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Research Article

Protective Effects of Nano-Curcumin on Inflammation and Apoptosis in the Liver of Arsenic Treated Rats

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Abstract

Arsenic is known to induce cytotoxicity through a couple of mechanisms viz. oxidative stress; mitochondrial dysfunction; inflammation; DNA damage and apoptosis. Several therapeutic agents including amino acids, vitamins, hormones, chelating agents and natural/herbal products derived from plants have been employed to reverse its toxicity. During present investigations, an attempt was made to observe the effects of nano curcumin (< 100 nm), synthesized from curcumin, an extract from a rhizome, *Curcuma longa* (Zingiberaceae) on pro-inflammatory cytokines (TNFα and IL-12), biomarkers of apoptosis (caspase-3 and caspase-9) and DNA damage (8-OHdG). Comet assay was employed to record DNA fragmentation. Female Wistar rats were administered 4 mg/kg b.w. arsenic trioxide and 50 mg/kg b.w. nano curcumin for sixty days. Results showed that nano curcumin could decrease the serum values of cytokines, inhibited activity of caspase-3 and caspase-9 in the liver, and decreased 8-OHdG values in the urine samples of arsenic treated rats. DNA damage studied through different parameters of Comet assay also exhibited protective effects of nano curcumin on apoptosis. It is hypothesized that biotransformed products of curcumin i.e. dihydroxycurcumin and tetrahydroxycurcumin contribute in protection against arsenicosis through autoxidation and double cyclization mechanisms. Smaller molecular size, enhanced bioavailability and sustained uptake of nano curcumin by hepatocytes further strengthen its antioxidative effects.

Keywords: *Arsenic; Nano-Curcumin; Inflammation; Apoptosis; DNA Damage*

Abbreviations

ROS: Reactive Oxygen Species; DNA: Deoxyribo Nucleic Acid; JNK: jun N-Terminal Kinase; TNFα: Tumor Necrosis Factor Alpha; Ils: Interleukins; CRP: c-Reactive Proteins; MAPK: Mitogen Activated Protein Kinase; Nrf2: Nuclear Factor Erythroid 2-Related Factor 2; 8-OHdG: 8-Hydroxy-2'-Deoxyguanosine; N-Cur: Nano Curcumin; CUR: Curcumin; As: Arsenic; As+Cur: Arsenic + Curcumin; As+Ncur: Arsenic + Nanocurcumin; As_2O_3: Arsenic Trioxide

Introduction

Arsenic is a widely distributed metalloid, present in earth crust, rocks, soil, air, aquatic systems and benthos. Ground water contamination by arsenic has been reported from a number of countries [1]. Thus, a vast human population, over the globe, drinks arsenic contaminated water consequently developing serious public health problems [2,3]. International Agency for Research on Cancer has declared arsenic as a class I human carcinogen [4]. Epidemiological reports further confirm that human exposure to arsenic manifests into cancer(s) of skin, liver, kidney, urinary bladder and lungs [5,6]. Arsenic induces cellular/molecular toxicity through different mechanisms viz. reactive oxygen species, oxidative stress, DNA damage, inflammation, mitochondrial dysfunction and cytoskeleton changes [7-9]. Redox disturbances further induce cell proliferation, differentiation and apoptosis [10,11].

Arsenic induced apoptosis has been attributed to accumulation of hydrogen peroxide, release of Cytochrome c from mitochondria, reduction of mitochondrial membrane potential and activation of caspases [12]. Recent studies show that JNK kinase also forms a key component in arsenic induced apoptosis. Arsenic trioxide can regulate pro-apoptotic proteins like bax, bad, bmf and down regulate bcl2, bcl-xl, mcl-1 [13]. Inflammation caused by arsenic has also been implicated in its toxicity. Up regulated pro-inflammatory mediators viz. tumor necrosis factor –α (TNF-α), interleukins (ILs) and c-reactive proteins (CRP) have been reported to induce systemic inflammation caused by low level exposure to arsenic [14]. Arsenic does modulate the expression of MAPK/Nrf2/NG-*k*B signaling molecules [15].

Chelation therapy has been traditionally used to treat arsenicosis. To name a few, British anti lewisite (BAL), dimercapto succinic acid (DMSA) and dimercapto-propane sulphate (DMPS) have been reported to reduce the body burden of arsenic [8]. However, several side effects of chelation do not encourage their clinical application. In recent years, attempts have been made to treat arsenicosis employing certain natural/herbal products derived from plants viz. *Allium sativum, Viscum album, Phyllanthus emblica*, *Ocimum sanctum and Curcuma longa* [16]. Curcumin (diferuloylmethane), an extract from the rhizome, *Curcuma longa* (Zingiberaceae) has been found to possess antioxidative, anti-inflammatory, antidiabetic and antiviral properties [17]. However, factors viz. low hydrophilicity, poor bioavailability, reduced absorption, diminished metabolism and rapid elimination have restricted its pharmacological applications [18]. Therefore, different formulations of curcumin i.e. adjuvants, stabilizers, conjugates and nanoparticles have been developed to enhance its therapeutic value [19]. Very few studies have been undertaken to study the effects of curcumin nanoparticles (N-Cur) on arsenic induced toxicity [20,21]. Nano curcumin was recently found to improve liver function of arsenic treated rats through its antioxidative properties [22]. Another study concluded that it modulated mitochondrial function in the liver of arsenic treated rats [23]. However, its ameliorative effects on inflammation and apoptosis, if any, are not known. Present study elucidates the effects of N-Cur on a few biomarkers of inflammation viz. tumor necrosis factor-α (TNF-α), interleukin-12 (IL-12) as well as on the activity of caspase-3 and caspase-9 as biomarkers of apoptosis. DNA fragmentation in hepatocytes has been studied through comet assay. 8-hydroxyl-2-guanosine (8-OHdG) has also been estimated to support results on comet assay. The results are expected to add in existing knowledge on pharmaco-therapeutic potential of nano curcumin

Materials and Methods

Chemicals and reagents

Curcumin was purchased from *Hi*-Media, Mumbai. Arsenic trioxide, dichloromethane, low melting agarose, normal melting agarose and ethidium bromide were supplied by Sigma Chemical Co., St Louis, Missouri (USA). Commercial kits for the estimation of caspase-3, caspase-9, TNF-α, IL-12 were procured from M/S Elabscience, Houston (USA). All other chemicals/reagents used in this study were purchased from *Hi*-Media, Mumbai.

Preparation and characterization of nano curcumin

N-Cur was prepared from curcumin powder following the method suggested by Bhawana., *et al*. [24]. Lyophilized N-Cur was characterized through a battery of methods viz. transmission electron microscopy (TEM), X-ray diffraction, zeta potential and fourier transform infra-red (FTIR) spectroscopy. These methods and results have been described in a previous report [22]. N-Cur used in present investigations possessed a size < 100 nm.

Animals and their treatments

Mandatory approval from Institutional Animal Ethical Committee was obtained to undertake present investigations. Three months old (170 ± 30g), healthy female Wistar rats were procured from the Animal Facility of All India Institute of Medical Sciences, New Delhi. They were maintained under standard laboratory conditions (room temperature- 25 ± 5°C; relative humidity- 50 ± 10% and 12h light and dark cycle) in the Animal House of Ch. Charan Singh University, Meerut. Each rat was housed in a polypropylene cage, offered commercial food

pellets (Golden Feeds, New Delhi) and tap water *ad libitum*. They were acclimatized to laboratory conditions for two weeks, prior to the treatments with arsenic and nano curcumin.

Rats were divided into six groups, each containing five rats. Rats of group A were administered a predetermined sub lethal dose of arsenic trioxide i.e. 4.0 mg/kg b.w. through gavage on each alternate day for 60 days. Rats of group B were administered As_2O_3 as the rats of group A, but also fed on the predetermined non-lethal dose of curcumin i.e. 50 mg/kg b.w. on each alternate day for 60 days. Whereas, rats of group C were treated with curcumin alone as the rats of group B. Rats of group D were treated with As_2O_3 and N-Cur while the rats of group E were treated with N-Cur alone as the rats of group D. Rats of group F were administered 4.0 mL/ kg b.w. of saline only to serve as controls.

On termination of the experiment, rats were anaesthetized with halothane and sacrificed to collect the blood and liver samples. Blood was collected through cardiac puncture and serum was separated by centrifugation. Urine samples from live animals were collected through metabolic cages.

Determination of TNF- α and IL-12

These cytokines were determined in serum samples of rats employing commercial kits procured from Elabscience, Houston (USA).

Determination of caspase-3 and caspase-9

The activity of caspase-3 was estimated in liver samples following the method of Gurtu., *et al.* [25]. The commercial kit was obtained from Elabscience, USA. The absorbance was recorded at 450nm using a micro plate reader (E.C. Hyderabad).

The activity of caspase-9 in liver samples was estimated by commercial kit supplied by Elabscience, USA. Caspase-9 catalyze the substrate Ac-LEHD-pNA to generate yellow colored pNA (p Nitroamidine). The absorbance was recorded at 405nm using a micro plate reader (EC, Hyderabad).

Single cell gel electrophoresis (Comet assay)

The method suggested by Singh., *et al.* [26] was followed to determine DNA fragmentation. Briefly, 10 µl of single liver cell suspension(s) mixed with low melting point agarose were spread over the frosted slides and subjected to electrophoresis for 30 min (24 V, 300 mA). The cells were washed with neutralizing buffer and stained with 1 X ethidium bromide for 5min. Scoring was performed under a florescent microscope (Nikon, Japan) using a VisComet software (GmBH, Germany).

8-hydroxyl-2-deoxyguanosine (8-OHdG)

A competitive ELISA technique was used for the estimation of 8-OHdG in urine samples using a commercial kit procured from Elabscience (USA). Absorbance of the final reaction product was recorded at 450 nm using a micro plate reader (EC, Hyderabad).

Statistical analyses

The results are expressed as mean ± standard error (SE). Comparison of data among different groups was made applying one way analysis of variance (ANOVA) with Duncan's post hoc test using SPSS version 2.0 software. $P < 0.05$ was considered to be statistically significant.

Results

As described in our earlier report [22], Nanoparticles of curcumin exhibited a spherical shape with an average diameter < 100 nm. Zeta potential was found to be 40.9 mV. X-ray diffractograms also qualified the nano size of these particles.

The serum values of TNF-α and IL-12 were found to be elevated after 60 days of treatment with As_2O_3. However, co-treatments with curcumin/ nanocurcumin decreased their values (Table 1).

	Cytokines	
Treatment	**TNF-α (pg/ml)**	**Interleukin-12 (pg/ml)**
Arsenic	381.75 ± 8.25*	0.633 ± 0.011*
As. + Cur.	248.25 ± 7.50*@	0.609 ± 0.019*@
Curcumin	120.00 ± 3.00*@	0.357 ± 0.020*@
As. + Ncur.	186.50 ± 7.00*@	0.550 ± 0.015*@
Nanocurcumin	81.75 ± 2.25*@	0.172 ± 0.078*@
Control	153.00 ± 8.50	0.499 ± 0.074

Table 1: *Status of cytokines in the liver of arsenic treated rats on simultaneously treatments with curcumin.*

Result are expressed as mean ± SE (n = 5).

**p < 0.05 significantly different in comparison to control rats.*

@p < 0.05 significantly different in comparison to arsenic treated rats.

 # Non significant in comparison to arsenic treated rats.

Present results on caspase-3, a reliable marker of apoptosis, suggested that As_2O_3 induced cell death by apoptosis. Enzyme activity increased in the liver of arsenic treated rats but declined in the liver of As+ Cur and As+ N-Cur treated rats. Percent enzyme inhibition was higher in As+ N-Cur treated rats (Figure 1). As_2O_3 activated caspase-9, however, its activity was inhibited by Cur/N-Cur. Percent inhibition was higher in As+ N-Cur treated rats (Figure 2).

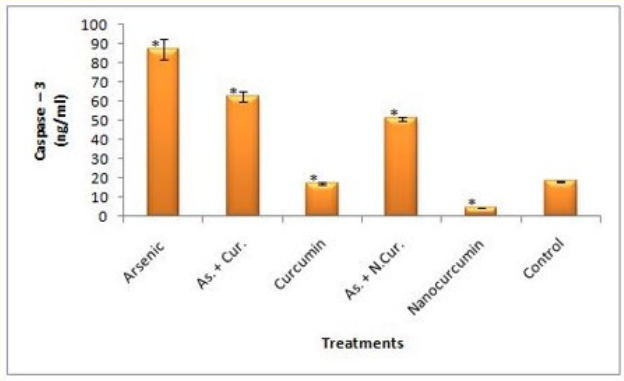

Figure 1: *Enzymatic activity of caspase – 3 of arsenic treated rats, simultaneously offered curcumin/nanocurcumin.*

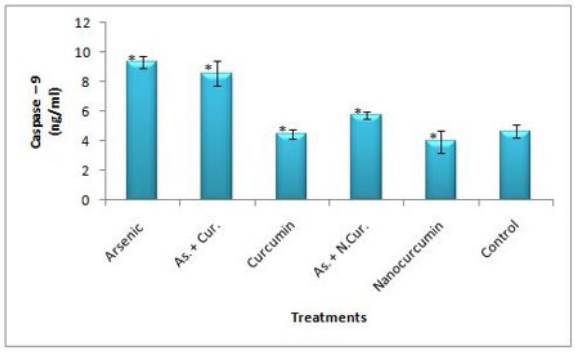

Figure 2: *Enzymatic activity of caspase – 9 of arsenic treated rats, simultaneously offered curcumin/nanocurcumin.*

To record the protective effects of curcumin(s) against arsenic induced genotoxicity, 8-OHdG was determined in the urine samples of rats of all the groups. It reflected significant DNA damage in the hepatocytes of arsenic treated rats. Results from arsenic + curcumin (s) treated rats showed protective effects. Lower values were recorded amongst As+ N-cur treated rats than As +Cur treated rats (Figure 3).

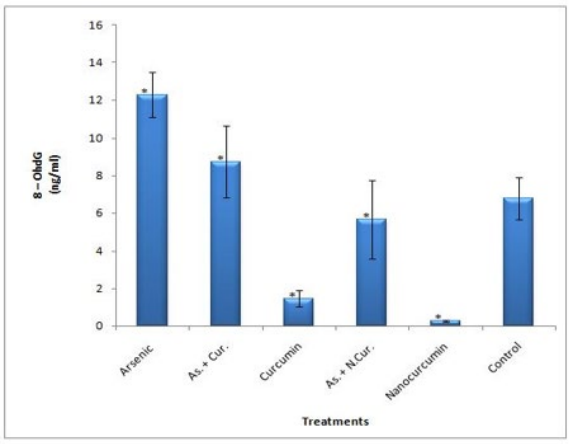

Figure 3: *8-OHdG in urine samples of arsenic treated rats, simultaneously offered curcumin/nanocurcumin.*

Results on Comet assay expressed higher percentage of DNA fragmentation in the hepatocytes of As treated rats. However, comet tail length decreased in As +Cur and As +N-Cur treated rats. The decrease in tail length was greater in As +N-Cur treated rats than As +Cur treated rats. These results reciprocated with head DNA (%) (Table 2 and figure 4-9).

Comet assay Parameters	Arsenic	As. + Cur.	Curcumin	As. + Ncur.	Nano curcumin	Control
Head DNA (%)	34.562 ± 5.569*	22.91 ± 0.934*@	76.312 ± 2.255*@	60.144 ± 1.920*@	44.428 ± 1.068*@	73.926 ± 2.300@
Tail DNA (%)	28.108 ± 5.524*	31.094 ± 0.452*@	6.402 ± 0.809*@	6.61 ± 0.435*@	14.426 ± 0.595*@	4.322 ± 0.629@

Tail Length	192.402 ± 27.752*	293.358 ± 7.164*@	50.98 ± 2.764*@	111.894 ± 2.704*@	139.138 ± 4.550*@	68.608 ± 4.172@
Tail Olive Moment	4238.064 ± 1159.564*	7029.614 ± 259.227*@	270.862 ± 30.565*@	584.848 ± 41.465*@	1563.264 ± 57.681*@	235.558 ± 27.424@

Table 2: *Effect of curcumin on apoptosis (comet assay) in the liver of arsenic treated rats.*

Result are expressed as mean ± SE (n = 5).

**p < 0.05 significantly different in comparison to control rats.*

@p < 0.05 significantly different in comparison to arsenic treated rats.

 # Non significant in comparison to arsenic treated rats.

Ns - Non significant in comparison to control rats.

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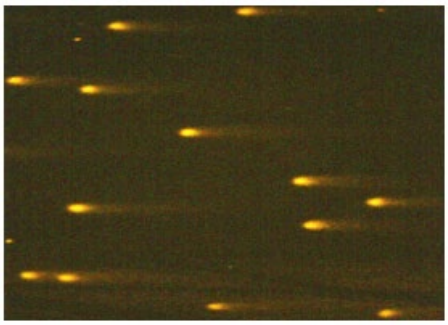

Figure 4: *Hepatocyte of As. treated rat shows the formation of a comet under electrophoretic conditions (24V/ 300 mA for 30 minutes).*

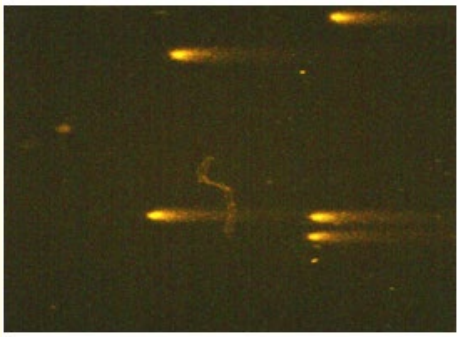

Figure 5: *Hepatocyte of As. + Cur treated rat shows the formation of a comet under electrophoretic conditions (24V/ 300 mA for 30 minutes).*

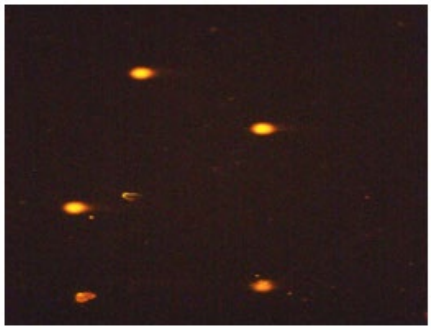

Figure 6: *Hepatocyte of curcumin treated rat shows the formation of a comet under electrophoretic conditions (24V/ 300 mA for 30 minutes).*

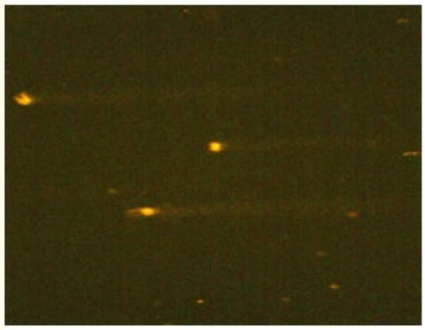

Figure 7: *Hepatocyte of As + Ncur treated rat shows the formation of a comet under electrophoretic conditions (24V/ 300 mA for 30 minutes).*

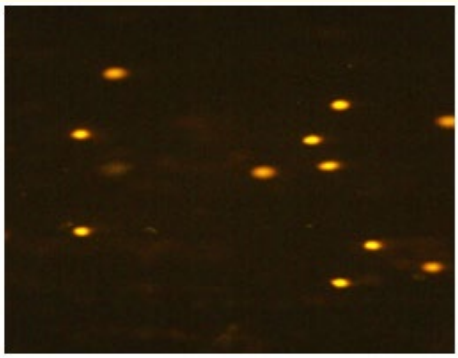

Figure 8: *Hepatocyte of nanocurcumin treated rat shows the formation of a comet under electrophoretic conditions (24V/ 300 mA for 30 minutes).*

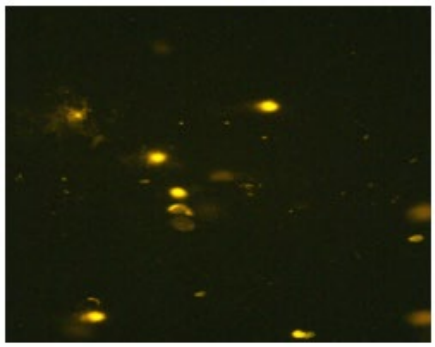

Figure 9: *Hepatocyte of control rat shows the formation of a comet under electrophoretic conditions (24V/ 300 mA for 30 minutes).*

Discussion

Protective effects of Cur and N-Cur against arsenic induced toxicity have been demonstrated earlier by a few reports [21,22,27]. It was also confirmed that Cur and N-Cur both ameliorate hepatotoxic effects of arsenic through its chelation from soft tissues, scavenging reactive oxygen species and improving the antioxidant status of liver parenchyma. Curcumin (s) are quickly metabolized by reduction and conjugation mechanisms to di, tetra, hexa and octa-hydrocurcumin(s) that manifests into their diverse biological functions [28]. Present investigations were made to record and compare their anti-inflammatory effects, if any, in the liver of arsenic treated rats. TNF-α and IL-12 were considered as reliable biomarkers of inflammation. Present results showed elevated values of these cytokines in the serum of arsenic treated rats. Acute inflammation by arsenic is known to increase the production of TNF-α by macrophages/ monocytes resulting into its increased level in the serum [29]. Recent studies made by Khan., *et al*. [30] and Duan., *et al.* [15] have also reported that arsenic increases the release of TNF-α in the blood of rats. Decreased values of TNF-α, registered after Cur/N-Cur treatments to arsenic treated rats thus confirmed ameliorative response through anti-inflammatory effects. Modulation of cytokines by nano-curcumin has been debated in the past. N-Cur regulated inflammatory pathways in Wistar rats during testicular trauma [31] and among covid -19 patients [32]. It is envisaged that curcumin(s) might mitigate arsenic induced liver injury by suppressing the secretion of TNF-α, along with other anti-oxidative mechanisms.

Results on another pro-inflammatory cytokine did show that exposure of Wistar rats to arsenic increased the serum level of IL-12. Earlier reports have suggested that arsenic promotes the release of IL-6 and IL-8 [33]. Enhanced generation of ROS by arsenic translated into increased release of interleukins [34]. Intriguingly, effects of curcumin(s) on proinflammatory cytokines have scarcely been studied. In certain experimental conditions i.e. during cyclophosphamide poisoning in rats, N-Cur caused suppression of interleukins [35]. Inhibition of inflammatory pathways, in general, might be executed by N-Cur to protect against cell death. Decrease in inflammatory cytokines caused by natural astaxanthin extracted from green algae, *Haematococcus pluvialis* attenuated arsenic induced hepatotoxicity via inhibition of cytokine mediated cell-cell interactions [36].

Apoptosis induced by arsenic and other toxic elements in different experimental models/ tissues was reviewed by Rana [37]. Apoptosis is a physiological process in a cell that maintains normal cellular development. Enhanced apoptosis, however, can induce cancer manifestations [38]. H_2O_2 accumulation, release of cytochrome c from mitochondria, decrease in mitochondrial membrane potential and caspase activation are common pathways that contribute to arsenic induced apoptosis [12]. Increased production of ROS in the liver of arsenic treated rats has also been linked to apoptosis [30]. Arsenic is also known to up regulate pro-apoptotic proteins like bax, bad, bmf,

bim, and down regulate bcl-2, bcl-xl and mcl-1 [13]. Present experimental evidence demonstrated that As_2O_3 activated initiator caspase-9 as well as the effector caspase-3. Certain adapter proteins function as the linkage between caspases and death receptors. Death receptors possess a death effector domain (DED) and a death domain (DD) and a caspase recruited domain (CARD). Internalized arsenic involves these domains to activate caspases that trigger apoptosis [38]. Activity of caspase-9 and caspase-3 decreased after the treatments with curcumin(s). N-Cur expressed better effects than Cur. Similar conclusions have been drawn by Basniwal., *et al*. [39].

Present results on comet assay showed that As_2O_3 induced DNA damage in hepatic cells. Several studies attribute DNA damage to ROS [40] whereas, others associate it with caspase mediated apoptosis and induction of inflammatory cytokines [30,41]. Cur/N-Cur is known to restore DNA fragmentation (DNA-DFF-40/CAD) interaction factor [42]. Moreover, its strong antioxidative function could inhibit DNA fragmentation. Present results on 8-OHdG also indicate DNA repair induced by Cur/N-Cur. Cur/N-Cur induced DNA repair enzymes involved in both base excision repair and non-homologous end joining pathways [43].

Conclusion

To conclude, under biological pH conditions, curcumin is unstable and is metabolized to dihydroxycurcumin and tetrahydroxycurcumin through reduction and glucuronidation mechanisms [44]. In addition, transformation products of autoxidation and double cyclization of curcumin contribute to its pharmacological activity [45]. N-Cur also undergoes similar mechanisms of biotransformation. However, better protection offered by N-Cur than Cur against arsenic induced inflammation, apoptosis and genotoxicity could be attributed to its molecular size, increased bioavailability, antioxidative properties and sustained uptake by hepatocytes. Further studies on biological activity N-Cur in hepatocytes are in progress.

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

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Author's Contributions

All authors contributed to the study, conception and design. Material preparation, data collection and analysis were performed by Lalit Mohan Vashishtha. Dr. Yeshvandra Verma supervised the experimental studies and prepared the first draft. Em. Prof. Suresh Vir Sing Rana prepared the final draft of the manuscript.

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Volume 12 Issue 10 October 2024