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Uncaria tomentosa extracts protect human erythrocyte catalase against damage induced by 2,4-D-Na and its metabolites

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ABSTRACT

The effect of ethanolic and aqueous extracts from leaves and bark of *Uncaria tomentosa* was studied, with particular attention to catalase activity (CAT – EC. 1.11.1.6).

We observed that all tested extracts, at a concentration of 250 μg/mL were not toxic to erythrocyte catalase because they did not decreased its activity.

Additionally, we investigated the protective effect of extracts on changes in CAT activity in the erythrocytes incubated with sodium salt of 2,4-dichlorophenoxyacetic acid (2,4-D-Na) and its metabolites i.e., 2,4-dichlorophenol (2,4-DCP) and catechol. Previous investigations showed that these chemicals decreased activity of erythrocyte catalase (Bukowska et al., 2000; Bukowska and Kowalska, 2004).

The erythrocytes were divided into two portions. The first portion was incubated for 1 and 5 h at 37 °C with 2,4-D-Na, 2,4-DCP and catechol, and second portion was preincubated with extracts for 10 min and then incubated with xenobiotics for 1 and 5 h. CAT activity was measured in the first and second portion of the erythrocytes.

We found a protective effect of the extracts from *U. tomentosa* on the activity of catalase incubated with xenobiotics studied.

Probably, phenolic compounds contained in *U. tomentosa* scavenged free radicals, and therefore protected active center (containing –SH groups) of catalase.

1. Introduction

Uncaria tomentosa (Willd.) DC is a medicinal herb that has been used by the indigenous people of Peru for over 2000 years. *U. tomentosa* was brought to Europe and in the 1990s it became a complementary treatment for AIDS and cancer. The extracts from this plant under the name of "vilcacora" or "cat's claw" are highly popular in many countries, due to its proven immunostimulatory and antiinflammatory activities as well as in respect to its anticancer and antioxidative effects.

Extracts from *U. tomentosa* and their protective properties are intensively studied in many aspects such as: reducing side effects caused by chemotherapy (Farias et al., 2012); endometriosis (Nogueira Neto et al., 2011); endodontic pathogens development (Herrera et al., 2010), and xenobiotics (Bors et al., 2011).

Our earlier studies showed that all tested extracts, even at a very high concentration of 500 μg/mL, were not toxic to the erythrocytes because they did not cause lipid peroxidation, methemoglobin and reactive oxygen species (ROS) formation, nor induced hemolysis. Additionally the extracts studied depleted the extent of hemoglobin oxidation and lipid peroxidation as well as decreased the level of ROS and hemolysis, which was induced by 2,4-DCP. Nevertheless, no protective activity of the extracts against catechol action, which is a precursor of semiquinones in cells was found (Bors et al., 2011).

However, we observed (Bors et al., 2012) disturbances in the size and shape of the erythrocytes incubated with ethanolic and aqueous extracts from the concentration of 100 and 250 μg/mL, respectively. We suggested that the observed changes were probably related with insertion of polyphenolic compounds contained in extracts of *U. tomentosa* into erythrocyte membrane.

Catalase (CAT – EC. 1.11.1.6; hydrogen peroxide: hydrogen peroxide oxidoreductase) is ubiquitously present in a wide range of aerobic cell types, with the highest activities in mammals being found in liver, kidney and red blood cells (Deisseroth and Dounce, 1970). In cells of liver and kidney, the enzyme occurs mainly in peroxisomes, and serves as a useful marker of this organelle; but it is also found as a soluble protein in the erythrocytes, where it may protect against the peroxidation of hemoglobin (Aebi, 1984). Catalase is important in elimination of potentially dangerous H_2O_2 in cells (Bartosz, 2009).

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Because of the important role of catalase in the process of scavenging of reactive oxygen species, the investigation of potential changes in the activity of this enzyme under the influence of the ethanolic and aqueous extracts from *U. tomentosa* seems to be essential.

We investigated also the protective effect of extracts on changes in CAT activity in the erythrocytes incubated with sodium salt of 2,4-dichlorophenoxyacetic acid (2,4-D-Na) and its metabolites i.e., 2,4-dichlorophenol (2,4-DCP) and catechol. Catechol and 2,4-DCP are intermediate products of decomposition of 2,4-dichlorophenoxyacetic (2,4-D), which is selective systemic herbicides commonly used for the post-emergency control of annual and perennial broad-leaved weeds in cereals, maize, grasslands, rice, forests, and non-crop lands. These compounds, emitted into the environment mainly from anthropogenic sources are characterized by high toxicity, persistence and ability to bioaccumulate (Michałowicz, 2005; Michałowicz and Duda, 2007; Aydin et al., 2005).

Previous investigations showed that these chemicals decreased activity of erythrocyte catalase (Bukowska et al., 2000; Bukowska and Kowalska, 2004). The concentrations of 2,4-D and their metabolites that may be potentially present in human organism during its acute intoxication (Michałowicz and Duda, 2007) and which provoked changes in human erythrocytes (Duchnowicz et al., 2005; Bukowska et al., 2000, 2008) were used in the experiments.

2. Materials and methods

2.1. Plant material and chemicals

The raw material (bark and leaves) of *U. tomentosa* originated from Instituto Peruano de Investigaciones Fitoterapia Andina in Lima Peru was kindly supplied by Wilcaccora Łomianki

Centre, Poland. The general characteristics of this material were described previously by Pilarski et al. (2006). The voucher material is deposited at the Laboratory of Phytochemistry, Institute of Bioorganic Chemistry, Polish Academy of Sciences, Poznań, Poland.

The 2,4-D and 2,4-DCP was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Catechol was obtained from Sigma (Poznań, Poland).

2.2. Preparation of samples

One gram of the raw material (bark and leaves) of *U. tomentosa* was extracted with 10 mL of water or 96% ethanol at 37 °C for 8 h. Then, the extracts were centrifuged at 4000 rpm for 15 min. Supernatants were evaporated on Speed-Vac to dry mass, and next exsiccated with P_2O_5. At these conditions, we recovered about 111 mg of preparation per gram of bark and about 158 mg of preparation per gram of leaves in case of aqueous extraction. During extraction with 96% ethanol we obtained 123 mg of preparation per gram of bark and 158 mg of preparation per gram of leaves.

Four extracts were obtained: B_{et} – ethanol extract of the bark; B_{aq} – aqueous extract of the bark; L_{et} – ethanol extract of leaves; L_{aq} – aqueous extract of leaves. Powdered preparations were stored in a refrigerator (– 20 °C) in tightly closed containers, from which stock solutions were prepared for each experiment.

2.3. Isolation of erythrocytes

Human erythrocytes were obtained from whole blood, taken from healthy donors in the Blood Bank of Łódź, Poland. The erythrocytes were centrifuged (3000 rpm/min) at 4 °C and washed three times with phosphate-buffer saline (PBS; 150 mmol L^{-1} NaCl, 1.9 mmol L^{-1} NaH_2PO_4, and 8.1 mmol L^{-1} Na_2HPO_4, pH 7.4). The erythrocytes of 5% hematocrit were incubated for 1 and 5 h at 37 °C with *U. tomentosa* (at concentrations of 250 μg/mL) both with and without xenobiotics: 2,4-D, 2,4-DCP and catechol (at a concentration of 500 μg/mL – 2,4-D-Na, 250 μg/ml – 2,4-DCP and 100 μg/ml – catechol). All of mentioned above compounds were dissolved in 30 μL DMSO, and than PBS was added to the final volume of 10 mL (the concentration of DMSO used in the study was not toxic to the cells). The erythrocytes, which were incubated with PBS and DMSO were used as controls. The analysis was performed using catalase, which was obtained from human erythrocyte hemolysate. Hemoglobin was obtained from hemolysates by the method of Drabkin (1946).

2.4. Catalase activity

Catalase activity in cells was determined by the method of Aebi (1984). To 3 mL H_2O_2 (54 mM H_2O_2 in 50 mM potassium phosphate buffer pH 7.0) 5 μL of hemolysate with catalase was added and the decrease in H_2O_2 was measured spectrophotometrically at 240 nm, at 25 °C in 60 s. In the erythrocyte preparations, hemolysates were centrifuged (12,000 rpm/min) and estimation of the activity was made on 1% hemolysate concentrations. One unit of catalase activity was defined as the activity required to degrade 1 μmol of hydrogen peroxide in 60 s.

At the first step, the erythrocyte suspension was incubated with *U. tomentosa* extracts (at concentration of 250 μg/mL – the concentration at which in earlier studies found a protective effect of the extracts against human erythrocyte hemolysis and hemoglobin oxidation – Bors et al., 2011) for 1 and 5 h. Then the activity of catalase (CAT) in human erythrocytes was determined.

After the exclusion of negative effect of *U. tomentosa* extracts on catalase activity, the erythrocytes were pre-incubated with the extracts studied (at concentration of 250 μg/mL) for 10 min, and then they were treated with xenobiotics: 2,4-D-Na and their metabolites, 2,4-DCP and catechol for 1 and 5 h, respectively. Then the activity of catalase was determined.

The applied xenobiotics concentrations (500 μg/mL for 2,4-D-Na, 250 μg/mL for 2,4-DCP and 100 μg/mL for catechol) have been selected on the basis of the our earlier investigation because they induced changes in human erythrocytes (Duchnowicz et al., 2005; Bukowska et al., 2000; Bukowska and Kowalska, 2004).

2.5. Statistical analyses

The statistical analysis was performed with STATISTICA 8 data analysis software (2000 StatSoft, Inc., Tulsa, OK, USA). In this study, one-way analysis of variance (ANOVA) with post hoc multiple comparisons procedure (Tukey test) was used to assess statistical differences in case of normal distribution. The difference was considered to be significant for $P < 0.05$ and highly significant for $P < 0.01$.

3. Results

3.1. Antioxidative enzymes activity in the erythrocytes incubated with *U. tomentosa* extracts

It was found that all extracts of *U. tomentosa* in concentration 250 μg/mL did not induced a statistically significant change in catalase activity after 1 h ($F_{4;40} = 1{,}01$; $P > 0.05$) and 5 h ($F_{4;40} = 0.57$; $P > 0.05$) incubation. Table 1 represent activity of catalase in human erythrocytes incubated with ethanolic and aqueous extracts from leaves and bark of *U. tomentosa*.

3.2. The protective activity of the extracts against sodium salt of 2,4-dichlorophenoxyacetic acid, 2,4-dichlophenol and catechol action

In this study was found that 2,4-D-Na after 5 h incubation ($F_{1;16} = 8.58$; $P < 0.001$) and 2,4-DCP (1 h – $F_{1;16} = 22.46$; 5 h – $F_{1;16} = 28.92$; $P < 0.001$) and catechol (1 h – $F_{1;16} = 25.62$; 5 h – $F_{1;16} = 54.00$; $P < 0.001$) in all investigated time were capable of inducing significant changes in CAT activity in human erythrocytes (Fig. 1–3).

It was observed a protective effect all extracts from *U. tomentosa* on the activity of catalase incubated with xenobiotics.

It was found that the ethanolic and aqueous extracts from *U. tomentosa* in a concentration of 250 μg/mL reduced changes in catalase activity induced by 2,4-D-Na (5 h – $F_{4;40} = 5.72$; $P < 0.001$),

Table 1
The effect of *U. tomentosa* extracts (250 μg/mL) on catalase activity (CAT) in human erythrocytes.

	1 h Activity of CAT	5 h (μmol/min/mg Hb)
Control	268.23 ± 24.44	257.81 ± 50.04
Let	256.95 ± 35.73	260.33 ± 45.66
Laq	276.28 ± 24.82	272.67 ± 37.47
Ket	266.79 ± 32.19	242.87 ± 49.63
Kaq	283.01 ± 29.16	246.84 ± 49.37
ANOVA I	$F_{4,40} = 0.71$; $P > 0.05$	$F_{4,40} = 0.27$; $P > 0.05$

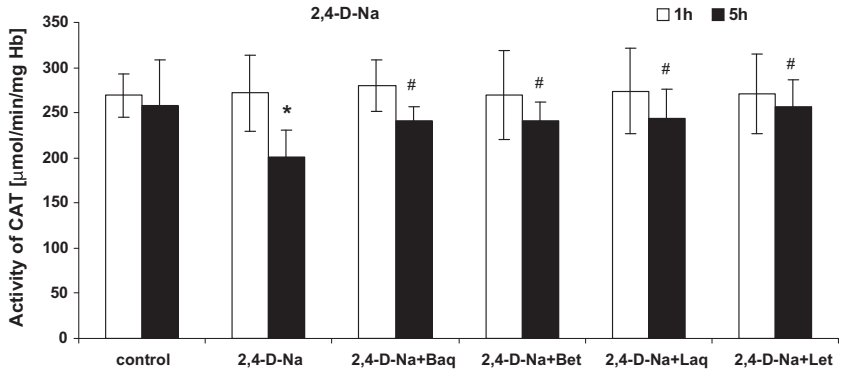

Fig. 1. The effect of *U. tomentosa* extracts (250 μg/mL) on the activity of catalase in human erythrocytes incubated with 2,4-D-Na (500 μg/mL) for 1 and 5 h of incubation. The results are presented in the form of mean values ± SD, $n = 9$. (∗) $P < 0.001$ for samples compared to the control (erythrocytes suspended in incubation buffer); (#) $P < 0.001$ for samples compared to 2,4-D-Na. B_{et} – ethanol extract of the bark; B_{aq} – aqueous extract of the bark; L_{et} – ethanol extract of leaves; L_{aq} – aqueous extract of leaves.

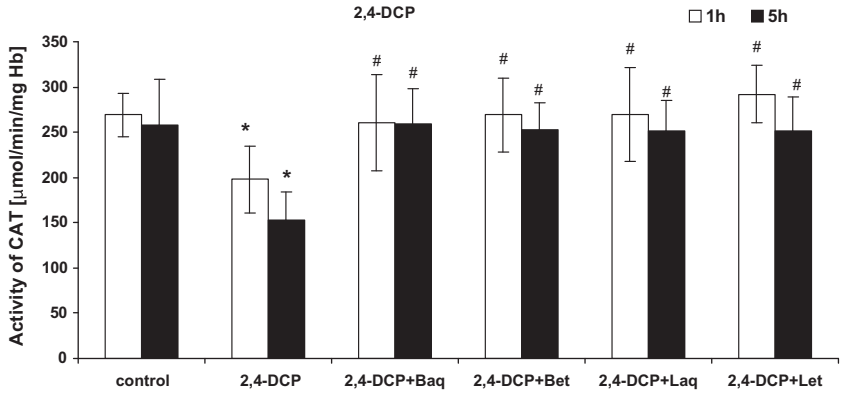

Fig. 2. The effect of *U. tomentosa* extracts (250 μg/mL) on the activity of catalase in human erythrocytes incubated with 2,4-DCP (100 μg/mL) for 1 and 5 h of incubation. The results are presented in the form of mean values ± SD, $n = 9$. (∗) $P < 0.001$ for samples compared to the control (the erythrocytes suspended in incubation buffer); (#) $P < 0.001$ for samples compared to 2,4-DCP. B_{et} – ethanol extract of the bark; B_{aq} – aqueous extract of the bark; L_{et} – ethanol extract of leaves; L_{aq} – aqueous extract of leaves.

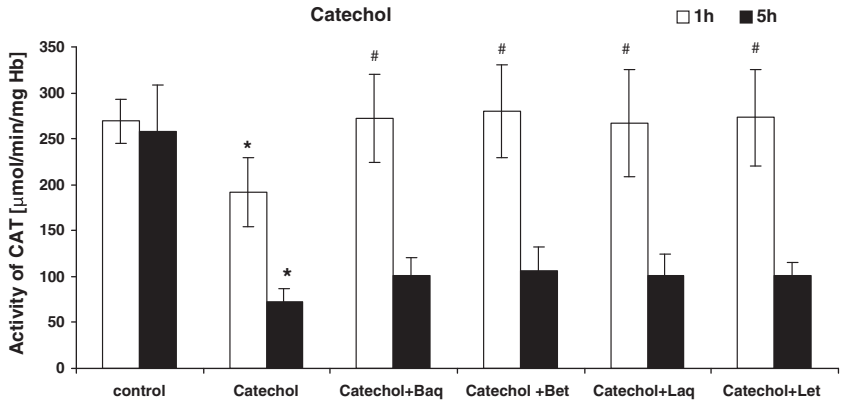

Fig. 3. The effect of *U. tomentosa* extracts (250 μg/mL) on the activity of catalase in human erythrocytes incubated with catechol (100 μg/mL) for 1 and 5 h of incubation. The results are presented in the form of mean values ± SD, $n = 9$. (∗) $P < 0.001$ for samples compared to the control (the erythrocytes suspended in incubation buffer); (#) $P < 0.001$ for samples compared to catechol. B_{et} – ethanol extract of the bark; B_{aq} – aqueous extract of the bark; L_{et} – ethanol extract of leaves; L_{aq} – aqueous extract of leaves.

2,4-DCP (1 h – $F_{4;40} = 5.76$; 5 h – $F_{4;40} = 15.75$; $P < 0.001$) and catechol (1 h – $F_{4;40} = 5.72$; $P < 0001$) (Fig. 1–3). However, *U. tomentosa* extracts did not modulate changes in catalase activity provoked by catechol in the erythrocytes incubated with this compound for 5 h ($F_{4;40} = 0.86$; $P > 0.05$) (Fig. 3).

No statistically significant differences in the activity between the different extracts studied was observed.

4. Discussion

Under physiological conditions, intracellular antioxidant enzymes, such as superoxidative dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-PX) eliminate ROS, thereby playing an integral role in the oxidative stress defenses of the cell. SOD catalyzes the conversion of superoxide radicals to hydrogen

peroxide, while CAT and GSH-PX convert hydrogen peroxide into water. Thus, these antioxidant enzymes can alleviate toxic effects exerted by ROS (Bartosz, 2009).

CAT is the main enzyme that plays a role in the response development to oxidative stress caused by pesticides (El-Demerdash, 2007; Sadowska-Woda et al., 2010).

Dreifuss et al. (2010) observed that hydroalcoholic extract from *U. tomentosa* successfully reduced tumor growth in rats. That extract increased CAT activity in liver, while decreasing it in tumor tissue. Yokoyama et al. (2004) also suggested that one of the mechanisms of the protective activity of extracts from *Uncaria sinensis* against transient brain ischemia in gerbil is their effect on CAT activity in the brain. The above mentioned studies were conducted *in vivo* in which the synthesis of the enzymes appears whereas *de novo* CAT synthesis cannot occur in human erythrocytes that are destituted of nucleus.

In our investigation the extract from *U. tomentosa* in a high concentrations of 250 μg/mL did not decrease the activity of this enzyme (Table 1).

Therefore, the protective effect of the extracts against the action of 2,4-D-Na phenoxyherbicide and its metabolites i.e., 2,4-dichlorophenol and catechol on human catalase activity was examined.

In previous studies, and in our investigation it was proven that 2,4-D-Na, 2,4-DCP and catechol decreased catalase activity (Figs. 2 and 3) (Bukowska et al., 2000; Bukowska and Kowalska, 2004).

Two main mechanisms may have played a role in a decrease in CAT activity. The first involves consumption of high level of H_2O_2 during the breakdown of free radicals and/or inhibition of the enzyme activity by these radicals, whereas the second involves direct inhibition of CAT activity by xenobiotics (Eraslan et al., 2007).

Sulfuric aminoacids play a very important role in the activity of catalase what was emphasized by Morikofer-Zwez et al. (1996). They reported that catalytic activity of bovine liver catalase was associated with disulfide bridge formation at the active site of the enzyme. Enzymatic activity supposedly being dependent on the number of disulfide bridges, as it was shown in catalase of horse erythrocytes. A decrease in the activity of human erythrocytes catalase was also noted when the effect of chlorophenols such as 2,4-dichlorophenol and 2,4,5-trichlorophenol was investigated, whereas no change was found under the influence of methylphenols. This effect may be explained by blockade of the –SH groups and, thus alterations in the special structure of the enzyme (Bukowska et al., 2000).

Catechol in biological systems may undergo metabolic activation under the influence of the enzymes. The oxidation of catechols *in vitro* is catalyzed by cytochrome P450/NADPH oxidase and other enzymes. The consequence of that oxidation is the formation of secondary semiquinone radicals, which may damage to the spatial structure of catalase as well as to other enzymes. Semiquinones are able to bind to nucleophilic residues such as –SH groups of proteins or $–NH_2$ groups of nucleic acids. As a result of binding, macromolecules may undergo inactivation (Głębska and Gwoździński, 1998).

It was found that ethanolic and aqueous extracts from *U. tomentosa* at the selected concentration of 250 μg/mL reduced changes in the activity of catalase provoked by 2,4-DCP.

In our earlier investigation, we concluded that the extracts in a wide range of concentrations from 5 to 500 μg/mL did not cause adverse changes in human erythrocytes, however, they exhibited a protective effect against free radicals generated as a result of 2,4-DCP action (Bors et al., 2011).

In the present study, the extracts of *U. tomentosa* protected the erythrocytes against inhibition of CAT activity induced by 2,4-D-Na, catechol and 2,4-DCP. Our earlier studies using HPLC method (Bors et al., 2011) and other studies conducted by Wirth and Wagner (1997), Heitzman et al. (2005) and Pilarski et al. (2006) showed that phenolic substances present in *U. tomentosa* extracts are mainly represented by tannins, flavonols derivatives (including campherol and dihydrocampherol), catechins (including catechin and epicatechin) and procyanides. Polyphenols possess the ability of free radicals scavenging and of inhibition of lipid peroxidation (Procházková et al., 2011), and therefore protect biomolecules such as catalase containing –SH groups in the active center against oxidative damage (Morikofer-Zwez et al., 1996).

Mi et al. (2007) suggested that quercetin, an antioxidant flavonoid, protected spermatogonial cells from 2,4-D-Na, which induced oxidative damage to chicken embryos.

Moreover, Bors et al. (2011) observed no protective effect of *U. tomentosa* extracts against organic radicals, such as semiquinones, which are usually formed as a result of catechol biotransformation in the organism. *U. tomentosa* extracts did not protect erythrocytes against catechol induced hemolysis, oxidation of hemoglobin and ROS formation.

U. tomentosa extracts exhibited a protective effect against catechol-induced decrease in CAT activity in human erythrocytes (Fig. 3).

Catalase-like properties consists both catalase and peroxidase activities of hemoglobin. Pseudoenzymatic hemoglobin activity cannot be eliminated because of the similarity structure of Hb and catalase (presence of heme group) and the same sensitivity to specific inhibitors (Giardina et al., 1995). Probably, the oxidation of hemoglobin to met-Hb resulted in peroxide decomposition, and therefore caused an increase in catalase activity. Previous studies have proven strong Hb oxidation after its incubation with catechol (Bukowska and Kowalska, 2004) and suggested an important role of hemoglobin in the activity of catalase (Giardina et al., 1995).

After 1 h incubation, the extracts studied protected catalase protein against oxidation caused by catechol, but prolonged incubation (5 h) caused very significant changes in catalase activity and the protective effect of the extracts did not appeared (Fig. 3).

Our investigations are model studies conducted *in vitro*, thus they did not include metabolism of the compounds, the effect of other cells or endogenous factors on *U. tomentosa* extracts biological activity *in vivo*. An isolated experimental system has both positive (elimination of other factors) and negative implications. Generally, the effects observed *in vitro* are stronger than that *in vivo*. In our investigations (*in vitro*) direct exposure of the examined cells on xenobiotics appeared, however, no protective systems such as serum albumins that bind various compounds were present in our samples, which usually protect the erythrocytes against toxic action of xenobiotics. The results of our investigation gives only the information about mechanism of action of the xenobiotics and extracts studied. It is difficult to determine the mechanism of action of the studied compounds *in vivo*, on the basis of the result obtained in *in vitro* investigations. Moreover, nowadays no information exists, which concentrations of these extracts may enter blood during their supplementation.

In summary, our results confirmed earlier reports that 2,4-D-Na, 2,4-DCP and catechol induced oxidative stress because they decreased CAT activity in human erythrocytes. The extracts of *U. tomentosa* studied protected activity of CAT erythrocyte against the inhibition induced by 2,4-D-Na, 2,4-DCP and catechol.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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