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DOXORUBICIN-INDUCED CENTRAL NERVOUS SYSTEM TOXICITY AND PROTECTION BY XANTHONE DERIVATIVE OF *GARCINIA MANGOSTANA*

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Abstract

Doxorubicin (Dox) is a potent, broad-spectrum chemotherapeutic drug used around the world. Despite its effectiveness, it has a wide range of toxic side effects, many of which most likely result from its inherent pro-oxidant activity. It has been reported that Dox has toxic effects on normal tissues, including brain tissue. The present study tested the protective effect of a xanthone derivative of *Garcinia Mangostana* against Dox-induced neuronal toxicity. Xanthone can prevent Dox from causing mononuclear cells to increase the level of tumor necrosis factor-alpha (TNF α). We show that xanthone given to mice before Dox administration suppresses protein carbonyl, nitrotyrosine and 4-hydroxy-2'-nonenal (4HNE)-adducted proteins in brain tissue. The levels of the pro-apoptotic proteins p53 and Bax and the anti-apoptotic protein Bcl-xL were significantly increased in Dox-treated mice compared with the control group. Consistent with the increase of apoptotic markers, the levels of caspase-3 activity and TUNEL-positive cells were also increased in Dox-treated mice. Pretreatment with xanthone suppressed Dox-induced increases in all indicators of injury tested. Together, the results suggest that xanthone prevents Dox-induced central nervous system toxicity, at least in part, by suppression of Dox-mediated increases in circulating TNF α . Thus, xanthone is a good candidate for prevention of systemic effects resulting from reactive oxygen generating anticancer therapeutics.

Keywords

Doxorubicin; xanthone; apoptosis; neurotoxicity; tumor necrosis factor-alpha

Doxorubicin (Dox), an antibiotic produced by the fungus *Streptomyces peucetius*, is a potent anticancer drug commonly used in the treatment of a variety of cancers including breast cancer (Hitchcock-Bryan et al., 1986). However, its clinical effectiveness is limited by its toxic effect on normal tissues (Singal et al., 2000; Oteki et al., 2005) including cumulative, dose-related cardiomyopathy (Singal and Iliskovic, 1998). Recent studies of breast cancer survivors have consistently shown similar changes in their cognitive function following chemotherapy, including memory loss, a tendency for lack of focus, and difficulty in performing simultaneous multiple tasks (Schagen et al., 1999; Brezden et al., 2000). These

cognitive problems, collectively called somnolence or cognitive dysfunction, are also reported in cancer patients, especially breast cancer patients, undergoing Dox-based chemotherapy (Schagen et al., 2001; Freeman and Broshek, 2002).

We have previously reported a significant increase in levels of protein oxidation and lipid peroxidation in brains isolated from Dox-treated mice (Joshi et al., 2005). We found that Dox induces circulating tumor necrosis factor- α (TNF α) which activates glial cells, leading to increases of TNF in the cortex and hippocampus (the memory function part of the brain). TNF induces mitochondrial dysfunction by its downstream consequences, resulting in further increases in oxidative stress, cytochrome c release, caspase 3 activity, and TUNEL-positive cell death, all of which are suggestive of apoptosis in brain following systemic Dox treatment. The levels of the known pro-apoptotic proteins p53 and Bax and the anti-apoptotic protein Bcl-xL were increased in brain mitochondria following Dox treatment. Blocking circulating TNF with anti-TNF antibody resulted in a significant decrease in TNF α levels and mitochondrial dysfunction in brain tissues (Tangpong et al., 2006).

Xanthones, a class of polyphenolic compounds, are biologically active antioxidant phytonutrients that neutralize free radicals and promote healthy cardiovascular, gastrointestinal, respiratory, immune and nervous systems (Jiang et al., 2003; Kondo et al., 2009; Marquez-Valadez et al., 2009; Pedraza-Chaverri et al., 2009). It has been shown that xanthones and xanthone derivatives have antioxidative and cardioprotective effects against isoproterenol-induced myocardial infarction (Devi Sampath and Vijayaraghavan, 2007; Kondo et al., 2009). Alpha-mangostin, an aprenylated xanthone derivative of mangosteen, has been shown to possess diverse pharmacological characteristics, including antioxidant and anti-inflammatory properties (Chen et al., 2008; Pedraza-Chaverri et al., 2008; Kondo et al., 2009). Xanthones have also been found to have the potential to combat Alzheimer's disease (Urbain et al., 2004).

The present study was undertaken to determine whether chemotherapy-induced neuronal injury can be prevented by xanthone supplementation. The neuroprotective effects of xanthone were investigated in mice by examining the level of TNF α , oxidative damage markers, and apoptotic markers in Dox based chemotherapy.

EXPERIMENTAL PROCEDURES

Animals

Eight-weeks-old male B6C3 mice (25–30 g) were divided into five groups of six mice each and maintained on a 12 h light/dark cycle in a temperature-controlled (23 $\pm$ 2 °C) room. The mice had free access to food and water. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Kentucky.

Experimental design

Mice were treated with a single dose of 20 mg/kg of Doxorubicin-Adriamycin (Dox) (DOXOrubicin HCl, Bedford Laboratories, Inc., Bedford, OH, USA) via i.p. injection. Xanthone (200 mg/kg, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 2% Gum acacia (Sigma-Aldrich, St. Louis, MO, USA) and administered as i.p. injection 15 min prior to the Dox treatment. Mice were euthanized 72 h after treatment. The experiments were conducted with five independent groups ($n=6$ per group) as follows:

Group 1: Control (Saline)

Group 2: Dox (20 mg/kg)

Group 3: Vehicle group (2% Gum acacia)

Group 4: Xanthone (200 mg/kg)

Group 5: Xanthone (200 mg/kg) + Dox (20 mg/kg)

Sample collection

Animals were anesthetized using Nembutal/sodium solution (65 mg/kg) (Abbott Laboratories, North Chicago, IL, USA) and blood was obtained via left ventricle puncture. Serum was collected for the macrophage stimulation experiments. Brain tissues were dissected from animals in all treatment groups and placed in ice-cold lysing buffer containing 4 g/ml leupeptin, 4 g/ml pepstatin, 5 g/ml aprotinin, 2 mM ethylenediaminetetraacetic acid (EDTA), 2 mM ethylene glycol-bis(tetraacetic acid) (EGTA), and 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), pH 7.4. The brain tissues were homogenized by 20 passes of a Wheaton tissue homogenizer, and the resulting homogenate was centrifuged at 20,000 g for 10 min and then assayed for protein concentration by the Pierce BCA method (Bradford, 1976).

Macrophage stimulation and cytokine assay

For experiments with macrophages, the mouse macrophage cell line J774A.1 (American Type Culture Collection) was cultured in DMEM supplemented with 10% (vol/vol) FBS, streptomycin (100 µg/ml) and penicillin (100 U/ml). All cultures were incubated at 37 °C in a humidified atmosphere with 5% CO₂. The cells were seeded onto a 48-well plate at 5×10^5 cells/well and allowed to grow overnight. Serum collected from the respective groups was inactivated at 56 °C for 30 min and filtered through 0.22 µm membrane and added to the cell cultures. After 24 h, incubation media were collected and TNFα levels were measured with an enzyme-linked immunosorbent assay (mouse TNF-α, R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions. The TNFα concentration in the sample was calculated from a recombinant mouse TNFα standard curve. The cells were also processed and measured for protein carbonyls 3-NT (nitrotyrosine) and 4-HNE (hydroxynonenal), as described below.

Measurement of protein carbonyls

Each sample (5 µl) of brain homogenate and macrophage cell lysate was mixed with 12% sodium dodecyl sulfate (SDS; 5 µl) and 10 µl 1:10 diluted 2,4-dinitrophenylhydrazine (DNPH) from 200 mM stock and incubated at room temperature for 20 min, followed by neutralization with 7.5 µl neutralization solution (2 M Tris in 30% glycerol). Protein (250 ng) was loaded into each well on a nitrocellulose membrane using a slot blot apparatus connected to a vacuum source. The membrane was blocked in blocking buffer (3% bovine serum albumin in PBS containing 0.01% (w/v) sodium azide and 0.2% (v/v) Tween 20) for 1 h and incubated with a 1:100 dilution of anti-DNP polyclonal antibody in PBS containing 0.01% (w/v) sodium azide and 0.2% (v/v) Tween 20 for 1 h. After incubation with the primary antibody, the membrane was washed three times in PBS at 5-min intervals then incubated for 1 h with an anti-rabbit IgG alkaline phosphatase secondary antibody diluted in PBS in a 1:8,000 ratio. The membrane was washed three times in PBS for 5 min and developed in substrate solution prepared with Sigma fast tablets (5-bromo-4-chloro-3-indolyl phosphate [BCIP]/nitro blue tetrazolium [NBT] substrate). The membranes were dried, scanned with Adobe Photoshop software, and quantified in Scion Image program (PC version of Macintosh-compatible NIH Image).

Measurement of 4-HNE and 3-nitrotyrosine (3-NT)

Each sample (5 μ l) of brain homogenate and macrophage cell lysate was mixed with 12% SDS (5 μ l) and 5 μ l modified Laemmli buffer containing 0.125 M Tris base, pH 6.8, 4% (v/v) SDS, and 20% (v/v) glycerol, incubated for 20 min at room temperature and loaded (250 ng) into each well on a nitrocellulose membrane in a slot blot apparatus connected to a vacuum source. The membrane was treated as described above except that 1-h primary antibody incubation was replaced with a 90 min incubation with a 1:5,000 dilution of anti-HNE polyclonal antibody in PBS.

Each sample (5 μ l) of brain homogenate was also mixed with 12% SDS (5 μ l), and modified Laemmli buffer (5 μ l) containing 0.125 M Tris base, pH 6.8, 4% (v/v) SDS, and 20% (v/v) glycerol, incubated for 20 min at room temperature and loaded (250 ng) into each well on a nitrocellulose membrane in a slot blot apparatus under vacuum. The membrane was treated as described above except that a 1:2,000 dilution of anti-3-nitrotyrosine polyclonal antibody was used for the primary antibody incubation.

Western blot analysis

Perfused brain homogenates were separated using 12.5% denaturing polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. The membrane was blocked for 1 h at room temperature in blocking solution consisting of 5% nonfat dried milk, 0.5% Tween-20, and Tris-buffered saline (TBST), pH 7.9. After blocking, the membrane was incubated overnight at 4 °C with primary antibodies against TNF (Upstate, Lake Placid, NY, USA), inducible nitric oxide synthase (iNOS), Bax, Bcl-xL (Santa Cruz Biotechnology, Santa Cruz, CA, USA), p53 (Cell signaling, Danvers, MA, USA) and β -actin (Clone AC-74, A5316, Sigma, St. Louis, MO, USA). The membrane was washed twice in TBST and incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies in blocking solution. After incubation with secondary antibodies, the membrane was washed twice with TBST and once in TBST without 0.5% Tween-20. Immunoreactivities of the protein bands were detected by enhanced chemiluminescence autoradiography (ECL, Amersham Pharmacia Biotech, Arlington Heights, IL, USA) as described by the manufacturer.

Brain nitric oxide levels

Nitric oxide (NO) production was measured based on the Griess reaction (Fox et al., 1982). A final volume of 200 μ l, including 40 μ l of sample, 40 μ l of nitrate/nitrite assay buffer, and 10 μ l each of nitrate reductase cofactor and nitrate reductase enzyme preparation, was incubated covered, at room temperature, for 3 h. After the addition of 50 μ l of Griess reagents, the samples were kept at room temperature for 10 min and formation of a deep purple azo compound was detected using a spectrophotometer at 540 nm. All reagents were obtained from Cayman Chemical (Ann Arbor, MI, USA).

Caspase 3 activity assay

Caspase 3 activity assay was performed with the use of a colorimetric substrate in accordance with the protocol supplied by the manufacturer (Sigma, St. Louis, MO, USA). In brief, mice were anesthetized and perfused with 1 $\times$ PBS to reduce any enzyme activity associated with intravascular blood components. Brain was dissected and homogenized in lysis buffer containing 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4, 5 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 5 mM Dithiothreitol (DTT) plus 1 μ g/ml aprotinin and pepstatin. The brain homogenates were incubated on ice for 15 min and centrifuged at 16,000 $\times$ g for 10 min. The protein concentration was determined by the Bradford method and the caspase 3 activity in

the supernatant was measured immediately. 50 µg protein samples in 10 µl were added to 980 µl assay buffer. The reaction was initiated by adding 10 µl of 20 mM of the caspase 3 substrate Ac-DEVD-pNA. The tubes were covered and incubated at 37 °C overnight. Cleavage of the chromophore from the substrate was detected spectrophotometrically at a wave-length of 405 nm.

TUNEL assay

The assay was performed following the manufacturer's instructions (Promega, Madison, WI, USA). Briefly, the cryosections of brain were fixed with 4% paraformaldehyde, permeabilized with Triton X-100, and incubated with biotinylated nucleotide and recombinant termination deoxynucleotidyltransferase (rTdT) for 1 h at 37 °C. The fragmented DNA labeled at the ends was coated with horseradish peroxidase-labeled streptavidin (streptavidin HRP) and detected as dark brown condensed nuclei, a positive indication of cell death. The sections were counterstained with Methyl-Green by incubating 5 min in Methyl Green staining dye followed by repeated rinsing in distilled water and subsequent quick dehydration using 95% alcohol (10 dips) and two changes of 100% alcohol (10 dips each). The sections were rinsed finally in xylene and mounted with mounting medium. Positive control samples were prepared by incubating sections with DNase I prior to treatment with terminal transferase. Negative controls consisted of specimens in which deoxynucleotidyltransferase were omitted.

Statistical analysis

Statistical analyses were performed using one-way ANOVA followed by Newman–Keuls post-test (GraphPad Prism-4). A *P*-value of less than 0.05 was considered a significant difference.

RESULTS

Dox-induced increase in TNF levels, iNOS protein levels and NO production is inhibited by xanthone

We previously have shown that Dox administration in mice increases the levels of the proinflammatory cytokine TNF α (Tangpong et al., 2006). As shown in Fig. 1a, TNF α levels were significantly increased in Dox-treated mice when compared to saline-treated controls. However, xanthone pretreatment for 15 min before Dox administration significantly decreased TNF α level when compared to Dox-only treatment (Fig. 1a, *P*<0.01). The increase in iNOS protein levels correlated with increased production of NO in brain tissues of Dox-treated mice. Xanthone ameliorated Dox-induced iNOS protein levels and NO production in the group treated with xanthone followed by treatment with Dox when compared to the group treated with Dox alone (Fig. 1b, c, *P*<0.01).

Xanthone reduces Dox-mediated increase in the levels of protein carbonyl, 3-nitrotyrosine and protein bound 4-HNE in brain tissues

Fig. 2 shows the levels of protein carbonyls (markers of protein oxidation), 3-NT (a marker of protein nitration) and protein-bound HNE (a lipid peroxidation product), respectively. In the Dox-treated group, levels of the above markers in brain tissues were significantly elevated as compared with the levels from saline- or xanthone-treated mice (*P*<0.01), confirming our previous findings (Joshi et al., 2005). Administration of xanthone significantly reduced the levels of protein carbonyls, 3NT, and protein-bound HNE (*P*<0.01) in brain isolated from mice injected with xanthone prior to the Dox treatment.

Xanthone inhibits DOX-mediated changes in pro- and anti-apoptotic proteins

Increase in oxidative stress leads to increase in the levels of pro-apoptotic protein, p53, Bax and anti-apoptotic protein, Bcl-xL, as detected by Western blot analysis (Fig. 3a–c). The increase of pro- and anti-apoptotic protein were significantly ($P<0.01$) inhibited by xanthone treatment prior to the Dox administration.

Xanthone reduces caspase 3 activity and TUNEL-positive apoptotic cells

Apoptotic cell death was represented by an increase in the activity of caspase 3 in Dox-treated mice when compared with the saline-treated mice ($P<0.01$). The apoptosis in brain was reduced by xanthone administration as revealed by a significant decrease in the elevated activity of caspase 3 following Dox treatment, as shown in Fig. 4a. Dox induces neuronal cell death via an apoptosis-like mechanism in brain tissues. Apoptotic cell death in brain was reduced when xanthone was administered prior to Dox, as revealed by a marked decrease in the elevated number of TUNEL-positive apoptotic cell deaths in comparison to the Dox-treated mice (Fig. 4b, c).

Xanthone inhibits Dox-mediated increases in TNF α , protein carbonyl, 3-nitrotyrosine and protein bound 4-HNE levels in macrophages

The mononuclear cell culture treated with serum isolated from Dox-treated mice showed increased levels of TNF, protein carbonyl, 3-NT, and protein bound 4-HNE (Fig. 5a–d [$P<0.01$]), all of which were significantly inhibited in the mononuclear cell culture treated with the serum isolated from the mice treated with xanthone prior to the Dox administration ($P<0.01$).

DISCUSSION

The neurotoxic effects of anticancer drugs that do not cross the blood brain barrier (BBB) have recently received considerable attention. Our group reported the first investigation of potential biochemical pathways for this event, using Dox as a prototype cancer therapeutic (Tangpong et al., 2006). We have also shown that increased protein oxidation and lipid peroxidation in brain isolated from Dox-treated mice could be involved in the symptoms of chemobrain observed in patients treated with Dox (Joshi et al., 2005). Dox does not cross the BBB (Bigotte and Olsson, 1982) but systemic administration of Dox significantly elevates the level of circulating TNF, which does cross the BBB to reside in neurons and increase neuronal-resident TNF α level to cause brain mitochondrial dysfunction (Tangpong et al., 2006, 2007). An increase of oxidative stress in neuronal cells leads to activation of pro-apoptotic protein and migration of p53 to brain mitochondria, which interacts with anti-apoptotic Bcl-xL (Tangpong et al., 2007). The interaction of p53 with anti-apoptotic protein and enhancement of pro-apoptotic protein can cause mitochondrial pore opening and cytochrome c release from mitochondria. Our results coupled with previous reports from other laboratories show that the release of cytochrome c from mitochondria to cytoplasm activates the apoptotic pathway and leads to increased activity of caspase 3 and more TUNEL positive cells (Brookes et al., 2000; Tangpong et al., 2007; Dohare et al., 2008). Our previous studies also demonstrate that the observed increase in 3-NT adducted protein after Dox treatment is mediated, in part, by an increase in NO level. It has been shown that NO-induced apoptosis is related to an increase in the Bax/Bcl-xL ratio, release of cytochrome c and caspase activation (Kolb, 2000). Our current data confirm these previous findings and demonstrate that this pathway may be preventable by systemic administration of xanthone.

The cytotoxic activity of Dox is partly related to its quinone structure. The drug is converted to a semiquinone free radical by NADPH-cytochrome P-450 that is subsequently reoxidized

and regenerated by oxygen, leading to the generation of superoxide anions (Goodman and Hochstein, 1977; Olson and Mushlin, 1990; Singal et al., 2000; De Beer et al., 2001). Increased superoxide anions may elevate the level of circulating TNF, which can directly pass through the BBB, as we have demonstrated in both the cerebral cortex and hippocampus, the memory and learning parts of the brain (Tangpong et al., 2007). Dox may exacerbate inflammatory processes, causing excessive release of TNF and resulting in cognitive disruption (Reichenberg et al., 2001). It is also possible that excessive inflammation activates microglia, causing further damage to neurons (Ahles et al., 2002).

The discovery that TNF mediates the toxicity of Dox in the brain suggests that an anti-TNF antibody could be a potential modality to quench oxidative stress in the brain, as recently demonstrated by the preliminary success of our study in a mouse model (Tangpong et al., 2007). Dox induces an imbalance between the generation of reactive oxygen species and reactive nitrogen species, ROS/RNS, and their cellular antioxidant system resulting in oxidative stress, which has been implicated in many neurodegenerative disorders, including Alzheimer's disease (Butterfield and Lauderback, 2002).

The results of the present study suggest that xanthone, the compound in *G. Mangostana*, is an antioxidant that acts as a scavenger of free radicals or anti-inflammation agent, which reduces the levels of TNF and prevents increase of iNOS and NO production. Previously, we showed that Dox treatment of mice lacking iNOS does not lead to brain mitochondrial dysfunction (Tangpong et al., 2006). Thus, it is interesting that administration of xanthone prior to Dox treatment significantly prevents an increase in the TNF, iNOS, and NO as compared with mice treated with Dox-only.

The efficacy of xanthone may be related to the anthocyanins found in the proanthocyaniclins in grape seed, a dietary antioxidant supplement that has been shown to enhance the anti-tumor activity of Dox and ameliorate Dox-induced myocardial oxidative stress in tumor-bearing mice (Zhang et al., 2005). The water and 50% ethanol extract of xanthone from *G. mangostana* exhibit antioxidative and neuroprotective activities in NG-108-15 neuroblastoma cells against H₂O₂-induced cell damage (Moongkarndi et al., 2004; Chen et al., 2008) and inhibit the lipopolysaccharide-stimulated NO production that inhibits iNOS expression and cytotoxicity in RAW 264.7 cells (Chen et al., 2008). Xanthone also shows a protective effect against lipid peroxidation during isoproterenol-induced myocardial infarction in rats (Devi Sampath and Vijayaraghavan, 2007). These data suggest that xanthone may protect against oxidative stress inducing agents via both direct and indirect action. Our results demonstrate that xanthone suppresses Dox-induced increases in circulating TNF α level and suggest that xanthone can exert an antioxidant effect via reduction of TNF α level. Our finding that serum obtained from animals pretreated with xanthone was inefficient for activating TNF α production by macrophage is consistent with this possibility.

CONCLUSION

In conclusion, our experimental paradigm provides a reproducible model to study the mechanisms of brain dysfunction caused by chemotherapy and to test the potency of possible preventive agents. Our findings suggest that a xanthone derivative isolated from the traditional Thai medicine, magosteen, may be effective for preventing tissue injury resulting from ROS generating chemotherapeutic drugs.

Abbreviations

BBB	blood brain barrier
BSA	bovine serum albumin
Dox	Doxorubicin
HRP	horseradish peroxidase
iNOS	inducible nitric oxide synthase
NO	nitric oxide
PBS	phosphate buffer saline
RNS	reactive nitrogen species
ROS	reactive oxygen species
rTdT	recombinant termination deoxynucleotidyltransferase
SDS	sodium dodecyl sulfate
TBS	Tris-buffered saline
TNF	tumor necrosis factor-alpha
3-NT	nitrotyrosine
4HNE	hydroxynonenal

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Fig. 1. Protein expression levels of TNF (a), iNOS (b) and NO (c) in brain tissue. Quantitative analysis represents the mean±SEM ($n=6$) in each group; * $P<0.01$ when compared to all other groups and @ $P<0.01$ as compared to Dox-treated group.

Fig. 2.

Oxidative stress analysis of brain homogenates showed significant increase in protein carbonyl (a), 3-NT (b) and 4HNE (c) 72 h after a single dose of 20 mg/kg Dox compared with control saline group (* $P < 0.01$) which was significantly decreased by xanthone treatment prior to Dox administration (@ $P < 0.01$). Analysis represents the mean $\pm$ SEM ($n=6$) in each group.

Fig. 3.

Xanthone inhibits Dox-induced apoptotic proteins. Pro-apoptotic proteins, p53 (a) and Bax (b), and anti-apoptotic protein, Bcl-xL (c), significantly increased in mice 72 h after treatment with a single dose of 20 mg/kg Dox as compared with saline control group (* $P < 0.01$). The levels of p53, Bax and Bcl-xL were inhibited in mice treated with xanthone 15 min prior to Dox administration. Analysis represents the mean $\pm$ SEM ($n=6$) in each group.

Fig. 4.

Xanthone inhibits Dox-induced increase in caspase 3 activity (a) and TUNEL-positive apoptotic cell death in cortex (b) and hippocampus (c). Caspase 3 activity was significantly increased in mice 72 h after treatment with a single dose of 20 mg/kg in Dox-treated mice as compared with the untreated mice (* $P < 0.01$). The caspase 3 activity in brain was rescued by xanthone (200 mg/kg) intraperitoneal injection prior to Dox treatment as compared to the mice treated with Dox-only (@ $P < 0.01$). Analysis represents the mean $\pm$ SEM ($n = 6$) in each group. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

Fig. 5. (a) TNF levels in macrophage cells showed a significant increase (* $P < 0.01$) with Dox-treated serum. Cells incubated in serum collected from mice treated with xanthone (@ $P < 0.01$) prior to Dox treatment showed a decrease in TNF production. A similar trend was observed in (b) protein carbonyls, (c) 3-NT and (d) 4-HNE levels. Analysis represents the mean $\pm$ SEM, $n=3$ in different experiments.