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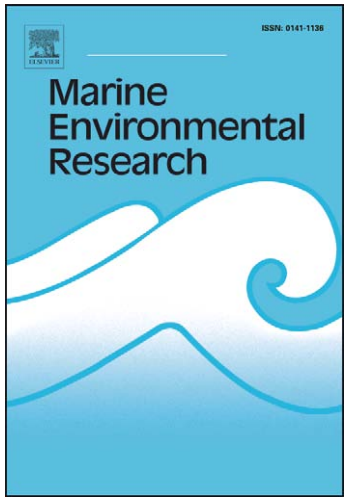


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# Sonophotocatalysis of endocrine disrupting chemicals

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*Key words*: Endocrine-disrupting chemical; Decomposition of EDC; Photocatalysis; Sonolysis; Oocyte maturation; Zebrafish

**Abstract**

Sonolysis and photolysis often exhibit synergistic effects in the degradation of organic molecules. An assay of fish oocyte maturation provides an appropriate experimental system to investigate the hormonal activities of chemical agents. Oocyte maturation in fish is triggered by maturation-inducing hormone (MIH), which acts on receptors on the oocyte surface. A synthetic estrogen, diethylstilbestrol (DES), possesses inducing activity of fish oocyte maturation, and a widely used biocide, pentachlorophenol (PCP), exhibits a potent inhibitory effect on fish oocyte maturation.

In this study, the effects of the combined treatment by sonolysis with photolysis (sonophotocatalysis) to diminish the hormonal activity of DES and the maturation preventing activity of PCP was examined. By sonophotocatalysis, hormonal activity of DES was completely lost within 30 minuets and the inhibiting activity of PCP was lost within 120 min. These results demonstrated that sonophotocatalysis is effective for diminishing the endocrine disrupting activity of chemical agents.

## 1. Introduction

Recently, the treatment of wastewater containing organic carbon has been one of the most important subjects in environmental protection. A rapidly increasing number of chemicals, or their degradation products, are being recognized as possessing estrogenic activity, albeit usually weak. It has been found that effluent from sewage treatment works contains a chemical, or mixture of chemicals, that induces vitellogenin synthesis in male fish maintained in the effluent, thus indicating that the effluent is estrogenic (Sumpter and Jobling, 1995). Thus, the development of effective treatment technologies for eliminating chemical agents from the waste stream at its source is now the subject of considerable concern.

An assay of fish oocyte maturation provides an appropriate experimental system to investigate the hormonal actions of chemical agents. Several factors responsible for the regulation of oocyte maturation in fresh water fish have been identified. These include the isolation and characterization of a fish maturation-inducing hormone (MIH), 17α,20β-dihydroxy-4-pregnen-3-one (17,20βDHP) (Nagahama et al., 1995), and the components of maturation-promoting factor (MPF) (cdc2, the catalytic subunit, and cyclin B, the regulatory subunit) (Yamashita et al., 1995). Oocyte maturation in fish is triggered by MIH, which acts on progestin receptors located on the oocyte membrane and induces the activation of MPF in the oocyte cytoplasm (Tokumoto et al., 2007). During the course of maturation, oocytes undergo drastic morphological changes associated with the progression of the meiotic cell cycle, among which breakdown of the oocyte nuclear envelope (germinal vesicle breakdown, GVBD) occurring at the prophase/metaphase transition is usually regarded as a hallmark of the progress of oocyte maturation.

Several endocrine-disrupting chemicals or EDCs, such as Kepon and *o,p*-DDD, have been reported to antagonize MIH-induced meiotic maturation of fish oocytes *in vitro* (Das and Thomas, 1999). EDCs such as methoxychlor and ethynyl estradiol also antagonize frog oocyte maturation. One EDC, diethylstilbestrol (DES), is a nonsteroidal substance, which was prescribed during the late 1940s to early 1970s to pregnant women to prevent abortion, preeclampsia, and other

complications of pregnancy. Male and female offspring exposed in utero to DES may develop multiple dysplastic and neoplastic lesions of the reproductive tract, along with other changes, during development (Bern, 1992). In a previous study, we found that treatment of oocytes with DES alone induced maturation in goldfish and zebrafish (Tokumoto et al., 2004). The maturation-inducing activity of DES was also reported in a marine fish, goby (Baek et al., 2007). Furthermore, a potent inhibitory effect of pentachlorophenol (PCP) was demonstrated on oocyte maturation induced by MIH and DES (Tokumoto et al., 2005). PCP is a widely used biocide that has been employed as a wood preservative, herbicide, and defoliant. Its extensive use and persistence have resulted in significant environmental contamination and potential exposure of the general population. Due to its highly persistent nature, PCP is still one of the dominant phenolic compounds in blood (Sandau et al., 2002). Recently, it was demonstrated that PCP was resistant for sulfonation, which is an important pathway in the biotransformation of a wide range of endogenous compounds and xenobiotics, in polar bears (Sacco and James, 2005). The carcinogenic effects of PCP have been evaluated in several animal bioassays (McConnell et al., 1991), and PCP levels in fish are used as a biomarker of contamination (Rogers et al., 1990). A relationship between PCP levels in women with reproductive and other endocrine problems was reported (Guvenius et al., 2003; Peper et al., 1999). Thus, PCP is an important organic chemical for environmental studies and various kinds of degradation technologies for PCP have been tried.

Photocatalysis is a harmless wastewater purification method. An ultraviolet light source and titanium oxide photocatalyst are commonly used in the photocatalytic process. Ultraviolet light has a disinfectant effect and decomposes organic compounds to inorganic materials such as carbon dioxide and water. Some researchers have studied the decomposition of dye by photocatalysis (Alessandra et al., 2001; Sökem et al., 2002). However, the decomposition efficiency is low when the organic concentration in the wastewater is high.

The decomposition of organic compounds using ultrasound (called sonolysis) is harmless purification method. Several researchers have studied the ultrasonic decomposition of organic

compounds such as alkyl ether (Suzuki et al., 1999), phenol (Weavers et al., 2005, Vassilakis et al., 2004), chlorofluorocarbon (Nagata et al., 1995), organohalogen compounds (Shemer et al., 2004, Wu et al., 2001), and azo dye (Okitsu et al., 2005). One of the problems with sonolysis is its low decomposition efficiency. However, it has been reported by several researchers that the decomposition efficiency increased with the combination of photocatalysis and sonolysis (sonophotocatalysis) (Maezawa et al., 2007, Entezari, et al. 2005, Chen et al. 2002 Suzuki et al., 2000). Some of the reasons for the enhancement of decomposition efficiency are reported as follows,

(1) Catalyst particles are physically dispersed by ultrasonic treatment (Shirgaonkar *et al*., 1998, Davydov *et al*., 2001, Ragaini *et al.*, 2001)

(2) Enhancement of mass transfer between the bulk liquid and the surface of the catalyst and renewal of the fluid film near the surface of the catalyst (Shirgaonkar *et al*., 1998, Stock *et al*., 2000)

(3) Formation of OH radicals from hydrogen peroxide, which is produced by the photocatalyst (Theron *et a*l., 1999, Selli, 2002)

In the present study, the effectiveness of sonophotocatalysis in diminishing the hormonal activity of DES and antagonistic activity of PCP on fish oocyte maturation as a model of water pollutant chemicals was examined.

## 2. Materials and methods

### *2.1 Materials*

The transgenic line *TG ( β-actin:EGFP)* was established by Hsiao et al. (Hsiao and Tsai, 2003). Although the cDNA integrated in this strain was constructed for expression of *EGFP* driven by the medaka *β-actin promoter*, the expression of *EGFP* is restricted to the oocytes in adult fish. Furthermore, the fluorescence is concentrated around the nucleus of immature oocytes (germinal

vesicle) and diffuses throughout the mature oocyte after germinal vesicle breakdown (GVBD). TG zebrafish were bred and maintained at 28.5 ˚C on a 14 h light/10 h dark cycle (Westerfield, 1995). 17,20βDHP and DES were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Pentachlorophenol was obtained from Wako Pure Chemical Industries (Osaka, Japan).

*2.2 Sonophotocatalysis*

Details of the reactor for sonophotolysis were described previously (Maezawa et al., 2007). Major components of the reactor were following. A low-pressure mercury lamp (Sen Tokushu Kougen Co. Japan, UVL20PS) was used as a light source. The main emission wavelength is 185 nm. The emission intensity of 254 nm is much lower than that of 185 nm. Fixed TiO2 catalyst prepared from titanium isopropoxide by dip-coating method was used as a photocatalyst. As the ultrasonic transducer, the 250 kHz commercial equipment from Kaijo Co. (Japan) was used. One liter of zebrafish Ringer's solution (116 mM NaCl, 2.9 mM KCl, 1.8 mM $CaCl_2$, and 5 mM HEPES, pH 7.2) was poured into the reactor. Test agents were dissolved in zebrafish Ringer's solution at 10 μM from a 10,000-fold stock in ethanol by stirring for 3 minutes. Sonophotocatalysis was carried out for 120 minutes under conditions of stirring at 500 rpm and aeration at 50 ml/min. During the treatment about 20 ml per time point of the solution was collected at 0, 5, 30, 60, and 120 minutes. Samples were stored at -20˚C until use.

*2.3 Spectral analysis*

The changes in compounds during the sonophotocatalysis were evaluated by the absorption intensity between 220 to 340 nm using a UV-VIS spectrophotometer (UV-2450, Hitachi CO., Japan).

*2.4 Oocyte preparation and in vitro culture*

Gravid female zebrafish, which possesses full-grown immature oocytes were selected from a group of mixture of 10-50 male and female that were kept in 20 cm x 25 cm square and 25 cm high acryl case with continuous out-flow water. Zebrafish were sacrificed within two hours after lights were turned on. Ovaries were isolated from sacrificed females and placed in fresh zebrafish Ringer's solution (116 mM NaCl, 2.9 mM KCl, 1.8 mM $CaCl_2$, and 5 mM HEPES, pH 7.2) and washed with the same solution. Ovaries were dissected into ovarian fragments (each containing 2-10 oocytes) manually by using fine forceps. Fully-grown immature oocytes were exposed *in vitro* by incubating ovarian fragments in 4 ml of zebrafish Ringer's solution containing each agent (from a 1000-fold stock in ethanol) at 25.0 ˚C or room temperature with gentle agitation (40 rpm). GVBD was assessed by scoring the oocytes that became transparent (Pang and Ge, 2002) or scoring the GFP-positive GVs using a fluorescence microscope (SZX12, Olympus, Japan). %GVBD was determined in more than twenty oocytes. The morphology of oocytes was recorded in photographs using a digital microscope (DP70, Olympus, Japan).

*2.5 Statistical analysis*

Summary data are presented as means ±S.D. The significance between multiple groups of data was evaluated using analysis of variance (ANOVA). Statistically significant differences between the percentage of GVBD were indicated as *; $P<0.05$ or **; $P<0.01$.

**3. Results and discussion**

Figure 1 show the morphology of oocytes after three hours treatment with Ethanol (EtOH), 17,20βDHP, DES and 17,20βDHP with PCP. Panels on the left show the images under normal lighting and panels on the right show fluorescence images. Previously, a transgenic zebrafish line with fluorescent oocytes was produced (Hsiao and Tsai, 2003). As shown in the figure, fluorescence in the transgenic oocytes is concentrated on the periphery of germinal vesicles.

GVBD in this strain is clearly observed using the fluorescence microscope. Germinal vesicles were seen near the center of oocytes after EtOH and 17,20βDHP with PCP treatment, whereas the signal disappeared after the 17,20βDHP and DES treatments (Fig. 1 B, D, F, and H). Also, 17,20βDHP and DES treated oocytes became transparent (Fig. 1 C, and E). DES induced GVBD in zebrafish oocytes in the same manner as the naturally occurring hormone 17,20βDHP. As shown in a previous report PCP completely prevented oocyte maturation. These compounds induce and inhibit oocyte maturation almost completely at concentrations of 10 μM.

To analyze the effect of sonophotocatalysis on hormonal activity of DES, DES-containing Ringer's solution was treated in a sonophotocatalysis chamber for 120 minutes and aliquots of the solution were collected at various times as samples for biological assaying. Oocytes were cultured in the samples of treated Ringer's solution. The sample collected before treatment (time 0) induced GVBD almost as completely as freshly prepared DES- and DHP-containing Ringer's solution. This result indicates that the hormonal activity of DES was stable in frozen Ringer's solution. As shown in figure 2A, the GVBD-inducing activity of DES was greatly reduced even by 5 minutes of treatment. The hormonal activity was almost lost after 30 minutes of treatment. When 17,20βDHP was added into sample, that had been treated for 120 minutes, the full magnitude of GVBD was induced. This result demonstrated that the decrease of GVBD-inducing activity of treated samples was not derived from the production of toxic substances by sonophotocatalysis, but was due to the breakdown of DES. This is supported by spectral analysis (Fig. 2B). Changes in the spectral pattern of samples, which suggested the degradation of DES, occurred after 5 minutes. These results indicate that DES was degraded by sonophotocatalysis rapidly and lost its hormonal activity. As described previously for compounds that lack ethyl groups and when hydroxyls were blocked by substitution, the hormonal activity of DES was greatly reduced. This result suggested that the structural changes following by sonophotocatalysis caused a reduction in the activity of DES. As expected, the activity of DES reduced within a short period.

In contrast to DES, a relatively long time was needed to degrade PCP for diminish its activity. As shown in figure 3, the inhibitory effect of PCP was begun to be lost after 60 min of treatment and lost completely after 120 min. This was supported by the spectral analysis (Fig.3B). Changes in the spectral pattern of samples suggested that the degradation of PCP occurred at 60 minutes. The transient peak at 280-300 nm could be derived from an intermediate of PCP degradation process as this peak decreased to 50% at 60 min and disappeared within 120 min. A correlation between the peak area and the inhibiting activity of the treated PCP solution was observed. This suggested that intermediates of PCP in the degradation process still possess inhibiting activity and 120 min was necessary to degrade PCP to inactive compounds. When oocytes were incubated in sample that was treated for 120 minutes, GVBD was not induced. This result demonstrated that the increase in GVBD-inducing activity of treated samples was not derived from the production of hormonally active substances by sonophotocatalysis of PCP. These results indicate that PCP was degraded by sonophotocatalysis and lost its inhibitory activity.

There have been various studies on the elimination of hormonally active chemicals from wastewater. Coleman et al. (2004) reported the degradation of estrogens by UVA photolysis and photocatalysis over an immobilized titanium dioxide ($TiO_2$) catalyst. They studied the loss of estrogenic activity of steroid estrogens constituting the main estrogenic component, 17β-estradiol (E2) and estrone (E1), and the synthetic steroid estrogen 17α-ethinylestradiol (EE2), in domestic sewage treatment work (STW) effluent. Estrogenic compounds lost their hormonal activity in a short period by photocatalysis over an immobilized $TiO_2$ catalyst. The results demonstrated that photocatalysis is effective at degrading estrogenic compounds.

PCP has been widely used as a wood preservative, herbicide, and insecticide all over the world. This has resulted in ubiquitous environmental pollution and the hazard risk of human exposure is now a matter of concern (Vartiainen et al., 1995). Biodegradation of PCP in water is slow and incomplete. There have been report on a variety of approaches to degrade and remove

PCPs from environment. For example biodegradation by bacterias or fungus, or enzymes isolated from these organisms, has been reported (Wu et al., 1993; Ye and Li, 2006). Although PCP is capable of biodegradation, the kinetics to complete its mineralization are slow. More recently, advanced oxidation processes were attempted for the degradation of PCPs such as ozonation, photolysis, or photocatalysis (Fukushima and Tatsumi, 2007; Hanna et al., 2004; Hong and Zeng, 2002; Quan et al., 2007). Hong and Zeng determined chemical structure of intermediates during degradation of PCP by ozonation in different pH conditions (2002). They demonstrated that 2,3,5,6-Tetrachloro-p-hydroquinone is an abundant intermediate at pH 7.0. Shoulder peak at 280-300 nm appeared during sonophotolysis of PCP in this study corresponded to the peak of the intermediate. It seems likely that the intermediate was produced during sonophotolysis and possessed inhibitory activity against induction of oocyte maturation. On the other hand, effective treatment technologies for eliminating chemical agents from wastewater have been developed. A recently developed technology, sonophotocatalysis, is an effective and economical method to degrade organic compounds in solution (Selli 2002; Maezawa et al., 2007). In the present study, the effectiveness of sonophotocatalysis for eliminating hazardous materials in the water was evaluated by a well-established biological assay.

## 4. **Conclusions**

It was demonstrated that DES lost its hormonal activity in a very short period and PCP was degraded and lost its inhibiting activity on fish oocyte maturation within 120 min. In sonophotocatalysis, undesirable production of compounds that have inhibiting or inducing activity on oocyte maturation was undetectable during DES and PCP degradation. As shown in this study, sonophotocatalysis is an effective technology for eliminating chemical agents, especially for endocrine-disrupting chemicals.

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**Figure legends**

Fig. 1. GVBD observation in transgenic zebrafish.
The morphology of oocytes after three hours of each treatment was recorded. Panels on the left indicate oocytes in zebrafish Ringer's solution. In panels on the right, oocytes were fixed in clearing solution for observation of germinal vesicles. Oocytes remained opaque after EtOH

treatment, whereas they became transparent after 17α,20βDHP or DES treatments. Germinal vesicles were seen near the center of oocytes after EtOH treatment whereas they disappeared after 17,20βDHP or DES treatments. The arrowhead indicates a germinal vesicle. The scale bar represents 1 mm.

Fig. 2. Decrease of maturation-inducing activity of DES.
One liter of DES containing medium (10 μM from 10,000 fold stock in EtOH) was treated with ultrasonic waves. During the treatment 20 ml samples of medium were collected at the indicated times. Oocyte maturation-inducing activity of DES in the ultrasonic treated medium was examined by adding ovarian fragments into the medium. Each value is the mean of three separate experiments using ovaries of three different females. To check for the production of toxic substances during ultrasonic treatment, GVBD was induced in 120 min-treated medium by adding 17,20βDHP (120 + DHP). %GVBD was calculated by determining the percentage of oocytes that had undergone germinal vesicle breakdown (GVBD) among more than 20 oocytes cultured for three hours. Vertical lines indicate standard deviation. * and ** indicate significant differences between the %GVBD induced by same concentration of DES at the $P<0.05$ and $P<0.01$ levels, respectively. Each line in panel B represents the following times: ~~–~~, 0 min; ~~–~~, 5 min; ~~–~~, 30 min; ~~–~~, 60 min; ~~–~~, 120 min.

Fig. 3. Decrease in maturation-inhibiting activity of PCP.
One liter of PCP containing medium (10 μM from 10,000 fold stock in EtOH) was treated with ultrasonic waves. During the treatment 20 ml samples of medium were collected at the indicated times. Oocyte maturation preventing activity of PCP in the ultrasonic treated medium was examined by adding ovarian fragments into the medium. Then 17,20βDHP was added to medium (0.1 μM from 10,000 fold stock in EtOH) to induce GVBD. To check for the production of GVBD-inducing substances during ultrasonic treatment, the effect of PCP on 120 min-treated medium was

examined (120 + PCP). Each value is the mean of three separate experiments using ovaries of three different females. %GVBD was calculated by determining the percentage of oocytes that had undergone germinal vesicle breakdown (GVBD) among more than 20 oocytes cultured for three hours. Vertical lines indicate standard deviation. ** indicates significant differences between the %GVBD induced by same concentration of PCP and 17,20βDHP at the $P<0.01$ level, respectively. Each lines in panel B represents the following times: —, 0 min; —, 5 min; —, 30 min; —, 60 min; —, 120 min.

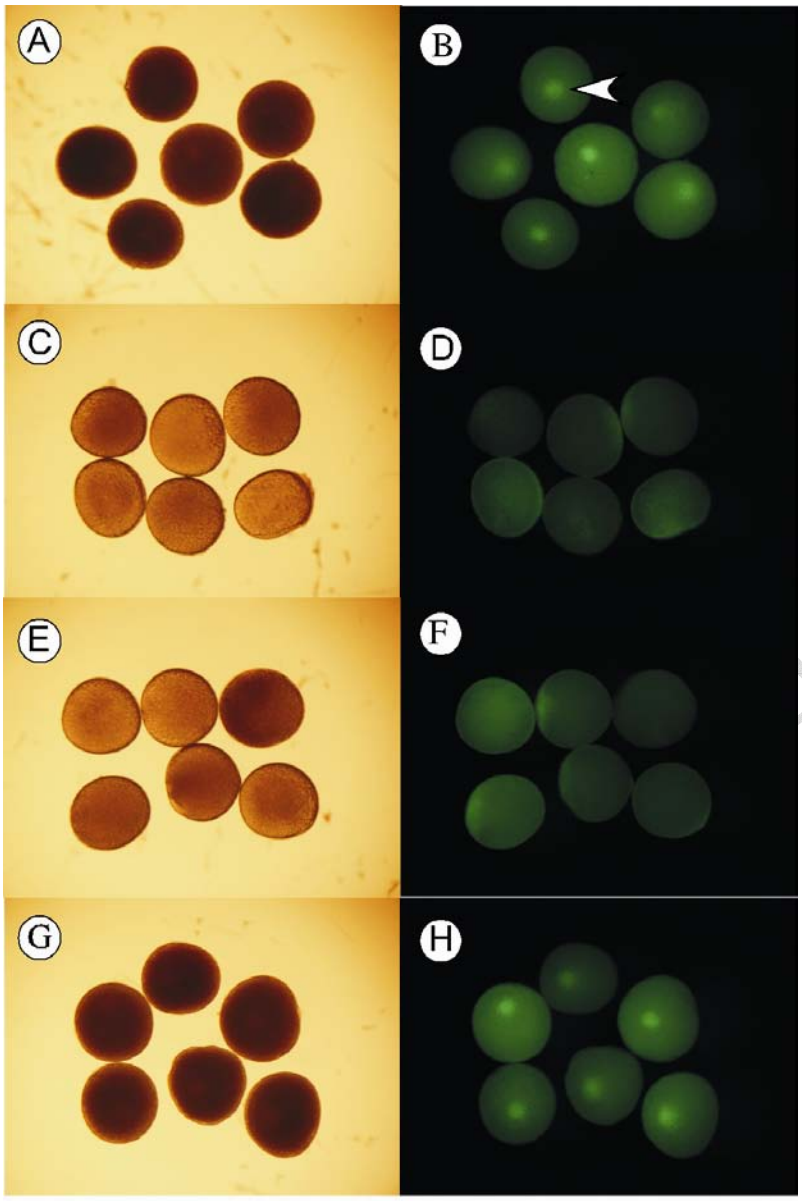


**Fig. 1 Tokumoto et al.**

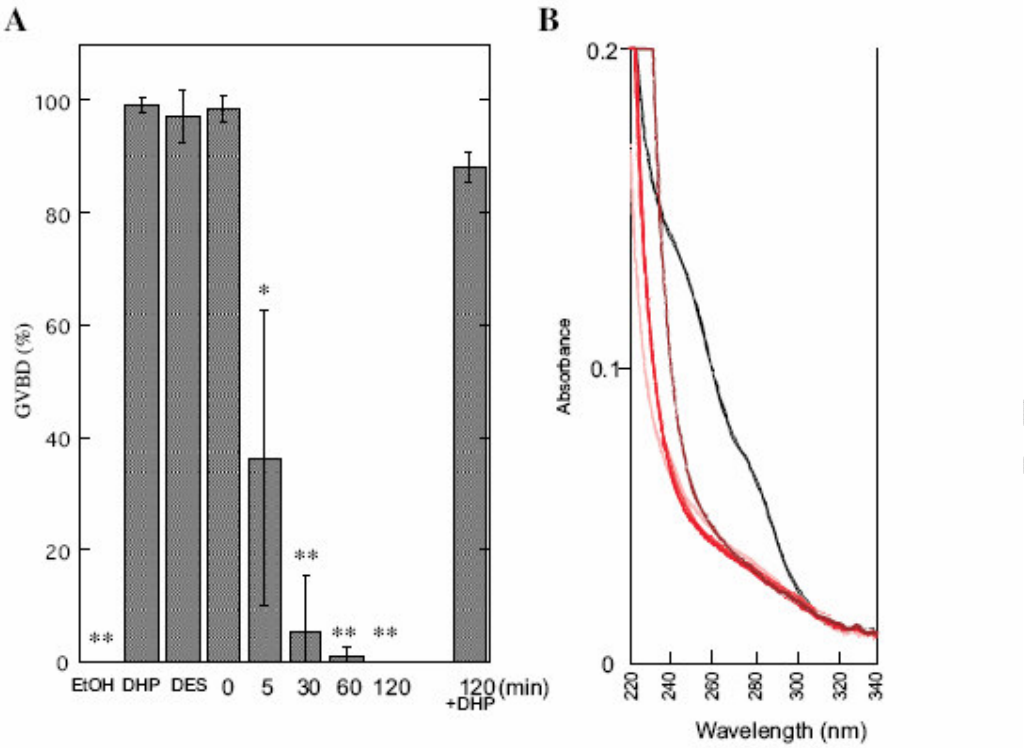


Fig. 2 Tokumoto et al.

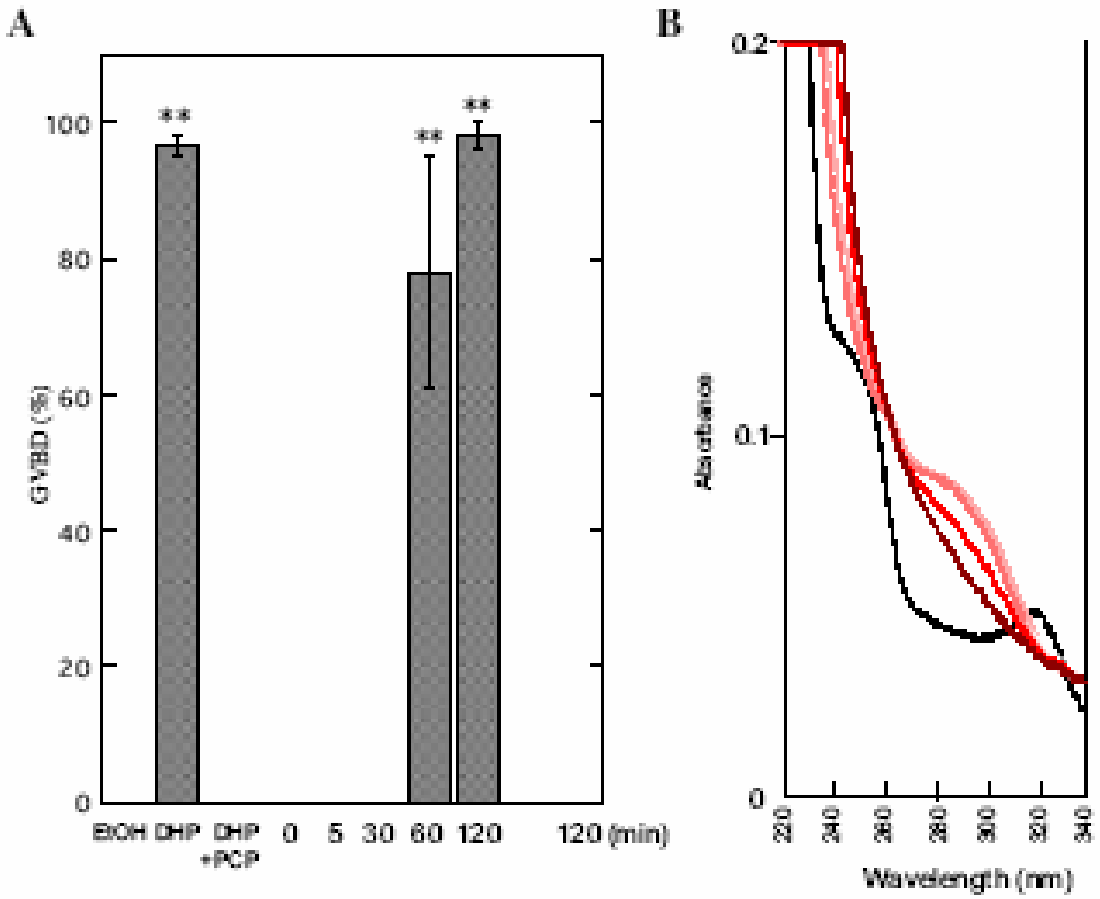


Fig. 3 Tokumoto et al.