (C) and betulinic acid (D). The detailed views of the binding areas between ellagic acid (E) and betulinic acid (F) with *Ec*NfsA; the 2D interactions between ellagic acid (G) and betulinic acid (H) with *Ec*NfsA, (yellow: ellagic acid, pink: betulinic acid, marine: nitrofurazone, orange: FMN, and blue: the residues of *Ec*NfsA)

cancer, bone cancer, liver cancer and lung cancer [50-54]. Ellagic acid induced apoptosis *via* metabolic enzymes and related cell pathways to exert its anti-cancer activity [55], while betulinic acid stimulated lipolysis in adipose tissue by inhibiting the activity of pancreatic lipase in a dose-dependent manner and accumulating lipid to achieve the anti-obesity effect [56]. Thus, the inhibitory mechanisms of both ellagic acid (**1**) and betulinic acid (**7**) toward *Ec*NfsA need to be clarified.

To explain the inhibition modes of ellagic acid and betulinic acid against *Ec*NfsA, the Lineweaver-Burk plots were constructed and the intersection points in the Lineweaver-Burk plots were calculated. As shown in Fig. 4 and Table 2, both ellagic acid (**1**) and betulinic acid (**7**) were

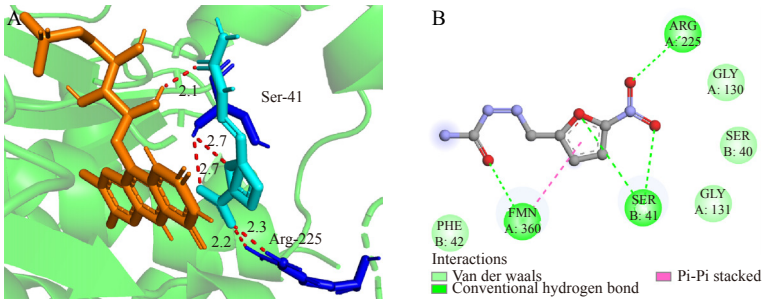

Fig. 6 The detailed views of the crystal structure of *Ec*NfsA (PDB ID: 1F5V) docked with the nitrofurazone (A), (marine: nitrofurazone, orange: FMN, blue: the residues of *Ec*NfsA). The 2D interactions between nitrofurazone and *Ec*NfsA (B)

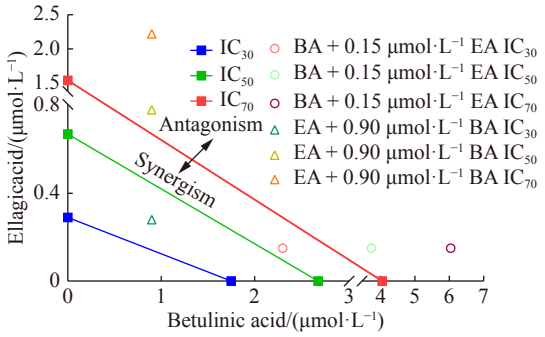

Fig. 7 Antagonism and synergism graphs of betulinic acid (BA) and ellagic acid (EA) against *Ec*NfsA-mediated reduction of nitrofurazone

Table 3 IC_{30}, IC_{50} and IC_{70} of ellagic acid (1) and betulinic acid (7) against *Ec*NfsA-mediated reduction of nitrofurazone

	IC_{30}	IC_{50}	IC_{70}
Ellagic acid/(μmol·L^{-1})	0.29	0.67	1.54
Betulinic acid/(μmol·L^{-1})	1.75	2.68	4.04

competitive inhibitors against *Ec*NfsA-mediated nitrofurazone reduction with K_i values of 0.40 and 0.68 μmol·L^{-1}. These results suggested that ellagic acid (**1**) and betulinic acid (**7**) can enter into the active pocket for reducting nitrofurazone catalyzed by *Ec*NfsA.

Finally, molecular docking simulation revealed that ellagic acid and betulinic acid bound on *Ec*NfsA at the catalytic sites of nitrofurazone. As showed in Figs. 5 and 6, Ser-41, FMN-360 and Arg-225 were the critical residues for *Ec*NfsA-mediated nitrofurazone reduction. Coincidentally, both ellagic acid (**1**) and betulinic acid (**7**) generated close interaction with the active residues. Ellagic acid closely bound to *Ec*NfsA *via* hydrogen bondings between residues Arg-225, Ser-41, Asn-134 and O-1 atom or hydroxyl groups on the C-4 and C-3 positions, respectively; while betulinic acid tightly interacted with *Ec*NfsA *via* hydrogen bondings between Ser-41 and hydroxyl group at the C-1 position. Moreover, the isobolographic plot and interaction index displayed that ellagic acid and betulinic acid exhibted little synergistic effects, which may be caused by the similar binding sites of the two inhibitors on *Ec*NfsA. Meanwhile, flavin mononucleotide was found as a potent competitive inhibitor towards *Ec*NfsA mediated NFZ reduction [57]. It bound three amino acids of *Ec*NsfA including Arg-225, Arg-133 and Glu-99 *via* hydrogen bondings to inhibit the reduction of NFZ, which was similar with the interactions of ellagic acid and betulinic acid. Compared with our findings, Arg-225 appeared to be the most important residue for the interactions between inhibitors and *Ec*NsfA. These findings were well consistent with in vitro experiments, which demonstrated the competitive modes of ellagic acid (**1**) and betulinic acid (**7**) against *Ec*NfsA-catalyzed nitrofurazone reduction. To our best knowledge, this is the first report on the evaluation and characterization of natural inhibitors of bacterial nitroreductase, which will be helpful for the interpretation of anti-mutagenic activity for *Syzygium aromaticum*.

It is important to mention that *Syzygium aromaticum* and its major constituents have regulatory effects on the composition of the gut bacteria. The extract of *Syzygium aromaticum* displayed antimicrobial action against *Escherichia coli*, *Clostridium perfringens*, and *Eimeria tenella* and prevented their adhesion in the gut broilers to protect gut villi [58]. In addition, urolithin M5, the main metabolite of ellagic acid in the human gut, effectively regulated the growth and composition

Table 4 Combination index (CI) of ellagic acid (1) and betulinic acid (7) against *Ec*NfsA-mediated reduction of nitrofurazone

Combination I (ellagic acid + betulinic acid 0.9 μmol·L^{-1})				Combination II (betulinic acid + ellagic acid 0.15 μmol·L^{-1})			
	Ellagic acid/(μmol·L^{-1})	Betulinic acid/(μmol·L^{-1})	CI		Ellagic acid/(μmol·L^{-1})	Betulinic acid/(μmol·L^{-1})	CI
IC_{30}	0.28	0.9	1.48	IC_{30}	0.15	2.3	1.73
IC_{50}	0.78	0.9	1.50	IC_{50}	0.15	3.73	1.61
IC_{70}	2.22	0.9	1.66	IC_{70}	0.15	6.04	1.58

of the gut microbiota [59]. In this study, it was found that the extract of *Syzygium aromaticum* and seven constituents inhibited the activity of *Ec*NfsA, which also verified that *Syzygium aromaticum* and its major chemical constituents can improve gastrointestinal health through maintaining the balance of the microbiota and regulating the activity of bacterial enzymes, so as to reduce intestinal toxicity and regulate human gut microbiota without inducing significant side-effects on host body.

In the future, more experiments should be developed to verify the binding sites for deeply understanding the inhibitory mechanisms. Detailed computational simulations (RMSD) will also be carried out to predict the dynamic deviations after the inhibitors are docked into the ligand-enzyme complex [24]. Furthermore, site-directed mutagenesis of amino acids residues will be performed to compare the variation of catalytic activity in reducting nitroaromatic compounds [60, 61]. The crystallization of ligand-enzyme complex should be obtained to identify the docking sites of inhibitor molecules into the enzyme [62]. Structure-activity relationship is also needed to be determined, so as to demonstrate the effects of substituted groups on the inhibitory activity.

In conclusion, this study investigated the inhibitory effect and mechanism of action of the major constituents in *Syzygium aromaticum* on gut bacterial nitroreductase (*Ec*NfsA), a key target to reduce nitroaromatics toxicity. The results clearly showed that seven active components in *Syzygium aromaticum* including ellagic acid (**1**), corilagin (**2**), betulinic acid (**7**), oleanic acid (**8**), ursolic acid (**9**), urolithin M5 (**22**) and isorhamnetin (**10**) displayed moderate to strong inhibitory effects towards *Ec*NfsA with IC_{50} values ranging from 0.67 to 28.98 μmol·L^{-1}. The inhibition kinetic analysis indicated that two potent inhibitors ellagic acid and betulinic acid were the competitive inhibitors against *Ec*NfsA-mediated nitrofurazone reduction, with K_i values less than 1.0 μmol·L^{-1}. Furthermore, molecular docking simulation confirmed that both ellagic acid and betulinic acid tightly bound on the Ser-41 and Arg-225 of *Ec*NfsA *via* hydrogen bondings, which were the essential residues for substrate reduction. Overall, these findings not only identified new natural *Ec*NfsA inhibitors for the treatment of nitroaromatics induced toxicity, but also provided new powerful evidence for explaining the anti-mutagenic activity of *Syzygium aromaticum*.

Supporting Information

Supporting information of this paper can be requested by sending E-mail to the corresponding author.

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