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Original Article

# Protective effects of NAT-CLW™ on acetaminophen-induced oxidative stress-mediated apoptosis in hepatic HepG2 cells

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## ABSTRACT

The hepatoprotective mechanism against drug-induced liver damage is the activity being searched to identify new therapeutic targets. Among them, the detoxifying effect of NAT-CLW™ is a plant formulation, which exhibits several pharmacological properties such as antioxidant, anti-inflammatory, anti-apoptotic activities, and inhibitory effect through its protective role in hepatic HepG2 cells. However, its therapeutic mechanism of liver damage is still unclear. The aim of the present study, we employed that the hepatoprotective effect and underlying mechanism of the NAT-CLW™ on acetaminophen (APAP) exposed hepatotoxicity were investigated in HepG2 cell line model. The results showed that NAT-CLW™ pre-treatment dramatically increased APAP-induced cell viability and inhibit increased ROS generation. These effects also showed strong antioxidants by an enhance in activity of GSH, SOD and by a diminished lipid peroxidation level compared to APAP induced group. Furthermore, pre-treatment with NAT-CLW™ drastically prevented mitochondrial alteration and protected morphological chances including (cell shrinkage, nuclear fragmentation, condensation, and cell blubbing) induced by APAP. These findings suggest that the hepatoprotective activity of NAT-CLW™ on APAP-induced hepatotoxic was closely associated with suppression of APAP-induced oxidative stress and mitochondrial dysfunction in HepG2 cells. These results strongly indicate that NAT-CLW™ has a significant protective effect against APAP induced hepatotoxicity.

**Keywords:** Acetaminophen, apoptosis, NAT-CLW™, oxidative stress, silymarin

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## INTRODUCTION

The liver is one of the important organs in the human body and actively involved in many metabolisms and major function of the liver are protein, carbohydrates, lipids and also detoxification of powerful toxic drug and metabolites, chemical, and environmental pathogens.[1] However, it is continuously and variedly to environmental toxins and abused by poor drug habits, alcohol, prescribed, and over the counter drug which can eventually lead to various liver diseases.[2,3] Acetaminophen (*N*-acetyl-*p*-aminophenol, APAP) is widely used as an analgesic and antipyretic agent is safe and effective at the therapeutic dose. However, APAP when taken high doses can cause serious liver injury, such as serve hepatic necrosis, hepatic lesion, cirrhosis, total malfunction, and death (). APAP-induced hepatotoxicity is metabolized to N-acetyl-p-benzoquinone imine (NAPQI) through cytochrome P450 enzymes including CYP2E1, CYP3A4, and CYP1A2.[4] NAPQI depletes hepatic cellular glutathione (GSH) levels and thereby adenosine triphosphate (ATP) causes mitochondrial function its leads to cellular damage and finally causes DNA fragmentation and apoptosis. APAP also contributes to enhanced ROS generation.[5,6] In addition, lipid peroxidation resulting from oxidative stress has been demonstrated to contribute to the initiation and progression of APAP-induced liver damage.[7]

The availability of synthetic drugs to treat several liver diseases in this state may further worsen the liver injury as well as need to get metabolized in the past liver damage.[8] This increased load on liver function and required action of the drug may not be observed. Vaccines, antiviral drugs, and steroids used as a treatment for liver pathologies have possible adverse effects, particularly if administrated chronically or

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sub chronically.[9] To date, numerous compounds, including polyphenols, flavonoids, quinines, coumarins, and carotenoids are derived from natural products widely distributed in fruits, vegetables, and well-known medicinal plants. These compounds have been extensively thought to be adjuvant and alternative medicines for the improvement of hepatic diseases by inhibited oxidative stress and mitochondrial dysfunction through antioxidants. Hence, developing pharmacology effective medicine from natural products has become an essential by the asset of its moderately low toxicity or fewer side effects. NAT-CLW™ is a patent pending formulated natural ingredients and developed by FFF BIO WORKS LLP as a hepatoprotective agent as used to treat APAP-induced liver disease. This contains curcuminoids and lutein combinations; these ingredients have established hepatoprotective activities in isolation. Individually single ingredients of the formulation have been investigated to have protective activity against different models of experimental hepatotoxicity. In the present investigation elaborated an *in vitro* model, an efficient research was undertaken to assess the potential effects of the formulation on the hepatotoxocity induced by APAP agent. The present communication substantiates the therapeutic utility of the formulation as hepatoprotective agents.

## MATERIALS AND METHODS

### Chemicals

Acetaminophen, curcuminoids, and lutein were obtained from Sigma-Aldrich, USA. NAT-CLW™ was developed by FFF BIO WORKS LLP, Bengaluru, India. Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), trypsin-ethylenediaminetetra acetic acid, phosphate-buffered saline (PBS), and antibiotic/antimycotic reagent were purchased from HiMedia. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), dichlorodihydrofluorescein diacetate (DCFH-DA), Rhodamine 123 (Rho 123), Ethidium bromide/acridine orange (EB/AO), and 6-diamidino-2-phenylindole (DAPI) were acquired from Sigma-Aldrich, USA. All the other chemicals were used in analytical grade.

### Cell Culture

We purchased the HepG2 cell line from the National Centre for Cell Science (NCCS), Pune, India. Human HepG2 cells were cultured in DMEM which is a basal medium supplemented with 10% FBS, followed by 5% antibiotic/antimycotic reagents were added to prevent bacterial and fungal contaminations. Cultured cells were maintained under the slandered condition at 37°C in an atmosphere of 5% $CO_2$.

### Cell Viability and Cytotoxicity Assay

Cell viability assay was used to determine the cytotoxic effects and protective effects against APAP-induced toxicity.[10] In brief, cells were seeded at a density of $3 \times 10^5$ cells/well and were cultured in 96 well plates followed by overnight incubation at 37°C. Followed by cells were treat with different concentration of NAT-CLW™ (3.9, 7.18, 15.62, 31.25, 62.50, 125, and 250 µg/ml) or APAP (20 mM) or silymarin (3.125, 12.5, 25, 50, 100, and 200 µg/ml) and incubated for 24 h. After incubation of respective time, 100 µl of MTT reagent (5 mg/ml) was added to each well and cells were incubated further 4 h at 37°C. The media were aspirated and the formazan crystals were dissolved in DMSO 100 µl/well and the absorbance was read with a microplate reader at 570 nm.

### Lipid Peroxidation and Antioxidant Enzyme Activity Assay

The amount of TBARS, SOD, and GSH in the cell suspensions was determined by measuring to the total lipid peroxidation, superoxide dismutase, glutathione levels, and the capability to chelate ROS in HepG2 cells using MDA, SOD, and GSH assay kits obtained from Sigma-Aldrich, USA. In briefly, the collected cells suspensions were measured the absorbance at 532 and 490 nm according to the manufactures instructions and TBARS, SOD, and GSH content were calculated as nmol/mL protein and U/mL.

### Detection of Intracellular ROS Levels

The levels of intracellular ROS generation were measured by DCFH-DA substrate as followed by according to the previous study with slight modifications.[11,12] In brief, HepG2 cells were seeded at a density of $1 \times 10^6$ cells per well in 6-well plates under the standard conditions. Cells were treated with the final concentration of 50 µg NAT-CLW™ and silymarin for 1 h, followed by APAP were exposed to HepG2 cells for 24 h. After treatment cells were incubated with 5 µM DCFH-DA at 37°C for 30 min in the dark place. Cells were washed with PBS buffer to remove excess dye and images were examined under the fluorescence microscopy.

### Determination of Mitochondrial Membrane Potential

A change in MMP was monitored by mitochondrial lipophilic cation dye Rho-123, according to the previous study.[13] Briefly, HepG2 cells were seeded at a density of $1 \times 10^6$ cells in six-well plates with a medium containing 20 mM APAP in the presence or absence of 50 µg NAT-CLW™ for 24 h. Cells were incubated with Rho-123 (5 µM) in DMEM at room temperature for 30 min and cells washed with PBS. Images were captured by fluorescence microscopy.

### Ethidium Bromide/Acridine Orange (dual staining)

The cell morphology was evaluated using AO/EB fluorescence staining was carried out by the method of Jimenez *et al*. (2008).[14] Briefly, the cells were plated at a density of $5 \times 10^4$ in six-well plates. They were grown at 37°C in humidifies

$CO^2$ incubator until they were reached at 70–80% confluent. Then, the cells were treated with NAT-CLW™/silymarin for 24 h. The cell suspensions incubated with 100 µg/ml of dye mixture AO/EB for 30 min in the dark. Then, the cells washed once with PBS to remove excess dye and cells were visualized immediately under a fluorescence microscope.

### DNA Fragmentation by DAPI Staining

Nuclear morphology was measured by fluorescence microscopy following DAPI sating. HepG2 cells were treated with APAP 20 mM 1 h before NAT-CLW™/silymarin for 24 h. Then, the cells were washed with PBS (pH 7.4) and cells were fixed with ice-cold 70% ethanol. Cells were incubated in DAPI for 15 min at 37°C in wrapped in aluminum foil. Then, the cells were washed with PBS and images viewed immediately by fluorescence microscopy.

### Statistical Analysis

All the values were analyzed as mean ± standard devotion (SD). The significant variance all the data are subjected to one-way analysis of variance (ANOVA) followed by Duncan multiple tests (DMRT) using SPSS software. $P \leq 0.05$ was considered to be significant.

## RESULTS

### Cytotoxicity Effects of NAT-CLW™ and Silymarin in HepG2 Cells

HepG2 cells were treated with different concentrations of NAT-CLW™ and APAP-induced cytotoxicity was determined by MTT assay for 24 h. Figure 1a and b shows the pretreatment with various concentrations of NAT-CLW™ and silymarin alone for 24 h, did not show any toxic effect in HepG2 cells. Further, the effect of NAT-CLW against APAP exposed HepG2 cells showed a significant percentage of growth in dose-dependent manner as compared to control cells. Whereas, similar results were observed when pre-treatment with silymarin in HepG2 cells was treat with APAP toxicity. From these results, we found significant lesser toxicity in HepG2 cell ($IC_{50}$ 62.50 µg/ml), hence the results we taken for further experiments.

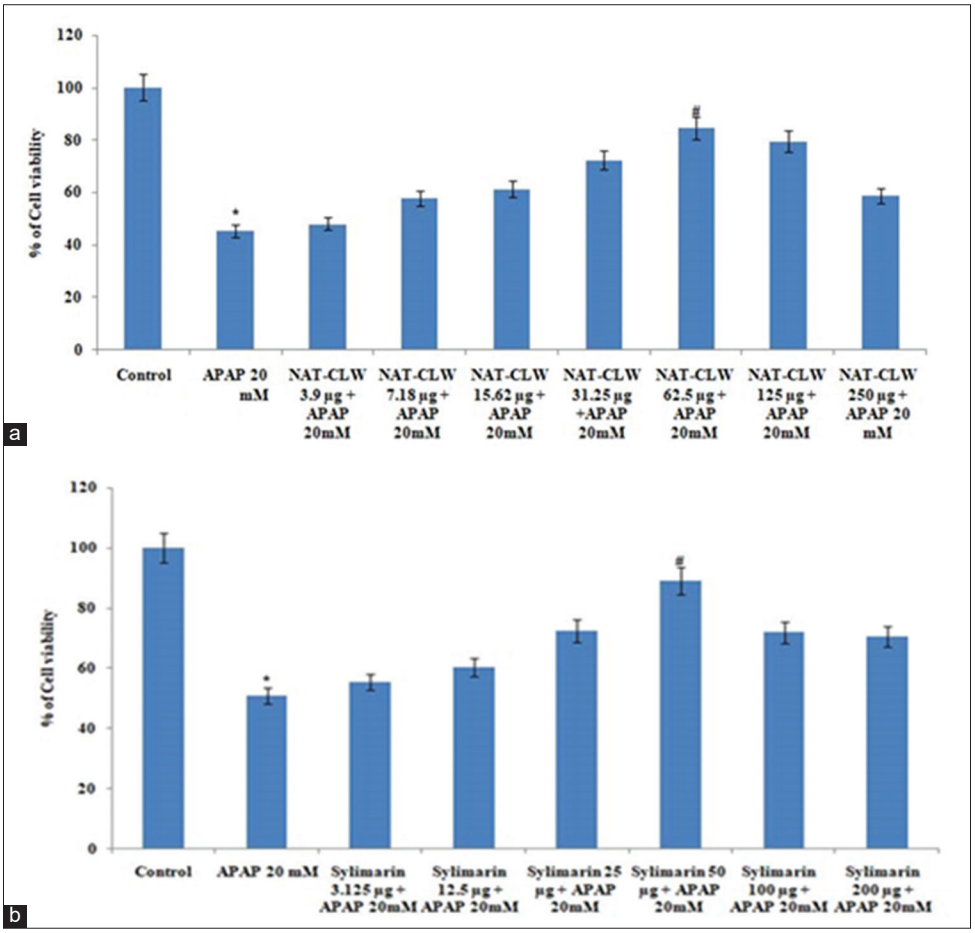


**Figure 1:** Displaying the MTT cell viability analysis of silymarin and NAT-CLW in the APAP-induced HepG2 cells. (a and b) NAT-CLW and silymarin were effectively increased the viability of hepatic HepG2 cells. Values are given as mean ± SD of three experiments in each group. Values not sharing a common marking (*, #) differ significantly at $P \leq 0.05$ (DMRT)

### Effects of NAT-CLW™ and Silymarin on TBARS and Antioxidant Enzyme Activity on APAP-induced HepG2 Cells

The results showed that the significant increase in TBARS level was observed in APAP-exposed HepG2 cells as compared to control cells. On NAT-CLW™ (62.50 µg/ml) and silymarin (50 µg/ml) pre-treatment significantly prevented lipid peroxidation formation in APAP-induced HepG2 cells (Figure 2a).

An antioxidants act as the most important defense against the free radical formation. Figure 2 shows APAP-induced HepG2 cells significantly diminished in SOD activity and GSH levels due to excessive ROS production. On the other hand, pretreatment with 62.50 µg of NAT-CLW™ significantly protected APAP-induced loss of antioxidant status (SOD activity and GSH levels) in HepG2 cells shows in Figure 2. From these results, we find that NAT-CLW has more potent antioxidant than silymarin.

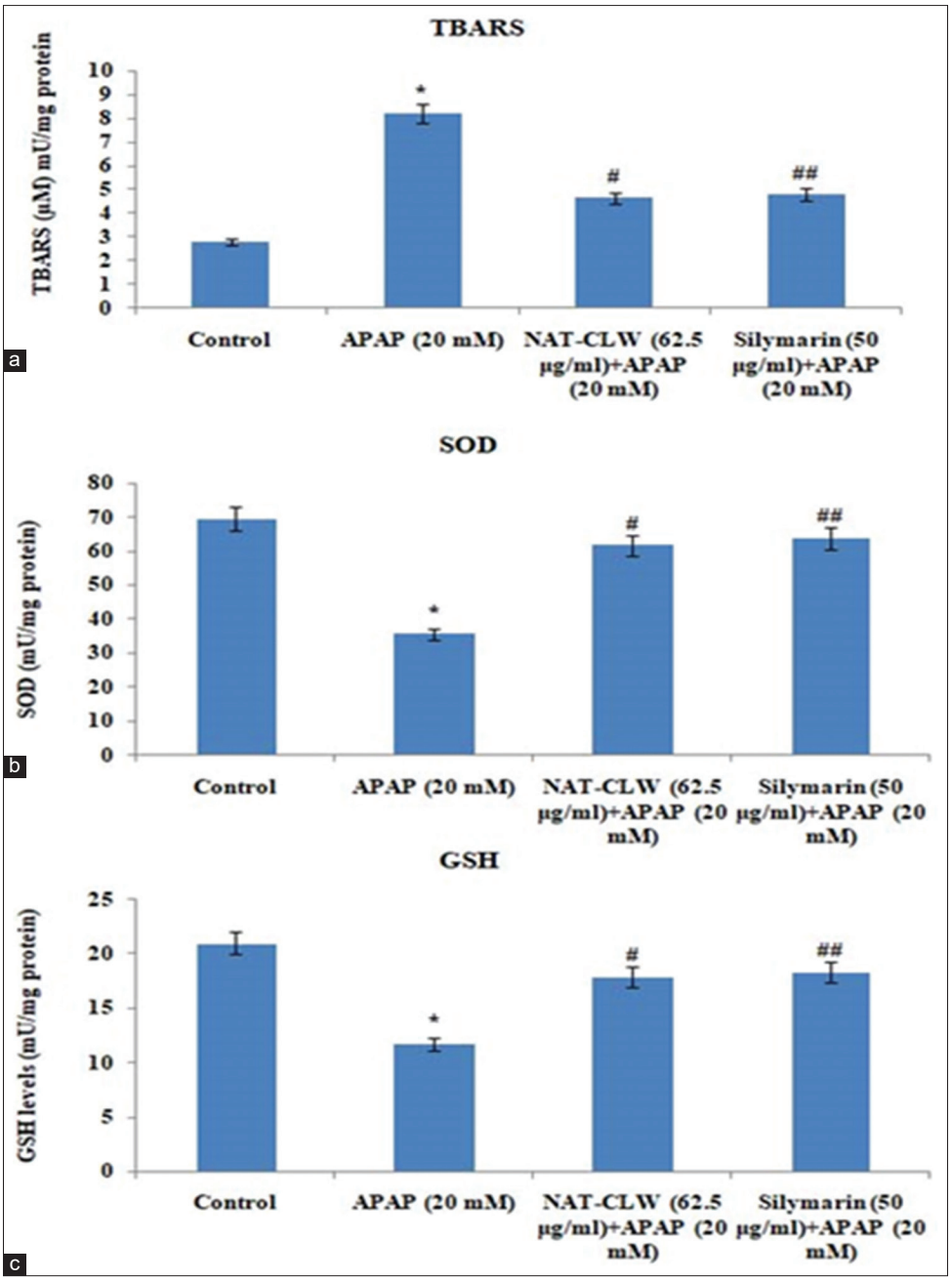


**Figure 2:** (a-c) Effect of NAT-CLW and silymarin on TBARS, GSH levels, and SOD enzyme activities in APAP-induced HepG2 cells. Values are given as mean ± SD of three experiments in each group. Values not sharing a common marking (*, #) differ significantly at $P \leq 0.05$ (DMRT)

### Impact of NAT-CLW™ and Silymarin on APAP-mediated Intracellular ROS Levels

Figure 3 shows a significant increase an intracellular ROS formation in APAP (20 mM) induced in HepG2 cells as compared to control cells. Whereas, pre-treatment with NAT-CLW™ (62.50 µg/ml) significantly decline the ROS formation and as it indicates the lesser APAP-induced free radical release as compared with APAP alone treated group. Silymarin treated cells dramatically less prevention as compared to NAT-CLW™.

### Effect of NAT-CLW™ and Silymarin on APAP-induced Reduction of MMP

In Figure 4, we examined that the APAP-induced cells are indicated polarized mitochondrial membrane and decreased green fluorescence as compared with untreated control cells. HepG2 cells were pre-treated with NAT-CLW™ shows an improved green fluorescence representing a depolarized state of the mitochondrial membrane as compared to APAP alone treated cells. Observation made from our findings study strongly indicated that NAT-CLW™ formulation exhibits more protective activity as compared to silymarin, it is indicated MMP protective activity.

### Effect of NAT-CLW™ and Silymarin on APAP-induced Nuclear Apoptosis

The apoptotic morphological was observed in APAP-induced in HepG2 cells showed that the green nuclei fragmented represented early apoptotic cells and yellow dots shows the condensed nuclei were of late apoptosis which was observed in fluorescent microscopy (Figure 5). On the other hand, HepG2 cells pre-treated with NAT-CLW™ (62.50 µg/ml) and silymarin 50 µg/ml showed increased green fluorescence and prevented morphological changes were observed in HepG2 cells treated with APAP, which indicate viable cells.

## EFFECT OF NAT-CLW™ AND SILYMARIN ON APAP-INDUCED NUCLEAR FRAGMENTATION

Figure 6 shows HepG2 cells treated with APAP showed characteristic changes associated with apoptosis, including shrinkage, chromatin condensation, nuclear fragmentation, and formation apoptotic bodies as compared to untreated control

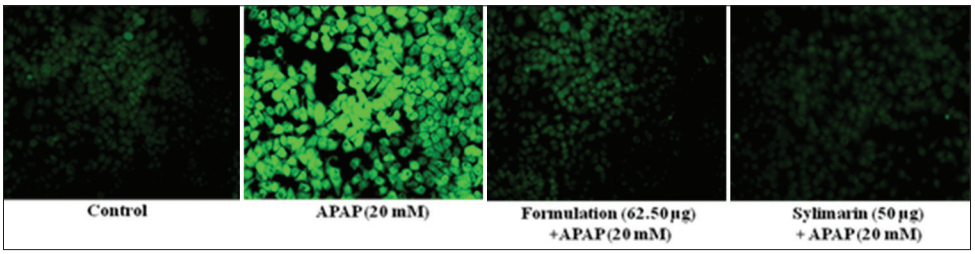


**Figure 3:** The NAT-CLW and silymarin reduce APAP-induced ROS generation in HepG2 cells. Intracellular ROS accumulation was measured using the fluorescence probe DCFH-DA staining

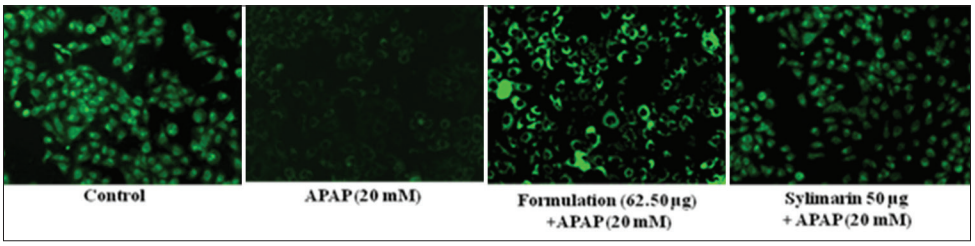


**Figure 4:** The effect of NAT-CLW and silymarin reduces APAP-induced ROS generation in HepG2 cells. Intracellular ROS accumulation was measured using the fluorescence probe DCFH-DA staining

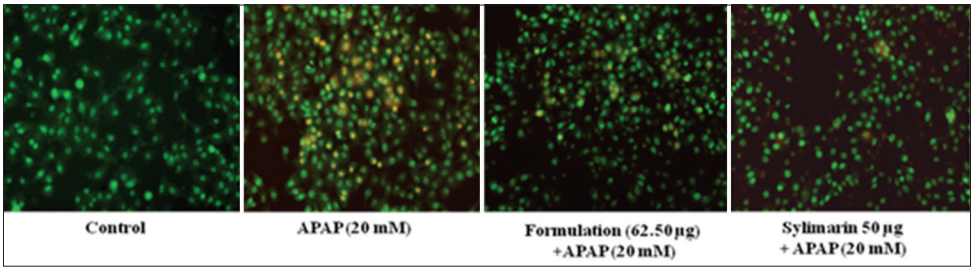


**Figure 5:** The effect of NAT-CLW and silymarin on APAP-induced apoptosis morphological changes as stained by AO/EB staining in HepG2 cells

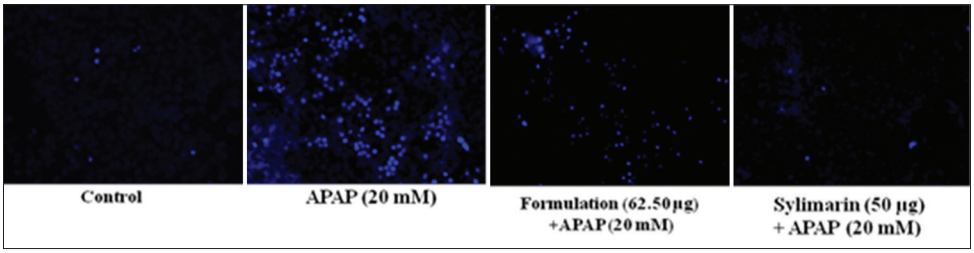


**Figure 6:** The effect of NAT-CLW and silymarin on APAP-induced apoptotic features in HepG2 cells by DAPI staining

cells. Whereas, treatment with NAT-CLW™ (62.50 µg/ml) showed inhibited condensation and fragmentation was observed in APAP-induced in HepG2 cells. Silymarin when compared to NAT-CLW™ had less prevention; these results clearly suggest that NAT-CLW™ exhibits more potent than silymarin.

## DISCUSSION

A significant number of plant extract and herbal formulations have been exhibit several biological properties to treat various life-threatening diseases.[15] To date, the available experimental studies reveal plants extract and phytochemicals that exhibit hepatoprotective effects that the phytoconstituents including phenyl compounds, coumarins, essential oils, monoterpenoids, diterpenoids, triterpenoids, steroids, alkaloids, and other nitrogenous compounds[16,17] that representation of hepatoprotective effects against APAP exposed to protect the liver disease from the harmful sound of drug-exposed intoxication.[18] Various formulations containing plant extracts of single or multiple known polyherbal formulations are available in the market for cure liver disease.[19-21] NAT-CLW™ is a natural plant-based formulation it contains curcumin and lutein, which is shows number of medicinal properties such as antioxidant, anti-apoptotic, and it possesses hepatoprotective properties. Previous studies have documented that the curcumin protects against APAP exposed hepatotoxicity through its antioxidant properties.[22] Another study indicated that curcumin was found to protect against genomic instability, cell death, and oxidative stress in liver.[23] Edakkadath *et al*. reported that the carotenoid lutein prevents hepatic damage induced by paracetamol or ethanol induced. Similarly, we find that HepG2 cells indicated cell death after induced overdose of APAP with a dose-response similarly that reported previously.[24,25] Our results strongly indicated that the pre-treatment of NAT-CLW™ formulation drastically increased cell viability by APAP induced cell death; these result suggesting that NAT-CLW™ is more potent than silymarin.

The formation of ROS results in oxidative stress, lipid peroxidation, DNA damage, and loss of cellular function; finally, it leads to apoptosis (). The major reactive species generated by oxidative stress which is responsible for the occurrence and progression of APAP toxicity.[26] Therefore, it is important to explore if NAT-CLW™ has been suppressed oxidative stress these findings clearly indicated that NAT-CLW™ could reduce APAP-triggered ROS levels due to its potent antioxidant. It is generally recognized that increased lipid peroxidation formation and reduced cellular function of enzymatic/non-enzymatic antioxidant defense systems are the most important characteristics of hepatotoxicity induced by APAP.[27] In the current study, we aimed to evaluate APAP induced to HepG2 cells significantly decrease liver antioxidant capacity, as characterized by dramatically enhance in MDA content and diminish in hepatic levels of SOD and GSH, which are often regard as indicator of oxidative stress response[6,28] However, pre-treatment with NAT-CLW™ markedly reversed the changes in parameters of lipid peroxidation and antioxidant status. Collectively, on the bases, our results demonstrated that the protective effect of NAT-CLW™ found more efficacy than silymarin on APAP-induced liver damage. This study coincident with the previous study demonstrated that the protective effects of curcumin against liver damage through attenuation of oxidative stress, inflammation, and cell death in both *in vitro* and *in vivo*. Further, NAT-CLW™ provides protection against APAP was established in rat hepatocytes mediated impaired lipid peroxidation, but no effect was found on depletion of GSH and LDH and in time-dependent action at low concentrations. However, overdoses have revealed the protective effects against APAP.[29]

Mitochondrial is a powerhouse of the cells for providing energy generating and also known as plays a key role in cellular signaling pathways. Their inmost role in energy metabolism, as well as their high abundance in hepatocytes, makes them important targets for drug-exposed hepatotoxicity. Mitochondrial membrane potential and mitochondrial permeability transition are biomarkers that have been used to examine mitochondrial dysfunction and damage.[30,31] Loss of mitochondrial function, through NAPQI binds to cellular protein as well as mitochondrial proteins and alters the mitochondrial ATP syntheses, could lead to ineffective ATP depletion, additionally opening mitochondrial membrane pore through which the release of cytochrome c into the cytosol, subsequently which forms apoptosome complex with other mitochondrial factors such as Apaf-1 which activate caspase.[32-34] APAP induced necrosis and apoptosis or both depending on the type of cells or other concentrations of APAP

treatment.[33-35] In the current study, APAP-induced cells showed reduced mitochondrial function, DNA fragmentation; however, NAT-CLW™ pre-treatment before APAP-induced effectively protected mitochondrial function and DNA fragmentation in HepG2 cells. Several reports clearly demonstrated that natural plant formulation occurring antioxidants could protect APAP-induced DNA fragmentation as well as early event of apoptotic formation (). Moreover, NAT-CLW™ prevented APAP exposed apoptosis through modulation of mitochondrial apoptotic signaling molecules; likewise, NAT-CLW™ has been reported to reduce apoptosis through restored mitochondrial function. Hence, we conclude that NAT-CLW™ protects APAP-induced apoptosis by preventing loss of MMP.

## CONCLUSION

In this current study stated that NAT-CLW™ protects APAP-exposed oxidative stress by inhibiting the ROS production and lipid peroxidation in HepG2 cells. Further, NAT-CLW™ modulates mitochondrial function thereby protects NAT-CLW™ exposed DNA fragmentation. Further, we find that NAT-CLW™ treatment to have protected APAP-exposed apoptosis by modulated mitochondrial complex and helped to protect the mitochondrial function. Finally, the observation made from our findings clearly indicated that plant-derived formulation of NAT-CLW™ exhibits more potent activity than silymarin administration. Further study needs to conventional medicinal formulation drug to treating various liver diseases.

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