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# Inhibition of oxidative stress, inflammation and apoptosis by *Terminalia arjuna* against acetaminophen-induced hepatotoxicity in Wistar albino rats

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Overuse of therapeutic drugs such as acetaminophen often affects liver, and may lead to inflammatory mediated liver cell death. Here, we studied the effect of *Terminalia arjuna* (TA) bark against acetaminophen (APAP) induced liver cell death/injury by testing the antioxidant levels, oxidative stress, and inflammation and apoptosis markers. Wistar albino male rats weighing 180-280 mg/kg were made into 5 groups of 6 animals each and were treated as follows: Gr. I, control; Gr. II, acetaminophen (APAP); GR. III, N-acetylcysteine (NAC); Gr. IV & V, *Terminalia arjuna* (TA) 250 and mg/kg. The antioxidant glutathione (GSH), lipid peroxidation (MDA), interleukin 1β (IL-1β) levels, caspase-9 levels, and Protein kinase B (P-AKT) gene expression levels were assessed. The rGr. V animals pre-treated with *Terminalia arjuna* high dose bark showed increased glutathione (GSH) levels, but decreased malondialdehyde (MDA) levels; inhibited IL-1β and caspase-9 levels; and also elevated gene expression level of P-AKT to regulate the cell signaling pathway. Apparently, the results demonstrated that a high dose of TA 500 mg/kg ameliorated acetaminophen-induced hepatotoxicity.

**Keywords**: Ayurvedic, Caspases, CYP2E1, DNA damage, Glutathione, Paracetamol, Proinflammatory cytokine

Liver intoxication occurs inherently during the process of xenobiotics in which lipophilic natures of drugs are converted into hydrophilic substances. This biotransformation is due to the mediation of cytochrome P450, glucuronidation and sulfation mechanisms. Popular acetaminophen is one of the responsible drugs for acute hepatotoxicity. The incidence of acetaminophen related hepatotoxicity has raised worldwide[1]. Acetaminophen is available over the counter (OTC) and its abuse is seen widely, at times deliberately for self-poisoning, which eventually leads to irreversible liver cell damage. Therefore, there is a need for developing a new therapeutic drug to prevent the irreversible hepatic injury.

*Terminalia arjuna* bark is a traditional ayurvedic medicine known for its cardiotonic effect. It is composed of many phytoconstituents, such as triterpenoids, flavonoids, saponins, alkaloids, phytosterol, tannins and phenolic compounds. These phytochemicals of *T. arjuna* have been shown to possess antioxidant and anti-inflammatory effects on acute liver injury. High dose of acetaminophen (APAP) alters the cell signaling pathway and damage cells by increasing the ODFR (oxygen damaged free radicals) and the levels of malondialdehyde (MDA), inflammatory caspases-1, and apoptotic caspase-9, but depleting glutathione (GSH) levels[2-6]. Therapeutic dose of acetaminophen is conjugated into glucuronide and sulfate which are eliminated through the excretory mechanism. In the condition of an excessive dose, acetaminophen (APAP) is converted into a toxic compound N-acetyl-p-benzoquinone imine (NAPQI) by cytochrome P450 (CYP450) enzyme which is detoxified by GSH[7-9]. Enormous accumulation of toxic N-acetyl-p-benzoquinone imine (NAPQI) occurs when there is an insufficient amount of GSH which ties up with macromolecules to increase lipid peroxidation and central lobular necrosis by the formation of tyrosine nitration[10]. Thiol-containing compound of N-acetylcysteine (NAC) is the main precursor of glutathione to detoxify the high dose of acetaminophen induced unconjugated toxic metabolite[11,12]. The excessive synthesis of glutathione reduces the oxygen damage free radicals (ODFR). Nevertheless, NAC does

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not solve the liver injury problem and may even require liver transplantation[13]. Hence, natural therapeutic agents such as *Terminalia arjuna* (TA) can be used to treat liver cell damage as it is more effective in enhancing bioactive compounds. Thus, in the present study, we explored the effect of *Terminalia arjuna* bark on glutathione, MDA levels, a pro-inflammatory cytokine, apoptotic caspases, and cell signaling pathway.

## Materials and Methods

**Source of chemicals**

Acetaminophen 99.0 was obtained from Sigma Aldrich, N-acetylcysteine was purchased from Samarth life sciences Pvt. QIA amp DNA Stool Mini Kit was purchased from Qiagen Inc., Hilden, Germany. Acids, bases, solvents, and salts used in the investigation were of analytical grade (AR) and were obtained from Glaxo Laboratories, SRL, Mumbai, India and Anilax chemicals, USA. The glutathione, MDA standards were purchased from Sigma Aldrich, USA.

**Instruments**

UV-Vis Spectrophotometer - Shimadzu UV1800
ELISA reader - MINDRAY 96A
RT-PCR - Applied Biosystems Veriti 96-Well Thermal Cycler

**Methodology for aqueous extract preparation**

The *T. arjuna* (TA) bark powder was purchased from Herbal Care & Cure Centre, Mylapore, Chennai-600004. One kg of the plant bark powder was mixed with 2 L of hot boiled water. The sterile conical flask containing 250 mL mixture was plugged with sterile cotton and kept in shaking incubator at 200 rpm for 24 h. The aqueous extracts were filtered with muslin cloths repeatedly for three times. The filtrated extracts were dried under reduced pressure at 40°C on a rotary evaporator and stored in a refrigerator at 4°C. The percentage of yield was – 17.9% (Fig. 1).

Fig. 1 — *Terminalia arjuna* bark, *Terminalia arjuna* bark powder, Aqueous extract preparation, Animal Groups

**Animals**

Wistar albino male rats (180-280 mg/kg) were used in the study. The animals were housed in five polypropylene cages (6 per cage) containing sterile paddy husk as bedding material with pellet diet, water *ad libitum* and maintained in the Centre for Laboratory and Animal Research (CLAR), SIMATS under standard conditions. The bedding material of the cages were changed every day. The whole experiment was carried out as per the approved guidelines of the Committee for Purpose of Control and Supervision of Experiments on Animals (CPCSEA, India) and the protocol was approved by the Institutional Animal Ethical Committee, Saveetha University (SU/CLAR/RD/005/2018 Dated 10/12/2018).

**Experimental design**

*Acute Toxicity study*

The animals were divided into five groups and each consists of six animals as follows: Group I, Normal control and received 0.5% of HPC (Hydroxypropyl cellulose) vehicle; and Group II, Acetaminophen (APAP) control and received 0.5% of HPC; Group III, N-acetylcysteine (NAC) @200 mg/kg/body wt.) once daily; Group IV & V, *Terminalia arjuna* (TA) @250 and 500 mg/kg/body wt.) once daily. All the treatments continued for 14 days. All the experimental groups but for Gr. I were administered with acetaminophen (750 mg/kg) as a single dose on day 14 after 1 h of treatment drug. All the test drugs and acetaminophen were administered orally. After 24 h of acetaminophen feeding, the animals were anesthetized with isoflurane; blood was collected by retro-orbital route into heparinized vacutainers. The blood was centrifuged at 3500 rpm for 10 min. Serum was collected and stored for liver function tests. The animals were sacrificed by using isoflurane anesthesia and liver tissue was removed, weighed and stored for further study.

*Estimation of glutathione*

GSH was estimated as the total non-protein sulfhydryl groups by the method described by Moron[14]. To precipitate the proteins, 25% TCA was added to the liver homogenate. TCA added mixture was centrifuged. The volume of the aliquot contained phosphate buffer, DTNB solution. The yellow colour formed was read with a spectrophotometer.

*Estimation of malondialdehyde*

The Hogberg method was followed to observe the pink color product formation after a heated mixture of

TBA and MDA[15]. The end product was measured by spectrophotometer.

*Hematoxylin & Eosin staining*

The collected fresh liver tissues were stored in the buffered formalin (10%) container about 24-48 h. The sequence methods of fixation, paraffin embedding bath, tissue blocking, tissue sectioning by rotary microtome were done for tissue processing. The mounted tissue slides were stained by hematoxylin and eosin stain. Alcohol, xylene were also used during tissue processing and staining. The prepared slides were examined under the microscope to observe the pathological changes in the liver tissues.

*IL-1β, Caspase-9, CYP2E1 estimation method*

Active IL-1β, caspase-9, and CYP2E1 in serum were measured using ELISA kit (Enzo Life Science) with manufacturer's instruction. The kit uses a double-antibody sandwich ELISA to assay the level of active IL-1β, caspase-9, and CYP2E1. The test samples, HRP-labeled monoclonal antibodies were added to the enzyme wells and then washed three times with PBS buffer to remove the uncombined enzyme after incubation. Added a chromogen substrate finally. The color of the liquid changed to yellow. The intensity of the color and the concentration of samples were positively correlated.

*Protein kinase B (PKB) or AKT gene expression*

Total RNA extracted from the homogenized liver tissue. The manufacturer's instructions were followed one step of RT-PCR analysis. RT-PCR reactions contained total RNA with primer, RT-Taq Mix, and 25 μL of the reaction mix. A thermal cycle of cDNA synthesis, denaturation, and PCR amplification cycles was done. Electrophoresed agarose gel medium was used to analyze the prepared RT –PCR products and visualized by ethidium bromide. The used primer sequences were mentioned below:

| Genes | Forward primer | Reverse Primer |
|---|---|---|
| Akt | 5′-GCTGGACGAT AGCTTGGA-3′ | 5′-GATGACAGAT AGCTGGTG-3′ |
| β-actin | 5′-GTGGGGCGCC CCAGGCACCA-3′ | 5′-CTCCTTAAGTC ACGCACGATTTC-3′ |

*Agarose gel electrophoresis*

Gel tray contained 1.5% agarose, 1X TAE buffer, PCR components, and marker ladder. The PCR mixture were run in gel at 50 V for 90 min and visualized. Gel pro analyzer software displayed the band intensity in digital images. The relative amount of each mRNA was normalized to the reference gene, β-actin mRNA.

**Statistical analysis**

Results of all the parameters were analyzed and expressed as the Mean ± Standard error of the mean (SEM). The statistical analysis of data was conducted with Statistical Package for Social Sciences (SPSS) software. Comparisons between groups that were more than two were performed using a one-way analysis of variance (ANOVA) followed by Dunnett's *t*- test.

## Results

**Inhibition of oxidative stress by *T. arjuna* bark extract**

*T. arjuna effect on antioxidant GSH level*

GSH levels were highly elevated in pretreated *Terminalia arjuna* (TA) high dose (500 mg/kg bw) group (Group V) compared to acetaminophen (APAP) toxic group (GroupII) and N-Acetylcysteine (NAC) standard group (Group III) (Fig. 2). But in TA Low

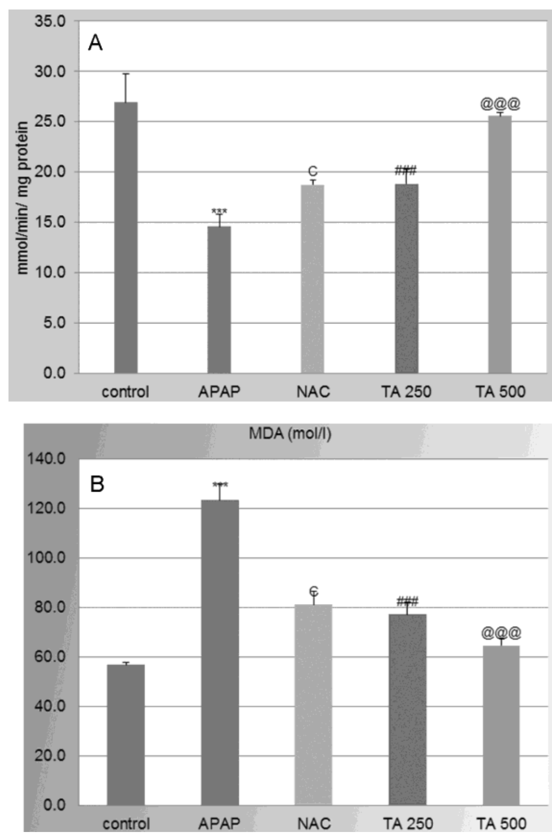


Fig. 2 — (A) Glutathione (GSH); and (B) MDA levels in the liver tissue of experimental rats. [$^{***}P$ <0.001 is statistically significant as compared with control group rats; $^{c,\ @@@}P$ <0.001 and $^{c,\ \#\#\#,\ @@@}P$ <0.001 is statistically significant as compared with APAP group rats, respectively. $^{\#\#\#}$ $P$ <0.05 is statistically significant as compared with APAP group rats. Results are expressed as Mean ± SEM for n=6 animals]

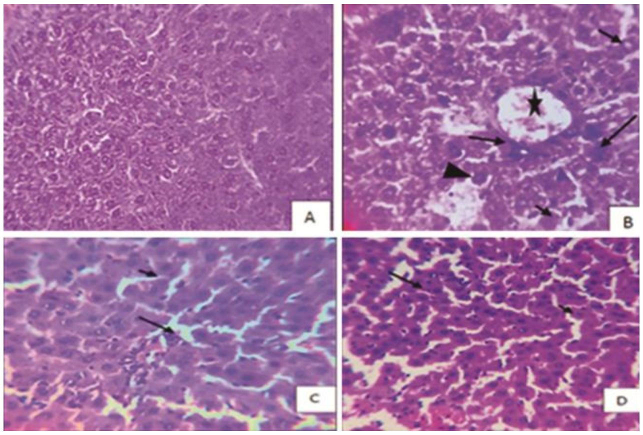


Fig. 3 — H&E stain of liver tissue. (A) Control; (B) APAP; (C) NAC; and (D) TA 500 mg group

dose group (group IV) levels were significantly lower than the standard drug NAC group.

*T. arjuna effect on lipid peroxidation level*

The pretreated TA high dose significantly attenuated the MDA levels compare with other groups (Fig. 2). Increased level of lipid peroxidation in the APAP toxic group can cause central lobular necrosis in the liver.

*T. arjuna effect on H & E stained liver tissues*

The recovery of hepatic cords with normal nuclei was appearing in TA 500 mg/kg group liver tissue (Fig. 3). It shows normal cords of hepatocytes which are bounded by an intact endothelium. Figure 3B, APAP treated rats showing extensive centrilobular necrosis (large arrows), hydropic degeneration (small arrows), severe hemorrhage (asterisks) with congestion of sinusoidal spaces, destruction of central vein (CV) karyorrhexis of nuclei (arrowheads). Figure 3C, NAC treated rats showed mild glycogen depletion (large arrow) and sinusoidal congestion (small arrow) was evident slightly on the hepatic lobule. Figure 3D TA (500 mg/kg) treated rats showed less sinusoidal congestion (small arrows), recovery of damaged hepatocytes and normal hepatic nuclei (large arrow).

*Inhibition of Pro-inflammatory cytokine of IL-1β level*

Acetaminophen (APAP) group had shown significantly increased the levels of IL-1β in serum compared with the control and other treatment groups (Fig. 4). TA 500 mg/kg group had shown alleviated IL-1β level than standard NAC group and APAP group. These results indicate that pre-treated TA 500 mg/kg remarkably inhibited the IL-1β to reduce the neutrophil and ROS production in the liver.

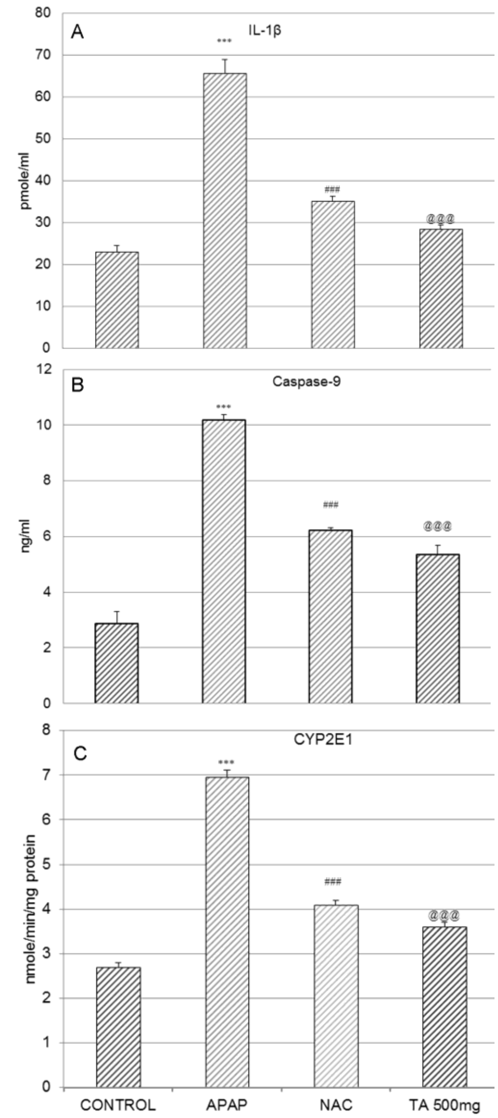


Fig. 4 — (A) Interleukin-1β; (B) caspase-9; and (C) CYP2E1 levels of experimental rats. [Results are expressed as Mean ± SEM for n=6 animals. $^{***}P$ <0.001 statistically significant as compared with Control rats; $^{\#\#\#,\ @@@}P$ <0.001 statistically significant as compared with APAP rats]

*Inhibition of Initiator Caspase-9 cysteine protease level*

Serum caspase levels were found reduced in the pretreated TA 500 mg/kg group compared to the standard NAC group (Fig. 4). The TA 500 mg/kg group was highly significant as compared with the APAP group suggesting that pretreatment with TA 500 mg/kg group may prevent the uncontrolled cell death.

*Inhibition of CYP2E1 enzyme expression*

CYP2E1 levels were elevated in the APAP group. But the pretreated TA 500 mg/kg group showed significantly attenuated CYP2E1 levels (Fig. 4).

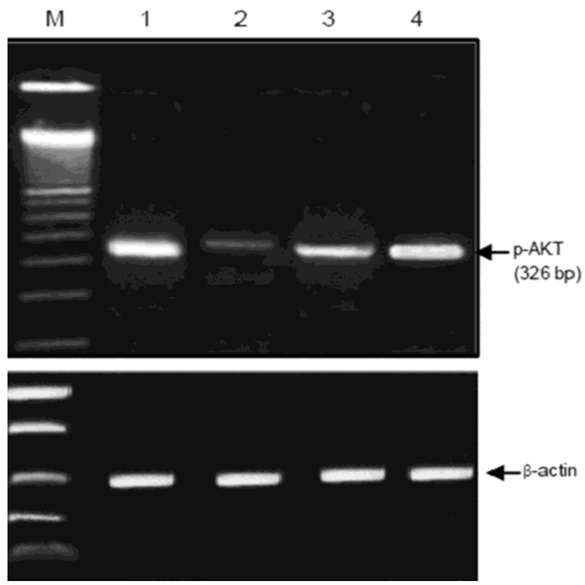


Fig. 5 — P-AKT gene expression of experimental rats. Lane 1- Marker lane; Lane 2 – Normal control; Lane 3 – APAP induced; Lane 4 – NAC ; Lane 5 – *Terminalia arjuna* (500 mg/kg)

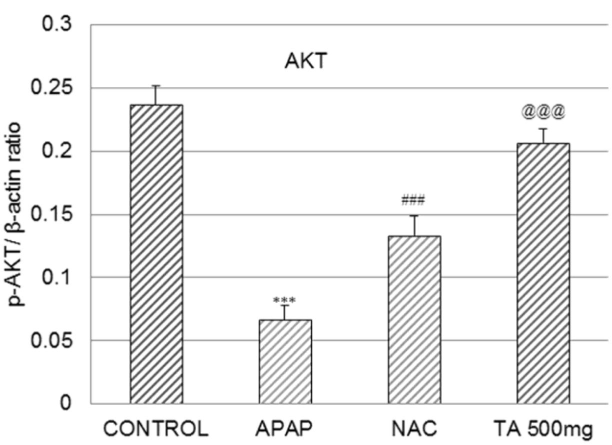


Fig. 6 — Shows the P-AKT gene expression levels of experimental rats. Results were expressed as Mean ± SEM for n=3 animals. $^{***}P$ <0.001 statistically significant as compared with Control rats; $^{\#\#\#,\ @@@}P$ <0.001 statistically significant as compared with APAP rats

CYP2E1, thus plays a key role in APAP detoxification. The decreased CYP2E1 levels in the TA 500 mg/kg group suggested that the TA bark was effective in the treatment of acute liver injury.

*Gene expression levels of P-AKT by RT-PCR*

The AKT gene expression was analyzed by RT-PCR (Figs. 5 & 6). The result indicated that the P-AKT levels in the APAP group was very low than the control group. But the gene expression levels were higher in the *T. arjuna* (TA) group which is almost similar to the control group.

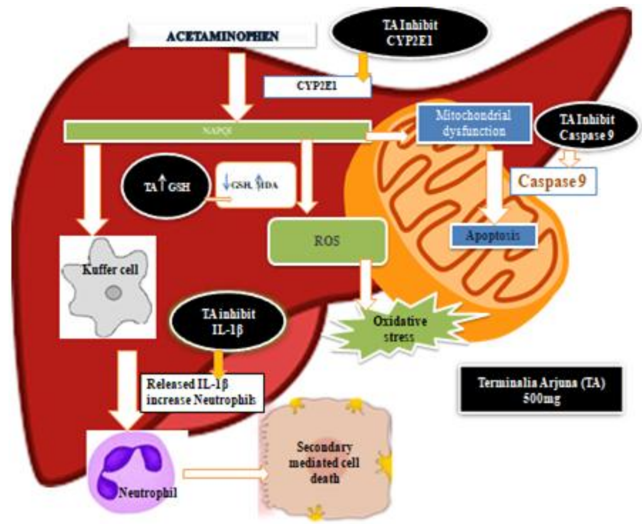


Fig. 7 — Restoration of liver tissue against acetaminophen-induced hepatotoxicity by *T. arjuna* bark extract

## Discussion

The liver function parameters, and antioxidants analyzed in the reports had shown that the high dose of *Terminalia arjuna* is more effective than the low dose. Hence, the only biochemical and antioxidant studies were performed in the low dose group in the present study and other studies were given up owing to futility. Pro-inflammatory cytokine, *Tumor necrosis facto*r TNF-α and Interleukin IL-1β are primary cytokines on Acetaminophen (APAP) induced Acute Liver Injury (ALI) and as well responsible for chronic inflammatory diseases[16,17]. Cytokines and chemokines are up-regulated in the innate immune cells which begin to infiltrate the neutrophils in the liver at the initial point of sterile inflammation[18].

Apoptosis is a well-programmed cell death to maintain normal cellular survival[19]. It works in different ways, such as intrinsic, extrinsic and stress-induced apoptosis *via* apoptotic caspases. Caspases are key cysteine proteases responsible for the execution of apoptosis[20]. There are initiator caspases (caspases 2, 8, 9 and 10), executioner caspases (caspases 3, 6 and 7), and inflammatory caspases (caspases 1, 4, 5, 11 and 12)[21,22]. The study explains the role of initiator caspase-9 on the intrinsic apoptosis pathway[23,24]. High dose of APAP activates the mitochondrial apoptosis by disrupting the mitochondrial membrane potential and activating the pro-apoptotic Bcl-2 group (Fig. 7)[25-27]. In another way, APAP indirectly stimulates the cytokine IL-1β through which it aggravates the neutrophil accumulation on the site of liver injury (Fig. 7)[28-30].

Nuclear protein high mobility HMGB1 and damage-associated molecular patterns (DAMP) were released after injection of a high dose of APAP in the necrotic cell[31,32]. These complexes activate inflammasomes *via* Toll-like receptors[33,34]. The inflammasomes are composed of the proteins Nalp3, caspase-1, and ASC[35]. The activated inflammasomes trigger the inactive pro-caspases-1 into mature caspases-1. The IL-1β converts enzyme (ICE), also known as caspase-1. The name indicates caspase-1 can cleave pro-IL-1β into mature IL-1β. Finally, IL-1β and TNF–α are released from kupffer cells. The released matured IL-1β is a key mediator for the release of an enormous amount of ROS by triggering neutrophil[29]. The experimental evidence indicates that a high dose of TA 500 mg/kg attenuates the ROS production by inhibiting IL-1β and preventing the secondary cell-mediated death. The experimental study revealed a low IL-1β levels in pre-treated *Terminalia arjuna* group than the acetaminophen group. The inhibited IL-1β levels in the *Terminalia arjuna* group leads to a reduction in neutrophil and ROS production.

Bcl-2 family proteins play a crucial role to initiate mitochondrial apoptosis through pro-apoptosis of BCL2-Associated X Protein (BAX) and Bcl2 Antagonist killer (BAK). The toxic dose of APAP creates mitochondrial permeability transition pore (MTP) by which translocation of the pro-apoptotic proteins from the cytosol to the mitochondrial membrane causes the release of cytochrome C[36]. The released cytochrome C oligomerizes with apoptotic protease activating factor 1 (Apaf-1) to form the apoptosome[37,38]. It activates the caspase-9 to cleave the executioner caspase-3 and caspase-7 for executing the uncontrolled cell death due to a toxic dose of APAP. In our data, TA 500 mg/kg group was found to alleviate the APAP induced increased levels of caspase-9. Therefore, the TA 500 mg/kg group prevents abnormal apoptotic pathway by inhibiting the caspase-9. Some other proteins also participate in the process of cell apoptosis such as endonuclease G, apoptosis-inducing factor (AIF), High-temperature requirement protein A2 HtrA2, a second mitochondria-derived activator of caspases (Smac) and direct IAP-binding protein with low pI (DIABLO)[39-42].

## Conclusion

The present animal study revealed in detail the effect of pre-treated *Terminalia arjuna* in the acetaminophen (APAP) induced inflammation, apoptosis, oxidative stress than the standard NAC group. TA 500 mg/kg group decreased the level of CYP2E1 expression, IL-1β levels, Caspase-9s level to control the oxidative stress, inflammation, and apoptosis. There is an elevated AKT gene expression level in the TA 500 mg/kg group to regulate the PI3k/AKT pathway.

## Conflict of Interest

Authors declare no conflict of interest.

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