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Presence of Fatty Acid Synthase Inhibitors in the Rhizome of *Alpinia officinarum* Hance

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The galangal (the rhizome of *Alpinia officinarum*, Hance) is popular in Asia as a traditional herbal medicine. The present study reports that the galangal extract (GE) can potently inhibit fatty-acid synthase (FAS, E.C.2.3.1.85). The inhibition consists of both reversible inhibition with an IC_{50} value of 1.73 μg dried GE/ml, and biphasic slow-binding inactivation. Subsequently the reversible inhibition and slow-binding inactivation to FAS were further studied. The inhibition of FAS by galangin, quercetin and kaempferol, which are the main flavonoids existing in the galangal, showed that quercetin and kaempferol had potent reversible inhibitory activity, but all three flavonoids had no obvious slow-binding inactivation. Analysis of the kinetic results led to the conclusion that the inhibitory mechanism of GE is totally different from that of some other previously reported inhibitors of FAS, such as cerulenin, EGCG (epigallocatechin gallate) and C75.

Keywords: Galangal; FAS; Inhibitor; Extract; Kinetics of Inhibition; Fatty Acid Synthase

Abbreviation: FAS, fatty-acid synthase; Ac-CoA, acetyl-CoA; Mal-CoA, malonyl-CoA; GE, galangal extract; EGCG, epigallocatechin gallate

INTRODUCTION

The animal fatty acid synthase (FAS), which plays an important role in energy metabolism, is the most sophisticated entry in the newly recognized class of multifunctional enzymes, and has two identical subunits. It synthesizes fatty-acid, mainly palmitate, *de novo* from the substrates Ac-CoA, Mal-CoA and NADPH by its seven active sites arranged in sequence.[1,2]

Recently, it has been found that FAS is related to various human diseases.[3] FAS is often highly expressed in human cancers but not in normal tissues. The differential distribution of FAS makes it possible that FAS inhibitors would target cancer cells, leaving normal cells unaffected. Thereby FAS can provide a novel target for therapeutic applications in human cancers.[3,4] Consistent with this conclusion, the three FAS inhibitors previously discovered, C75, cerulenin and EGCG were reported to have positive effects on human cancer cell *in vivo* or *in vitro*.[4–6] In addition, FAS inhibitors can reduce body weight, for example, mice treated with C75 had dramatic decreases in food intake and body weight. Therefore, FAS is also a target for both appetite and weight control.[7,8] Overall, FAS is drawing more and more attention world-wide for its association with human diseases. It is necessary and significant to discover new types of FAS inhibitors, which would be useful clinically for the treatment of obesity and cancer, but it would also be helpful to further understand the reaction and inhibition mechanism(s) of FAS.

Herbal medicines are traditional, popular and low-cost, and contain many chemicals, and so are an ideal resource from which inhibitors of FAS are searched for. During the course of our investigation of many herbs, the 40% ethanol extract of the rhizome of *Alpinia officinarum* Hance, whose common name is galangal, was found to inhibit FAS, which hitherto had been unexplored. The galangal is a resource for flavonoids, and in the present work, we describe two flavonoids, quercetin and kaempferol, existing in the galangal, which inhibit animal FAS, which has not been previously reported.

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The galangal has been used as a spice, and is thus considered to be nonpoisonous, and as a herbal medicine for a variety of ailments in Asia for centuries,[9] which is a favourable aspect for the future application of the effective extract.

MATERIALS AND METHODS

Materials

Acetyl-CoA (Ac-CoA), malonyl-CoA (Mal-CoA), NADPH, galangin, quercetin and kaempferol were obtained from Sigma. All other reagents were local products of analytical grade. The galangal was purchased from Beijing Tong Ren Tang Corp. Ltd. Galangin, quercetin and kaempferol were used as solutions in DMSO.

Preparation of GE

1 g dry herbs were smashed, and extracted with 10 ml of 40% ethanol. The mixture was stirred for 2 h, then centrifuged at 4000 × g for 15 min, and the supernatant, which was used in all the following experiments, was the GE sample that was divided into aliquots and stored at 4°C. The dried weight of 1 ml GE was 13.3 mg.

Preparation of FAS and Substrates

The FAS used was extracted from chicken liver, since the amino acid sequence of human FAS has 63% identity with the sequences of chicken enzymes.[10] The preparation, storage and use of FAS were performed as described previously.[11] The preparation was homogeneous on polyacrylamide gel electrophoresis (PAGE) in the presence and absence of SDS. The enzyme and substrates concentrations were determined by spectrophotometry using the following extinction coefficients: Chicken liver FAS, $4.83 \times 10^5\,M^{-1}\,cm^{-1}$ at 279 nm; Ac-CoA, $1.54 \times 10^4\,M^{-1}\,cm^{-1}$ at 259 nm, pH 7.0; Mal-CoA, $1.46 \times 10^4\,M^{-1}\,cm^{-1}$ at 260 nm, pH 6.0; NADPH, $6.02 \times 10^3\,M^{-1}\,cm^{-1}$ at 340 nm and $1.59 \times 10^4\,M^{-1}\,cm^{-1}$ at 259 nm, pH 9.0.

Assay of FAS Activity

FAS activity (for the overall reaction) was determined using a Hitachi UV557 Double wavelength/Double beam spectrophotometer at 37°C by following the decrease of NADPH at 340 nm. The reaction mixture contained potassium phosphate buffer, 100 mM, pH 7.0; EDTA, 1 mM; Ac-CoA, 3 μM; Mal-CoA, 10 μM; NADPH, 35 μM and chicken liver FAS 15 μg in a total volume of 2.0 ml. The β-ketoacyl reduction and enoyl reduction activities were determined at 37°C by measuring the change of absorption at 340 nm. The ketoacyl reduction reaction mixture contained ethyl acetoacetate, 40 mM; NADPH, 35 μM; 1 mM EDTA and the enzyme 15 μg in 100 mM phosphate buffer, pH 7.0. The enoyl reduction reaction mixture contained ethyl crotonate, 40 mM; NADPH, 35μM; 1 mM EDTA and 100 μg FAS in 10 mM phosphate buffer, pH 6.3.[12,13]

Assay of Fast-binding Reversible Inhibition Activity and Slow-binding Inactivation

Reversible inhibition was determined by adding the inhibitors in DMSO or 40% ethanol to the reaction system before FAS initiated the reaction. The final concentration of DMSO or ethanol didn't exceed 0.2% (V/V) in the reaction mixture, and this concentration didn't affect FAS activity. After the enzyme solutions were mixed with various concentrations of inhibitors, aliquots were taken to measure the relative activity at the indicated time intervals to measure slow-binding inactivation.[6]

Substrate Protection

GE was added to FAS solutions mixed with one of three substrates respectively, and then the relative activities of FAS at different time intervals were measured. The same concentration of GE and FAS solutions without any substrate added was used as the control. The first order rate constants of these reactions were compared to determine the substrate protective effects.

RESULTS

Reversible Inhibition of FAS by GE

The catalyzed reaction and reversible inhibition occurred simultaneously during the assay of reversible inhibition. GE showed potent reversible inhibition to FAS. The data of Figure 1 showed that 0.013% (v/v) GE, equivalent to 1.73 μg dried GE/ml reaction solution, could inhibit 50% of the overall reaction activity of FAS, and that 0.03% (v/v) GE, equivalent to 3.99 μg dried GE/ml, could inhibit 50% of the β-ketoacyl reductase activity of FAS. Interestingly, inhibition of the β-ketoacyl reduction was considerable, whereas inhibition of the enoyl reduction was nearly negligible. So it was a reasonable conclusion that the reversible inhibition by GE was related to the β-ketoacyl reductase but little related to the enoyl reductase. In order to explore the effect on the β-ketoacyl reductase and the inhibition mechanism of GE, some kinetic parameters of the β-ketoacyl reduction

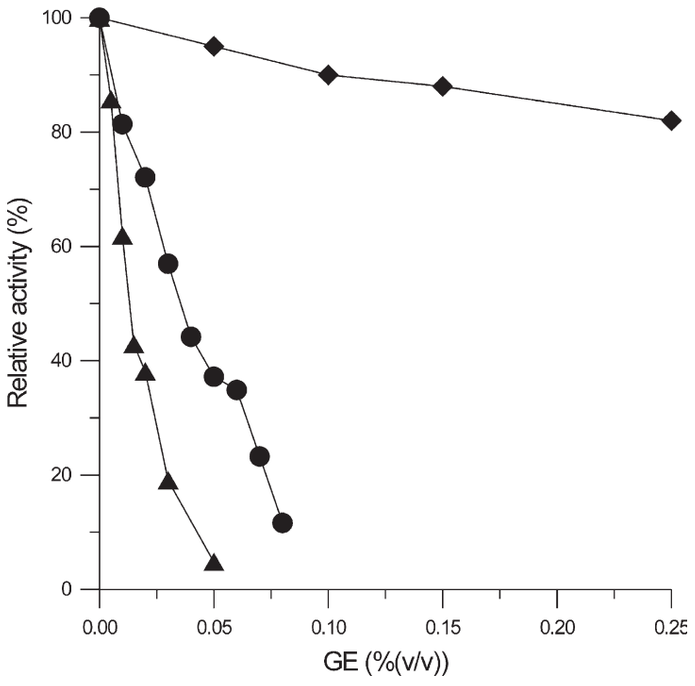

FIGURE 1 Effects of GE on FAS activity. The inhibition of FAS in the presence of various concentrations of GE. Inhibition of the overall reaction (▲); Inhibition of β-ketoacyl reduction (●); Inhibition of enoyl reduction (◆).

were measured. FAS ketoacyl-reduction activity was studied in the presence of increasing GE concentrations and NADPH as the variable substrate. The double-reciprocal plot (Figure 2) indicated that GE inhibited the β-ketoacyl reductase in an uncompetitive manner, and the inhibition constant obtained from the experimental data is recorded in Table I. Furthermore, the inhibition types of GE to the overall reaction of FAS were kinetically examined. Lineweaver-Burk analysis of the kinetic data showed that GE inhibited the overall reaction of FAS uncompetitively with respect to Mal-CoA, and noncompetitively with respect to Ac-CoA (not shown). The obtained inhibition constants are summarized in Table I.

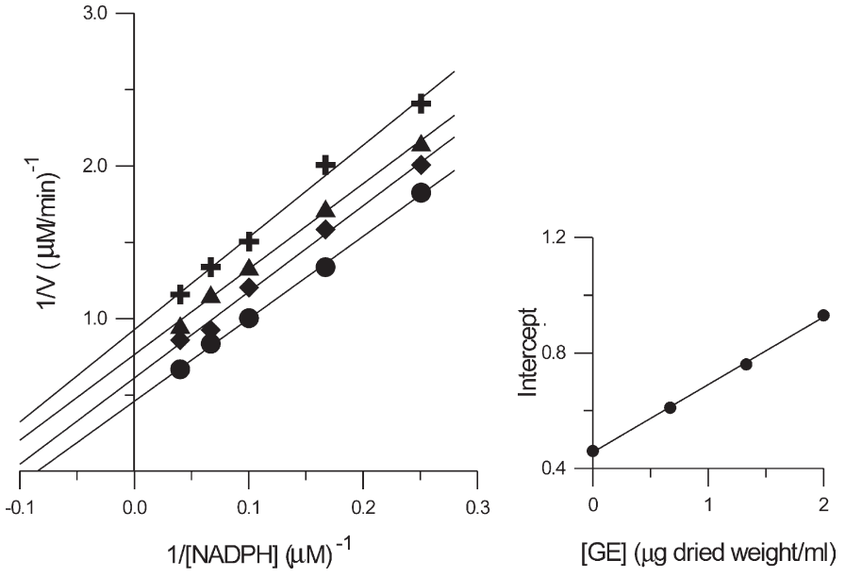

FIGURE 2 Lineweaver-Burk plot for inhibition of the β-ketoacyl reduction of FAS by GE. The fixed concentration of ethyl acetoacetate is 40 mM and NADPH is the variable substrate. The concentration of GE used are; 0 μg dried weight/ml (●); 0.67 μg dried weight/ml (◆); 1.33 μg dried weight/ml (▲); 2.00 μg dried weight/ml (✚).

TABLE I Inhibition types and inhibition constants for the inhibition of FAS by GE for each substrate

Substrate	Inhibition type	Inhibition constant (K_i) (μg dried GE/ml)
NADPH	Uncompetitive*	1.95*
Ac-CoA	Noncompetitive	1.13
Mal-CoA	Uncompetitive	0.72

*The inhibition type and inhibition constant of the β-ketoacyl reduction of FAS.

Stability of GE

The stability of GE is important for its development as a therapeutic agent and some further experiments were designed to examine it. The IC_{50} of a GE sample was measured after it had been stored for 82 days at room temperature, and then compared with its IC_{50} from its immediate use. As a result, the IC_{50} value of 0.015% (v/v) after 82 days was very similar to its initial value of 0.013% (v/v) (Figure 3), which showed that the inhibition activity of the GE sample had not changed on storage.

Slow-binding Inactivation by GE

The FAS solutions were mixed with GE and aliquots were taken to measure the relative activity of the overall reaction, at pre-determined time intervals. GE showed slow-binding inactivation of FAS. Figure 4A showed that the time course of the overall reaction of FAS in the presence of GE of 0.22 mg dried weight/ml underwent three processes. In the first process that lasted a period of 12 seconds, the plot was initially sharp concave to approximate 40% loss of activity. However, when the FAS solution mixed with GE is added to the reaction system, GE is diluted 200 times, and its concentration is sequently reduces to 1.1 μg dried weight/ml. At this concentration, about 35% of the overall reaction of FAS can be inhibited reversibly. Therefore it suggests 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, which follows, includes clearly two phases; a fast phase and a slow phase.

The semi-logarithm plot in Figure 4B, where the contribution of the reversible inhibition has been subtracted, showed good straight lines for both phases, and the first order rate constant of 0.15 min^{-1} for the fast phase, from which the contribution of the slow phase is subtracted, is much higher than that of the slow phase (4.1×10^{-3} min^{-1}).

Furthermore, the results of substrate protection indicated that none of the three substrates could protect FAS against inactivation by GE in both phases (data not shown). It can be concluded that the binding site of GE in the slow-binding inactivation of FAS is different to that of the substrates.

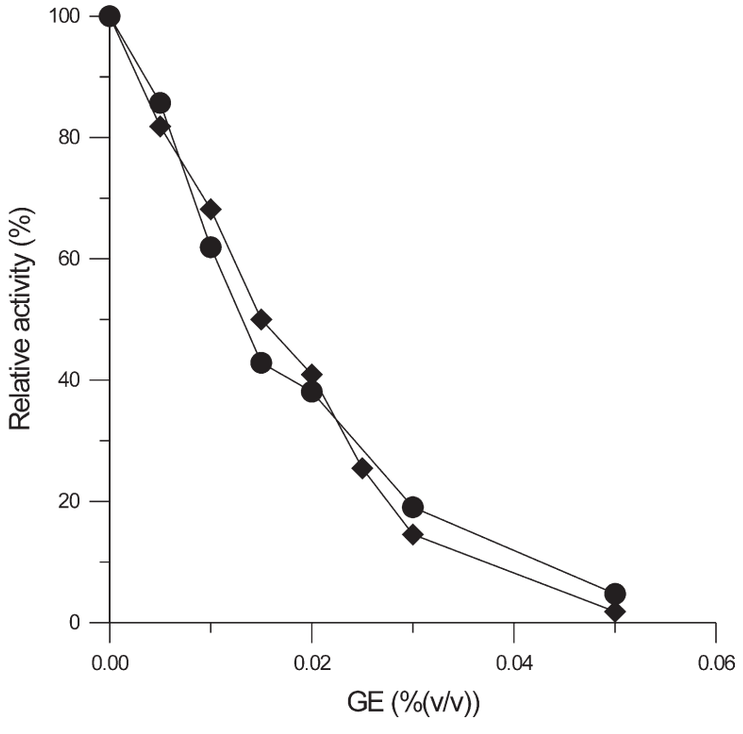

FIGURE 3 Effects of GE stored for different time on FAS activity. Inhibition of the FAS overall reaction in the presence of various concentrations of GE. (●) the initial GE reaction; (◆) reaction after GE stored for 82 days.

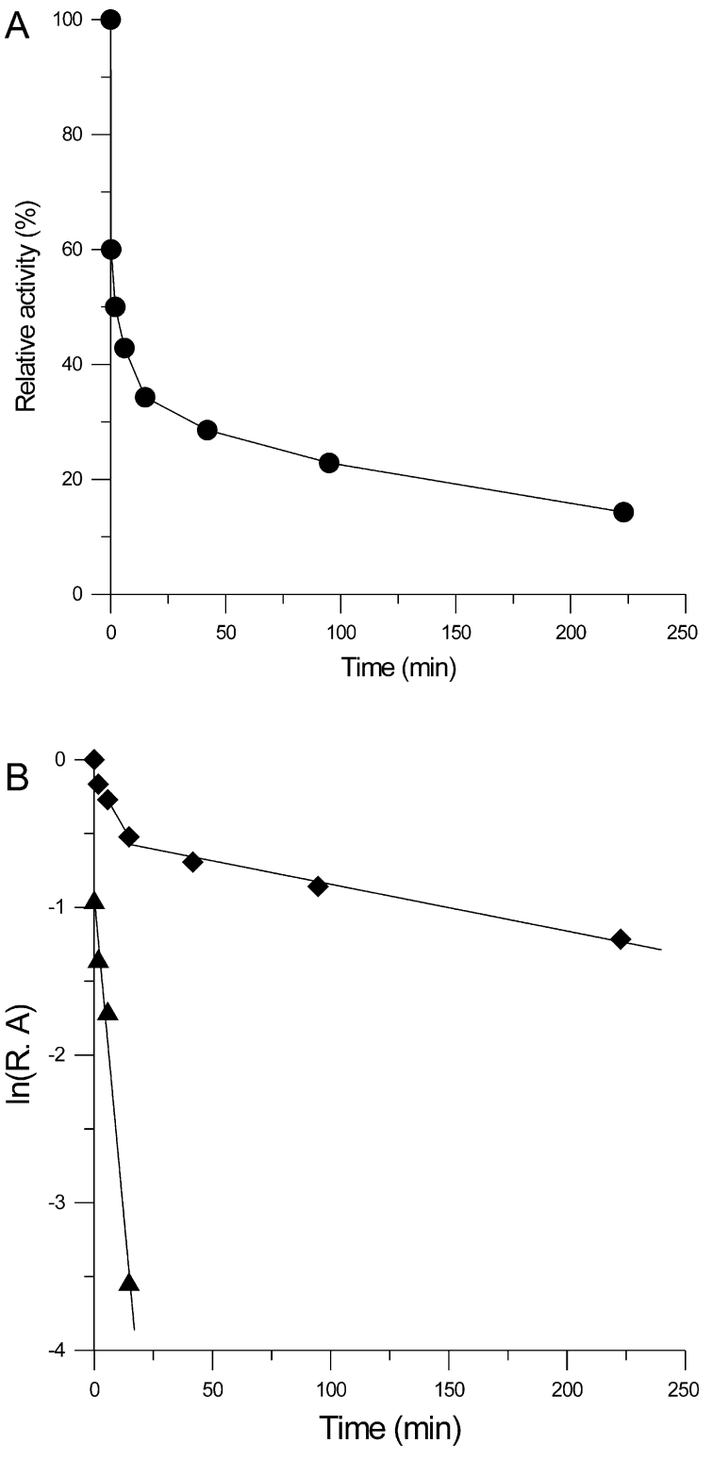

FIGURE 4 Kinetic time course of inhibition of FAS activity. Slow-binding inactivation of the overall reaction of FAS is measured in the presence of GE. (A) Experimental data (●). (B) Plot of ln(R.A.) *vs* time calculated from (A), with subtraction of the reversible inhibition contribution. The plot has two phases: the fast phase (▲) and the slow phase (◆). The FAS solution (1.8 μM) was mixed with GE (1.65%(v/v), equivalent to 0.22 mg dried GE/ml), and aliquots were taken and assayed for relative activity at the indicated time intervals (R.A. = relative activity).

TABLE II IC_{50} values for inhibition by GE and flavonoids of the overall reaction of FAS

Inhibitor	IC_{50}*
Galangin	Poor inhibition
Quercetin	2.40 ± 0.10 μg/ml (7.09 ± 0.30 μM)
Kaempferol	2.98 ± 0.09 μg/ml (10.53 ± 0.28 μM)
Extract	1.73 ± 0.06 μg/ml

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

Some Flavonoids Existing in Galangal can Inhibit FAS

It has been reported that galangal is rich in flavonoids,[14] and moreover, that these flavonoids can be easily extracted. For example, the ethanol extract of galangal contains abundant flavonoids.[9]

The effects of galangin, quercetin and kaempferol, the major flavonoid compounds in galangal,[15] on FAS activity was examined. As shown in Table II, of the three flavonoids, galangin was a very poor inhibitor, but the other two flavonoids potently inhibited FAS activity. The IC_{50} value of kaempferol is 10.53 μM (2.98 μg/ml), while that of quercetin is 7.09 μM (2.40 μg/ml). However, quercetin was found to inhibit the β-ketoacyl reaction of FAS with an IC_{50} value of about 280 μM (estimated from Figure 5), which was much higher than that for the overall reaction, which suggested that the effect of quercetin on FAS was unrelated to inhibition of the β-ketoacyl reductase. Further kinetic work on quercetin showed that quercetin inhibited FAS competitively with respect to Ac-CoA, noncompetitively with respect to Mal-CoA and in a mixed manner (combining the competitive and noncompetitive forms of inhibition) with respect to NADPH (see Figure 6). The inhibition constants for quercetin are summarized in Table III. The inhibition kinetic patterns for inhibition of FAS by kaempferol (data not shown) were similar to those for quercetin.

DISCUSSION

In the present work, we have demonstrated that GE could inhibit the overall reaction and β-ketoacyl reduction of FAS markedly, and that inhibition of the overall reaction included both reversible inhibition and slow-binding inactivation.

GE is a very potent inhibitor of the overall reaction of FAS. Compared with the reported previously FAS inhibitors, EGCG[6] and cerulenin,[16] the IC_{50} value, 1.7 μg dried weight/ml, of GE is much lower than that of EGCG (23.8 μg/ml) or cerulenin (20 μg/ml). The strong inhibitory activity of GE towards FAS, plus its excellent stability, opens up excellent prospects for its application as an anti-cancer agent.

GE shows potent reversible inhibition to the overall reaction of FAS mainly by inhibiting the β-ketoacyl reduction, and the IC_{50} values of the two reactions are kinetically similar, but the site GE attacks isn't the binding site of the substrate NADPH, since GE is an uncompetitive inhibitor of

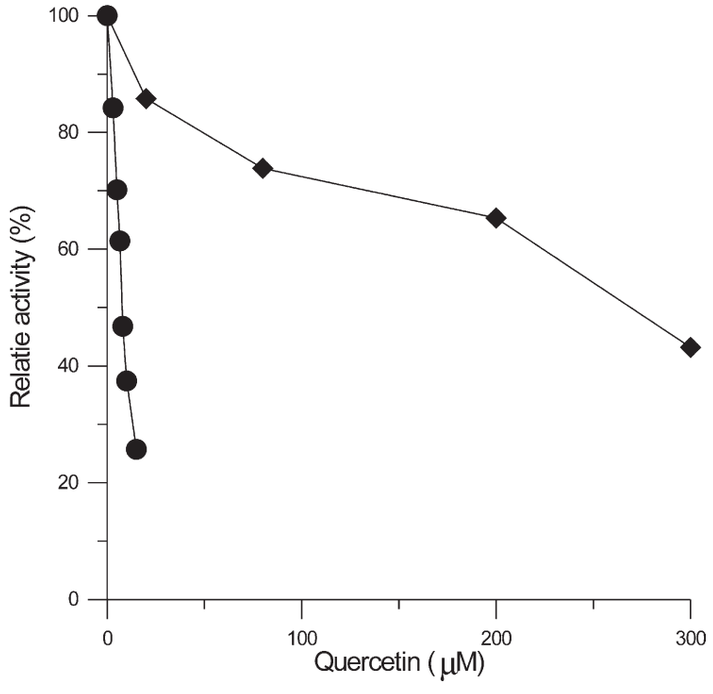

FIGURE 5 Effects of quercetin on FAS activity. The inhibition of FAS in the presence of various concentrations of quercetin. Inhibition of the overall reaction (●); Inhibition of the β-ketoacyl reduction (◆).

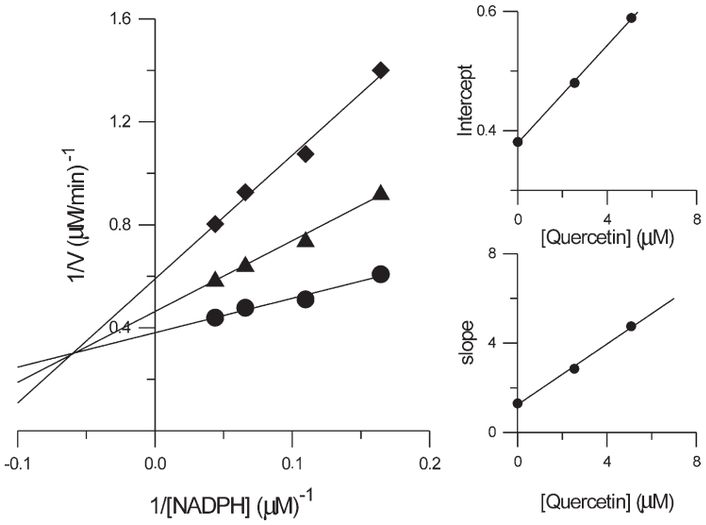

FIGURE 6 Lineweaver-Burk plot for inhibition of the overall reaction of FAS by quercetin. The fixed concentrations of Ac-CoA and Mal-CoA are 3 μM and 10 μM respectively and NADPH is the variable substrate. The concentrations of quercetin were; 0 μM (●); 2.5 μM (▲); 5.0 μM (◆).

FAS with respect to NADPH. EGCG also inhibits mainly the ketoacyl reductase of FAS, but it differs from GE in that EGCG and NADPH bind to the same site competitively.[6] As for C75 and cerulenin, two other known FAS inhibitors, it has been reported that they inhibit FAS by irreversibly binding to the the ketoacyl synthase of FAS, and that their inhibition of FAS is related to the binding site of the substrate Mal-CoA or Ac-CoA.[6,17,18] Therefore, as regards inhibition mechanism, GE is totally different from the other three inhibitors previously reported.

In addition, GE can possibly affect other sites of FAS besides the ketoacyl reductase, because the IC_{50} values for the overall reaction and ketoacyl reductase are not identical. Furthermore these sites are independent of the three substrates of FAS, a hypothesis that was confirmed by substrate protection experiments that showed that none of three substrates has protective effects against inactivation by GE.

Although the composition of galangal is complex, the flavonoids account for the majority of all the bioactive components,[19] so we investigated some flavonoids existing in galangal. It was found that some of them, such as quercetin and kaempferol, showed potent inhibition of FAS, but their inhibition effects on FAS were poorer than that of GE, i.e. for the overall reaction the IC_{50} value of GE was lower than that of these flavonoids. Considering that GE is a mixture of compounds, the effective component of GE should be much more potent against FAS than is shown by these flavonoids. Moreover, these flavonoids show no slow-binding inactivation towards FAS. Their binding sites are also different. Quercetin and kaempferol could affect some site other than the ketoacyl reductase, since their IC_{50} values for the overall reaction and ketoacyl reduction are very different. Quercetin and kaempferol possibly affected the ketoacyl synthase or the acetyl transferase because they inhibited FAS competitivly with respect to Ac-CoA, while GE was a noncompetitive inhibitor of FAS against Ac-CoA. This also further suggests that the inhibition mechanisms of these flavonoids and GE are different. Further kinetic experiments with the two flavonoids confirmed this conclusion, because the inhibition types shown by them clearly differed from those of GE. All the above data suggested that GE contains at least some other inhibitory component(s) different from these flavonoids. The active components are possibly more potent inhibitors of FAS than these flavonoids, or alternatively there are several chemicals cooperating in the inhibition of FAS.

It has been reported that inhibition of FAS is selectively cytotoxic to human cancer cells because FAS activity is vital for the growth and survival of these cells.[3] The two flavonoids, quercetin and kaempferol, are well-known for their antitumor effects, but their mechanisms still remain

TABLE III Inhibition types and inhibition constants for inhibition of FAS by Quercetin for each substrate

		Inhibition constant (μM)	
Substrate	Inhibition type	K_i	K_{is}
NADPH	Mixed*	1.83	9.24
Ac-CoA	Competitive	3.01	–
Mal-CoA	Noncompetitive	–	7.44

*Mixed competitive and noncompetitive inhibition.

unknown.[20,21] We now speculate that they possibly perform, at least in part, by affecting FAS activity.

Our present work not only demonstrates that there are inhibitors of FAS existing in GE, but also provides a method by which other herbs could be examined and found to contain inhibitors of FAS, which is a promising way forward in the discovering of new drugs for the treatment of obesity and cancer.

Acknowledgement

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