Electronic Supplementary Material (ESI) for Green Chemistry.

Sono-transformation of tannic acid into biofunctional ellagic acid micro/nanocrystals with distinct morphologies

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Contents:

1. Experimental Procedures

Materials

Tannic acid (TA), gallic acid, ellagic acid (EA), phosphoric acid solution (35 wt% in H_2O), DMSO, 1-diphenyl-2-picrylhydrazyl (DPPH), hydrogen peroxide (35 wt%) and3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Aldrich. Di-sodium hydrogen orthophosphate (98%) and sodium dihydrogen orthophosphate (99%) were purchased from Chem-Supply; iron(II) chloride tetrahydrate was purchased from Fluka, hydroquinone was purchased from BDH AnalaR®, HPLC grade methanol (99.9%) was purchased from Fisher chemicals. Sulphuric acid (95-97%) was purchased from Scharlau, hydrochloric acid (36%) and pyridines were purchased from Univar. Doxorubicin hydrochloride (~99%) was purchased from OChem Inc. (USA). All solutions were prepared in high purity water extracted from a Millipore system with resistivity of 18.2 MΩ/cm

Sonication of TA: The sonication of 1 mM tannic acid solution prepared in PBS of pH 7 was carried out in a sealed glass vial. The glass vial was inserted into 200 ml water contained in a double walled glass cell, the base of which was mounted on a stainless steel ultrasonic plate transducer of diameter 5.4 cm. The plate transducer used was ELAC Nautik USW 51-052 transducer which was powered by a T&C Power Conversion, Inc. The unit was operated at 355 kHz and 1058 kHz frequencies and at different ultrasonic power levels from 5.5 W/cm^3 to 20 W/cm^3). All reactions were performed at constant temperature of 37 ± 2 ˚C.

Characterisation

Fluorescence spectroscopy: Fluorescence spectra were obtained using a Shimadzu RF-5301PC fluorescence spectrophotometer equipped with a xenon lamp and 1.0 cm optical length quartz cell. All the measurements were carried out in PBS buffer (pH 7). Samples were excited at different wavelengths (from 320 nm to 460 nm) and the fluorescence spectra were recorded from 340 to 600 nm using slit widths of 5 nm for excitation and 10 nm for emission spectra. UV- visible absorption spectra were recorded on a Cary Bio 50 UV-spectrophotometer

HPLC: Shimadzu SCL-10AVP high-performance liquid chromatography (HPLC) equipped with a Phenomenex column model “Jupiter 5u C18 300A” and with an UV detector set at 280 nm was used to perform HPLC analysis. All chromatograms were generated by LabSolution software (Shimadzu). The injection volume was 20 µl and the flow-rate used was 1 mL/min.

For the analysis, a gradient elution was carried out with solvent A (0.5 % phosphoric acid) and solvent B (methanol with 0.5% phosphoric acid). The gradient used was 10% solvent B from 0 -5 min, then increased to 50 % solvent B from 5-10 min and then 100 % solvent B from 10-30 min.

NMR: ^{1}H and ^{13}C NMR spectra of TA and sonicated TA particles was conducted in d_6-DMSO on Varian MR400 NMR spectrometer at 400 MHz at 25 ˚C.

SEM: The morphology of the particles was examined by high-resolution field emission environmental Scanning Electron Microscope (Quanta 200 FEI). The particles were sputter-coated with the thin layer of gold during the sample preparation.

TGA: Thermogravimetric analysis was carried out by TGA/SDTA8511e (Mettler Toledo). The analysis was conducted under the nitrogen flow at a heating rate of 10˚C/min within

Electrospray Mass spectrometry (ESMS): The ESMS analyses were performed using an Agilent 6500 Q-TOF LC/MS system. Scans were performed in both negative and positive ion modes. Also, MALDI-TOF analysis was carried out. The sample (10 mg/ml) was mixed with the matrix solution (2,5-Dihydroxybenzoic acid) and cationization reagent (Sodium trifluoroacetate) in 10:1:1 ratio. The matrix and the cationization reagent used were 2, 5-dihydroxybenzoic acid and sodium trifluoroacetate, respectively.

Powder X-ray diffraction (PXRD): The PXRD analysis was performed by Bruker D8 Advance X-ray diffractometer with Ni-filtered Cu kα radiation (1.54 Å). Data were collected between 5 –85° 2θ, with a step size of 0.02° and a scan rate of 1.0 s per step. An anti-scatter blade was used to reduce the diffracted background intensity at low angles. An incident beam divergence of 1.0° was used with a 2.5° soller slit in the diffracted beam. The sample was spun at 15 revolutions per minute.

DPPH Assay: The antioxidant activity of TA, TAS and EA was estimated from their radical scavenging activity. The 1-diphenyl-2-picrylhydrazyl radical (DPPH) was utilised for this purpose.

To 3 ml of 250 μM solution of DPPH were added different concentrations of EA, TA and TAS solutions. The absorbance was noted at 520 nm as function of time. The radical scavenging activity was determined using following expression:

$$Radical\ scavenging\ activity\ (\%) = \left[\left(A_{DPPH} - A_{sample}\right)/A_{DPPH}\right] \times 100$$

Where A_{DPPH} is the absorbance of blank DPPH solution and A_{sample} is the absorbance of DPPH solution after the addition of sample.

Cell Viability Assay: The cytotoxicity of the TA and TAS was estimated using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay.MDA-MB-231 (human breast adenocarcinoma) were plated on 96-well plates (Costar 3596, Corning, MA, USA) with seeding density of 7000 cells per well in 100 μL of DMEM medium supplemented with 10% fetal bovine serum (FBS) and 1% of Penicillin/ Streptomycin antibiotic mixture. Cells were incubated overnight in 37°C, 5% CO_2. Particles were added to culture media at various concentrations in quadruplicates followed by incubation for 24h. Cell viability was assessed by the addition of 10 μL of 5 mg/mL MTT reagent and further incubation for 4 hours. Resulted formazan crystals were dissolved in 70 μL DMSO and absorbance was measured at 554 nm and 670 nm by Infinite M200 microplate reader (Tecan, Switzerland).

2. HPLC and Mass spectrometry of TA sonicated solution

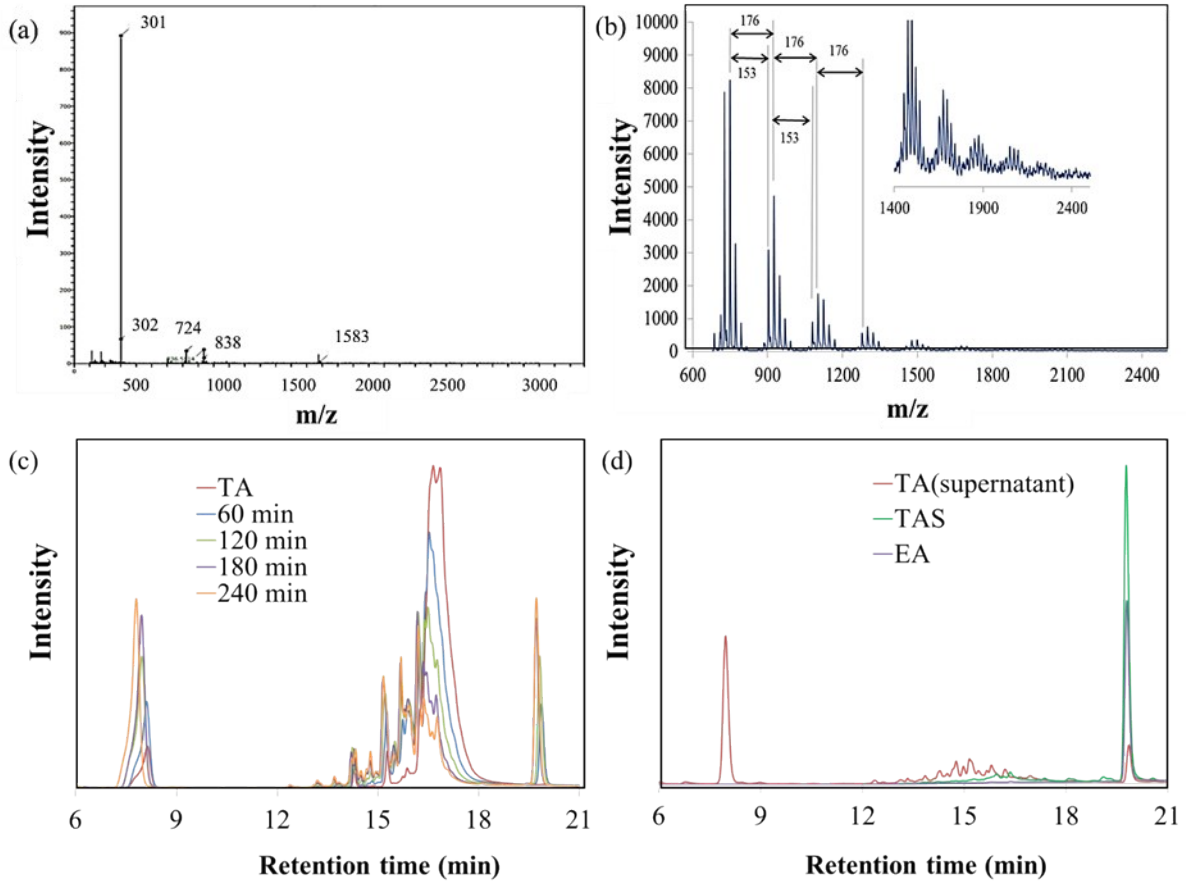

Figure S1. Analysis of sonicated TA solution and TAS particles using Mass spectroscopy and HPLC (a) ESMS analysis of TAS particles (b) MALDI-ToF spectra of TAS particles: to identify the minor high molecular weight component of microparticles, MALDI-ToF analysis was performed, which revealed the formation of some high molecular weight products with repeating unit of m/z 153 and 176 corresponding to galloyl moiety and galloyl + Na (Supplementary Fig 1b), respectively. (c) HPLC analysis of sonicated TA solution after different sonication times at 355 kHz and 5.5 W/cm^3 and (d) HPLC profile of standard EA, TAS particles and supernatant.

HPLC chromatograms indicate:

(i) The disappearance of the peak assigned to TA at 17. 5 min as a function of sonication time and the appearance of gallic acid peak at retention time 8 min which increases with an increase in sonication time (Figure S1c). This indicates the ultrasonic conversion of TA into gallic acid by hydrolysis of the ester linkages,

(ii) The appearance of a peak at 20 min which increases with an increase in sonication time. This peak could be attributed to EA as the standard EA also has a similar HPLC profile as shown in Figure 1c. This clearly suggests that major component of the particles generated by sonication was ellagic acid.

(iii) The presence of some other species in the supernatant, eluted between 12-18 min was also observed (Figure S1d), could be attributed to soluble ellagic acid derivatives.

3. UV-vis spectra of sonicated TA solution at different sonication times and of TA, TAS and EA

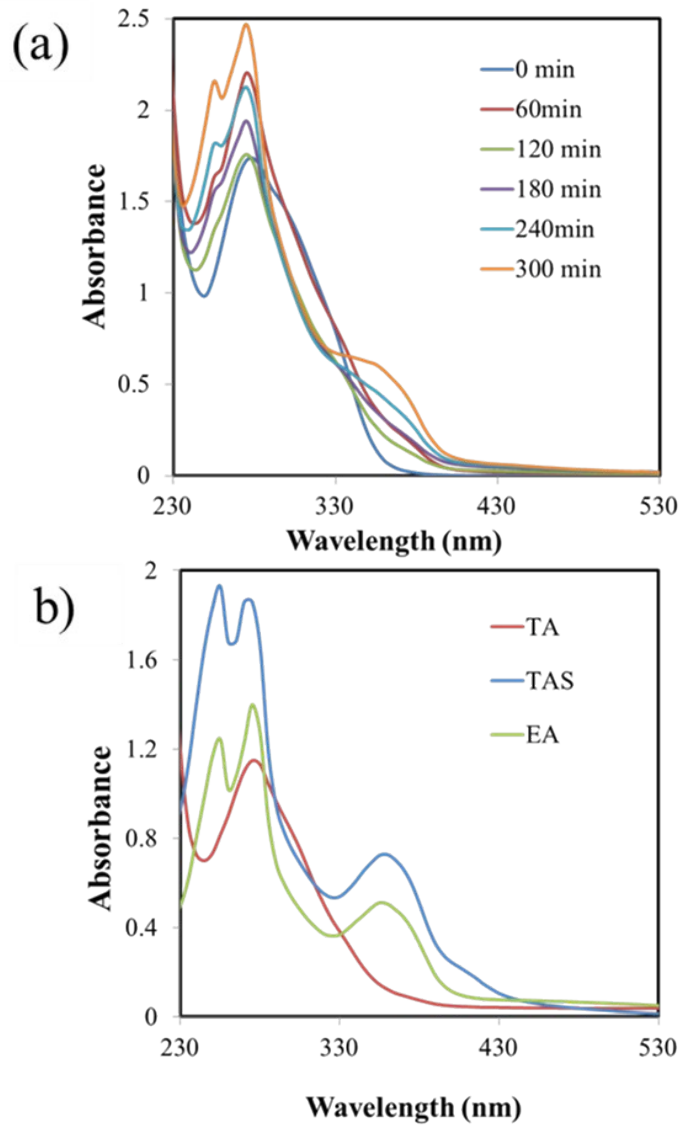

Figure S2. (a) Absorption spectra of sonicated TA solution (355 kHz and 5.5 W/cm3 after different sonication times, (b) Absorption spectra of TA, EA and TAS after dissolution in water. The absorption spectra of sonicated TA solution as a function of sonication time and TAS particles dissolved in water revealed a spectral profile similar to that of a standard EA solution with a new peak appearing at 370 nm.

4. Experimental procedure for determination of ellagic acid content

The Ellagic acid content of the sonicated tannic acid was determined using the well-established Ellagic acid assay [1].

The method is based on the formation quinone oxime of the ellagic acid nitrosylation product [1]. The ellagic acid standrad solution in pyridine forms red color nitrosylated product when incubated in $NaNO_2$ solution and gives a characteristic absorbance band at around 515 nm that can be further used to quantify the concentration of ellagic acid

For hydrolysis, 10 mg of the TAS was added to 2 N H_2SO_4 solution in a test tube. The reaction mixture was frozen in liquid nitrogen and was vacuum sealed. The content was heated for 24 hr at 100 ˚C. After the hydrolysis, the content was cooled at room temperature and filtered using vacuum filtration. The residue was then dissolved in 10 ml pyridine. 1.1 ml of pyridine and 1ml of sample solution also in pyridine were mixed and 0.100 ml concentrated HCl solution was added and the mixture was heated for 5 min at 30 ˚C. This sample was quickly mixed with 0.100 ml of 1% $NaNO_2$ (w/v in water) solution and absorbance was immediately recorded. The absorbance was again noted after incubating the sample with $NaNO_2$ at 30 ˚C for 36 min. Difference between the absorbance at 0 min and 36 min is proportional to the Ellagic acid concentration.

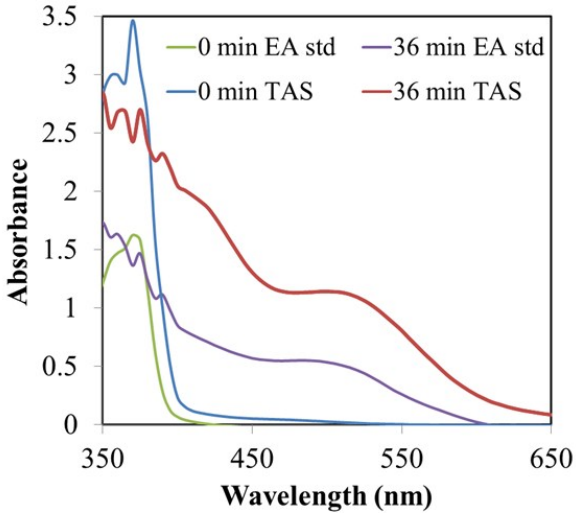

Figure S3. The absorbance spectra of nitrosylated product of EA and TAS in pyridine and after 36 min of incubation with $NaNO_2$. The appearance of the band at 515 nm indicate the presence of ellagic acid.

5. ^{1}H NMR of TAS particles

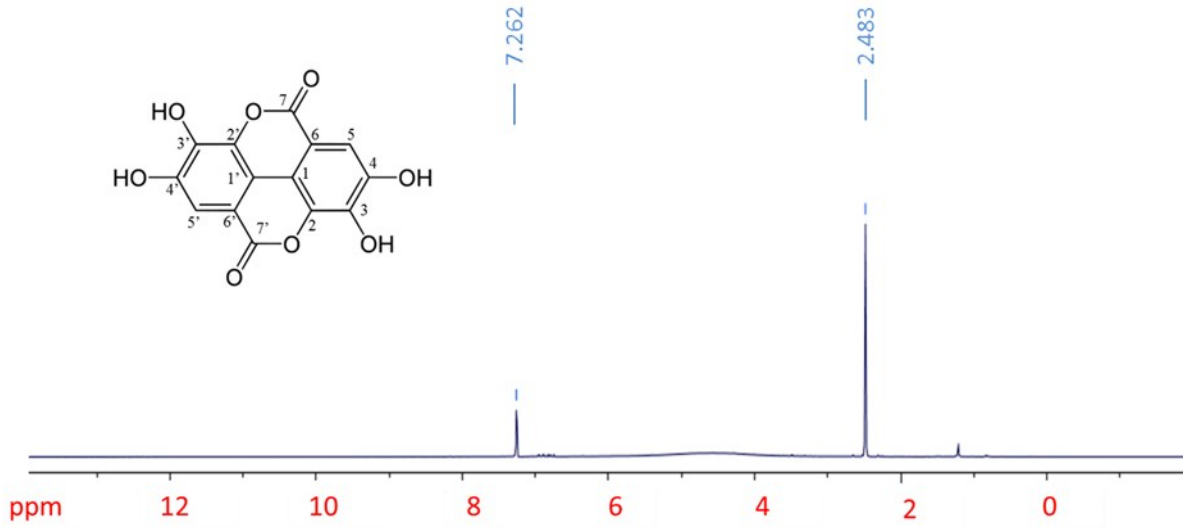

Figure S4. ^{1}H NMR spectrum of the TAS particles dissolved in DMSO represents peak at δ 7.262 due to 5, 5’ proton

6. ^{13}C NMR of TAS particles

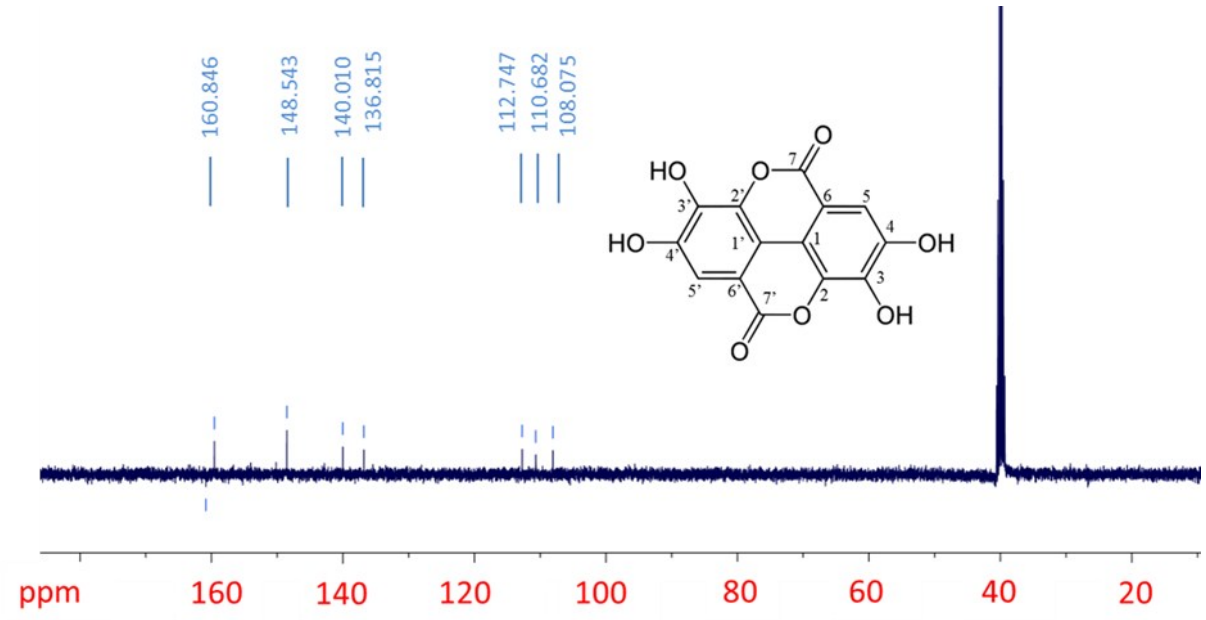

Figure S5. Figure S5: ^{13}C NMR spectrum of the TAS particles dissolved in DMSO. The peaks are assigned as follows δ 160.846 ppm- C7, 148.08 ppm- C4, 140.010 ppm - C3, and 136.35 ppm - C2. 112.747 ppm -C1, 110.682 ppm -C5, 108.075 ppm - C6

7. PXRD diffraction pattern of TA and TAS particles

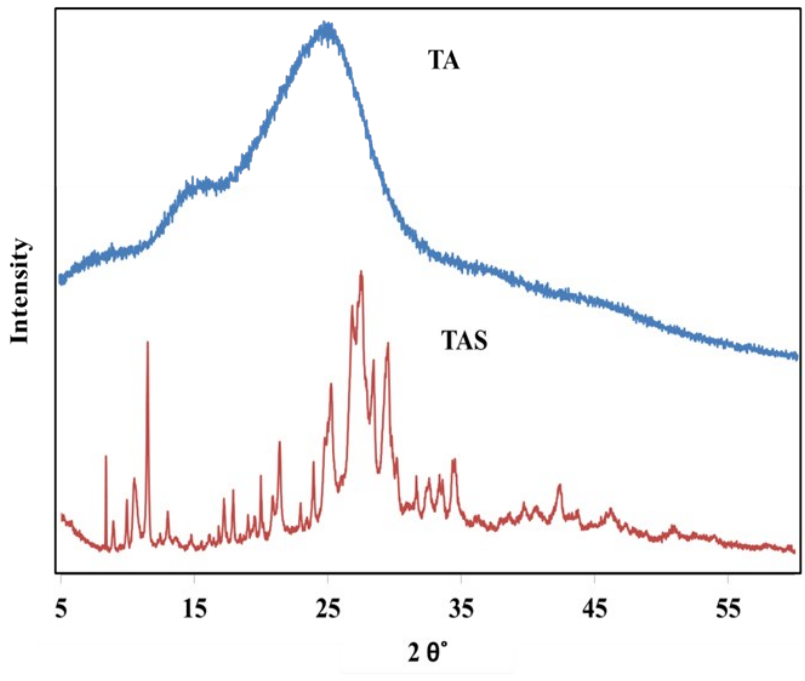

Figure S6. X-ray diffraction patterns of TA and TAS particles

8. Fluorescence spectroscopy of TA solution

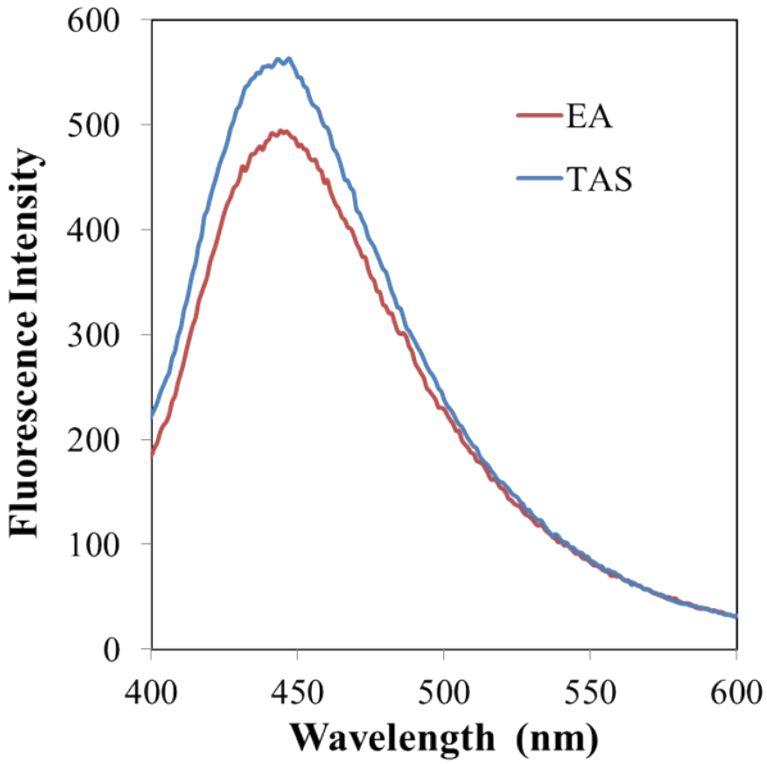

Figure S7. Fluorescence emission spectra of TAS and EA particles after dissolution in 0.1 N NaOH obtained at excitation wavelength of 360 nm

9. Thermogravimetric analysis of TA and TAS particles

Figure S8 illustrates thermal degradation curves of TA and TAS particles formed by using ultrasound. The weight loss of TA molecules started at about 80 °C and continued up to 200 °C by slight degradation with 2% weight loss. This could be due to the loss of moisture in the sample. The decomposition temperature of the tannic acid was around 230 ˚C. However, TAS particles prepared by ultrasound have different degradation temperatures. The initial weight loss was measured between 80 and 171 °C; again, this could be due to the loss of adsorbed water, and the onset for the decomposition of TAS particles was estimated around 430 ˚C. Therefore, the tannic acid nanoparticles obtained after sonication are also more thermostable compared to tannic acid.

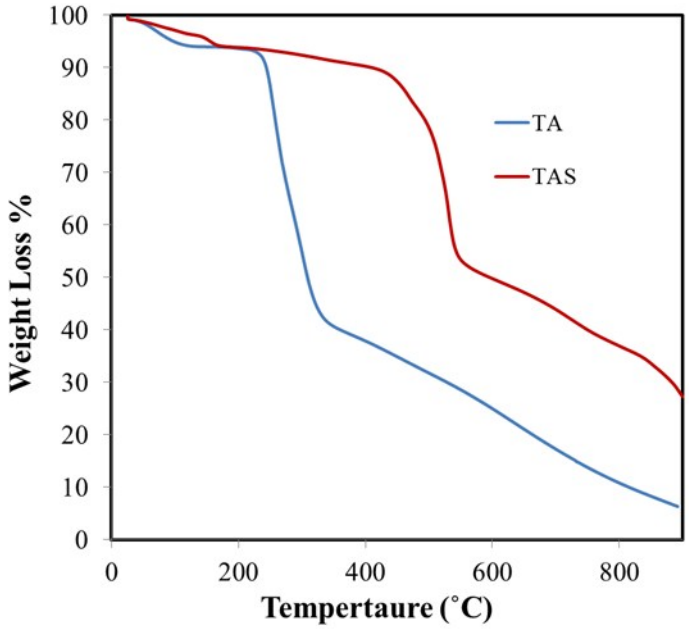

Figure S8. TGA profile of TA and TAS particles as a function of increasing temperature showing the decomposition of TA at around 200 °C and of TAS at 430 °C

10. References

[1] TC. Wilson, AE. Hagerman. *J. Agric. Food Chem.*, 1990, **38**, 1678-1683.