Evaluation of detoxifying activity of ORGCHP against acetaminophen induced hepatotoxicity in Sprague Dawley rats

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Received: 27 February 2020
Accepted: 27 April 2020

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ABSTRACT

Background: Accumulation of toxins in the body over a period of time interferes with the normal body functioning. The removal of toxins from the body is called ‘detoxification’. The liver is the main organ involved in detoxification and damage to the liver may impede the removal of toxins. The present study assessed the hepatoprotective activity of an organic Chlorella vulgaris in acetaminophen-induced liver damage model.

Methods: A total of 35 animals were randomized to five groups with seven animals in each group. The test drug, organic Chlorella vulgaris (ORGCHP; 350 mg/kg and 700 mg/kg) was compared with a reference standard (200 mg/kg). The positive control group and the vehicle control group were administered 0.5% w/v carboxy methyl cellulose. All groups received dose volume of 10 ml/kg for 10 days. Acetaminophen was given on day 8 and day 9. Blood collections were done at baseline day 1, day 8 and day 10. Outcome measures were the change in body weight, oxidation biomarkers, histopathological evaluation, gross pathological evaluation and relative body weight.

Results: Test drug ORGCHP Chlorella vulgaris showed dose dependent reduction in hepatotoxicity with high reduction in aspartate transaminase (AST) and modest lowering of alanine transaminase (ALT) (350 mg/kg; p.o.); while at high dose (700 mg/kg; p.o.) there was significant reduction in alkaline phosphatase (p<0.05), ALT (p<0.001) and AST (p<0.001) levels. No pathological or histopathological abnormality was seen in control group. In drug group, one animal showed minimal necrosis, while mild and moderate necrosis was seen in three animals each respectively.

Conclusions: The test drug exhibits detoxifying and hepatoprotective activity against acetaminophen induced hepatotoxicity.

Keywords: Hepatotoxicity, Acetaminophen, Detoxification, Antioxidant, Hepatoprotective

INTRODUCTION

The use of therapies to remove or eliminate toxins from one’s body is called ‘detox’.¹ These toxins could be one’s own metabolic products, outcomes of an unhealthy lifestyle, or environmental toxins accumulated in the body over a period of time.² The concept of detoxification or removal of all toxins, which are rather innocuous at significantly high concentration from the body is an integral part of traditional systems of medicine - Ayurveda, yoga, and naturopathy.³ Recently, several studies have shown that persistent accumulation of toxins in the body over a long period of time may result in several chronic problems.⁴ The liver plays an important role in the body in ‘detoxification’ by metabolizing most of these toxic chemicals and facilitating their elimination from the body.² The ‘toxic chemicals’ that have the potential to damage the liver could be certain hepatotoxic drugs like allopurinol, efavirenz and erythromycin, or even organic solvents like dimethylacetamide or dimethylformamide. The hepatotoxins can be broadly classified into intrinsic and idiosyncratic hepatotoxins.
The intrinsic hepatotoxins have a dose-dependent and predictable response unlike the idiosyncratic toxins that can occur in very small number of individuals. This ability of the liver to detoxify is largely due to the presence of xenobiotic bio-transforming enzymes and the ability to release bile. These bio-transforming enzymes mainly act on xenobiotics by the following four processes: hydrolysis, reduction, oxidation and conjugation reactions. Cytochrome (CYP) 450 are the most common enzyme systems involved in oxidation reactions. There are more than 50 cytochrome p450 enzymes however, six key enzymes are involved in biotransformation of 90% of the drugs. The highest levels of these enzymes are found in the liver, apart from small intestine, lungs, placenta and kidney. The hydroxylation, reduction and oxidation are Phase I reactions that play a crucial role in increasing the hydrophilicity of the drug by facilitating its elimination in the urine. The conjugation pathway is also a crucial pathway in metabolism of various xenobiotics and the enzymes involved namely glucuronosyl (or glucuronyl) transferases, sulfotransferases, GSH S-transferases and acetyl and amino acid N-transferases are part of the liver microsomes. Thus, any damage to the liver may greatly compromise its ability to remove the toxins from the body and may also cause damage to the body. Studies have shown that not only environmental toxins but also some drugs have the potential to cause liver injury. The need to screen and study hepatoprotective agents has largely come from this concern. The study of hepatoprotective agents in vivo is done using an animal model (preferably rats) developed by inducing liver damage. The induction of liver damage is can be through various chemical agents which include drugs like acetaminophen and carbamazepine.

A detoxifying and hepatoprotective agent named ‘ORGCHP’ was developed by EID parry nutraceuticals from the marine algae Chlorella vulgaris. This algae extract has shown good hepatoprotective activity in carbon tetrachloride induced hepatotoxicity. However, no data are available on whether this natural extract exhibits hepatoprotective activity against acetaminophen-induced damage. The present study was carried out to assess the detoxification potential and extent of hepatoprotection manifested by this test compound relative to N-acetyl cysteine in an acute acetaminophen-induced hepatotoxicity model.

METHODS

Animals

Male Sprague Dawley rats of about 8-12 weeks and weighing between 180.0-280.0 g were procured from Palamur Biosciences Private Limited, Hyderabad, India. The study was conducted at Laila Pharmacology Laboratories, Vijayawada, India after obtaining approval from the Institutional Animal Ethics Committee (IEAC) of the said facility. The animals were housed in a room of the research facility with temperature and relative humidity maintained at 22±3°C and 30-70%, respectively. Illumination was controlled to give 12 hours light and 12 hours dark cycle, and free access to food and water was provided to the animals. A total of 40 rats were acclimatized for a period of three days before enrolment in the study.

Materials

Hepatotoxicity inducing agent

Acetaminophen in a dose of 3 g/kg body wt. in 0.5% w/v carboxymethyl cellulose, sodium (CMC-Na) was administered orally one hour after the test dose. The solution of acetaminophen in a dose volume of 10 ml/kg body weight was given on day 8 and day 10.

Vehicle

The vehicle (0.5% w/v CMC-Na) was administered in dose volume of 10 ml/kg body weight to the vehicle and positive control group.

Test and reference drug

The hepatoprotective activity of organic Chlorella vulgaris (ORGCHP), provided by EID parry nutraceuticals, Chennai, India, was compared with N-acetyl cysteine. The test drug was administered in two doses, 350 mg/kg and 700 mg/kg body weight to group 3 and group 4, respectively. The reference drug (N acetyl cysteine) was administered in a dose of 200 mg/kg to group 5. The animals in the test and reference groups were dosed at a volume of 10 ml/kg body weight for 10 days. Biomarker estimation: 0.1 M phosphate buffer and Dounce homogenizer were used for biomarker estimation.

Blood collection

The blood was collected by retro-orbital plexus method under mild isoflurane anesthesia with the help of a fine capillary tube. The collection was done on day 1 (baseline), day 8 (post 12 hour fasting) and day 10. On day 10, the collection was done four hours after the dosing with acetaminophen. The analysis was performed in a laboratory of the research facility (Laila Pharmacology Laboratories, Vijayawada, India).

Drug dosing schedule

The dosing of the animals with the drugs was started after blood collection on day 1. The test and reference groups were administered the drug while the vehicle and positive control groups were administered 0.5% w/v of CMC-Na. All the study groups were dosed for ten days at a dose volume of 10 ml/kg body wt. The animals were administered only drugs in the first seven days to assess the detoxifying effect.
Hepatotoxigen dosing schedule

Acetaminophen was administered in oral dose of 3 g/kg body wt. one hour after drug administration on days 8 and 10 to all study groups except the vehicle control. At the end of the treatment period, rats were sacrificed using carbon dioxide inhalation followed by exsanguination. The liver, brain and kidneys were excised, weighed and preserved in 10% buffered formalin for further evaluation. The detailed experimental design with dosing is given in (Figure 1).

Randomization and study groups

After the acclimatization period of three days all the animals were examined and 35 were selected for enrollment in the study. These 35 animals were further divided into five groups, with seven animals in each group.

The study groups were as below: group 1: normal control/vehicle control (CMC-Na), group 2: acetaminophen control/positive control, group 3: ORGCHP (350 mg/kg) and acetaminophen, group 4: ORGCHP (700 mg/kg) and acetaminophen, group 5: N-acetyl cysteine (200 mg/kg) and acetaminophen.

Outcomes measured were

Assessment of detoxifying effect of the test drug for the first seven days

Clinical signs of toxicity were assessed for the animals daily. Measurement of body weight on day 1, 7, and 10 and comparison between the groups.

Assessment of the hepatoprotective effect of the test drug

Biochemical parameters: Aspartate transaminase (AST) alanine transaminase (ALT), alkaline phosphatase (ALP), total bilirubin (TB), gamma glutamyl transferase (γ-GT), albumin (ALB) and total protein (TP) were assessed on day 7, 8 and 10, and compared with the baseline values.

Gross pathological examination: After intervention period, the animals were euthanized with carbon dioxide intubation, and necropsy and gross pathological examinations were performed.

Percentage relative organ weight: The percentage relative organ weight was calculated using last day body weight and organ weight after sacrificing the animals using the following formula:

\[ \% \text{ relative organ weight} = \frac{\text{organ weight}}{\text{body weight}} \times 100 \]

Histopathology: Analysis of the excised liver tissues was performed using haematoxylin and eosin (H and E) stain mounted slide preparations of liver. H and E staining was performed on formalin-fixed, paraffin-embedded liver sections and microscopic lesions were observed and scored manually. Criteria for scoring of the lesions was as follows: minimal-1, mild-2, moderate-3, marked-4 and severe-5. Total score or parameter or group is the product of the score of lesion and number of animals that exhibited that lesion scoring.

Biomarker estimation: The brain, kidney and liver tissue samples were homogenized in ice cold 0.1M potassium phosphate buffer (pH 7.4) using Dounce homogenizer and the cell-free supernatant was assessed for antioxidant level using glutathione (GSH), catalase (CAT) and malondialdehyde (MDA) assays.

Statistical analysis

Data were expressed as Mean±SEM for the body weights. The comparison for the liver function tests between the groups was done using two-way ANOVA with Bonferroni correction. The organ function indices (glutathione, malondialdehyde, catalase levels) between the groups were compared using one-way ANOVA with Dunnett’s test. Significance was assumed at p<0.05. All the analyses were done using statistical package for social sciences (SPSS), manufactured by IBM corporation Ltd, USA.

Ethical approval

Approval was obtained from the Institutional Animal Ethics Committee of Laila Pharmacology Laboratories, Vijayawada, India (IAEC protocol approval number: LI/IAEC/LI171210) for the conduct of the study.

RESULTS

Clinical signs

All the animals were apparently healthy and normal throughout the study duration.
Effect of ORGCHP on body weight

There was no difference in body weight among the study groups. Individual animal body weights of the animals are given in (Table 1).

Effect of ORGCHP on detoxification and hepatoprotection

During detoxification phase (day 1 to 8) no significant or palpable changes were observed in either ORGCHP-treated group (groups 3 and 4) or in the N-acetyl cysteine treated group (group 5) in comparison with day 1 (baseline). Significant elevation in ALP, AST, ALT and total bilirubin levels were observed in disease control group on day 9 (group 2), in comparison with normal control group (group 1; p<0.01, p<0.001, p<0.001 and p<0.001, respectively), indicating hepatotoxicity. Between the test groups (groups 3 and 4) and the reference standard comparator, N-acetyl cysteine (group 5), at 200 mg/kg, body wt. p. o, a significant alleviation of hepatotoxicity as evident by reduction in ALP (p<0.01), ALT (p<0.001) and AST (p<0.01) levels. Test drug ORGCHP showed dose dependent reduction in hepatotoxicity with high reduction in AST and modest lowering of ALT (350 mg/kg; p.o.); while at high dose of the test compound (700 mg/kg; p.o) there was significant reduction in ALP (p<0.05), ALT (p<0.001) and AST (p<0.001) levels (Table 2 and 3).

Table 1: Effect of the extract (ORGCHP) on the body weight of the animals.

| Group                        | Body weight (g) |            |            |            |
|------------------------------|-----------------|------------|------------|------------|
|                              | Day 1           | Day 7      | Day 10     |
| G1-normal control            | 236.37±8.81     | 255.49±9.42| 259.49±8.93|
| G2-vehicle control           | 238.29±9.12     | 256.17±9.73| 229.83±10.01|
| G3-ORGCHP (350 mg/kg)        | 241.49±9.95     | 254.74±12.59| 240.00±10.40|
| G4-ORGCHP (700 mg/kg)        | 243.06±9.34     | 259.74±9.70| 240.49±9.31|
| G5-N-acetyl cysteine (200 mg/kg) | 245.31±10.79   | 263.71±13.19| 249.43±12.03|

Table 2: Effect of ORGCHP on ALB, ALP and ALT on day 1, 8 and 9.

| Group                        | ALB (g/dl) | ALP (U/l) | ALT (U/l) |
|------------------------------|------------|-----------|-----------|
|                              | Day 1      | Day 8     | Day 9     | Day 1 | Day 8 | Day 9 |
| G1-normal control            | 3.16±      | 3.54±     | 202.7±    | 174.7±| 191.3±| 66.57±|
| G2-vehicle control           | 0.06±      | 0.11      | 15.3±     | 15.0±| 21.3±| 8.86±|
| G3-ORGCHP (350 mg/kg)        | 3.14±      | 3.63±     | 299.8±    | 259.2±| 340.8±| 75.67±|
| G4-ORGCHP (700 mg/kg)        | 0.09±      | 0.14      | 20.2±*    | 21.0±*| 27.7±***| 9.08±|
| G5-N-acetyl cysteine (200 mg/kg) | 3.16±      | 3.59±     | 302.0±    | 259.1±| 334.4±| 80.00±|
|                              | 0.04±      | 0.12      | 15.3±     | 15.7±| 18.6±| 6.44±|
|                              | 0.07±      | 0.13      | 26.7±     | 16.0±| 21.4±*| 5.78±|
|                              | 3.13±      | 3.70±     | 246.2±    | 202.0±| 251.3±| 74.00±|
|                              | 0.07±      | 0.23      | 18.6±     | 23.0±| 28.8±**| 6.68±|
|                              | 3.09±      | 3.70±     | 246.2±    | 202.0±| 251.3±| 74.00±|
|                              | 0.07±      | 0.23      | 18.6±     | 23.0±| 28.8±**| 6.68±|
|                              | 3.13±      | 3.70±     | 246.2±    | 202.0±| 251.3±| 74.00±|
|                              | 0.07±      | 0.23      | 18.6±     | 23.0±| 28.8±**| 6.68±|

Table 3: Effect of ORGCHP on AST, TB and TP on day 1, 8 and 9.

| Group                        | AST (U/l) | BIL-T (mg/dl) | Total Protein (g/dl) |
|------------------------------|-----------|---------------|----------------------|
|                              | Day 1     | Day 8         | Day 9                | Day 1 | Day 8 | Day 9 |
| G1-normal control            | 151.00±   | 143.57±       | 135.71±              | 0.10± | 0.13± | 0.04± | 6.30± | 6.19± | 6.74± |
| G2-vehicle control           | 9.87      | 9.23          | 8.13                 | 0.00± | 0.01± | 0.00± | 0.10± | 0.05± | 0.12± |
| G3-ORGCHP (350 mg/kg)        | 164.67±   | 140.17±       | 1391.33±             | 0.10± | 0.13± | 0.10± | 0.10± | 0.16± | 0.07± | 0.15± |
| G4-ORGCHP (700 mg/kg)        | 9.78      | 8.32          | 476.13***            | 0.00± | 0.01± | 0.01*** | 0.16± | 0.07± | 0.15± |
| G5 - N-acetyl cysteine (200 mg/kg) | 169.50±   | 143.83±       | 813.17±              | 0.11± | 0.10± | 0.09± | 6.19± | 6.03± | 6.70± |
|                              | 5.99      | 8.02          | 72.85***             | 0.02± | 0.01± | 0.02± | 6.30± | 6.27± | 6.60± |
|                              | 167.83±   | 176.00±       | 680.17±              | 0.10± | 0.11± | 0.09± | 6.10± | 6.14± | 6.69± |
|                              | 14.76     | 13.18         | 312.03**             | 0.00± | 0.01± | 0.01± | 0.13± | 0.08± | 0.12± |

Values are represented as mean ±SEM; n=7 animals per group *p<0.05, **p<0.01, ***p<0.001 as compared to vehicle control (G2), *p<0.05,**p<0.01, ***p<0.001 as compared to normal control (G1) group using two-way ANOVA followed by Bonferroni post-hoc test.
Table 3: Effect of ORGCHP on relative liver weight.

| Group                          | Relative liver weight | Relative brain weight | Relative kidney weight |
|-------------------------------|-----------------------|-----------------------|------------------------|
| G1 - Normal control           | 4.07±0.16             | 0.65±0.04             | 0.69±0.02              |
| G2 - Vehicle control          | 4.24±0.12             | 0.76±0.03             | 0.82±0.03 **           |
| G3 - ORGCHP (350 mg/kg)       | 4.23±0.17             | 0.69±0.02             | 0.79±0.02              |
| G4 - ORGCHP (700 mg/kg)       | 4.16±0.21             | 0.75±0.04             | 0.81±0.03              |
| G5 - N-acetyl cysteine (200 mg/kg) | 4.33±0.15             | 0.71±0.04             | 0.84±0.03              |

Values are represented as mean ±SEM; n=7 animals per group. ##: p<0.01 as compared to normal control (G1) group using one-way ANOVA followed by Dunnett’s post-hoc test.

Effect of ORGCHP on morphometry and oxidative stress

Except for kidney catalase levels (p<0.01), no significant changes were observed between vehicle control and normal control groups. The results are represented in (Figure 2).

Effect of ORGCHP on gross pathology and histology of liver

We observed that five out of seven animals in vehicle control showed varying degrees of paleness and discolored liver. None of the treated and vehicle control groups showed any abnormality. The percent liver weights between the groups did not vary significantly (Table 4).

Histological examination of the liver

Microscopic examination of livers of control group (G1) rats did not reveal any abnormal histopathological changes (score: 0).

In the second group (G2) i.e., the positive control group, one animal showed minimal necrosis, while three animals showed mild and three animals showed moderate necrosis, respectively. Marked perportal necrosis was not observed in any animal in group 2. There was no fatty change in the group (score in positive control group: 2.29±0.29 vs test drug group: 1.57±0.37 (p<0.001)), indicating hepatotoxicity.

Hepatocellular degeneration was mostly observed in perportal zone of hepatic lobules. This was slightly reduced by low dose of ORGCHP in terms of severity (score: 1.57±0.37). High dose (700 mg/kg; p.o.) of ORGCHP protected 3 out of 7 animals from hepatocellular damage and the severity was less in remaining animals (score: 1.29±0.52). However; reference standard showed less efficacy compared to that of ORGCHP (score: 2.00±0.49). Data for each group are shown in (Figure 3A and B).

Figure 2: Organ indices, catalase activity in (A) liver, (B) brain and (C) kidney.

One-way ANOVA followed by Dunnett’s test; *p<0.05, ***p<0.001 vs vehicle control; ## p<0.01 vs normal control.
The hepatoprotective effect was measured by administering the treatment after challenge with a high bolus dose of acetaminophen (3 g/kg, p.o.) to induce acute hepatotoxicity. Acetaminophen is a widely used antipyretic agent and is safe for use in therapeutic doses. However, at very high doses it can induce centrilobular hepatic necrosis which can be fatal. The mechanisms by which it induces damage to the liver include depletion of glutathione and increased oxidative stress. Liver damage induced by the acetaminophen is thus a classical model for screening hepatoprotective activity and hence was preferred for this study.

In the present study we observed hepatotoxicity at a dose of 3 g/kg ORGCHP as evinced by significantly elevated levels of ALP, ALT, AST and total bilirubin. The oral administration of higher dose (ORGCHP 700 mg/kg; p.o.); significantly decreased the elevated levels of ALP, ALT and AST. At low dose treatment, reduction in only AST was seen. The efficacy of the extract in hepatoprotection was however found to be better than N-acetyl cysteine during histopathological evaluation.

The primary mechanism of inducing damage of several hepatotoxicogens including acetaminophen is the increase in oxidative stress. The dose dependent reduction in hepatotoxicity by the extract indicates that the activity is possibly related to the concentration of the active phytoconstituents present. A study done by XiXi Cai and colleagues found that a protein pigment complex isolated from Chlorella vulgaris had hepatoprotective activity against carbon tetrachloride induced hepatotoxicity. The hepatoprotective effect seen in our study is corroborated by the evidence from another study by Peng et al that assessed the hepatoprotective effect of Chlorella vulgaris against carbon tetrachloride induced oxidative damage. An alternative mechanism that could be involved in hepatoprotective activity of the extract could be the antioxidant potential, as evidenced by a study done by Serie et al that found that the extracts of Chlorella vulgaris had antioxidant and anti-inflammatory potential.

Interestingly, the study identified some important phytoconstituents in the extract which include the different types of phenolic compounds. Phenolic compounds or flavonoid have well established antioxidant potential and a correlation could exist between the flavonoid content of extract and the magnitude of hepatoprotection however, this needs verification.

Gopal et al state that the presence of flavonoids, phenols, terpenoids, glycosides, tannins and triterpenes in the marine algae Chlorella vulgaris. Many of these compounds have been reported to have a strong antioxidant potential. The extract also showed better macroscopic and microscopic scoring in the acetaminophen-induced acute hepatotoxicity, which warrants the need for quantification of the active constituents in the extract to further explore the possible mechanisms of action. Studies have shown that during liver damage or hepatotoxicity the levels of liver enzymes are altered. Therefore, evaluating the activity of various enzymes of this family could help in building a more

![Figure 3: (A) Gross pathology score and (B) histopathology score.](image)

One-way ANOVA followed by Dunnett’s test. ***p<0.001 vs. vehicle control; ### p<0.01 vs. normal control.

**DISCUSSION**

Complementary and alternative medicine (CAM) considers detoxification as the cleansing of the body to rejuvenate, recharge and renew in order to attain the complete potential of an individual. This process is essential to prevent diseases and degenerative changes that lead to disease in both healthy as well as diseased individuals. The present study was carried out to evaluate the detoxifying and hepatoprotective effect of ORGCHP (Chlorella vulgaris) in Sprague Dawley rats. The detoxifying effect was measured by initial treatment for a period of seven days in the normal animals. There were absence of abnormal signs, macroscopic changes and weight variations during treatment with ORGCHP for initial seven days which indicates its safety. The safety profile of the treatment was further corroborated by the unaltered biochemical parameters observed during the detoxification phase.

The hepatoprotective effect was measured by administering the treatment after challenge with a high bolus dose of acetaminophen (3 g/kg, p.o.) to induce...
credible evidence to support the hepatoprotective potential of the extract.

CONCLUSION

The present study extends the liver protective effect of organic Chlorella vulgaris in oxidative stress induced animal model and the overall results indicate that the study extract was efficacious in reducing ALP, ALT and AST enzyme levels relative to the reference drug (N-acetyl cysteine). Additional proof of concept studies is required to understand the receptor mediated molecular level mechanism of action and clinical studies to note considerable biomarker change with dose and duration specific designs.

Funding: The study was funded by E.I.D Parry (India) limited, N.S.C Bose road
Conflict of interest: None declared
Ethical approval: The study was approved by the Institutional Ethics Committee

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Cite this article as: Vasudevan SK, Seetharam S, Poongavanam A. Evaluation of detoxifying activity of ORGCHP against acetaminophen induced hepatotoxicity in Sprague Dawley rats. Int J Basic Clin Pharmacol 2020;9:912-8.