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Antimicrobial effect by extracts of rhizome of *Alpinia officinarum* Hance may relate to its inhibition of β-ketoacyl-ACP reductase

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Abstract

Inhibitory effects on bacterial growth showed that 40% ethanol extract of galangal (rhizome of *Alpinia officinarum* Hance) can inhibit *Staphylococcus aureus, α-Hemolytic streptococcus*, *β-Hemolytic streptococcus* and *Streptococcus pneumoniae*. β-ketoacyl-ACP reductase (FabG, EC.1.1.1.100) is a key enzyme in type II fatty acid synthase system in bacteria and catalyzes β-ketoacyl-ACP reduction. The galangal extracts inhibited FabG with an IC_{50} value of only 4.47 ± 0.10 μg/mL and is more potent than other previously published inhibitors. Kinetics studies showed that the inhibition consisted of both reversible and irreversible inhibition. The extracts of galangal inhibit FabG in a competitive pattern against NADPH. So far, no inhibitor has been reported to exhibit irreversible inhibition of FabG, whereas the galangal ethanol extract can inhibit FabG irreversibly. The irreversible inhibition presented two phases. It is probable that the galangal extract inhibit FabG, thereby displaying antibacterial ability.

Keywords: *Galangal, β-ketoacyl-ACP reductase (FabG), antibacterial, inhibition, kinetic*

Introduction

The emergence of multidrug resistance in pathogenic bacteria is a global problem that calls for the development of new antibiotics with unique cellular targets [1]. Bacterial fatty acid synthase (FAS II) is consisted of seven individual enzymes, each encoded by a discrete gene [2,3] in contrast of mammalian fatty acid synthase (FAS I, EC 2.3.1.85), which is a homodimer of single multifunctional polypeptide derived from a single gene [4]. The difference between the bacterial and mammalian fatty acid synthase made fatty acid synthease becoming a potential target for antibacterial drug discovery [5,6]. β-ketoacyl-ACP reductase (FabG, EC 1.1.1.100) is the only enzyme without any isoenzymes in the FAS II synthesis pathway, moreover *fabG* gene exists in all bacterial genomes which had been studied, and the predicted protein sequences are all highly homologous. In addition, there are also evidences demonstrating that *fabG* gene is necessary and vital to bacteria survival. [7] Therefore, FabG appears to be a perfect drug target for antibacterial development [5], and FabG inhibitors could be exploited into antibacterial drugs. However, the shortage of efficient and selective FabG inhibitors made the work hard to process for a time.

Rhizome of *Alpinia officinarum* Hance (galangal) has been widely used as a condiment for foods in Thailand (galangal, Yang), and as a traditional herbal medicine for curing various ailments in Asia for centuries [8]. Galangal extract (GE) at higher concentrations of 0.05% and 0.10% (wt/wt) were found to extend the shelf-life of minced beef [9]. Acute (24 hours) and chronic (90 days) oral toxicity studies on the ethanolic extracts of the rhizomes of Alpinia galangal had been carried out in mice and no significant mortality was observed compared with the control [10]. Thus, galangal is considered to be innocuous. It has been found that 100% ethanol extracts can ruptured the outer membrane of *Staphylococcus aureus* and induced the loss of cell permeability properties [11], while the mechanism

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have not been interpreted. In this paper, we demonstrated that 40% ethanol extracts of galangal (GE) can inhibit some bacteria proliferation, and it also displayed strong inhibition to FabG. For the sake of explaining the manner of GE action with FabG, we also assayed some kinetic parameters.

Materials and method

Materials

Ethyl acetoacetate, NADPH, galangin, quercetin and kaempferol were obtained from Sigma. All other reagents were local products of analytical grade. Galangal was purchased from Beijing Tong Ren Tang Corp. Ltd. Galangin, quercetin and kaempferol were used as solutions in DMSO (dimethyl sulfoxide).

Preparation of GE

1g galangal dry herbs (Beijing Tong Ren Tang Corp.) were smashed, soaked in 20mL ethanol for 2hr with magnetic stirring, centrifuged at 4000 × g for 15 min. The supernatant was stored at 4°C and subsequently used in all the experiments described in this paper. We treated galangal with five different percent ethanol solutions, double-dilute water, 20%, 40%, 70% and 100% ethanol. Then we assayed inhibition ability of those extracts to the FabG. Before we determined the minimal inhibitory concentrations (MICs), the GE sample was sterilized by filtration using 0.2 μm Acrodisc Syringe Filters (PALL).

Preparation of FabG

The recombinant *E. coli* strain over-expressed FabG was kindly provided by Dr. Charles Rock (St. Jude Children Hospital, USA.). FabG was expressed and purified as previously described [12–14]. Briefly, His-tagged FabG were expressed in *E. coli* strain BL21 (DE3) and purified by nickel chelation affinity chromatography. The protein purity and quantity were determined by SDS-PAGE and Bradford Assay respectively. Purified protein was stored in 50% glycerol flash-frozen at − 80°C.

Assay of FabG activity

The FabG assay reaction mixture contained 200 mM ethyl acetoacetate (Sigma, dissolved in 50% methanol), 35 μM NADPH (Sigma) and 10–20 μg FabG within a total volume of 2.0 mL reaction buffer (100 mM phosphate buffer, pH 7.0). The reaction was initiated by the adding of enzyme, and the activity was determined using an Amersham Pharmacia Ultrospec 4300 pro UV-Vis spectrophotometer at 37°C by following the increase of absorption at 340 nm due to NADPH oxidation.

Assay of fast-binding reversible inhibition activity and slow-binding inactivation

Fast-binding inhibition was determined by adding the inhibitors to the reaction system before the reaction was initiated by the addition of FabG. FabG's activities in the presence and absence of inhibitor were assigned as A_i and A_0 respectively. A_i/A_0 was defined as the relative activity (R.A.). GE was diluted with reaction buffer if necessary. The final concentration of ethanol in the reaction mixture did not exceed 0.4% (V/V) and had no effect on the FabG activity (data not shown). In our experiments the largest volume added to the reaction was far less than 0.4% (V/V), thus the ethanol existing in the GE had no influence to the results of activity assay. The concentration of inhibitor for 50% inhibition (IC_{50}) was obtained from a plot of R.A. versus inhibitor concentration. The less the IC_{50} value was, the stronger inhibitory activity the inhibitor had.

Competitive parameter assay: FabG activity was studied in the presence of increasing GE concentrations and NADPH as the variable substrate, keeping concentration of other substrates invariable. A Lineweaver-Burk double-reciprocal plot was constructed for 1/ [NADPH] versus 1/reaction velocity to estimate the relation between inhibitor and variable substrate NADPH. For the substrate ethyl acetoacetate, the same procedure as above was adopted but just change concentration of GE and ethyl acetoacetate.

Slow-binding inhibition: after the enzyme solutions were mixed with the inhibitor, the aliquots were taken to measure the relative activity at the indicated time intervals to determine the slow-binding inactivation. The degree of slow-binding inhibition is dependent on GE and FabG concentration. However, the value of $k_{obs}/[I]$ is independent of GE and FabG concentration.

Bacterial strains

Staphylococcus aureus ATCC25923, *Pseudomonas aeruginosa* ATCC 27853, *α-Haemolytics Streptococcus* 32213-7, and *β-Haemolytics Streptococcus* 32210-20 were purchased from National Center for Medical Culture Collection in Beijing, P.R.China. *Streptococcus pneumoniae* was separated and identified by Chinese Navy General Hospital, Beijing, P. R. China.

Minimum inhibitory concentration (MIC) determination

The MICs were determined using the broth macrodilution method according to NCCLS [15]. *Streptococcus pneumoniae* was cultured in 50mL Mueller-Hinuton broth medium (MH medium), other strains were cultured in MH medium contained 2–5% aseptic defibrinated sheep blood, incubated in a shaker incubator at 35°C for 18–24 h. Then those bacteria were transferred to MH agar plates or MH agar plates containing 5% sheep blood that were

incubated at 35°C overnight. We picked several colonies, transferred to 3-5mL sterile sodium chloride solution, and adjusted the optical density (OD) of the bacterial solution to the 0.5 McFarland standard. Working suspension was made by 1:10 dilution of the adjusted suspension with MH medium the concentration of the cell number of approximately 10^7 CFU/mL.

The galangal ethanol extract was diluted by sterile MH medium to 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024, 1:2048, 1:4096, the final dilution ranged from 0.312 μg/mL to 1280 μg/mL. Those different concentration galangal ethanol extracts were added to tube 2 mL/tube respectively, then 100μl bacterial solution prepared was added (the final concentration to 5×10^5 CFU/mL) and incubated without shaking at 35°C for 18–24 h. The MIC of the GE was regarded as the lowest concentration of extracts that were not permitted for any turbidity of the tested microorganism.

Positive control was monitored by using *Streptococcus aureus* (ATCC 25923) and Ciprofloxacin. *Streptococcus aureus* and Ciprofloxacin were treated as mentioned above, but the final concentration of Ciprofloxacin ranged from 0.0312 μg/mL to 128 μg/mL. The MIC value of test group was not credible, unless the MIC value of Ciprofloxacin was among the standard range. Negative control was only bacteria without sample. Those controls make sure the results of test reliable.

Results

Antimicrobial effect of GE

The MIC values of the GE tested for antibacterial activity are given in Table I. Positive control showed the MIC value of Ciprofloxacin to *Streptococcus aureus* is 0.25 μg/mL to 0.125 μg/mL and negative control manifested bacteria growth normal mean that the assay system was effective. Lower MIC values mean stronger inhibition effect. As table shown, GE exhibited stronger inhibition to *Streptococcus pneumoniae* and *α- haemolytics Streptococcus* 32213-7, whose MIC values are around 10 ~ 20 μg/mL and 25 ~ 50 μg/mL respectively. However, the MIC values to *β-haemolytics Streptococcus* 32210-20 and *Staphylococcus aureus* ATCC25923 are higher and the inhibition to *P. aeruginosa* ATCC 27853 is the weakest with MIC value more than 1280 μg/mL. For the inhibition of GE to *P.aeruginosa* was so weak that the MIC value exceeded the maximum concentration chose.

Inhibition of different concentration ethanol extraction of galangal

We compared the IC_{50} values of the different concentration ethanol extractions, found that 40% ethanol GE had the strongest inhibition to FabG as shown in Figure 1. So we choose 40% ethanol galangal extraction as sample.

Reversible inhibition of FabG by GE

GE showed potent reversible inhibition on FabG as Figure 2 showing, the IC_{50} value of GE obtained from three parallel experiments was 4.47 ± 0.10 μg/mL (1 mL 40% GE ethanol extraction's dried weight was 13.3 ± 0.4 mg). We studied several kinetic parameters of GE to identify its possible reaction site on FabG.

Competitive parameters always are used to describe the mechanism of inhibitors to enzyme. k_{is} represents the dissociation constant of enzyme-inhibitor complex, was calculated from the secondary plot of slopes versus GE concentration. And k_{ii} means the dissociation of enzyme-inhibitor-substrate complex, was calculated from the secondary plot of intersections versus GE concentration.

$$E + I \overset{k_{is}}{\longleftrightarrow} EI, \quad ES + I \overset{k_{ii}}{\longleftrightarrow} EIS$$

The Lineweaver-Burk double-reciprocal plot (Figure 3A) showed when changing the concentration of NADPH and inhibitor, the plots intersect on Y axis, which illustrated that GE was a competitive inhibitor to NADPH (k_{is} value is 3.64 μg/mL). In another words GE and NADPH might compete for the same binding site of FabG, GE combined to the FabG forming enzyme-inhibitor thus impeding NADPH binding to the enzyme. In addition, the kinetic results (Figure 3B) showed that when ethyl acetoacetate as variable substrate, the plots intersect in second quadrant, which represented that GE inhibited FabG in a mixed competitive and noncompetitive manner with substrate ethyl acetoaceteat (k_{is} value of 3.99 μg/mL, k_{ii} value of 13.73 μg/mL). On one hand GE compete with the binding site of ethyl acetoacetate, and on the other hand it also combined to FabG after enzyme-substrate complex formation.

Table I. Inhibitory effects of GE (μg/mL) on bacterial growth.

Bacterial strains	*Pseudomonas aeruginosa* ATCC 27853	*Staphylococcus aureus* ATCC25923	*Streptococcus pneumoniae*	*α- haemolytics Streptococcus* 32213-7	*β- haemolytics Streptococcus*32210-20
MIC	>1280	320 ~ 640	10 ~ 20	25 ~ 50	200 ~ 400

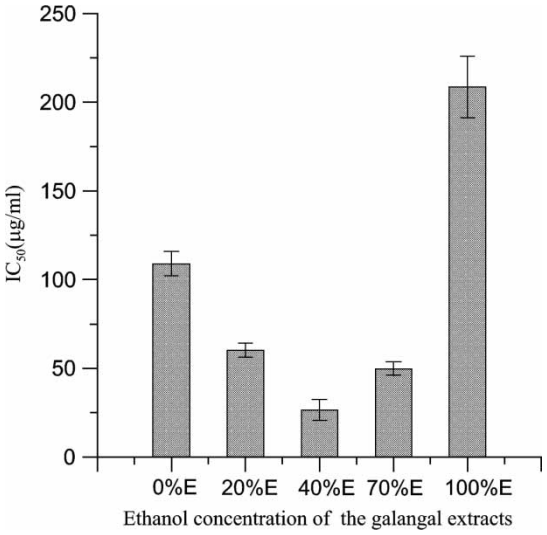

Figure 1. Inhibition of different GE extracts to FabG. Galangal was extracted with 0% ethanol, 20% ethanol, 40% ethanol, 70% ethanol and 100% ethanol respectively and then the five galangal extractions' inhibition effects to FabG were assayed and the IC_{50} values (μg dried leaves weight/mL) were determined.

Slow-binding inactivation of FabG by GE

The FabG solutions were mixed with GE and aliquots were taken to measure the relative activity of the reaction, at pre-determined time intervals. GE displayed a time-dependent inactivation on FabG. The semi-logarithmic plot of Figure 4A showed that the time course of the FabG reaction with the presence of GE of 3.99 μg dried weight/mL in the enzyme-inhibitor mixture. In the first process that lasted a period of 12 seconds the plot was initially sharp concave to approximate 30% loss of activity, which suggested that the reversible inhibition mostly accounts for the loss of FAS activity in the first process. It can be seen from Figure 4 that the slow-binding inactivation followed including two phases: a fast phase and a slow phase. As showing in Figure 4B, the apparent first-order rate constant k_{obs} of 0.109 min^{-1} for the fast phase (the contribution of slow phase was subtracted); and k_{obs} of 0.005 min^{-1} for the slow phase were calculated from the plot.

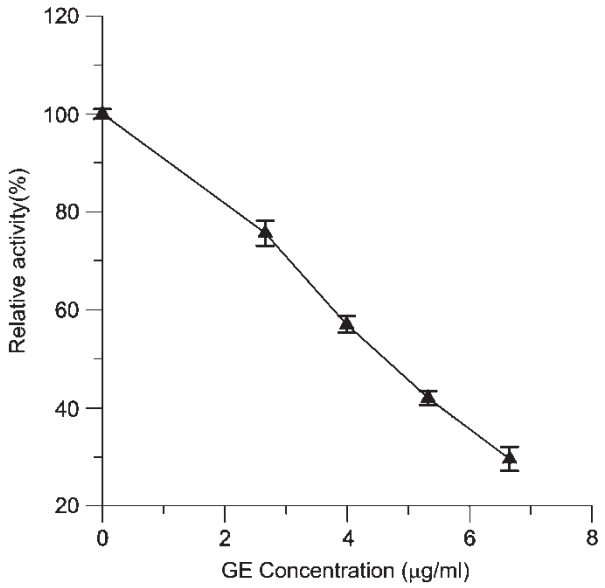

Figure 2. Inhibitory activities of GE on FabG.

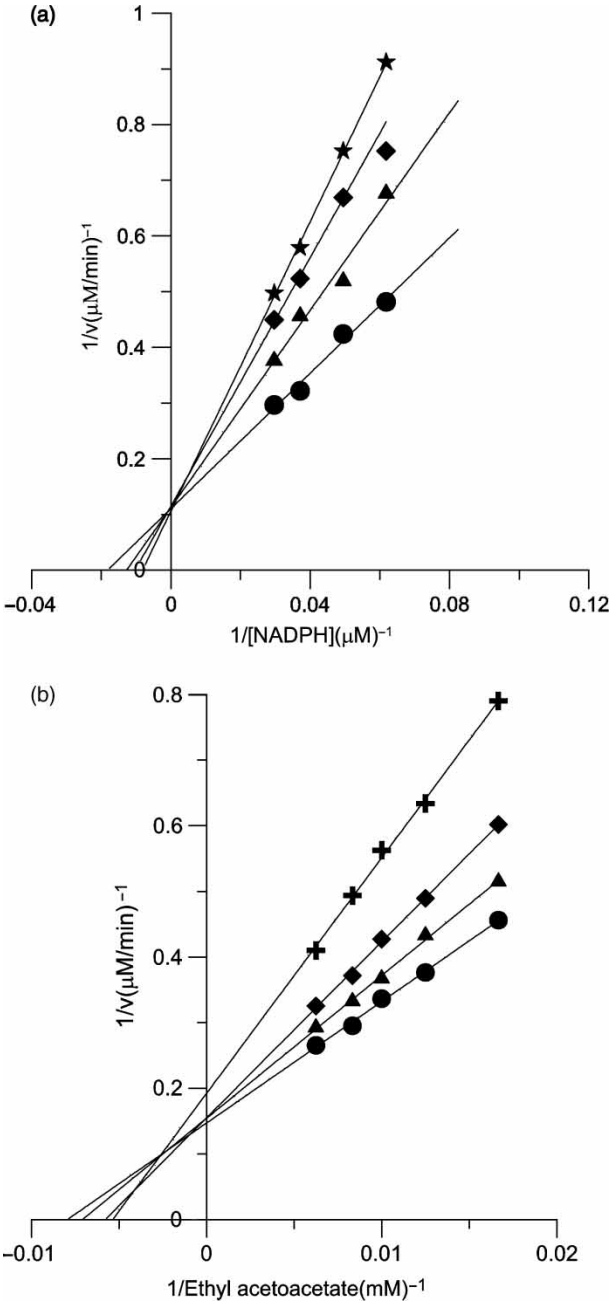

Figure 3. Lineweaver-Burk double-reciprocal plot for inhibition of FabG by GE. (A). NADPH is the variable substrate, and the concentration of ethyl acetoacetate is fixed at 200 mM. The concentrations of GE used were 0 μg/mL (●), 1.33 μg/mL (▲), 2.66 μg/mL (◆), 3.99 μg/mL (★) respectively. (B). Ethyl acetoacetate is the variable substrate, and the concentration of NADPH was fixed at 35 μM. The concentrations of GE used were 0 μg/mL (●), 1.33 μg/mL (▲), 2.66 μg/mL (◆), 3.99 μg/mL (★) respectively.

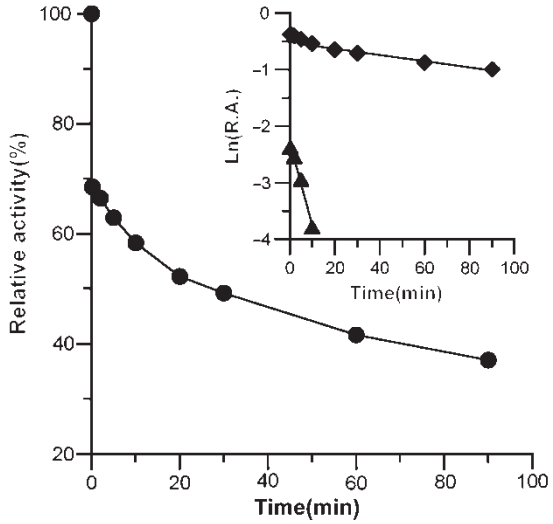

Figure 4. Time-dependent inhibition of FabG in presence of GE. FabG activity was measured at the indicated time intervals after the FabG solution (8 nM) was mixed with GE (2.86% (V/V), equivalent to 3.99 μg dried GE/mL) (●). A semi-logarithmic plot of the experimental data (INSET) shows two phases: the fast phase (▲) and the slow phase (◆). The linear slopes of the plot can be obtained as the apparent first-order rate constants.

Furthermore, The k_{obs} /[I] value of fast-phase is 0.42 min^{-1} mg^{-1} mL; the k_{obs} /[I] value of slow-phase is 0.02 min^{-1} mg^{-1} mL. The concentration of GE in reaction mixture was 3.99 μg/mL; under this concentration, ethanol did not exceed 1% (V/V) in the reaction mixture, and thus ethanol did not affect the FabG activity.

Some flavonoids existing in galangal have inhibition to FabG

We assayed some flavonoids that were abundant in galangal such as galangin, quercetin and kaempferol. As shown in Table II, quercetin has the strongest inhibition, and the effects of the kaempferol and ganlangin are weak.

Discussion

Antimicrobial effect of GE

Galangal is known to contain various antimicrobial agents and have inhibition to some gram-positive bacteria [11]. The basic steps of the cyclical pathway of the fatty acids synthesis are unanimous in almost all bacteria, and the genes encoding these enzymes can generally be identified by homology to the corresponding *E. coli* protein [5]. Due to fatty acids are component of cell membrane lipids, they are vital to the proliferation and survival of bacteria, then prohibiting the pathway of fatty acid synthesis is one mechanism for development new antibiotics. There have been reported some antibiotics which are inhibitors of bacteria fatty acid synthase system such as isoniazid a front line treatment for tuberculosis [16] through inhibiting enoyl-ACP redutase; triclosan inhibiting lipid synthesis in *E. coli*, with the probable target enoyl-ACP reductase [17]; platensimycin a newly reported antibiotics separated from *S. platensis* by Jun Wang etc. through the selective targeting of β-ketoacyl-ACP synthase I/II [18] having potential to becoming novel antibacterial drug. We assayed different concentration ethanol galangal extracts and found that 40% ethanol GE has the strongest inhibition to FabG. What's more, those abstrcts also exhibit antibacterial activity, even with the lower MIC value to *Streptococcus aureus* compared to the 100% GE [11]. It has been reported that 100% GE can inhibit *Streptococcus aureus* through destroying bacterial cell membrane [11]. From the results, we speculate that 40% GE may like 100% GE rupturing bacteria cell membrane, and they may both by the means of inhibiting FabG thereby inhibiting lipids of bacterial cell membrane synthesis.GE have antimicrobial activities against *Streptococcus aureus* ATCC 25923, *P. aeruginosa* ATCC 27853, *α-haemolytics, Streptococcus* 32213-7, *β-haemolytics, Streptococcus* 32210-20, and *Streptococcus pneumoniae*, especially have strongest inhibition effect to *Streptococcus pneumoniae* with the lowest MIC value. We suppose that one reason why GE prohibiting bacteria growth may be that GE can inhibit the activity of FabG. Additionally, *Streptococcus pneumoniae* the most common pathogen of community-acquired pneumonia (CAP), has acquired antimicrobial resistance to many antibiotics, including penicillin, cephalosporins, macrolides, tetracyclines, and trimethoprim-sulfamethoxazole. The GE may become new drug candidate effective in treating the disease induced by *Streptococcus pneumoniae*.

Table II. IC_{50} values for inhibition of FabG by GE and flavonoids.

Inhibitor	IC_{50}*
Galangin	No inhibition
Quercetin	6.46 ± 0.41 μg/mL (21.2 ± 1.0 μM)
Kaempferol	87.2 ± 2.7 μg/mL (304.8 ± 9.1 μM)
GE	4.47 ± 0.10 μg/mL

*The values shown are the mean ± SD for three experiments.

Reversible inhibition of FabG by GE

Our work demonstrates that GE is a very potent inhibitor of FabG with reversible inhibition *in vitro*. The IC_{50} value of 40% ethanol GE is 4.47 ± 0.10 μg dried galangal/mL, compared with previously reported FabG inhibitor higher than (-)-Epigallocatechin gallate (EGCG) whose IC_{50} value of 2.29 μg/mL (5 μM) [19] but lower than chlorogenic acid's (31.21 μg/mL, 88.1 μM) [20]. Rock and his comrade also proved that EGCG had inhibition to the growth of E.*coli* may through disturbing bacteria lipid synthesis [19]. However, the GE sample in our

experiment is crude herbal extracts, while plant polyphenols used in above studies were refined compounds, which means that GE may contain more efficient inhibitors of FabG. There has been reported that galangin, quercetin and kaempferol were the major flavonoid compounds in galangal [21], and moreover existing in the ethanol extract of the galangal [22]. For sake of searching for the main active compound in the galangal, we assayed the inhibition ability of the three compounds the galangal mainly contained mentioned above. As the results shown, quercetin with the IC_{50} value almost to the 40%GE,which might play important role in the reversible inhibition, while for its low content there might exist other active compounds. To prove this, further work we will isolate the effective component from GE by phytochemical methods. It has known that many China traditional medical plants have antibacterial effect and contain many compounds which reported can impede bacteria proliferation [23].

In addition, it has been reported that GE showed potent reversible inhibition to FAS I with IC_{50} value of 1.73 μg dried galangal/mL reaction solution. Interestingly, GE's inhibition of β-ketoacyl reductase of FAS I (KR) was considerable, whereas inhibition of the enoyl reduction was nearly neglectable [24]. Our work demonstrated that GE as EGCG and chlorogenic acid, has inhibitory ability to both FAS I and FabG. Since FabG has significant similarities to a segment of KR [25], that may be explain it. Furthermore, we speculated that some medical herbs especially reported have inhibition to KR of FAS I may be ideal sources for screening the FabG inhibitors, which is especially important when very few FabG inhibitors had been discovered so far.

In order to explore the possible inhibition mechanism of the GE to FabG, we investigated several kinetic parameters. To the substrate NADPH, GE inhibited FabG in a competitive manner, different from inhibiting KR in an uncompetitive manner [24]. Therefore, we assumed that the active compounds in GE which inhibit to FabG and KR are not the same. It is possible to find inhibitors exclusively action on FabG. On the other hand, the active configuration of FabG is tetrameric, while the two KR domains of mammalian FAS do not interact, which indicated that FabG and KR may have different catalysis mechanism. Thus we also speculated that there maybe the same compounds with different inhibitory mechanism. The competitive inhibition results also suggested that GE primarily acts on the site where NADPH bonds. While to NADPH, EGCG was a mixed type inhibitor of FabG [19], GE is different from EGCG.

Slow-binding irreversible inhibition of FabG by GE

Irreversible inhibitors always through covalent bond react with enzyme, and display slow-banding inhibition effect to the enzyme. It has been previously reported that FabG displays a functional conformational flexibility [26], which represents both an impediment and an opportunity for drug discovery. On one hand, it is difficult to model drugs into a flexible substrate binding pocket; on the other hand, it offers the prospect of discovering slow-binding inhibitors [27]. In the few FabG inhibitors reported, none had been found had slow-binding inhibition to FabG. Thus our discovery of GE with slow-binding inhibitive ability made it possible to find constituent in GE which can make FabG irreversible inactivation. We also assayed slow-binding inhibition of the there major flavonoids, however they all didn't exhibit irreversible effect. So the irreversible inhibition of the GE isn't determined by the there flavonoids, it can be speculated that there might exist other compounds worked as the irreversible inhibitor(s) and offered us an orient to find irreversible inhibitor.

In conclusion, Bacterial membrane like animal cell membrane also needs fatty acids as component. FabG has great prospect to become a novel target of antibacterial. So far, the only reported FabG inhibitor is polyphenol [19]. In our research, we found that galangal extracts inhibits the reaction of the enzyme FabG in vitro and it also can effectively inhibit proliferation of some gram positive bacteria. Besides some flavonoids acting as active compounds, there also exists other category active components action as irreversible inhibitors. It was reported that the galangal extracts penetrate into the bacterial cell, can made the membrane of bacteria rupture [25] and result in bacterial death. Therefore, we speculate that the galangal extracts may through inhibiting fatty acids synthesis thereby destroy membrane of the bacterial, ultimately prevent bacterial propagation.

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