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Low concentration of condensed tannins from catechu significantly inhibits fatty acid synthase and growth of MCF-7 cells

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ABSTRACT

Tannins exist widely in plants, but because they precipitate proteins, scientists frequently ignore them in search of bioactive components. Catechu, a traditional astringent, is rich in tannins. In this study, we found that condensed tannins from catechu potently inhibited animal fatty acid synthase (FAS). Among them, trimeric condensed tannin showed the most potent inhibition with IC_{50} of 0.47 μg/ml and it also exhibited strong time-dependent inhibition. Its inhibitory kinetics and reacting sites on FAS were obviously different from the known inhibitors of FAS. Furthermore, condensed tannins were found to suppress the growth of MCF-7 breast cancer cells, and the effect was related to their activity of FAS inhibition. The inhibition of both FAS activity and MCF-7 growth was exhibited by low concentrations of condensed tannins without FAS being precipitated. These results suggest tannins would be a valuable resource of bioactive substances.

Catechu is a widely-used herbal medicine; it is typically used as a clotting agent for the treatment of diarrhea, bloody stool, uterine bleeding, hemorrhoids, and canker sores [1–3]. It is an extract prepared with boiling water from the heartwood of *Acacia catechu* (L.) Willd. Catechu is rich in tannins [1,4]. The main property of tannins is to bind and precipitate proteins, which is called astringency [5]. High tannin content makes catechu an excellent astringent agent.

However, precipitating proteins is not the only capacity of tannins. In fact, tannins exhibit many biological activities. In previous studies, tannins have been evaluated as antibacterial, antiviral, radical scavenging, and complement modulating agents; they have also been reported to have antitumor activities and to inhibit enzymes [6,7].

In this study, we demonstrated for the first time that condensed tannins from catechu were potent and novel inhibitors of animal fatty acid synthase (FAS, EC 2.3.1.85). Animal FAS is a key enzyme participating in the *de novo* synthesis of long chain fatty acids *in vivo* [8]. In most human tissues, except for liver and adipose tissue, the expression of FAS is low. However, FAS expression is surprisingly high in a variety of common human cancers, such as cancer of the breast, prostate, ovary, and lung [9]. Inhibitors of FAS, such as C75, cerulenin, and EGCG, specifically induce apoptosis in cancer cells [10–12]. All of these features highlight FAS as a therapeutic target for the treatment of cancer [9]. Here we found that condensed tannins from catechu inhibited the growth of breast cancer cells, while they had no effect on normal cells, and the inhibition was related to their ability to suppress FAS. More importantly, the inhibition of both FAS activity and the growth of breast cancer cells by the tannins from catechu was exhibited without proteins being precipitated.

Materials and methods

Materials. Acetyl-CoA, malonyl-CoA, NADPH, ethyl acetoacetate, EDTA, dithiothreitol, and bovine serum albumin were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Sephadex LH-20 was from General Electric Co. (Shanghai, China). Methanol, acetone, *n*-butanol, petroleum ether, ethyl acetate, and acetic acid were purchased from Beijing Chemical Reagent Co. (Beijing, China). All other chemicals and reagents were local products of analytical grade.

Extraction and separation of condensed tannins from catechu. Catechu was obtained from the Tongrentang Chinese Medicine Co. Ltd. (Beijing, China). Eighty grams of catechu was milled into powder. The powder was extracted with 400 ml acetone–water (7:3, V/V), and the mixture was stirred continuously for 2 h at room temperature. Then the mixture was filtrated and the supernatant was collected. This procedure was repeated two more times and the combined supernatant was evaporated under vacuum at 40 °C to remove acetone. The remaining solution was washed with petroleum ether to remove lipid-soluble substances. After that, the solution was further extracted with ethyl acetate at a ratio of 1:1 (V/V). The water layer was separated and extracted twice more similarly. Then the resulting water layer was evaporated to dryness, and the resulting substance was referred to as water extract.

Two and a half grams of water extract was dissolved in 3 ml water and applied to a 20 × 400 mm Sephadex LH-20 column equilibrated with water. The column was successively eluted with water (120 ml), methanol–water (1:9, 100 ml; 3:7, 100 ml; 2:1, 210 ml; 6:1, 100 ml), methanol (250 ml) and acetone–water (7:3, 600 ml). The elution rate was 0.5 ml/min and fractions of 20 ml each were collected.

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Fractions were monitored by thin layer chromatography (TLC). Fractions having similar TLC profiles were combined and then they were analyzed by the subsequent chemical assays. The IC_{50} values of fractions on FAS were measured.

Analysis of the fractions. Protein precipitable phenolics assay and acid-butanol assay were carried out to determine the presence of condensed tannins in the fractions, according to the procedure described by Hangerman and Butler [13] and the procedure reported by Porter et al. [14], respectively. The relative degree of polymerization of condensed tannin was estimated according to the method of Butler [15]. The electrospray ionization (ESI) mass spectra of the fractions were recorded on a Bruker APEX II instrument (Bremen, Germany). The detection was carried out in a negative-ion mode.

Preparation of FAS and substrates. The preparation, storage and use of FAS from chicken liver were performed as described previously [16]. The purified FAS was homogeneous on polyacrylamide gel electrophoresis in the presence and absence of SDS. The concentrations of the enzyme and substrate were determined by spectrophotometer using the following coefficients: FAS, $4.83 \times 10^5\ M^{-1}\ cm^{-1}$ at 279 nm; Acetyl-CoA, $1.54 \times 10^4\ M^{-1}\ cm^{-1}$ at 259 nm, pH 7.0; Malonyl-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 [17].

Assays of FAS activity. The FAS activity for the overall reaction was measured with an Amersham Pharmacia Ultrospec 4300 pro UV–Vis spectrophotometer (England, UK) at 37 °C by detecting the decrease of NADPH absorption at 340 nm. The assay system contained 100 mM KH_2PO_4–K_2HPO_4 buffer (pH 7.0), 1 mM EDTA, 1 mM dithiothreitol, 3 μM acetyl-CoA, 10 μM malonyl-CoA, 35 μM NADPH, and 10 nM FAS in a total volume of 2 ml as previously described [16,17].

The assay for β-ketoacyl reduction activity of FAS was also carried out at 37 °C by detecting the decrease of NADPH absorption at 340 nm. The assay mixture contained 200 mM ethyl acetoacetate, 35 μM NADPH, and 10 nM FAS in 100 mM KH_2PO_4–K_2HPO_4 buffer, pH 7.0.

Assays of FAS inhibition. For fast-binding reversible inhibition assay, a sample (inhibitor) was added to the assay system before FAS initiated the reaction. The activity of FAS in the presence of sample was designated as A_i, and the control activity of FAS in the absence of sample was designated as A_0. A_i/A_0 was the remaining activity (RA) of FAS that was less than 1 for the inhibition of FAS.

For time-dependent inactivation assay, the FAS solution was mixed with a sample first and then aliquots of the mixture were taken to assay the RA of FAS at a series of time intervals. The control contained only the solvent of the inhibitor.

Growth inhibition of breast cancer cells and normal endothelial cells. Human breast cancer MCF-7 cells and human microvascular endothelial cells, HMEC, were applied to test the growth inhibition by fractions from catechu. The MTT cell viability assay was performed according to the manufacturer's instructions. Briefly, both MCF-7 cells and HMEC were harvested and washed with 1× PBS, and then were diluted to a final concentration of 1×10^5/ml in an assay medium. One hundred microliters of cell suspension (10^4 cells per well) were dispensed into 96-well plates. The plates were incubated at 37 °C for 24 h in a humidified CO_2 incubator. One hundred microliters of complete culture media with different concentrations of samples were added to the wells. Sequentially the plates were incubated at 37 °C for 24 h in a humidified CO_2 incubator. Then cultured cells were washed with warm PBS. For the color development, dye solution (0.5 mg/ml MTT in DMEM without phenol red) was added to each well and the plates were incubated at 37 °C for 4 h. After the dye solution was removed, 100 μl solubilization/stop solution was added to each well. The plates were kept at 4 °C overnight and then the absorbance at 570 nm was recorded by the 96-well plate reader. Experiments were performed in triplicate.

Detection of protein precipitation. FAS solution was incubated with sample solutions of various concentrations and the absorbance of the mixture at 400 nm was monitored. The decrease of the absorbance showed the precipitation of FAS.

Results

Separation and analysis of condensed tannins from catechu

The separation of condensed tannins from catechu was carried out by Sephadex LH-20 chromatography. During isolation, we found that the fractions listed in Table 1 exhibited a positive reaction in both the protein precipitable phenolics assay and the acid-butanol assay, which indicated the presence of condensed tannins in those fractions. Fig. 1A shows that only a single molecular ion peak of 577 appears in the negative-ion ESI mass spectrum for Fraction 1, which indicates that Fraction 1 is a dimeric condensed tannin. Under the separation procedure adopted, the following fractions must also be condensed tannins [18]. We found that all of these tannins greatly inhibited FAS (Table 1).

Table 1
IC_{50} values on FAS of the fractions from catechu

Elution solvent	Fraction name	IC_{50} (μg/ml)
Methanol:water = 6:1	Fraction 1	7.5
Methanol	Fraction 2	1.5
Acetone:water = 7:3	Fraction 3	0.7
	Fraction 4	0.5

Then the relative degrees of polymerization of Fractions 1 and 4 were estimated by acid-butanol/modified vanillin ratio assay, the results of which are displayed in Fig. 1B. It could be calculated that the relative degree of polymerization of Fraction 1 is 0.219 and that of Fraction 4 is 0.329. Fraction 1 is a dimer. So the average degree of polymerization of Fraction 4 should be three. Thus, the most potent inhibitors in catechu are condensed tannins with an average degree of polymerization of three.

Reversible inhibition of FAS by Fraction 4

Fig. 2A shows that 0.47 μg/ml of Fraction 4 inhibits 50% of the overall reaction activity of FAS and 2.36 μg/ml of Fraction 4 inhibits 50% of the β-ketoacyl reduction activity of FAS.

For the overall reaction activity of FAS, the inhibition kinetics was studied in the presence of increasing Fraction 4 concentration with acetyl-CoA as the variable substrate. The double-reciprocal plot (Fig. 2B) indicates that Fraction 4 is a competitive inhibitor of FAS to acetyl-CoA. The inhibition constant K_i obtained from the plot of the slope vs. Fraction 4 concentration is 0.40 μg/ml. For the β-ketoacyl reduction activity of FAS, the inhibition was studied in the presence of increasing Fraction 4 concentration with NADPH as the variable substrate. Fig. 2C indicates that Fraction 4 is also a competitive inhibitor of FAS to NADPH. The inhibition constant K_i obtained from the plot of the slope vs. Fraction 4 concentration is 1.55 μg/ml.

Time-dependent inactivation of FAS by Fraction 4

The time courses of inactivation on the overall reaction activity and the β-ketoacyl reduction activity of FAS by Fraction 4 were determined, respectively. Fraction 4 exhibited the capability of inactivating the FAS activities in a time-dependent manner. The pseudo-first-order rate constants, k_{obs}, were obtained from the slope of the plot of ln (RA) vs. time (Fig. 2D). The k_{obs} for the inactivation of the overall reaction and the β-ketoacyl reduction by 0.048 mg/ml Fraction 4 were 0.0045 and 0.0021 min^{-1}, respectively. This result suggested that the suppression of FAS activity by Fraction 4 partially involved the inactivation of the β-ketoacyl reductase domain on FAS. Also, there may be some other reaction sites for the inactivation.

Effects of extract and condensed tannins from catechu on MCF-7 cells

A common breast cancer cell line, MCF-7 cells, was used to investigate whether the components of catechu influenced the cancer cell viability. The results are shown in Fig. 3. Water extract of catechu exhibited weak inhibition on the proliferation of MCF-7 cells. Fractions 1 and 4 inhibited the growth of MCF-7 cells obviously and the inhibition was dose-dependent. With similar doses, Fraction 4 inhibited cancer cell proliferation much more significantly than Fraction 1 did. Human microvascular endothelial cells, HMEC, were treated accordingly as the normal cell control, and their growth was not observably influenced by any of the samples (data not shown).

Detection of protein precipitation by Fraction 4

The defining characteristic of tannins is their ability to precipitate proteins. The next question is whether Fraction 4 inhibited

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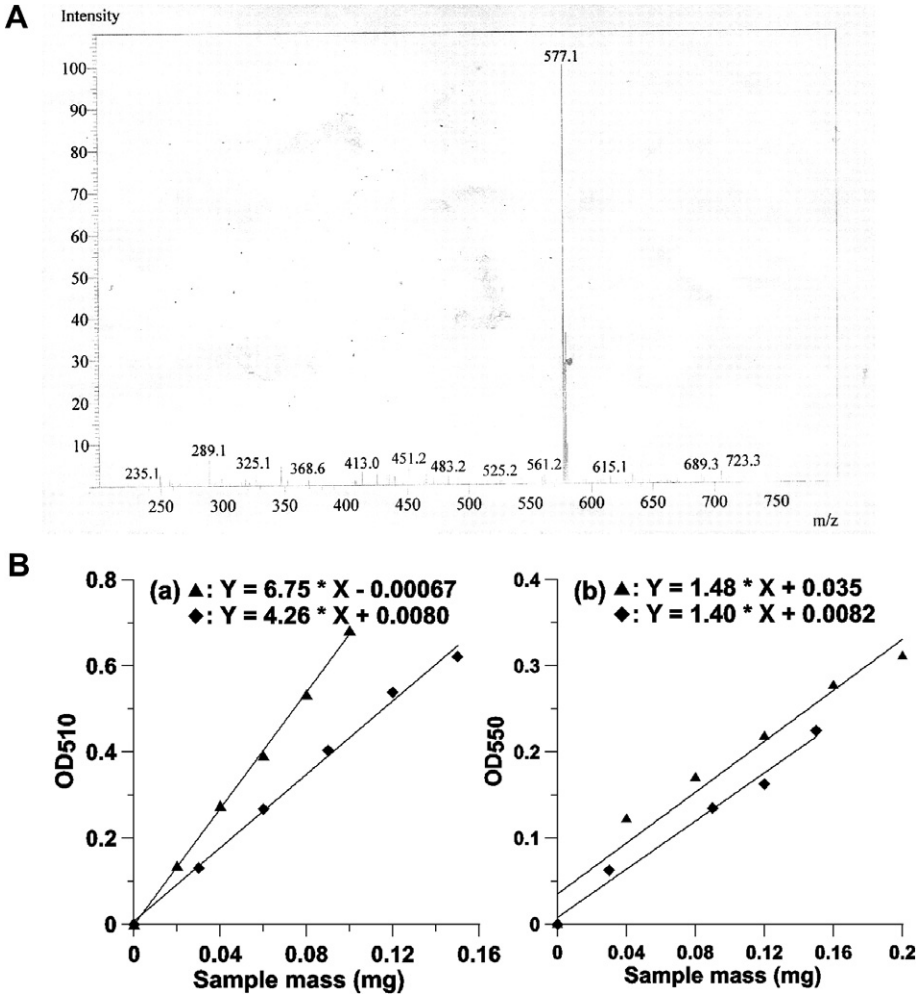

Fig. 1. Analysis of condensed tannins from catechu. (A) Negative-ion ESI mass spectrum of Fraction 1. (B) Determination of the relative degrees of polymerization of Fractions 1 and 4. (a) Modified vanillin assay according to the method of Butler [15]. (b) Acid-butanol assay according to the procedure reported by Porter et al. [14]. (▲) Fraction 1, the concentration of the Fraction 1 solution to be assayed was 0.2 mg/ml. (♦) Fraction 4, the concentration of the Fraction 4 solution to be assayed was 0.3 mg/ml.

and inactivated FAS by precipitating FAS. To answer this question, FAS solutions were mixed with various concentrations of Fraction 4 and the absorbance of the mixtures at 400 nm was monitored to detect precipitation (Fig. 4).

The reduction of the absorbance of the mixtures indicates the decreasing of Fraction 4 in the solution, which means that Fraction 4 binds with FAS and becomes a precipitate. Results showed that obvious precipitation took place when FAS was mixed with 0.24 mg/ml Fraction 4. However, when FAS was incubated with 0.048 mg/ml or 0.14 mg/ml Fraction 4, no apparent precipitation was observed (Fig. 4). Then the mixtures were centrifuged. Congruously, there was an obvious deposit at the bottom of the tubes with 0.24 mg/ml Fraction 4, little deposit with 0.14 mg/ml Fraction 4 and no deposit with 0.048 mg/ml Fraction 4. Therefore, FAS was not precipitated obviously until the concentration of Fraction 4 was beyond 0.24 mg/ml.

Discussion

The condensed tannins from catechu are potent and novel inhibitors of FAS

Tannins are polyphenol compounds that exist widely in plants. Non-specific precipitation of protein is the common characteristic of tannins. Catechu is rich in tannins [1,4]. In this work, it was found that low concentrations of condensed tannins from catechu potently inhibited FAS, which has been reported as a novel potential therapeutic target for cancer [9]. The condensed tannins, with an average degree of polymerization of three (Fraction 4), exhibited the most potent inhibitory activity. It blocked most enzyme activity of FAS at a concentration of less than 1 μg/ml. Its IC_{50} value, 0.5 μg/ml, is about 40-fold lower than that of EGCG and cerulenin, the IC_{50} values of which were reported to be 24 and 20 μg/ml respectively [19,20].

Trimeric condensed tannins inhibited the β-ketoacyl reductase domain of FAS with an IC_{50} value of 2.36 μg/ml, and the inhibition was competitive to NADPH, with a K_i value of 1.55 μg/ml. This suggests that the NADPH loading site in the β-ketoacyl reductase domain of FAS is a reaction site for the tannins. For trimeric condensed tannins, the ratio of IC_{50} for the ketoacyl reduction to IC_{50} for the overall reaction is about five, which is obviously higher than that for EGCG (about two) [19]. For dimeric condensed tannins (Fraction 1), this ratio is even higher than 10. These results indicate that the β-ketoacyl reductase domain is not the major reaction site for condensed tannins, but it is for EGCG. The competitive inhibition to acetyl-CoA suggests that condensed tannins may react on the acyl transferase domain of FAS, which is similar to flavonoids in this respect. However, flavonoids do not inhibit β-ketoacyl reductase or show

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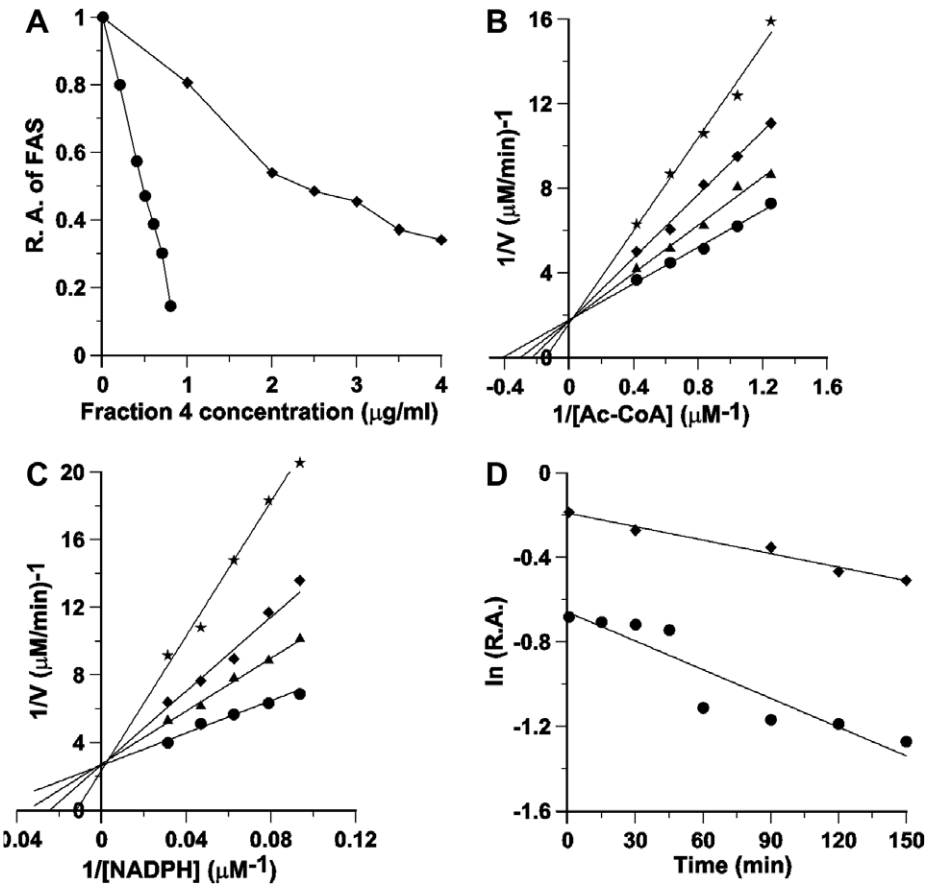

Fig. 2. Inhibitory kinetics of FAS by Fraction 4. (A) Inhibitory effects on FAS activities by Fraction 4. The inhibition of FAS by various concentrations of Fraction 4 was measured. (●) Inhibition of the overall reaction; (♦) Inhibition of the β-ketoacyl reduction. (B) Double-reciprocal plots for inhibition of the overall reaction activity of FAS by Fraction 4. The concentrations of malonyl-CoA and NADPH were fixed at 10.0 and 32.3 μM, respectively. Acetyl-CoA was a variable substrate. The concentrations of Fraction 4 were 0 (●); 0.15 μg/ml (▲); 0.30 μg/ml (♦); 0.45 μg/ml (★). (C) Double-reciprocal plots for inhibition of the β-ketoacyl reduction activity of FAS by Fraction 4. The concentration of ethyl acetoacetate was fixed at 200 mM. NADPH was a variable substrate. The concentrations of Fraction 4 were 0 (●); 1 μg/ml (▲); 2 μg/ml (♦); 3 μg/ml (★). (D) Semi-logarithmic plot of time-dependent inactivation of FAS by Fraction 4. The FAS solution (0.048 mg/ml) was mixed with Fraction 4 (0.048 mg/ml) and the aliquots were taken to assay the remaining activity of the overall reaction (●) and the β-ketoacyl reduction (♦) at predetermined time intervals.

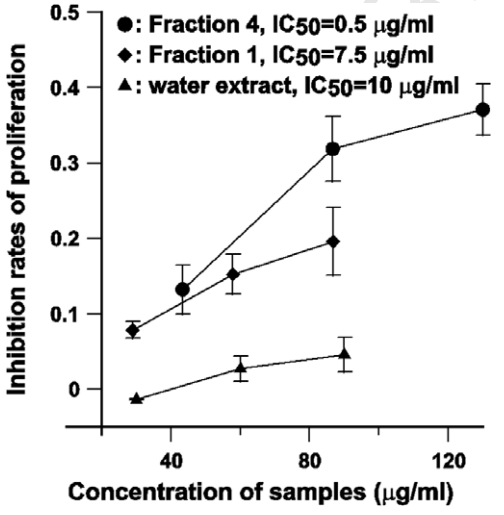

Fig. 3. Impact of proliferation of MCF-7 cells by different fractions from catechu. After been seeded in the 96-well plate, the breast cancer MCF-7 cells were incubated with different concentrations of water extract, Fraction 1 and Fraction 4 for 24 h. The percentage of viable cells was determined by MTT assay. Data are represented as means ± SD ($n = 3$). *Significantly different ($p < 0.05$) from control cells (no samples) by Tukey test. **Significantly different ($p < 0.01$) from control cells (no samples) by Tukey test.

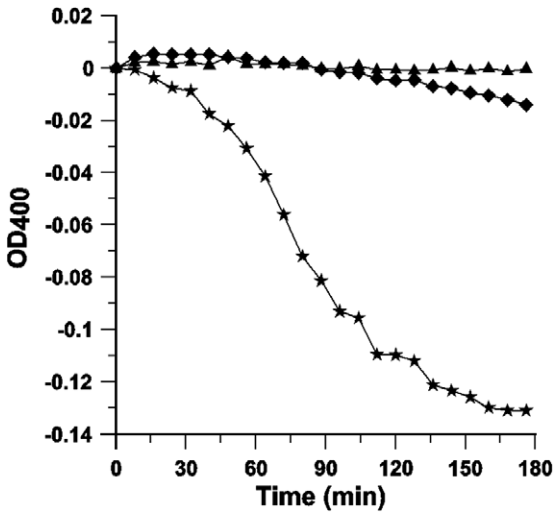

Fig. 4. Time course for absorbance at 400 nm of mixed FAS and various concentrations of Fraction 4. The concentration of FAS was 0.048 mg/ml. The concentration of Fraction 4: (▲) 0.048 mg/ml; (♦) 0.14 mg/ml; (★) 0.24 mg/ml. Q1

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time-dependent inhibition of FAS, while condensed tannins do. It had been reported that C75 and cerulenin react on the ketoacyl synthase domain of FAS [11,20]. Therefore, the inhibition of condensed tannins is different from those of the main known FAS inhibitors.

The condensed tannins from catechu inhibit the growth of MCF-7 cells and the effect is related to their inhibition of FAS

As shown in Fig. 3, condensed tannins exhibit strong inhibition of the growth of MCF-7 cells in a dose-dependent manner. The water extract from catechu, which contained 22% condensed tannins in our study, exhibits only a weak inhibition. The abilities of these samples to inhibit MCF-7 cells are positively related to their FAS inhibition activity. It is suggested that the suppression of MCF-7 cells by these samples may result from their inhibition of the enzyme activity of FAS.

Studies have shown that FAS is up-regulated in many kinds of tumors and its function has been strongly linked to the proliferation of tumor cells [9]. This investigation provides a new example for this linkage.

At low concentrations, condensed tannins from catechu act as bioactive substances rather than astringent agents

The competitive inhibition of FAS against acetyl-CoA and NADPH, together with the very low inhibition constants, indicates the strong affinity of condensed tannins with the substrate loading sites on FAS. For the precipitation of FAS by condensed tannins, the required concentration was at least 240 μg/ml. However, the IC_{50} for inhibiting FAS by condensed tannins is about 500-fold lower than that for precipitating FAS. Therefore, at very low concentration, condensed tannins inhibit FAS not due to the general affinity of their hydroxyl groups with peptides but because of their specific inhibition of the enzyme.

The concentrations of condensed tannins (Fraction 4) for inhibiting MCF-7 cells ranged from 40 to 130 μg/ml, which were lower than 240 μg/ml, the concentration for precipitating FAS. Therefore, the effect of condensed tannins on MCF-7 cells has nothing to do with their ability to precipitate FAS. At the proper concentration, condensed tannins exhibit biological activity rather than astringency.

In summary, we found that condensed tannins at low concentration were excellent inhibitors of fatty acid synthase and exhibited significant cytotoxicity against the human breast cancer cell line without protein precipitation. Tannins are naturally occurring plant metabolites widely available in fruits, vegetables, nuts, seeds, and medicinal herbs. Therefore, if tannins are removed from the plant extracts, some important biological effects may be nullified.

Acknowledgments

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