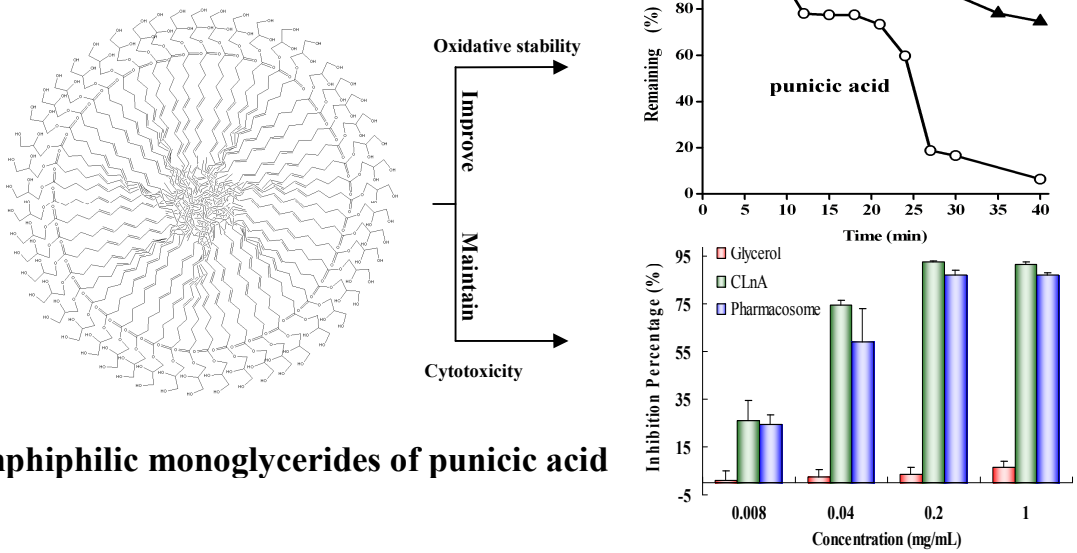
RSC Advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances



amphiphilic monoglycerides of punicic acid

Interest in the biological activity of punicic acid (9-trans, 11-cis,13-trans, octadecatrienoic acid) is growing now. Since punicic acid is extremely susceptible to oxidation, antioxidants were often added to increase its stability. However, sometimes such method resulted in the great decrease of its antitumor activity. In addition, punicic acid is quite insoluble in water and then only a minor fraction can be absorbed. **Therefore, the present study aims to find a method, which can maintain the cytotoxicity when increases the oxidative stability, solubility of punicic acid.** In brief, free punicic acid was modified with glycerol and the amphiphilic monoglycerides of punicic acid was obtained, which was further dispersed to nanodispersions. Oxidative stability, cytotoxicity to NIH3T3 cells and digestion in intestinal juice of such nanodispersions under different conditions were studied. It was found that such nanodispersions showed higher oxidative stability, higher solubility in water. The most important, its cytotoxicity to NIH3T3 cells was also as strong as that of free punicic acid. Since it can be hydrolyzed by pancreatin in intestinal juice, the nanodispersions formed by monoglycerides of punicic acid can be used as nutrient precursor of free punicic acid.

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

Nanodispersions of monoglycerides of punicic acid: a potential nutrient precursor with higher oxidative stability and cytotoxicity

Ying Cao, Lina Wang, Mingli He, Yanhua Zhang and Huajie Wang*

Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX

DOI: 10.1039/b000000x

Punicic acid belongs to one of conjugated linolenic acids isomers (CLnAs) and contains many outstanding functions related to human health, such as anti-carcinogenic, anti-diabetes, anti-hyperlipidemia, anti-obesity and anti-atherosclerotic properties. Interests in the punicic acid are growing now. Since punicic acid is extremely susceptible to oxidation, antioxidants were often added to increase its stability. However, sometimes such method resulted in the great decrease of its antitumor activity. In addition, punicic acid is quite insoluble in water and then only a minor fraction can be absorbed. **Therefore, the present study aims to find a method, which can maintain the cytotoxicity when increases the oxidative stability, solubility of punicic acid.** In brief, free punicic acid was modified with glycerol and the amphiphilic monoglycerides of punicic acid was obtained, which was further dispersed to nanodispersions. Oxidative stability, cytotoxicity to NIH3T3 cells and digestion in intestinal juice of such nanodispersions under different conditions were studied. It was found that such nanodispersions showed higher oxidative stability, higher solubility in water. The most important, its cytotoxicity to NIH3T3 cells was also as strong as that of free punicic acid. Since it can be hydrolyzed by pancreatin in intestinal juice, the nanodispersions formed by monoglycerides of punicic acid can be used as nutrient precursor of free punicic acid.

1. Introduction

Punicic acid (9-trans, 11-cis, 13-trans, conjugated linolenic acid) is contained about 72% in pomegranate seed oil and belongs to one of conjugated linolenic acids isomers (CLnA).[1] It was found that punicic acid and other CLnA isomers, such as α-eleostearic acid (9-cis, 11-trans, 13-trans-CLnA), catalpic acid (9-trans, 11-trans, 13-cis-CLnA), calendic acid (8-trans, 10-trans, 12-cis-CLnA) all contain many outstanding functions related to human health, such as anti-carcinogenic, anti-diabetes, anti-hyperlipidemia, anti-obesity and anti-atherosclerotic properties both *in vitro* and *in vivo*.[2-5]

For example, Suzuki et al reported that α-eleostearic acid and punicic acid showed much higher cytotoxicity.[6] Arao et al. reported the hypolipidemic effect of punicic acid in human liver derived HepG2 cells.[7] Punicic acid induced obesity and insulin resistance in mice, independent of changes in food intake or energy expenditure.[8] Punicic acid can also alleviate hepatic triacylglycerol accumulation in obese, hyperlipidemic OLETF rats.[9] Pomegranate seed oil decreases weight gain and type 2 diabetes risk in CD-1 mice.[10] In a word, punicic acid has already gained increased attentions now.

However, there are two main problems in the studies of punicic acid. The first, the highly unsaturated structure, conjugated triene system, makes it extremely susceptible to oxidation, light or thermal treatments. For example, we previously found that punicic acid could be completely oxidized after exposure to air for 30 min at 50 □.[11] Therefore, it becomes very difficult for the storage and carriage of punicic acid products.

To solve this problem, many methods were used, including mixing with edible oils and fats, microencapsulation, cyclodextrin inclusion, addition of antioxidants, etc.[12,13] However, the protective effects of the first three methods were not very well and the encapsulation rate was also low. For the method of addition of antioxidants, although the oxidative stability increases greatly, the antitumor activity of conjugated fatty acid decreased sharply sometimes.[14] Therefore, it is very important to find a more appropriate method, which can maintain the biological activity of punicic acid and also increase its oxidative stability at the same time.

The second problem is, punicic acid is quite insoluble in water and then only a minor fraction can be absorbed. Owing to this characteristic, the bioavailability of punicic acid is low and the range of application is also limited.

Recently, many kinds of micro or nano delivery systems, including liposome, solid lipid nanoparticles, noisome, polymeric micelles and inorganic drug carrier etc. were designed and established. Nutrients or drugs with high unstability and high lipid solubility can be carried and protected by such delivery systems. For example, astaxanthin nanodispersions increase the dissolution rate and saturation solubility due to a reduced size and increased surface area.[15] In a word, with this modern nano carrier technology, solubility, stability, and bioavailability of bioactive molecule can be considerably improved. These nano delivery systems were established on the basis of the amphiphilic molecules, such as phospholipid, amphiphilic block copolymer, modified protein, polysaccharose and so on.

Inspired by the delivery systems and carrier materials, an amphiphilic monoglycerides of punicic acid was synthesized as a

precursor, which was then subsequently dispersed to nano scale in water. The physicochemical structures, oxidative stability, micro-morphology, cytotoxicity to NIH3T3 cells and digestibility in vitro of both monoglycerides of punicic acid and nanodispersions were observed in this study.

The present study had two objectives. The first is to increase the oxidative stability of punicic acid by establish the nanodispersions on the basis of amphiphilic monoglycerides of punicic acid. The second is to estimate the cytotoxicity to NIH3T3 cells of nanodispersions. The possibility of nanodispersions used as a nutrient precursor was also discussed.

2. Material and methods

2.1 Materials

Punicic acid (purity≥90%) was purified from pomegranate seed according to our previous work.[16] The mouse embryonic fibroblast cell line NIH3T3 was purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. Pancreatin (USP grade) was purchased from Aladdin. All other solvents and chemicals were analytical grade. TLC plate is 20×20 cm (Macherey-Naged, Duren, Germany).

2.2 Synthesis and purification of monoglycerides of punicic acid

Punicic acid acyl chloride was firstly prepared by treating punicic acid with molar equivalent of thionyl chloride in the presence of pyridine under N_2 protection. Then the obtained punicic acid acyl chloride (10 mmol) and molecular sieve (1 g) were added into acetone containing glycerol (50 mmol). After the mixture was kept static at 45 □for 4 h under N_2 protection, the crude product was obtained by a reduced pressure rotary evaporator. After washing with 1 mol/L HCL and then distilled water to neutrality, the crude product was purified by TLC using petroleum ether/ethyl ether/methanol (4:2:0.5) and stored at -20 □ for future use.

2.3 Preparation of nanodispersions of monoglycerides of punicic acid

20 mg/ml of the monoglycerides of punicic acid ethanol solution (0.1 ml) was quickly injected into 1.9 ml of distilled water (or cell nutrient solution) under high speed stirring. Nanodispersions of monoglycerides of punicic acid were prepared by mixing the O/W emulsion in a high-speed disperser (HSD) followed by passing of ultrasonic waves by ultrasonic processor (Biosafer 400UP, 80W). The temperature throughout the process of ultrasonication was maintained at 10 □. After ultrasound treatment for 20 min, the solution was filtrated by 0.22 um microfiltration membrane.

2.4 Physicochemical characterization of monoglycerides of punicic acid

2.4.1 Fourier transform infrared spectra

For FT-IR analysis, the purified monoglycerides of punicic acid was mixed with KBr powder. It was then pressed into pellet directly for FTIR determination (Bio-Rad FTS-40 Fourier transform infrared spectrograph) in the wavenumber range of 4000-400 cm^{-1}. The spectra were collected at 2 cm^{-1} resolution with 128 scans.

2.4.2 Nuclear magnetic resonance analysis

NMR experiment was recorded on a Bruker AV-400 Fourier transform NMR spectrometer (Bruker, Fallanden, Switzerland). Tetramethylsilane (TMS, 0.2-0.3 mM) in deuteriochloroform was used as an internal reference standard. Chemical shifts were given in δ-values in ppm downfield from TMS (δ_{TMS}=0). ^{1}H-NMR was conducted to assign the chemical shifts of proton of samples.[17]

2.4.3 UV spectra analysis

The ultraviolet–visible absorption property of monoglycerides of punicic acid and its nanodispersion in water were measured by an UV-Vis spectrophotometer (TU-11900, Beijing Purkinje General Instrument Co., Ltd., China) with the scope of wavelength ranging from 200 nm to 400 nm.

2.5 Oxidation of monoglycerides of punicic acid in air.

To evaluate the modification of glycerol to the oxidative stability of punicic acid, we oxidized the monoglycerides of punicic acid in air at 50 ℃. The remaining amounts of monoglycerides of punicic acid were analyzed by UV analysis. The free punicic acid with the same molar concentration was used as the control. Briefly, 1.5 mg monoglycerides of punicic acid was dissolved in 6 ml of the water/ethanol solution. 300 μL of such solution (75 μg monoglycerides of punicic acid) were dropped into 20 test tubes. After drying by N_2 termovap sample concentrator, the samples were oxidized in air at 50 ℃. At the given time intervals, 2 mL ethanol was added and was then taken to UV analysis. The UV adsorption of unoxidized punicic acid at 273 nm was measured and the amount of punicic acid was quantified according to the standard curve of pure punicic acid solution. All the experiments were performed in triplicates.[18]

2.6 Morphology observation of nanodispersions

Morphologies of monoglycerides of punicic acid nanodispersions were observed by HRTEM (JEOL-100CX, JEOL, Japan). Briefly, droplet of dispersions (5 μL) was dripped onto a holey carbon-coated formvar support and observed by HRTEM. In addition, the mean nanodispersions droplet sizes were determined by using the laser light-scattering method with a Zetasizer 2000 (Malvern Instruments, Worcestershire, UK).

2.7 Oxidation of nanodispersions in cell nutrient solution

It is already found that the anticancer mechanism of punicic acid is associated with its high unstability. Since the monoglycerides of punicic acid was firstly dispersed to nano scales and then cultured with the NIH3T3 cells, it was also necessary to measure the oxidative stability of nanodispersions in cell nutrient solution except in air. In brief, nanodispersions (0.1 mg/mL in cell nutrient solution) were dropped into 20 test tubes (1mL per tubes). These test tubes were oxidized under continuous shocking at 37 ℃. At the given intervals, one tube was taken and 3 mL acetic ether was added. After centrifugation (4000 rpm/min), the top acetic ether layer was saved and subjected to UV analysis. The amount of punicic acid was quantified according to the standard curve of pure punicic acid solution.

2.8 Survivability of nanodispersions to the simulated intestinal fluid

Whether used as a nutrient precursor or a potential carrier, it was

very important to understand the survivability of nanodispersions to digestive enzyme. The simulated intestinal fluid (SIF) was then prepared and mixed with the nanodispersions. The remaining amount of monoglycerides of punicic acid and the free punicic acid hydrolyzed by enzyme were analyzed by HPLC.

In brief, SIF was prepared as follows: monopotassium phosphate (6.8 g) was dissolved in 500 mL water and the PH was adjusted to 6.8 by 0.2 M NaOH. After dissolving the pancreatin (10 g) in moderate water, monopotassium phosphate and pancreatin solution were then mixed and diluted with water to 1000 mL. The nanodispersions of monoglycerides of punicic acid (0.2 mg/mL in SIF) were dropped into several test tubes (1mL per tubes). These test tubes were shaken at 37 ℃ and one test tube was taken at the given time intervals. 1 mL 0.5M HCl and 1 mL acetic ether were then added to terminate the digestion reaction. After centrifugation (3500 rpm/min), the top acetic ether layer was saved and subjected to HPLC analysis (Agilent-1260 HPLC). The separated monoglycerides of punicic acid and free punicic acid was monitored at 273 nm. A RP-HPLC was performed at 27 ℃ using a ZORBAX SB-C18 (150×2.1 mm i.d., 1.8 μm, Agilent, USA). A gradient system with the mobile phase consisting of A (0.1% formic acid in H_2O) and B (methanol) was chosen at a flow rate of 200 μL/min. The gradient programme used was: initial 30% A and 70% B; linear gradient 100% B in 10 min; hold for 10 min, return to initial conditions in 1 min, followed by equilibration for 5 min. Free punicic acid and monoglycerides of punicic acid eluted at 14.39 and 15.24 min, respectively.

2.9 Cytotoxicity of nanodispersions

2.9.1 Exposure of NIH3T3 cells to nanodispersions.

Mouse embryonic fibroblast cell line NIH3T3 was routinely cultured in tissue culture flasks with RPMI-1640 medium, containing 10% fetal bovine serum and incubated at 37 ℃ in a humidified atmosphere with 95% air and 5% CO_2. The culture medium was refreshed every two days. When the cells became almost confluent after 5 days, they were released by treatment with 0.25% trypsin. Then the cells were counted to 104 cells/cm^2 and 200 μL of the cells suspension was pippetted into 96-well tissue culture plate.[19] After 12 h of culture, the medium was replaced with the fresh RPMI-1640 medium containing 0.008-1 mg/ml of nanodispersions of monoglycerides of punicic acid. In addition, the normal group without any additive was chosen as the control group.

2.9.2 Cell viability

The metabolic analysis was performed by a 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyl tetrazolium bromide assay (MTT) based on succinic dehydrogenase activity at OD 490 nm (n=3); 630 nm was chosen as the reference wavelength.[20] Briefly, after 48 h of cell culture, the wells were carefully washed with PBS. 20 μL of MTT was added in 180 μL of culture medium, and cell culture was continued for another 4 h. Then the solution was removed and the wells were washed twice with PBS. 200 μL of dimethyl sulfoxide was pipetted into each well and optical density (OD) values were read on a microplate reader (Multiskan MK3, Thermo Labsystems, USA). The inhibition rate of heavy metal sulfides on cells was calculated as follows:

Inhibition rate=(ODc-ODt)/ODc ×100%

In this equation, the ODc and ODt represent the OD 490 nm values of control group and the treatment group, respectively.

2.9.3 Cell proliferation

The number and distribution of attached cells on plate treated with different additives were measured by the cell counting method. Briefly, after 48 h of cell culture, all substrates were clearly rinsed in phosphate buffered solution (PBS, pH 7.2, 0.1 mol/L) and then the cells were counted on the microscope (Axioskop 40, ZEISS, Germany).

2.10 Statistical analysis

The number of independent replica was listed individually for each experiment. Where applicable, all data were mean ± SD. The analysis of data for cell proliferation was performed by one-way factorial analysis of variances (ANOVA) and multiple comparisons (Fisher's method as Post-Hoc test, $p<0.05$).[21]

3. Results and discussion

3.1 Synthesis and characteristics of monoglycerides of punicic acid

The structure of the as-prepared sample was characterized by FTIR, NMR and UV spectra, respectively. Fig. 1 shows the FTIR spectra of punicic acid, glycerol and monoglycerides of punicic acid.

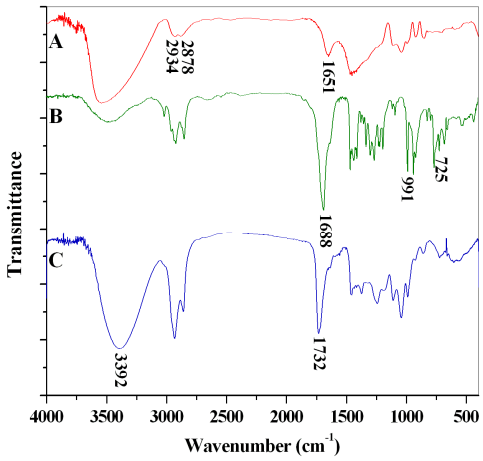


Fig. 1 FTIR spectra of glycerol (A), punicic acid (B) and monoglycerides of punicic acid(C).

The corresponding infrared adsorptions arising from the functional groups in samples are shown in Table 1. The characteristic peak of hydroxide group in glycerol appears at 1038 cm^{-1}. Punicic acid shows the characteristic peaks of carboxyl at about 1688 cm^{-1}. Moreover, the typical adsorption peaks of conjugated double bond appear at 991 and 725 cm^{-1}, respectively. By comparison with the spectrum of punicic acid, the disappearance of carboxyl at 1688 cm^{-1} and the appearance of ester bond stretching peak at 1732 cm^{-1} could demonstrate the occurrence of condensation reaction between glycerol and punicic acid.

Table 1. Some of the infrared band assignments of glycerol, punicic acid and monoglycerides of punicic acid

Frequency (cm^{-1})	Functional group and mode of vibration	glycerol	punicic acid	monoglycerides of punicic acid
725, 991	Conjugated =CH bending	-	+	+
1038	-O-H stretching	+	-	+
1463	-CH (-CH_2-, CH_3) bending	+	+	+
1688	-COOH stretching	-	+	-
1732	-C=O ester Fermi resonance	-	-	+
2857-2934	CH (-CH_2-) stretching	+	+	+

Fig. 2 indicates the ^{1}H-NMR spectrum of monoglycerides of punicic acid. It shows signals at δ_H 5.0-7.0 ppm, which correspond to the shifts of olefinic protons. The signals at δ_H 3.894-3.945 ppm can be assigned to chemical shifts of protons in the ester bonds, suggesting the successful condensation between glycerol and punicic acid. In addition, it is clear that both hydroxyl protons appear at δ_H 2.319 ppm (-CH_2OH-) and δ_H 4.113-4.205 ppm (-CHOH-), respectively. It proved the formation of monoglycerides of punicic acid.

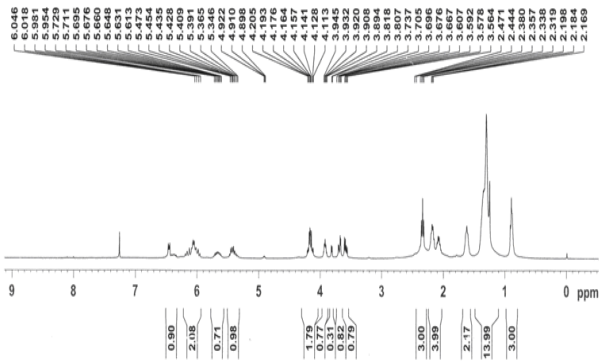


Fig. 2 ^{1}H-NMR of the monoglycerides of punicic acid.

Additionally, the UV spectra of punicic acid and monoglycerides of punicic acid nanodispersions in water were compared as shown in Fig. 3A. Punicic acid exhibits UV adsorption peaks at 263 nm, 273 nm and 284 nm, respectively. It is notable that the UV adsorption properties of conjugated ester bonds in monoglycerides of punicic acid are not affected after being dispersed to nanoscale.

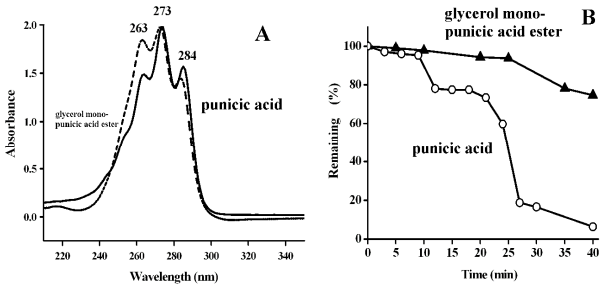


Fig. 3 (A) UV spectra of punicic acid and monoglycerides of punicic acid; (B) Oxidative stability comparison of free punicic acid (closed circles) and monoglycerides of punicic acid (open triangles).

3.2 Morphology of nanodispersions

The morphology and mean particle sizes of dispersions from glycerol mono-punicic acid in water were observed by HRTEM and laser light-scattering method (Fig. 4). It can be seen that monoglycerides of punicic acid nanodispersions are granular and have about 23 nm size in diameter.

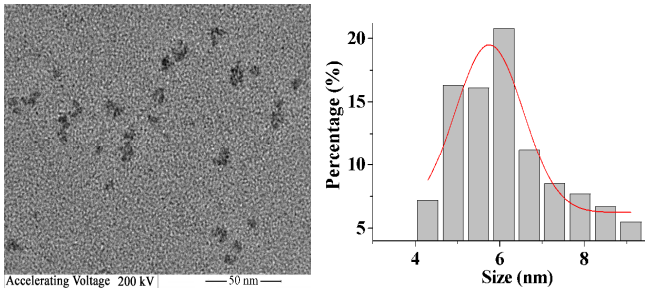


Fig.4 HRTEM (A) of the corresponding particle size distribution (B) of monoglycerides of punicic acid.

In view of the amphipathicity of glycerol mono-punicic acid, the possible structure of such global nanoparticle was speculated in Fig.5. We speculate the long hydrophobic alkyl tail with conjugated triene system faces inward and the glycerol polar hydroxyl group head stretches outside in water (Fig. 5). The whole system then can form a spherical particle in nano scales. It looks that the long hydrophobic alkyl tail aggregates together and is protected by the glycerol polar hydroxyl group.

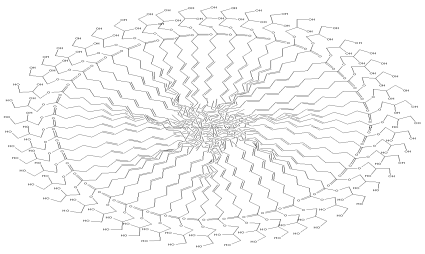


Fig. 5 The possible structure of monoglycerides of punicic acid nanoparticle in water.

3.3 Stability of monoglycerides of punicic acid in air at 50 ℃

In order to assess the effects of glycerol modification on the oxidative stability of punicic acid, monoglycerides of punicic acid was oxidized in air at 50 ℃ and the free punicic acid was used as the control. It was found that under the present oxidative condition (in air at 50℃), free punicic acid is unstable. About 40.3% of free punicic acid was lost for 25 min (Fig. 3B). The percentage of the oxidized punicic acid got to 93.3% only when oxidizing for 36 min. After the modification of glycerol, the oxidative stability of conjugated triene stucture is significantly improved comparatively. It can be seen that only 6.3% and 25.3% of monoglycerides of punicic acid were oxidized in 25 min and 40 min, respectively (Fig. 3B). That was, the formation of the ester group based on the modification of glycerol increased the oxidative stability of conjugated triene system of punicici acid greatly.

3.4 Responses of NIH3T3 cells to nanodispersions

In this study, we exposed mouse embryonic fibroblast cell line NIH3T3 to nanodispersions of monoglycerides of punicic acid. Cells responses were then studied by metabolic and morphological methods. Fig. 6 shows the inhibition rate of glycerol, punicic acid and monoglycerides of punicic acid nanodispersions on the metabolism of NIH3T3 cells measured by the MTT method, which is associated with the function of mitochondria. It was found glycerol has a slightly inhibition effect on the metabolism of NIH3T3 cells. The inhibition rate of glycerol increases with the increase of sample content, but the maximum value is only about 6.6% when the sample content gets to 1 mg/ml. The IC50 is calculated as the killing concentration inducing 50% inhibition. As for glycerol, the IC50 for NIH3T3 cells is 8896.0 mg/ml, suggesting almost no toxicity. After exposure to punicic acid or monoglycerides of punicic acid nanodispersions, the metabolism of NIH3T3 cells is significantly inhibited. The IC50 value reaches 134.7 μg/ml and 167.1 μg/ml, respectively. Moreover, almost a complete inhibition on the metabolism of NIH3T3 cells can be observed when the content of punicic acid or monoglycerides of punicic acid nanodispersions gets to 0.2 mg/mL.

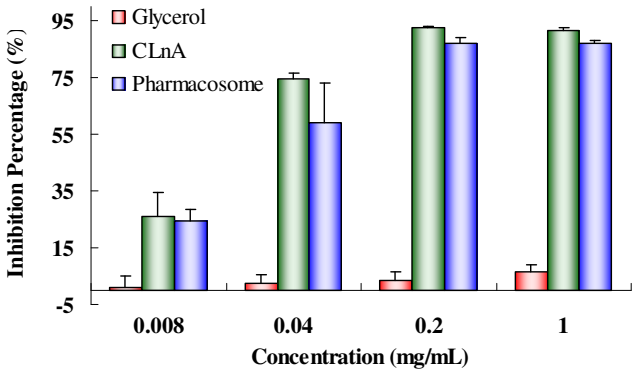


Fig. 6 The inhibition rate of glycerol, punicic acid and monoglycerides of punicic acid nanodispersions on the metabolic activity of NIH3T3 cells measured by the MTT method based on sucoino dehydrogenase activity.

The distribution and morphology of cells were observed by an optical microscope. Fig. 7 shows the number and distribution of NIH3T3 cells after exposure to glycerol, punicic acid and monoglycerides of punicic acid nanodispersions for 48 h, respectively. The cells grow well in the absence of any additive and the cell number is the most compared with that in other groups (Fig. 7A). Besides, cells develop a spindle like morphology and produce long neuritis. In the presence of glycerol, the cells morphology has no significant change (Fig. 7B). But when the glycerol content reaches 0.2 mg/ml, the cells number slightly decreases (Fig. 7B_3). As for punicic acid and monoglycerides of punicic acid nanodispersions, both the number and morphology of cells obviously change (Fig. 7C and 7D). Generally speaking, the cells number gradually decreases with the increase of the additive content and only several cells could be observed when the content gets to 0.2 mg/ml. Besides, cells contract and form a round shape after exposure to punicic acid or monoglycerides of punicic acid nanodispersions for 48 h, respectively. Contractive cells also gradually increase with the increase of the sample concentration. However, there are no great difference between punicic acid group and monoglycerides of punicic acid nanodispersions group.

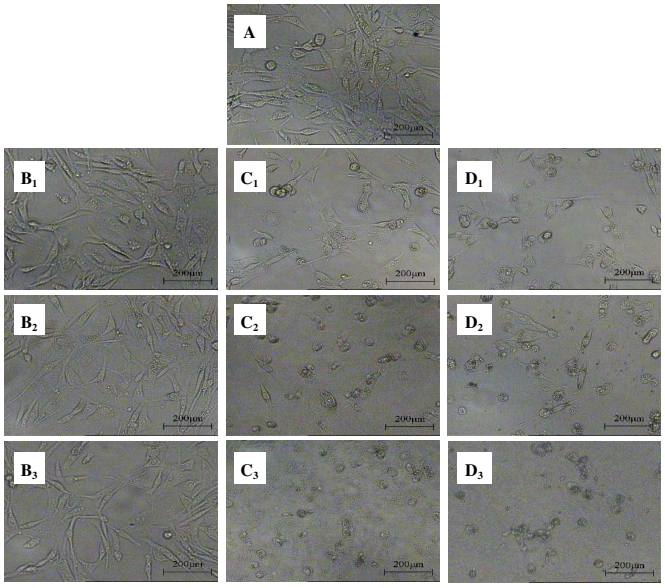


Fig. 7 The morphology and distribution of NIH3T3 cells after exposure to control (A), glycerol (B), punicic acid (C) and monoglycerides of punicic acid nanodispersions (D) with different concentrations for 2 days. Concentration "1": 8 μg/ml; "2": 40 μg/ml and "3": 200 μg/ml.

3.5 Stability of nanodispersions in cell nutrient solution and simulated intestinal fluid at 37 ℃

Since both nanodispersions and free punicic acid had special effects on cytotoxicity in solutions but not in air, it was necessary then to study their oxidative stability in cell nutrient solution at 37 ℃. It was found that under the present condition, oxidative speed of free punicic acid was not very fast, about 54.24 % of free punicic acid was lost for 38 h (Fig. 8). It looked

that the temperature had great effects on its oxidation speed. In addition, after modification and dispersion into nanoscales, the nanodispersions showed higher stability than that of free punicic acid. It was found that only 25% monoglycerides of punicic acid was lost under the same condition. That was, the nanodispersions can also protect conjugated triene system from oxidation. According to its microscopic structure described above, we deduced the possible reason was as below: the glycerol polar hydroxyl group of monoglycerides of punicic acid can stand outside in water and then protect partly long hydrophobic alkyl tail containing conjugated triene system inside the nano particles.

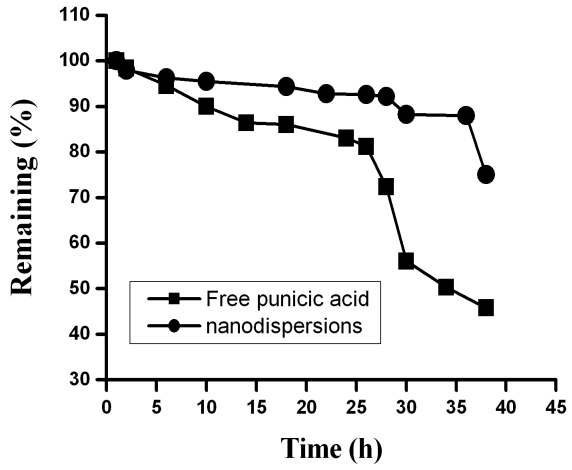


Fig. 8 Oxidative stability comparison of free punicic acid (closed square) and nanodispersions (closed circles)

As one kind of polyunsaturated fatty acids, it was important to realize the characteristics of digestion and absorption of punicic acid and its modification products. In this study, their digestion in simulated intestinal fluid was also studied. It was found the nanodispersions of monoglycerides of punicic acid can be hydrolyzed by pancreatin to free punicic acid. It took about 2 hours to hydrolyze 0.2 mg monoglycerides of punicic acid completely (Fig. 9 水解).

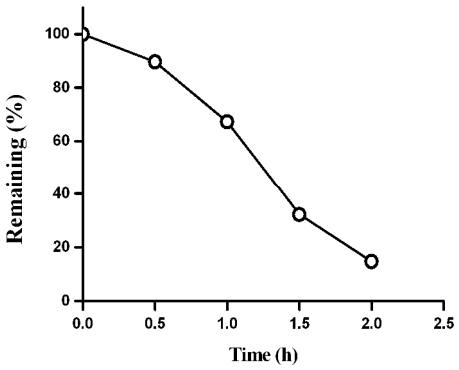


Fig. 9 The remaining amount of monoglycerides of punicic acid in the simulated intestinal fluid

According to the present results, both monoglycerides of punicic acid and its nanodispersions were more stable than free punicic acid in air and solution. Such a modification method then can provide a new way of protection and will be very helpful for its production, storage, carriage etc. Since the monoglycerides of punicic acid can be hydrolyzed to free punicic acid by intestinal juice, it then can be used as a precursor of free punicic acid.

Since hydrophilic glycerol exhibited a slight inhibition on the metabolism of NIH3T3 cells in a dose-dependent mode and had no effect on the cells' spread under the tested concentrations (Fig. 5 and Fig. 6), it was chosen to modify the punicic acid. As the increase of oxidative stability, the cytotoxicity of nanodispersions to NIH3T3 cell was not weakened. The possible reasons perhaps were as below: (i) the interaction of nanodispersions with cell membrane is different from free punicic acid, considering its nano particle structure and two hydroxyl groups; (ii) the bioavailability of nanodispersions increased as the increase of solubility in body fluid.

Conclusions

In a word, the oxidative stability of free punicic acid increased by modifying the carboxyl using glycerol. The obtained amphipathic monoglycerides of punicic acid can be dispersed and form a kind of nanodispersions. Such nanodispersions showed higher oxidative stability, higher solubility in water and also can be hydrolyzed by pancreatin. Therefore, this nanodispersions and monoglycerides of punicic acid can be used as precursor of free punicic acid.

In addition, the most important was, the cytotoxicity of such precursor to NIH3T3 cells was also as strong as that of free punicic acid. The present study finds one way which can increase the oxidative stability and also maintain the cytotoxicity simultaneously. Consequently, the biological activity of monoglycerides of punicic acid nanodispersion *in vivo* is worth expecting.

Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (31000774 and 20971039) and the Program for Innovative Research Team in University of Henan Province (2012IRTSTHN006) and Program for Chang-jiang Scholars and Innovative Research Team in University (IRT1061). Additionally, we also thank Xinxiang R&J Chemistry and Biotechnology Com. Ltd. for their help at the biological activity analysis.

Notes and references

Collaborative Innovation Center of Henan Province for Green Manufacturing of Fine Chemicals, Key Laboratory of Green Chemical Media and Reactions, Ministry of Education, School of Chemistry and Chemical Engineering, Henan Normal University, Xinxiang, Henan 453007, P. R. China. Fax: +86-373-3328507; Tel: +86-373-3326335; E-mail:wanghuajie972001@163.com

† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

‡ Footnotes should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

1 H. Abbasi, K. Rezaei and L. Rashidi, *Journal of the American Oil Chemists'Society*, 2008, **85**, 83-89.

2 Y. Cao, J. Chen, L. Yang and Z. Y. Chen, *J. Nutr. Biochem.*, 2009, **20**, 685-693.

3 J. Chen, Y. Cao, H. Gao, L. Yang and Z. Y. Chen, *J. Chem. Phys. Lipids*, 2007, **150**, 136-142.

4 Y. Yasui, M. Hosokawa, H. Kohno, T. Tanaka and K. Miyashita, *Anticancer Res.*, 2006, **26**, 1855-1860.

5 K. Koba, J. Imamura, A. Akashoshi, J. Kohno-Murase, S. Nishizono, M. Iwabuchi, K. Tanaka and M. Sugano, *J. Agric. Food Chem.*, 2007, **55**, 3741-3748.

6 R. Suzuki, R. Noguchi, T. Ota, M. Abe, K. Miyashita, T. Kawada, *Lipids*, 2001, **36**, 477-482.

7 K. Arao, H. Yotsumoto, S.Y. Han, K. Nagao and T. Yanagita, *Biosci Biotechnol Biochem*, 2004, **68**, 395-397.

8 Irene O. C. M. Vroegrijk, J. A. Diepen, S. Berg, I. Westbroek and H. Keizer, *Food and Chemical Toxicology*, 2011, **49**, 1426-1430.

9 K. Arao, Y. M. Wang, N. Inoue, J. Hirata, J. Y. Cha, K. Nagao and T. Yanagita, *Lipids in Health and Disease*, 2004, **3**, 24.

10 B. K. McFarlin, K. A. Strohacker and M. L. Kueht, *Br. J. Nutr.*, 2009. **102**, 54-59.

11 L. Yang, Y. Cao, J. N. Chen and Z. Y. Chen, *J Agric. Food Chem.*, 2009, **57**, 4212-4217.

12 I. Lalush, H. Bar, I. Zakaria, S. Eichler and E. Shimoni, *Biomacromolecules*, 2005, **6**, 121-130.

13 C. W. Park, S. J. Kim, S. J. Park, J. H. Kim, J. K. Kim, G. B. Park, J. O. Kim and Y. L. HA, *Journal of Agricultural and Food Chemistry*, 2002, **50**, 2977-2983.

14 P. Bougnoux, N. Hajjaji, K. Maheo, C. Couet and S. Chevalier, *Progress in Lipid Research*, 2010, **49**, 76-86.

15 N. Anarjan, I. A. Nehdi and C. P. Tan, *Chemistry Central Journal*, 2013, **7**, 127.

16 Y. Cao, L. Yang, H. L. Gao, J. N. Chen, Z. Y. Chen and Q. S. Ren, *J Chem. Phys. Lipids*, 2007, **145**, 128-133.

17 H. J. Wang, Y. Cao, C. Cao, Y. Y. Sun, X. H. Yu, L. F. Zhu and L. Yang, *ACS Appl. Mater. Interfaces*, 2011, **3**, 2755-2763.

18 Y. Cao, M. L. He, Y. H. Zhang and H. J. Wang, *Micro. Nano. Letters*, 2011, **6**, 874-877.

19 H. J. Wang, X. H. Yu, Y. Cao, B. Zhou and C. F. Wang, *J. Inorg. Biochem.*, 2012, **113**, 40-46.

20 M. C. Alley, D. A. Scudiero, A. Monks, M. L. Hursey, M. J. Czerwinski, D. L. Fine, B. J. Abbott, J. G. Mayo, R. H. Shoemaker and M. R. Royd, *J. Cancer Res.*, 1988, **48**, 589-601.

21 Y. Cao, H. J. Wang, C. Cao, Y. Y. Sun, L. Yang, B. Q. Wang and J. G. Zhou, *J. Nanopart. Res.*, 2011, **13**, 2759-2767.