Role of nanocurcumin on experimentally induced alpha-amanitin toxicity in rats

Khumtya Debbarma, Jadav Sarma, Rohini Kumar Roy, Himangshu Baruah and Biswajit Dutta

Abstract

Alpha-amanitin is considered to be the main toxin present in mushroom species of Amanita phalloides and it is possibly the most deadly of all amatoxins. The aim of this study was to test whether nanocurcumin could revert the hepatic damage in experimentally induced Alpha-amanitin rats, tested with nanocurcumin in following protocols: Group I served as negative control group (PBS @1 ml/kg p.o), Group II served as positive control group (α-AMA @0.2 ml/kg p.o single dose), Group III served as Standard group (α-AMA @0.2 ml/kg p.o single dose + silymarin @100 mg/kg p.o.), Group IV (α-AMA @0.2 ml/kg p.o single dose + curcumin @800 mg/kg p.o), Group V (α-AMA @0.2 ml/kg p.o single dose + nanocurcumin @200 mg/kg p.o.), Group VI (α-AMA @0.2 ml/kg p.o single dose + nanocurcumin @400 mg/kg p.o), Group VII (α-AMA @0.2 ml/kg p.o single dose + nanocurcumin @800 mg/kg p.o) for four consecutive weeks. Our results showed that after 24 hours of Alpha-amanitin administration serum transaminase, urea, BUN and creatinine was found to be increased whereas, serum Total protein (TP) and haemoglobin decreased significantly. This study of treatment with Nanocurcumin were also compared with curcumin and silymarin. The overall results suggest that Nanocurcumin induces a beneficial effect by significantly improving the functional parameters particularly in alpha-amanitin induced hepatotoxicity in rats.

Keywords: Alpha-amanitin, amatoxin, amanita phalloides, curcumin, silymarin, liver, hepatotoxicity, hepatomegaly

Introduction

Mushrooms have generated a lot of interest in respect of food and medicines (Chang and Bussell, 1993; Starnes, 1993) [5, 24]. Mushroom is the source of income as well as source of fuel of most of the tribal people in India particularly of Northeastern region. Northeastern region due to its high rainfall, high moisture content accompanied by moderate to high temperature during summer and rainy season, favors the growth many mushroom species, both edible and poisonous. Moreover, mushroom is one of the preferred delicacies among many ethnic tribal communities of Northeastern region. Surveys on edible and inedible mushrooms have been conducted in different reserve forests of Assam such as Jeyapore, Jalubari, Amsing etc. Identification of有毒 mushrooms in Assam was conducted by some mushroom research workers on the basis of morphology and spore prints (Gogoi and Sharma, 2012; Tapwal et al., 2013; Devi and Srivastava, 2016) [12, 25, 26]. Seasonal incidences of mushroom poisoning were reported in different locations of Assam viz., Tinsukia, Dibrugarh, Sivasagar, Jorhat, Lakhimpur, Golaghat districts and few locations in Lower Assam (Sharma et al., 2013) [23]. Amanita phalloides, which is also known as ‘death cap’. These species contains three main group of toxins: amatoxins, phalloxins, and virotoxins. From these, amatoxins, especially alpha-amanitin, is mainly responsible for the toxic effects in human and animals (Garcia J et al., 2015) [9]. Amatoxins are potent and selective inhibitors of RNA polymerase II, causing disruption of transcription of mRNA. As a result hepatocytes cannot synthesize key protein coding genes, leading to the disintegration of nucleoli and pathologically centrilobular hepatic necrosis. The liver is the primary target organ of toxicity of amatoxins, and hepatocellular effects represent the most lethal and the least treatable manifestation of toxicity (Karlson-Stibor and Persson, 2003) [23].

Curcumin is a yellow pigment found in the rhizome of the spice turmeric (Curcuma longa), a member of the Zingiberaceae family. Curcumin has antioxidant, anti-inflammatory, and anticarcinogenic pharmacological effects. It acts by either interacting with molecular targets directly or altering gene expression and signaling pathways.
Curcumin is a free radical scavenger and hydrogen donor and exhibits antioxidant activity. The main colouring principle of turmeric rhizome was isolated in 19th century and named as ‘Curcumin’.

Nanocurcumin is a modified form of curcumin in which the particles of curcumin are transformed into nanoparticles that are more soluble and deliverable in the body. Nanocurcumin has been showed to have better bioavailability and efficacy than curcumin due to its smaller size, higher aqueous solubility and higher tissue penetration ability.

Despite reports of many incidences of mushroom poisoning cases (particularly Amanita spp.) in human and animals (Sharma et al., 2013) [23] in Northeastern region, there is lack of detailed systemic study on mushroom poisoning and its possible ameliorative alternative needs investigation. Hence, the present study was undertaken to evaluate the effect of nanocurcumin in experimentally induced alpha-amanitin toxicity in rats.

Materials and Methods

Materials

Alpha-amanitin was purchased from Sigma-Aldrich Chemicals Pvt. Ltd., Bengaluru and Curcumin was purchased from HIMEDIA. Silymarin suspension from Microlabs Ltd, Bengaluru and Curcumin was purchased from Sigma-Aldrich Chemicals Pvt. Ltd., Bengaluru and Curcumin was purchased from HIMEDIA. Silymarin suspension from Microlabs Ltd, Bengaluru and Curcumin was purchased from Sigma-Aldrich Chemicals Pvt. Ltd., Bengaluru and Curcumin was purchased from HIMEDIA. Silymarin suspension from Microlabs Ltd, Bengaluru and Curcumin was purchased from Sigma-Aldrich Chemicals Pvt. Ltd., Bengaluru and Curcumin was purchased from HIMEDIA. Silymarin suspension from Microlabs Ltd, Bengaluru and Curcumin was purchased from Sigma-Aldrich Chemicals Pvt. Ltd., Bengaluru and Curcumin was purchased from HIMEDIA. Silymarin suspension from Microlabs Ltd, Bengaluru.

Preparation of nanocurcumin

Evaporative precipitation of nanosuspension

As per the method described by Kakran et al. (2012) [14], the solution of original curcumin was prepared in ethanol and then a nanosuspension was formed by adding hexane (antisolvent). Drug particles in the nanosuspension were obtained by quick evaporation of the solvent and antisolvent, under vacuum using a rotary evaporator. This was followed by vacuum drying of the nanoparticles to completely evaporate all the solvents. The drug concentrations used were 5, 10, 15 mg/mL and the solvent to antisolvent (SAS) ratios were varied from 1:10, 1:15 and 1:20 (v/v). For 20 ml of the drug solution, 200–400 ml hexane was used.

Experimental protocol and procedure

Animals

The study was performed in accordance with the guidelines for the use and care of laboratory animals approved by Institutional Animal Ethical Committee (Approval no. 770/GO/Re/S/03/CPCSEA/FVSc/AAU/IAEC/19-20/780). Healthy Wistar rats of either sex weighing 150-200 gm was taken during the experiment. All the animals were kept in polypropylene cages in a small group of 6 rats per cage. Animals had free access to standard balanced ration and clean drinking water ad libitum and were maintained in a standard laboratory conditions (12:12 hours light/dark cycle at ambient temperature ranging between 22-25°C). The animals were acclimatized to surround for 1 week before experiment.

Experimental Design

Acute oral toxicity of nanocurcumin

In accordance to OECD Test Guideline 425 (Up and Down procedure), nulliparous and non-pregnant female albino mice, weighing 28 ± 4 g having age of 8-10 weeks were randomly selected. Animals were kept under standard conditions for seven days. Limit test was performed at 2000 mg/kg p.o. as single dose (Table 1) and mice were kept without food for 3-4 hours prior to dosing but had access to water ad libitum. The dose was administered to a single female mice according to body weight. The animals were closely observed for first 30 minutes, followed by 4 hours. Food was provided after 1-2 hours of dosing. After survival of the nanocurcumin administered mice, 4 additional mice were administered with the same dose under same condition.

Table 1: Acute oral toxicity studies with different doses:

| Test          | DOSE (mg/kg) | No. of animals | Sex |
|---------------|-------------|----------------|-----|
| Limit test    | 2000        | 1              | Female |
| Main test     | 2000        | 4              | Female |

In rats, liver injury was experimentally induced by alpha-amanitin. The mixture of alpha-amanitin and distilled water (1:1) with the dose of 0.2 ml/kg body weight was given per orally.

Rats were randomly divided as follows:

Group I: Received phosphate buffer saline (PBS) 1ml/kg per day for 28 days
Group II: Received alpha-amanitin 0.2 ml/kg body weight single dose
Group III: Received alpha-amanitin 0.2 ml/kg body weight single dose and silymarin 100 mg/kg body weight once in a week for 28 days
Group IV: Received alpha-amanitin 0.2 ml/kg body weight single dose and curcumin 800 mg/kg body weight once in a week for 28 days
Group V: Received alpha-amanitin 0.2 ml/kg body weight single dose and nanocurcumin 200 mg/kg body weight once in a week for 28 days
Group VI: Received alpha-amanitin 0.2 ml/kg body weight single dose and nanocurcumin 400 mg/kg body weight once in a week for 28 days
Group VII: Received alpha-amanitin 0.2 ml/kg body weight single dose and nanocurcumin 800 mg/kg body weight once in a week for 28 days

Body weight and organ weight

The body weights of all the rats were taken on 0 and 28th day. The liver weight was taken immediately after sacrificing the animals on 28th day. The variations in the body weight and liver weight among the groups were analyzed statistically.

Blood sample collection

The blood was collected from tail vein on 0, 7th, 14th, 21st, 28th day. Blood samples were collected into EDTA vials and clot activator vials for whole blood and serum preparation respectively. For serum preparation, blood samples were centrifuged at 3500 rpm for 15 min. The separated serum was used to measure all biochemical parameters such as Alanine transaminase (ALT), Aspartate transaminase (AST), Total Protein, Urea and BUN. The whole blood was used for the estimation of haemoglobin (Hb).

Preparation of Liver homogenate

Whole liver was miniced and randomly a portion was taken, weighed and was homogenized in 0.154 M potassium chloride at the rate of 10 mg/ml following the procedure of Glock et al. (1956) [11]. The homogenate was centrifuged at
3000 rpm for 10 minutes and the supernatant were kept in vials and in the same day enzymes were estimated.

**Histopathological Investigation**
Rats were sacrificed on 28th day of the experiment by cervical dislocation under light ether anesthesia. The liver tissue was collected and washed with cold saline. The excised liver was fixed in 10% formalin solution and taken to the Department of pathology, College of veterinary science, Khanapara, Guwahati, for histopathological study. The extent of experimentally alpha-amanitin induced liver injury was evaluated by assessing the morphological changes in liver sections stained with Haematoxylin and Eosin stain.

**Statistical analysis**
The data obtained from the study was analyzed using software GraphPad Prism 5. All parameters were expressed as Mean±SEM. The evaluation of differences between recorded data, $p<0.05$ was regarded as statistically significant.

**Results And Discussion**
**Results**
The resultant nanocurcumin was prepared by Evaporative Precipitation of Nanosuspension (EPN) method from the curcumin, had the particle size of 69.30nm (Fig. 1a) respectively, while the original curcumin had the particle size of 4706 nm (Fig. 1b).
Alpha-amanitin was given orally single dose on the first day of experiment; there was rise in AST, ALT, urea and BUN. However, different scenarios were seen in negative controls. When Silymarin was given to alpha-amanitin treated groups, blood parameter such as Hemoglobin and all biochemical parameters such as AST, ALT, Urea, BUN and total protein (TP) are normalized. Similar activity were seen in all treatment groups such as Nanocurcumin (200, 400, 800 mg/kg body weight) treated groups. In curcumin treated group, there was rise in AST, ALT, Urea and BUN, whereas reduced Total protein and hemoglobin level. Changes in hemoglobin level were predicted in Table 2. Changes in various serum parameters in rats exposed to alpha-amanitin, silymarin, curcumin and nanocurcumin is shown in Table 3. Similarly, changes in serum transaminase levels in the liver tissue on 28th day is shown in Table 4. Changes in body weight of rats treated with alpha-amanitin, silymarin, curcumin and curcumin was taken on 0 day and 28th day of experiment, whereas liver weight taken on 28th day of experiment after sacrificing the experimental rats, was predicted in Table 5.

Table 2: Changes in hemoglobin (Hb) level in rats exposed to alpha-amanitin, silymarin, curcumin and nanocurcumin

| Test Groups                        | Hemoglobin (Hb) Level       |
|------------------------------------|----------------------------|
| Negative control group (PBS)       | (p<0.001) 12.76±0.246       |
| Alpha-amanitin treated group       | 8.420±1.047                 |
| Silymarin treated group            | (p<0.001) 12.68±0.203       |
| Curcumin treated group             | (p<0.05) 10.58±0.549        |
| Nanocurcumin (200 mg/kg) treated group | (p<0.05) 10.88±0.411    |
| Nanocurcumin (400 mg/kg) treated group | (p<0.01) 11.40±0.488     |
| Nanocurcumin (800 mg/kg) treated group | (p<0.001) 12.86±0.426 |

Data were expressed as mean±SEM (n=6). Significant differences were indicated by p<0.05, p<0.01, p<0.01
Table 3: Changes in various serum parameters in rats exposed to alpha-amanitin, silymarin, curcumin and nanocurcumin

| Test Groups                  | Ast     | Alt     | Total Protein (Tp) | Urea     | Bun     |
|------------------------------|---------|---------|--------------------|----------|---------|
| Negative control group       | 86.18±3.973 | 70.02±1.381 | 6.906±0.3246 | 23.51±1.447 | 10.96±0.6834 |
| Alpha-amanitin treated group | 1357±322.2 | 1494±439.0 | 3.761±0.912 | 41.62±1.573 | 19.44±0.7345 |
| Silymarin treated group      | 173.4±477.95 | 352.4±105.8 | 6.818±1.144 | 23.76±1.477 | 11.09±0.6898 |
| Curcumin treated group       | 716±170.1 | 693.4±169.9 | 5.682±0.4247 | 33.04±2.149 | 15.43±1.003 |
| Nanocurcumin (200 mg/kg) treated group | 648.8±146.5 | 568.5±133.9 | 5.718±0.2772 | 29.92±3.442 | 13.97±1.607 |
| Nanocurcumin (400 mg/kg) treated group | 464.9±108.9 | 477.3±118.4 | 6.491±0.5963 | 24.99±2.286 | 11.67±1.068 |
| Nanocurcumin (800 mg/kg) treated group | 171.3±55.78 | 230.0±63.0 | 7.604±1.189 | 20.66±2.242 | 9.741±0.09793 |

Data were expressed as mean±SEM (n=6). Significant differences were indicated by p<0.05, p<0.01, p<0.001.

Table 4: Changes in serum transaminase levels in liver tissue on 28th day

| Test Groups                  | AST     | ALT     |
|------------------------------|---------|---------|
| Negative control group       | 70.29±2.29 | 60.67±1.83 |
| Alpha-amanitin treated group | 70.29±2.29 | 60.67±1.83 |
| Silymarin treated group      | 94.79±0.77 | 92.06±0.01 |
| Curcumin treated group       | 87.15±0.50 | 85.57±169.9 |
| Nanocurcumin (200 mg/kg) treated group | 84.85±0.65 | 80.09±0.70 |
| Nanocurcumin (400 mg/kg) treated group | 88.58±0.51 | 86.87±0.84 |
| Nanocurcumin (800 mg/kg) treated group | 96.53±0.33 | 94.08±0.39 |

Data were expressed as mean±SEM (n=6). Significant differences were indicated by p<0.05, p<0.01, p<0.01.

Table 5: Changes of bodyweight and liver weight in rats treated with alpha-amanitin, silymarin, curcumin and nanocurcumin

| Test Groups                  | Body Weight | Liver Weight |
|------------------------------|-------------|--------------|
|                             | 0 DAY       | 28th DAY     | 28th DAY    |
| Negative control group       | 135.36±1.62 | 136.54±1.12 | 136.54±1.12 |
| Alpha-amanitin treated group | 136.86±0.86 | 139.01±12.82 | 137.03±1.15 |
| Silymarin treated group      | 135.14±0.93 | 133.12±0.78 | 132.91±1.57 |
| Curcumin treated group       | 136.30±1.19 | 129.16±0.85 | 132.91±1.57 |
| Nanocurcumin (200 mg/kg) treated group | 136.25±1.48 | 129.19±1.02 | 132.91±1.57 |
| Nanocurcumin (400 mg/kg) treated group | 136.25±1.48 | 131.04±0.86 | 132.91±1.57 |
| Nanocurcumin (800 mg/kg) treated group | 137.03±1.37 | 132.91±1.15 | 132.91±1.57 |

Data were expressed as mean±SEM (n=6). Significant differences were indicated by p<0.05, p<0.01, p<0.01.

Histological findings

The negative control rats did not revealed any pathological alterations (Fig. 2). In positive control rats, the hepatic parenchyma showed multiple focal necrosis (Fig 3a). The cellular details were totally lost where as details persist. There was fragmentation of the nuclear materials. Fatty changes characterized by focal accumulation of fat droplets (Fig. 3b) was noticed. In some areas the hepatic parenchyma showed moderate to severe haemorrhages (Fig. 3c). Where as in silymarin treated group the hepatic parenchyma showed degeneration (black arrow) with no accumulation of fat and mild haemorrhages (yellow arrow). In Curcumin treated group, the hepatic parenchyma showed distortion of the hepatic cord (Fig. 4a) with necrosis (Fig. 4b). In Nanocurcumin treated group, @200 mg/kg (Fig. 5a), @400 mg/kg (Fig. 5b) and @800 mg/kg body weight (Fig. 5c), hepatocytes are normal and arranged in a cord. No fat droplets and degeneration of the hepatocytes were observed. But in Nanocurcumin 200 mg/kg body weight and Nanocurcumin 400 mg/kg body weight, mild congestion of sinusoidal capillaries were seen.
Fig 3: Photomicrograph Showing (A) Multiple Focal Areas of Necrosis In Liver, (B) Accumulation of Fat Droplets In Liver, (C) Moderate To Severe Haemorrhages In Liver (Group II), H&E, x40

Fig 4: Photomicrograph Showing (A) Degeneration With Mild Haemorrhages In Liver (Group III), H&E, X40 (B) Distortion Of The Hepatic Cord In Liver, (C) Necrosis In Liver (Group IV), H&E, X10

Fig 5: Photomicrograph Showing (A) & (B) Normal Hepatocytes Arranged In A Cord With Mild Congestion Of Sinusoidal Capillaries In Group V And VI, Normal Hepatocytes Arranged In A Cord With No Congestion Of Sinusoidal Capillaries In Group VII, H&E, X10h&E, X10

Discussion
Liver is the target organ for toxicity because of its role in clearing and metabolizing chemicals through the process called Detoxification.

Estimation of nanocurcumin particle size:
The nanocurcumin with various advantages in drug delivery and medicinal values were compared with Kakran et al. (2012) [14] who studied nanoparticles by two methods: antisolvent precipitation with a syringe pump (APSP) and evaporative precipitation of nanosuspension (EPN) and resultant particle size was 330nm and 150nm respectively. In 2008, International organization of Standardization (ISO) defined nanoparticles as a discrete nano-object where all the three dimensions are less than 100nm and the size can range from 1 nm to 100 nm. Hence we can conclude that the resultant nanocurcumin prepared will come under that range specified by ISO and can be used for further studies by considering the advantages of nano-drug preparations in drug delivery system and as a very good hepatoprotective agent.
Acute oral toxicity study of nanocurcumin
Acute oral toxicity study was conducted according to OECD Test Guidelines 425 (Up and Down procedure) in mice for nanocurcumin at 2000 mg/kg BW. There was no mortality, no change in behavior and body weight reported in the 14 day study period. The safe nanocurcumin on oral treatment was compared with the studies of Gopi et al. (2016) [13] where they conducted acute oral toxicity on curcuminoids and concluded it to be safe. Sankar et al. (2013) [22] and Bisht et al. (2007) [2] concluded that the highest dose 2000 mg/kg BW and lowest dose 50 mg/kg BW of nanocurcumin did not show any signs of toxicity and mortality and its safe for oral administration. By our current study, it can be concluded that nanocurcumin is safe for oral consumption without any toxicity symptoms.

Hemoglobin (Hb)
The haemoglobin level showed normal in negative control group, whereas decreased in positive control group and also showed increasing trend (normal) ranging from 12.68±0.2035 in silymarin treated group, curcumin treated group, nanocurcumin (200, 400, 800 mg/kg) treated group. The lowest level of haemoglobin level was recorded in alpha-amanitin treated group. Analysis of the data revealed significant difference (P<0.05) between the groups. From the results, it could be observed that the concentration of haemoglobin depended on the treated groups. Alpha-amanitin treated group revealed significant (p ≤ 0.05) decrease in concentration of haemoglobin which might be due to destruction of the red blood cells (Coles, 1986) [6]. Destruction of the red blood cells corroborates with the histopathological findings of haemorrhages in different organs mainly liver and kidneys. This result was in agreement with the findings of Zhao et al.(2006) [28], Cao et al.(2009) [1] and Wu et al.(2013) [26].

Serum aminotransferase enzymes
Upon cellular membrane damage and leakage serum activities were increased. It is seen that, in all types of liver injury, serum aminotransferase activities were increased. Serum estimation of SGPT was specific for the liver tissue so in liver injury serum SGPT was of greater value. Whereas, SGOT level may increase in acute necrosis or ischemia of other organs such as myocardium, besides the liver cell injury. In this study, serum AST, ALT, urea, BUN and creatinine activities were greatly increased (p<0.05) in rats exposed to alpha-amanitin as compared to negative control. The increased serum levels of hepatic markers have been attributed to the liver injury because these enzymes are mainly placed in cytoplasmic area of the cell and are released into circulation seen incase of cellular damage. The liver marker enzymes (AST, ALT) used as the most common biochemical markers to evaluate liver injury (Kozier et al., 2003; Girish et al., 2009) [17, 10] because these enzymes occur in the cytoplasmic area of the cell and released into circulation in case of cellular damage (Brent and Rumack, 1993) [3]. Thus, the activities of these enzymes in serum reflected the severity of liver injury (Zhang et al., 2005) [27]. The elevated activities of these enzymes were indicative of cellular leakage and loss of the functional integrity of the cell membranes in liver observed pathologically as hepatonecrosis (Rajesh and Latha, 2004 and Naik and Panda, 2008) [21, 20]. Hepatic necrosis in the liver were noticed in alpha-amanitin, curcumin, silymarin, nanocurcumin (@200mg/kg) treated groups with variations depending upon the dose.

Total Protein
The Total protein levels for the alpha-amanitin treated group were found to be decreased and subsequently all the animals of this group died. The Total protein values of silymarin showed nearly normal level as compared to that of the positive control group. Reduction in total protein content can be deemed as a useful index as the severity of hepatocellular damage (Kumar et al., 2007) [19]. In the present study, amanitin intoxication reduced the serum total protein level which is attributed to the initial damage produced and localized in the endoplasmic reticulum which results in the loss of cytochrome P-450 enzymes leading to its functional failure with a decrease in protein synthesis and accumulation of triglycerides leading to fatty liver (Bishayee et al., 2009) [1]. The pretreatment of silymarin, curcumin and nanocurcumin treated groups restored the total protein level that suggests the stabilization of endoplasmic reticulum leading to protein synthesis (Kumar and Mishra, 2006) [18].

UREA
The Urea levels showed a gradual increase in alpha-amanitin treated group as compared to negative control group; silymarin treated group and curcumin treated group. Whereas, showed decreasing trend (normal level) with different doses of nanocurcumin (200, 400, 800 mg/kg) as compared to positive control group.

Blood Urea Nitrogen (Bun)
The BUN level showed a gradual increase in alpha-amanitin treated group as compared to negative control group, silymarin treated group and curcumin treated group. Whereas, BUN Level showed decreasing trend (normal) with different doses of nanocurcumin (200, 400 and 800 mg/kg) as compared to alpha-amanitin treated group. The increased BUN level in all the groups with varied significant differences was in agreement with the findings of Kaya et al. (2014) [16], Ergin et al. (2015) [10]. The histopathological lesions observed in the kidney in our present investigation also support increase in BUN level. In the present study, blood creatinine level was increased in all the groups with variations in values. It was an indication of glomerular dysfunction, which support changes in biochemical estimation depending on different dose rates.

Histopathological examination
The histopathological result of the present study also revealed that treatment of alpha-amanitin induced hepatotoxicity in rats with nanocurcumin showed moderate improvement of necro-inflammatory changes and fatty changes caused by alpha-amanitin. These results suggested that treatment with nanocurcumin attenuated the severity of inflammation and necrosis induced by alpha-amanitin, which might be due to their antioxidant effect.

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