IJPSR (2014), Vol. 5, Issue 6 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 18 December, 2013; received in revised form, 18 February, 2014; accepted, 12 May, 2014; published 01 June, 2014

TRIDAX PROCUMBENS ATTENUATES ACETAMINOPHEN - INDUCED FREE RADICAL REACTION AND CELL NECROSIS IN CULTURED MOUSE HEPATOCYTES

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Keywords:

Tridax procumbens, Hepatocyte culture, Acetaminophen, Hepatocyte necrosis, Free radical reactions

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ABSTRACT: Drug-induced hepatotoxicity represents a major clinical problem and an impediment to new medicine development. *In vitro* evaluation of hepatotoxicity is an essential stage in the research and development of new pharmaceuticals as the liver is one of the most commonly impacted organs during preclinical toxicity studies. The effect of an aqueous leaf extract of *Tridax procumbens* was evaluated against acetaminophen-induced free radical reaction and liver cell necrosis in mouse primary hepatocyte culture. The liver was excised from the male albino mouse and cells were isolated and cultured. After monolayer developed, cells were treated with acetaminophen and different concentrations of *Tridax procumbens* aqueous extract. After the treatment for 3 to 24 h, free radical reaction, mitochondrial and extramitochondrial dehydrogenase activity, lactate dehydrogenase release, glutathione level, trypan blue uptake and haptocyte morphology were determined. Increased free radical reactions, LDH release, trypan blue uptake, liver cell necrosis and decreased levels of cellular glutathione, mitochondrial and extramitochondrial dehydrogenase activity were detected in acetaminophen-treated groups. Pretreatment of hepatocytes with *Tridax procumbens* extract caused attenuation of acetaminophen induced toxicity in various extents of oxidative stress, increased cell viability, glutathione level and mitochondrial and extramitochondrial dehydrogenase in a dose dependant manner.

INTRODUCTION: Adverse drug reactions are a clinical concern and cause attrition in drug development with hepatotoxicity being a major contributor. Metabolic activation of drugs is an important mechanism in drug-induced liver injury. Drug-induced liver injury is a potential complication of many prescribed medications.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.5(6).2185-92

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.5(6).2185-92

Acetaminophen [*N*-acetyl-*p*-aminophenol] (APAP) is a widely used analgesic and antipyretic drug. However, overdose of APAP is a frequent cause of acute hepatic failure and death[1].

At recommended therapeutic doses APAP is conjugated by glucuronic acid and eliminated in bile without any toxicity. However, levels of APAP over the limit of detoxification by glucronic acid conjugation are metabolized by cytochrome P-450 and form a chemically reactive metabolite, N-acetyl-p-benzoquinone imine (NAPQI) [2], which is normally detoxified by reduced glutathione (GSH) and eliminated as a mercapturic metabolite [3].

Higher doses limit the ability of GSH to detoxify the excess NAPQI, resulting in the depletion of liver GSH. GSH depletion then causes generation of reactive oxygen species (ROS) [4]. The excess NAPQI can covalently bind to cysteine residues (CYS) of critical hepatocyte cell proteins, forming APAP-CYS adducts, resulting in inactivation of these proteins [5]. Removing oxidized and damaged proteins from cells is essential for homeostasis. Oxidized proteins are degraded via the proteasome and lysosomal pathways. The proteasome is the major pathway for regulating mildly oxidized proteins by degrading them to short peptides. Ubiquitination allows the target protein to be recognized by the proteasome and targeted for protein degradation.

Oxidized proteins can also be degraded by the proteasome in the absence of ubiquitin [6]. Moreover, these highly reactive molecules have toxic effects on membrane phospholipids, resulting in lipid peroxidation, and cause oxidation of protein thiols, DNA fragmentation, cell necrosis and cell death [7, 8].

Plant-derived natural products have received considerable attention in recent years due to their diverse pharmacological propertie[9] Herbal drugs containing antiradical constituents are gaining importance in prevention and treatment of disease processes involving oxidative stress [10].

Tridax procumbens (TP) is used for various problems related to the liver such as jaundice, hepatitis, cirrhosis, and heart burn. It has been demonstrated that the chloroform insoluble fraction of ethanol extract of *Tridax procumbens* (TP) is most potent in alleviating the oxidative stress/liver injury caused by factors similar to viral hepatitis, drug intoxication, and lipid peroxidation due to reactive oxidative species [11- 16].

The aim of this study was to investigate the toxic effect of APAP and protective effect of the aqueous extract of TP on mouse primary hepatocyte cell culture.

MATERIALS AND METHODS:

Animals: Male healthy albino mouse (20 g) was purchased from Tamil Nadu Veterinary and Animal Sciences University, Madhavaram, Chennai, India. The animal was housed in an environmentally controlled room with 12-h light/dark cycle and allowed free access to food and water. All experimental protocols followed the criteria of the Central Leather Research Institute, Chennai, India for the care and use of laboratory animals in research.

Hepatocyte isolation: Primary hepatocytes were isolated from mouse anesthetized with pento-barbital sodium solution as previously described [17, 18] with minor modifications.

Animal cell culture medium and chemicals: Dulbecco's Modified Eagle's Medium with glutamine, without sodium bicarbonate and with 10% Fetal Bovine Serum, Penicillin and Streptomycin used for growing the hepatocyte primary culture medium was obtained from Invitrogen, USA. 0.25% Trypsin-EDTA, Hank's Balanced Salt Solution and Trypan Blue dye used for testing the viability was obtained from Hi-Media Laboratories, Mumbai. 0.2 % Collagenase (Type IV) and 2', 7'-dichlorofluorescin-diacetate (DCFH-DA) was obtained from Sigma, USA. All other chemicals used in the present investigation were of analytical grade from SRL Mumbai, India.

Acetaminophen (APAP): A stock solution of 0.2 mg/ml of APAP was prepared in PBS. Aliquots of stock were used for the treatment of cell cultures. An aliquot of this solution (175 µl) was added to the culture to obtain a final concentration of 0.5 mM APAP [19].

Preparation of extracts: TP leaves were collected from the Indian Institute of Technology, Chennai, Tamil Nadu, India. The extract was prepared from fresh leaves of TP by following the reported procedure of Diwan *et al.*, [20]. For the experimental study, a stock solution (in water 1mg/ml) of the extract was prepared. Aliquots of stock were used for the experiments. An aliquot of 40, 80 and 160 µl was added to the whole culture medium to obtain 3.2, 6.4 and 12.8 µg final concentrations of extract.

Primary Hepatocyte Cultures: Approximately 5 × 10^4 cells were transferred to T-25 flasks in 1 ml of Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin, streptomycin, and glutamine.

For APAP/ control/ TP treatments ,4 ml of this medium was added to the cells to bring the volume to 5 ml, and the hepatocytes were placed at 37°C in a 5% CO_2 / 95% O_2 atmosphere in a humidified incubator and allowed to adhere to flasks for 2 hr. After the adhesion period, cultures were washed to remove dead and non-adherent cells. Then the cultures were returned to the incubator with fresh medium.

Cell viability: Cell viability was assessed by trypan blue uptake and LDH release. After removal of the cell medium, hepatocytes were incubated with 0.8% trypan blue solution for 3 min at room temperature. Trypan blue-positive cells were counted in 4 different microscopic fields (X10; a total of approximately 1500 cells). For LDH release measurements, medium was removed from cells and lysis buffer containing 25 mM HEPES, 5 mM EDTA, 0.1% CHAPS, and 1 mg/ml each of pepstatin, leupeptin, and aprotinin, p^H 7.5, was added to the hepatocytes for 5min. Cells were removed from wells with a cell scraper and placed into a test tube. After sonication, cells were centrifuged for 20 min at 15,000 rpm at 4°C. Aliquots of the cell lysate or medium added to a reaction mixture in potassium phosphate buffer (60 mM, pH 7.5) containing 0.72mM pyruvate and 16mM NADH. The decrease in absorbance at 340 nm was measured with a spectrophotometer (UV-1601PC, Shimadzu Scientific Instruments).

Determination of hepatic GSH levels and SDS-PAGE protein separation: Total live cell glutathione (GSH + GSSG) levels were determined as described previously [21]. The liver cell proteins were separated by SDS-PAGE as described previously [22].

Measurement of reactive oxygen species: The non- fluorescent probe, 2′, 7′- diacetyl dichlorofluorescein (DCFH-DA) penetrates the intracellular matrix of cells and becomes oxidised by ROS to fluorescent dichlorofluorescien (DCF). DCFH-DA will cross the cell membrane where it undergoes hydrolysis by intercellular esterases to form the nonfluorescent DCFH. Upon oxidation, the highly fluorescent dichlorofluorescein (DCF) forms. Various pro-oxidants and oxidants ($ONOO^-$, AAPH, SIN-1 etc) can trigger DCF fluorescence [24]. Endogenous amounts of ROS were measured according to the method described by Pereira [25] with some modifications. Briefly in our study an aliquot of cells (40 µl) was diluted with 360 µl of normal phosphate buffered saline (pH 7.4) to which 100 µl of DCFH-DA (10 µM) was added and incubated at 37°C for 30 min. Fluorescent measurements (Perkin Elmer LS 45 model) were made with excitation and emission filters set at 485 nm and 530 nm respectively.

Mitochondrial and extra mitochondrial dehydrogenase (XTT) assay: Cell viability was also determined using the 2, 3-bis [2- Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide inner salt (XTT) system according to the manufacturer's instructions (Sigma). The XTT assay measures the activity of mitochondrial and extra mitochondrial dehydrogenase[26] and therefore provides an indicator of overall functional cell viability. The tetrazolium ring of XTT is cleaved by dehydrogenases of viable cells to produce soluble orange formazan, which can be detected spectrophotometrically. After adding XTT, the cells were incubated for 2 h and the increase in formazan absorbance was read at a wavelength of 450 nM on a microplate reader (Spectra Max 190, Molecular Devices.).

Morphological examination: After monolayer formation (36 h period of culture) the monolayer was examined microscopically using a phase contrast microscope (OLYMPUS model CK 40). Similarly, the monolayer treated with APAP and TP for 24 h period also was examined.

Statistics: All data is expressed as mean $\pm$ SD. Comparisons of the determined variables among the groups were conducted by using analysis variance (ANOVA). When the ANOVA showed a significant difference the group means were compared by Duncan's Multiple Range Test (DMRT). Values of $p<0.05$ were considered statistically significant. The data were analyzed using version 7.5 of SPSS.

RESULTS:

Effect of TP extracts on APAP-induced free radical reactions: Measurement of DCF fluorescence as indicator of reactive oxygen formation after exposure to APAP showed no significant change at 3 h (**Figure 1**).

However, between 6 and 24 h after treatment with 0.5Mm APAP, DCF fluorescence was increased > 10-fold above values of untreated cells (**Figure 1**).

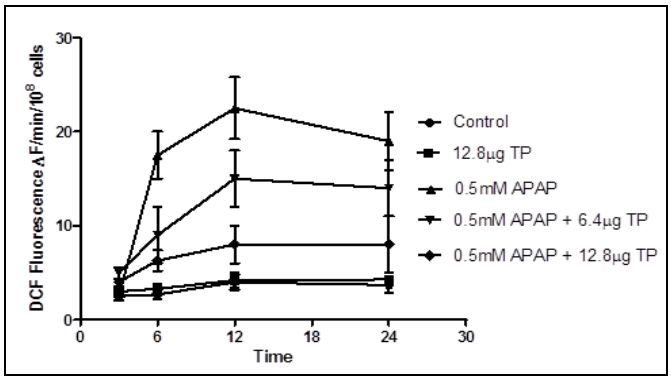

FIGURE 1: TIME COURSE OF 2′, 7′-DIACETYL DICHLOROFLUORESCEIN (DCF) FLUORESCENCE IN CULTURED MOUSE HEPATOCYTES. Time course of 2',7'-dichlorofluorescein (DCF) fluorescence in cultured mouse hepatocytes exposed to 0.5 mM acetaminophen (APAP), control, and some cells were treated with *Tridax* for , 6, 12,18 and 24h: After removal of the cell culture medium, cells were loaded with 5- and 6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (dissolved in PBS) for 30 min, and the increase in fluorescence was monitored. Data are given as mean of six values ± SD for each time period of 4 separate time-course experiments; $p < 0.05$ (compared to control at each time period).

The oxidant stress was first detectable when hepatocellular GSH levels reached their lowest values at 6 h after APAP exposure (**Figure 5**). Evaluation of LDH release and trypan blue uptake as indicators of cell injury in these experiments showed no significant increase in cell membrane permeability up to 6 h after APAP exposure (**Figure 2 & 3**). More than 50% of all cells were trypan blue-positive and 45% of the entire cellular LDH content was released into the cell culture medium at 12 h after APAP treatment (**Figure 2**).

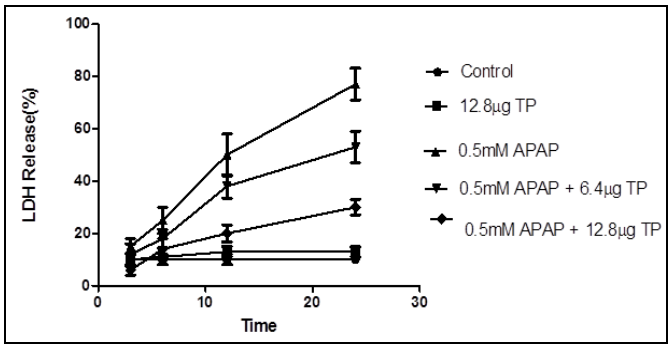

FIGURE 2: RELEASE OF LACTATE DEHYDROGENASE (LDH) IN CULTURED MOUSE HEPATOCYTES. Time course of cell injury as indicated by release of lactate dehydrogenase (LDH) in cultured mouse hepatocytes exposed to APAP and attenuated by *Tridax procumbens* in dose dependant manner. LDH was measured in the culture supernatant. Data are given as mean of six values ± SD for each time point of 4 (3, 6, 12 & 24 h) separate time-course experiments; $p < 0.05$.

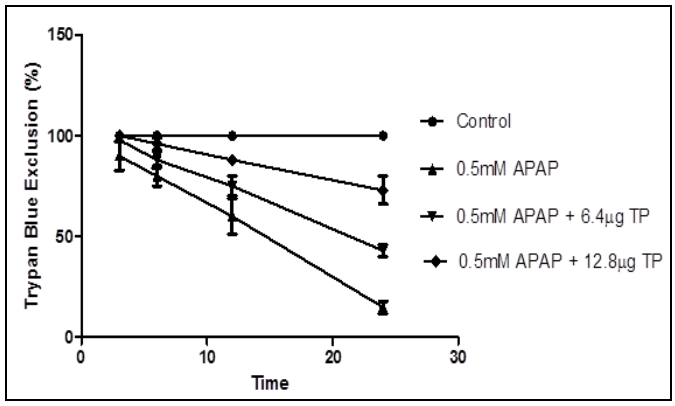

FIGURE 3: TRYPAN BLUE EXCLUSION. Time course of cell injury as indicated by trypan blue exclusion in cultured mouse hepatocytes exposed APAP and attenuated by *Tridax procumbens* in dose dependant manner. The percent of viable (trypan blue-negative) cells are shown for each time point. Data are given as mean of six values ± SD for each time point of 4 (3, 6, 12 & 24 h) separate time-course experiments; $p < 0.05$

Since trypan blue uptake and LDH release assess the permeability of the cell membrane, we used the XTT assay to evaluate cell viability with a functional parameter. This assay depends on cellular respiration, i.e., the activity of mitochondrial and extra-mitochondrial dehydrogenases. At various times after APAP treatment, XTT was added to the cell culture medium, and the cells were incubated for an additional 2 h. Compared to time-matched control cells, the capacity to reduce XTT was unchanged during the assay period between 1 and 3 h after APAP treatment (**Figure 4**).

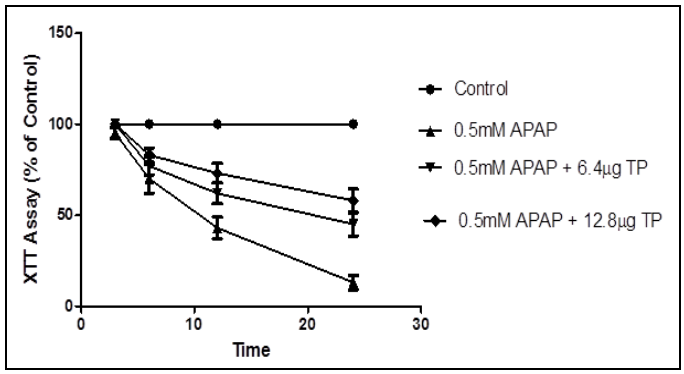

FIGURE 4: XTT ASSAY. Attenuation of *Tridax procumbens* on acetaminophen induced reduced functional viability (XTT assay) in dose dependant manner: Data are given as mean of six values ± SD for each time point of 4 (3,6,12 & 24 h) separate time course experiments; $p < 0.05$.

However, cellular respiration was reduced by 35% between 3 and 6 h, by 55% between 6 and 12 h, and by 84% between 12 and 24 h. Taken together, these time course experiments indicate that the onset of a functional deterioration of the cell correlates with the nadir of cellular glutathione levels, which is followed by increases in intracellular oxidant stress and later by cell necrosis as indicated by the permeability increases of the cell membrane and cell content release.

Glutathione depletion leads to increase in ROS production, mitochondrial impairment, cell damage and, finally, liver injury [23]. Since our data showed that the oxidant stress preceded loss of cell viability, we assessed whether enhanced recovery of cellular glutathione levels could reduce cell injury. Cells pretreated with TP had significantly higher hepatocellular GSH levels than control (untreated APAP) cells. Exposure of hepatocytes to APAP resulted in a time- dependent decline of GSH levels in controls (**Figure 5**) but not in TP - treated cells.

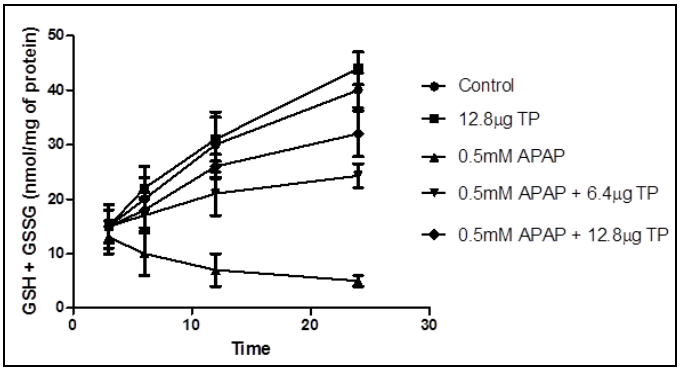

FIGURE 5: CHANGES IN INTRACELLULAR GLUTATHIONE. Time course of changes in intracellular glutathione (GSH + GSSG) levels in cultured Mouse hepatocytes exposed to 0.5 mM paracetamol , control and some cells were treated with *Tridax* (6.4 µg and 12.8 µg of fresh leaf extracts) for 6–24 h:Data are given as mean of six values ± SD for each time point of 4 separate time course experiments; $p < 0.05$.

Effect of TP extracts on APAP-induced protein degradation: Figure 7 shows the electrophoretic profiles of liver cell protein in different experimental groups. The result indicates that in APAP-treated cells 43KD and 36.22KD proteins were decreased. However TP treatment restores APAP affected protein similar to the control. Exclusively in the cells treaded with TP alone the protein density were found to be higher than the control.

Effect of TP extract on hepatocyte cell viability: The protective effects of TP extract against APAP-induced toxicity in hepatocytes are shown in **Figure 6**. The morphological examination of cells clearly depicts that TP could suppress the toxic insults of APAP (**Figure 6D and E**) in a concentration-dependent manner. At lower concentration (3.2 µg/ml), TP did not show a protective effect against APAP toxicity (data not shown). The TP extract alone did not confer any toxicity on hepatocytes, which appeared normal (**Figure 6 B**). However, most of the cells treated with APAP were completely disrupted due to toxicity. In the APAP-treated culture the cells were easily detached, showing fragmentation (**Figure 6.C**). In addition, blabbing and cell shrinkage could also be seen in these cells.

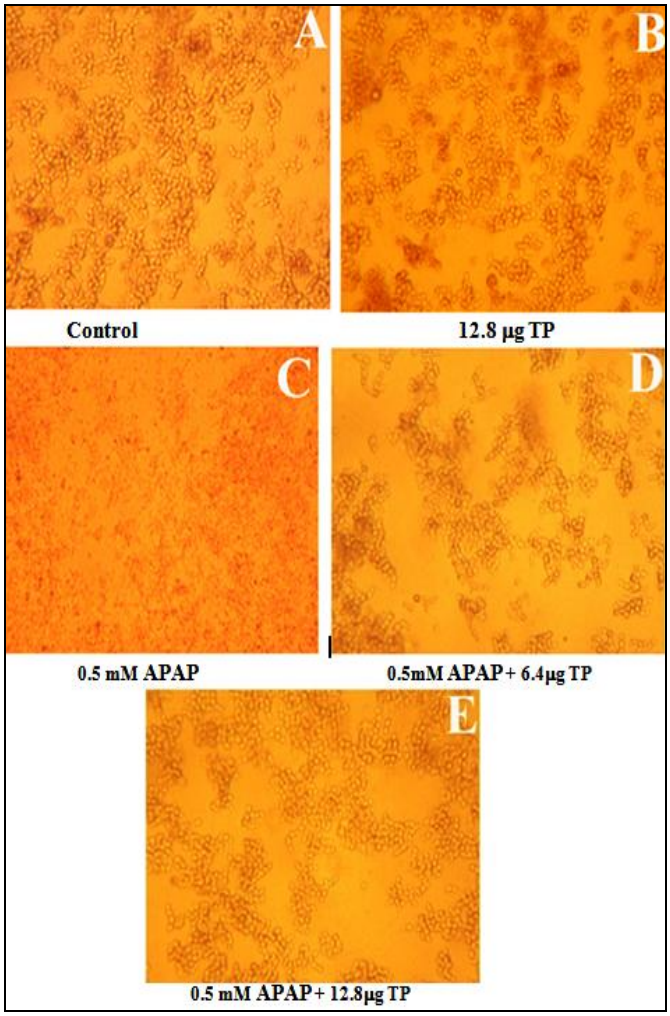

FIGURE 6: LIVER CELL MORPHOLOGY OF DIFFERENT EXPERIMENTAL GROUPS. ATTENUATION OF *TRIDAX PROCUMBENS* ON APAP-INDUCED LIVER CELL NECROSIS OF MOUSE PRIMARY HEPATOCYTE MONOLAYER CULTURE

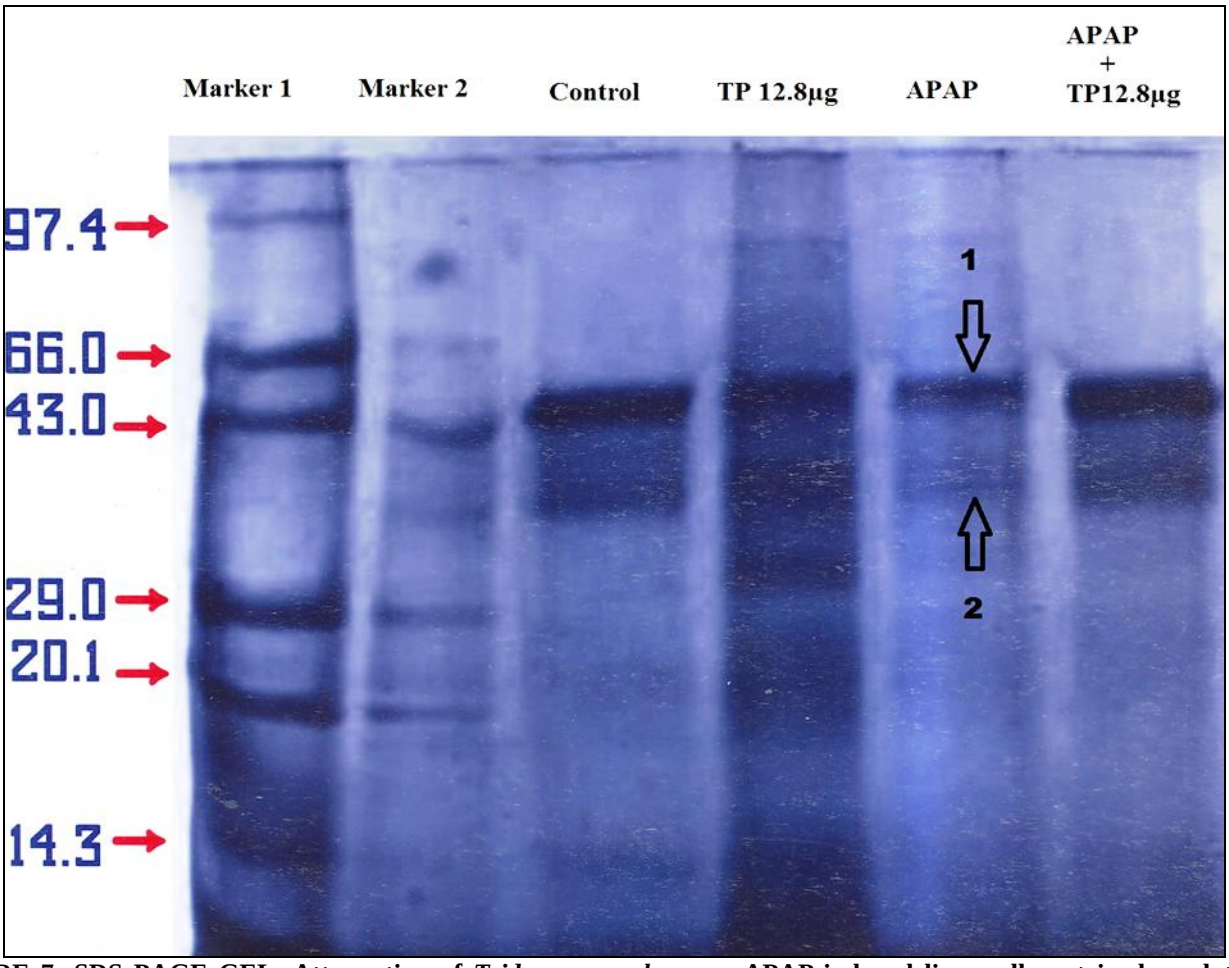

FIGURE 7: SDS PAGE GEL. Attenuation of *Tridax procumbens* on APAP-induced liver cell protein degradation of mouse primary hepatocyte. (Arrow mark 1 & 2 indicates that protein density decreased due to APAP toxicity)

DISCUSSION: APAP is a widely used analgesic and antipyretic. When taken in high doses, it becomes a potent hepatotoxin and can cause fatal hepatic necrosis [24] and is employed as an experimental hepatotoxic agent. Oxidative stress caused by APAP results in the release of LDH, a marker of cell damage [25]. GSH is the major non-enzymatic antioxidant and regulator of intracellular redox homeostasis, ubiquitously present in all cell types. Glutathione is an important antioxidant that protects the liver against acetaminophen induced damage. The depletion of GSH in liver cells and increase in ROS production in APAP toxicity [26].

At the cellular level, when proteins are exposed to reactive oxygen species, modifications of amino acid side chains occur and, consequently, protein structure is altered. These modifications lead to functional changes that disturb cellular metabolism [27]. A strong correlation was demonstrated between increased hydrophobicity on the protein surface and the recognition and proteolytic degradation of oxidatively modified proteins [28].

The mechanism of hepatoprotection by TP against APAP toxicity might be due to restoration of the GSH level and prevention of protein degradation. Many factors can influence the rate of glutathione synthesis and therefore the amount of glutathione available in the cell for drug detoxification [29].

Previous phytochemical results showed that TP contains alkaloids, flavonoids, saponins, carotenoids, tannins and other components [30]. Xu *et al*., reported that two new flavones, 8,3′-dihydroxy-3,7,4′-trimethoxy-6-*O*-*β*-D-gluco-pyranosyl flavone and 6,8,3′-trihydroxy-3,7,4′-trimethoxy flavone also presented in TP [31].

It has been reported that flavonoids are able to inhibit D-galactosamine and CCl_4-induced hepatotoxicity in experimental models due to their potent anti-oxidant or free radical scavenging properties [32, 33]. This active principle may account for the pharmacological properties of TP extracts. In addition, there is evidence that, alkaloids elicit hepatoprotective activity by strongly inhibiting

lipid peroxidation and cell membrane disruption in cultured human liver-derived HepG2 cells against CCl4-induced damage [34]. Moreover the administration of biological antioxidants such as β-carotene suppresses lipid peroxidation in the plasma of rats**.** β -carotene is a quencher of free radical induced oxidative stress [35]. The previous studies have been reported that the TP are able to inhibit paracetamol and 2-Acetylaminoflourene induced hepatotoxicity [36, 37]. The present study has demonstrated that the TP exerts an attenuated against APAP-induced toxicity in mouse primary hepatocyte culture. Increased levels of GSH and XTT assay and a reduction in the amount of LDH release, oxidative stress, protein degradation and increased cell viability are likely to be the major mechanisms by which TP prevents development of liver cell damage induced by APAP.

CONCLUSION: The protective activity of the aqueous extract of *Tridax procumbens* against APAP-induced injury in mouse hepatocytes may be due to its antioxidative properties, possible through restoration of GSH levels. Further safety and efficacy studies are needed to elucidate its mechanism of action in detail that plant itself reacts with the reactive oxygen species or boosting the antioxidant enzyme and GSH production.

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How to cite this article:
Gnanasekaran N, Murugan P and Seifu D: *Tridax procumbens* attenuates acetaminophen - induced free radical reaction and cell necrosis in cultured mouse hepatocytes. Int J Pharm Sci Res 2014; 5(6): 2185-92.doi: 10.13040/IJPSR.0975-8232.5(6).2185-92