Evaluation of Hepatoprotective and Antidote Potential of Orthocetamol: A Novel Alternative Antidote of Paracetamol Toxicity

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ABSTRACT

Background: Paracetamol is well known analgesic and antipyretic drug. It is used as over the counter drug throughout the world. Prolonged use of paracetamol in higher doses is associated with severe risk of hepatotoxicity. Paracetamol induces oxidative stress in liver, which mimic the generation of free radicals, inflammatory prostaglandins, and cytokines. The documented antidote for paracetamol in clinical practice is N-acetylcysteine, which acts as an endogenous antioxidant. N-acetylcysteine is associated with several adverse effects, and it can induce reverse effects on glutathione and ultimately produce toxicity. Therefore, we aim to evaluate the effects of Orthocetamol, as 2-Actamidophenol and used against paracetamol toxicity instead of N-acetylcysteine.

Methods and findings: In the current research work, orthocetamol was analyzed as an antidote against paracetamol toxicity. The hepatoprotective and toxicological effects of orthocetamol in different doses were evaluated using both pre-treated and post treated parameters with drug induced hepatotoxicity by standard paracetamol. Furthermore, the lethal dose toxicity of

orthocetamol was also analyzed. The biochemical and histopathological methods were performed using standard protocols including liver function test as biochemical findings and using H & E staining methods for histopathological findings for liver. It was found that orthocetamol has antidote potential against paracetamol induced oxidative stress. It was also found effective in minimizing the toxic effects of paracetamol in both pretreated and post-treated group animals. Furthermore, it was found non-hepatotoxic in its lethal doses.

Conclusion: Orthocetamol suitable alternative to N-Acetyl cysteine in paracetamol induced hepatotoxicity.

Key words: Antipyretic, Paracetamol toxicity, Antidote, Orthocetamol

INTRODUCTION

Liver is a metabolic organ which is involved in several metabolic and biochemical functions [1]. Drug induced liver disease is a fatal challenge in world population. Several drugs are found in the form of prodrugs which are converted into their active forms following hepatic metabolism [2, 3]. Paracetamol possess analgesic and antipyretic and mild anti-inflammatory effects [4, 5]. It is easily available as over-the-counter *(OTC)* drug worldwide and one of the largest selling drug all over the world [2]. The prolonged use of paracetamol is associated with severe adverse effect. Many suicidal attempts are being made by this safe drug by taking lethal dose of 4g/day [6-8]. The most common antidote used for paracetamol poisoning is N-acetyl cysteine *(NAC)*. Historical use of *NAC* is in the management of paracetamol induced poisoning. The most common mechanism of *NAC* to overcome hepatotoxicity is its use as an antioxidant enzyme Glutathione precursor. That enhances the concentration of *NAC* with increase the cysteine protein. Cysteine amino acid is the integral part in synthesis of Glutathione and inhibits paracetamol toxicity. However, *NAC* possess several adverse effects which may warrant its therapeutic use. The common adverse effects include short half-life, anaphylactic reactions,

neurotoxicity, and hepatotoxicity. Its continuous use enhances the chances of drug resistance development [9]. Therefore, there is a need to search for a novel alternative antidote drug compound to combat paracetamol induced toxicity. In this context, several compounds are under pre-clinical investigation.

The current study has been aimed to find out hepatoprotective substitute for *NAC* in paracetamol poisoning. That would be more effective at lower doses with minimum side effects. In this regard the current research work is focused on the study of the pharmacological and toxicological effects of orthocetamol, also known as 2-Acetamidophenol *(2-AMP)* which is the; ortho positional isomer of paracetamol which was used against paracetamol induced hepatotoxicity. This compound was used in both pre-treated and post-treated animal groups. In Pre-treated group, orthocetamol was injected intraperitoneal (i.p) prior to 30 minutes administration of paracetamol (800mg/kg). In post-treated group, orthocetamol was injected through intraperitoneal rout after the 30 minutes administration of paracetamol toxic dose *i.e.*,800mg/kg

METHODS

Preparation and characterization of chemicals & experimentation in Albino Rats

The solvents used for experiment were olive oil, formalin, saline solution, hematoxylin & eosin stain, paraffin wax and all other reagents were of analytical and highest purity grade. Orthocetamol *(2-AMP)*, Paracetamol *(4-AMP)* was purchased from Sigma Chemical Company, St Louis, USA, and other leading suppliers. Experiments were performed on white male albino rats weighing 150-200g (8-10 weeks). The animal protocol was approved by the Ethics Committee. The animals were kept at 21 ± 2ºC and on a 12-h light/dark cycle with free access to

standard laboratory rat food pellets and water. Animals were distributed to treatment groups and toxicological studies groups. The animals included in the groups that were weighed daily, and accordingly the dose to be administered as per body weight; was calculated and given by intra peritoneal (i.p) route. This route was more accurate and safer. The animals were randomly divided into following 5 groups contain 7 animals per each group *(n=7/group)*

Group I: Negative control and untreated group used for solvent control; this animal group was administered with 2 ml/kg solvent for orthocetamol.

Group II: Standard drug induced hepatotoxicity; the positive control group were used to induce paracetamol hepatotoxicity using a dose of 800mg/kg.

Group III: Orthocetamol pretreated group was used to determine standard drug induced hepatotoxicity; each animal was first administered with orthocetamol test doses from 10mg/kg-100mg/kg, and then administered with toxic dose of paracetamol 800mg/kg after each 30 min.

Group IV: Orthocetamol post treated group was used to determine counteracting & antagonizing effects of drug induced hepatotoxicity by paracetamol; each animal was initially administered with paracetamol 800mg/kg toxic dose to induced hepatotoxicity then treated with orthocetamol test doses range from 10mg/kg-100mg/kg after each 30 min

Group V: Orthocetamol lethal dose toxicological studies group was used to determine lethal dose toxicity (LD_{50}), by administered high doses of orthocetamol ranging from 200mg/kg to 2000mg/kg.

After 24 hours, each group animals were anesthetized, the blood samples were collected in silica gel coated tubes and liver samples were fixed in 10 % buffer formalin solution, and then

performed biochemical and histopathological analysis. After completing the experiment on each group, the animals were sacrificed.

Toxicological studies

Liver biochemical analysis was performed on each group of animals for hepatotoxicological evaluations in albino rat model. The standard protocols described for biochemical assays were used [10].

Immunohistological studies

Control and drug treated rat liver tissues were obtained by sacrificing the animal, cutting of liver sections and stored in 10 % formalin were processed and put in paraffin bed blocks, and then section cutting was done by microtone section machine. Finally, their hematoxylin & eosin *(H & E)* staining was done to observe the morphological changes in rat hepatocytes, and compared them with untreated group [11].

Statistical Analyses

The statistical analysis represents by columnar graph hepato-biochemical parameters. The orthocetamol biochemical result compared to normal hepatocyte of experimental animals and analyzed decrease of paracetamol hepato-toxicity from toxic range. Throughout this study mean ± S.D were used to describe the data. The columnar graphs prepared by used of MS-excel worked sheet 2020 version.

RESULTS

1. Pharmacological Effects of Orthocetamol on pretreated animals

The biochemical assay of animals with paracetamol induced hepatotoxicity at 800mg/kg showed toxic SGPT level *i.e.*, 162.2u/l and ALP level 250.17u/l *(Table 1 & N=7*; N is number of animals used per each group

Table 2 *N=7*; N is number of animals used per each group*)*; while histopathological analysis showed moderate inflamed hepatocytes *(fig.4A)*. It is found that animals which were pretreated with orthocetamol in dose range between 10 mg/kg up to 100mg/kg showed increase in SGPT and decrease in ALP (Table 1 & Table 2 N=7; N is number of animals used per each group). It showed that orthocetamol lowers the SGPT & ALP levels of paracetamol induced hepato-toxicity. Histomorphological analysis in pretreated animals revealed that orthocetamol in dose range from 10mg/kg-100mg/kg exhibited normal to mild inflamed hepatocytes, respectively *(fig.4, B-D)*. The results of statistical analysis supported these results. It is evident *(fig.1)* that the animals pretreated with orthocetamol that their biochemical parameters were remained within normal range following paracetamol induced hepato-toxicity.

Table 1. Serum Glutamic Pyruvic Transaminase (SGPT) biochemical analysis of 2-AMP administered.

Groups and parameters		**SGPT (U/L) Mean± S.D**
Group I control group	(2mg/kg)	13.2±0.03 U/L
Group II 4-AMP induced hepatotoxicity	(800mg/kg)	162.2±0.31 U/L
Group III 2-AMP pretreated	(10mg/kg)	10.1±0.06 U/L
	(50mg/kg)	12.2±0.08 U/L
	(100mg/kg)	18.1±0.51 U/L
Group IV 2-AMP post-treated	(10mg/kg)	110.0±0.08 U/L
	(50mg/kg)	106.2±0.07 U/L
	(100mg/kg)	104.3±0.08 U/L

Group V 2-AMP Lethal dose toxicity (LD_{50})	(200mg/kg)	8.2±0.08 U/L
	(1500mg/kg)	10.3± 0.27 U/L
	(2000mg/kg)	12.3± 0.10 U/L

N=7; N is number of animals used per each group

Table 2. Alkaline Phosphatase (ALP) biochemical analysis of 2-AMP administered.

Groups and parameters		**ALP (U/L) Mean± S.D**
Group I control group	(2mg/kg)	116.8±0.04 U/L
Group II 4-AMP induced hepatotoxicity	(800mg/kg)	250.1±0.03 U/L
Group III 2-AMP pretreated	(10mg/kg)	106.2± 0.08
	(50mg/kg)	85.3±0.10
	(100mg/kg)	70.2±0.1
Group IV 2-AMP post-treated	(10mg/kg)	115.3±0.08
	(50mg/kg)	178.3±0.09
	(100mg/kg)	195.3±0.08
Group V 2-AMP Lethal dose toxicity (LD_{50})	(200mg/kg)	106.2±0.06
	(1500mg/kg)	96.3± 0.11
	(2000mg/kg)	90.3± 0.08

N=7; N is number of animals used per each group

Pretreated biochemical assay showed that orthocetamol has inhibitory potential and effects that hold the SGPT & ALP level within the normal range and prevent paracetamol induced hepatotoxicity. The histomorphological analysis revealed that in the orthocetamol pretreated animals followed by in paracetamol hepatotoxicity there were minor to mild toxicity appeared in pretreated animal hepatocytes. The biochemical, histomorphological and graphical analysis represent the parallel result, *i.e.,* the therapeutic effect is much better in low dose *(table.1 & 2, fig.1 & fig.4, B-D).* In summary, it was demonstrated in this experiment that orthocetamol acts as hepatoprotective agent against paracetamol induced hepatotoxicity in very low doses in pretreated animals

2. ***Pharmacological Effects of orthocetamol in post-treated animals: After paracetamol induced hepatotoxicity***

The post-treated dose of orthocetamol was increased from 10 mg/kg-100mg/kg. This showed a gradual decrease in SGPT and increase in ALP as shown in *(table.1 & 2).* The biochemical results showed that the orthocetamol have antagonizing and counter acting effects to decrease SGPT and ALP from toxic level *i.e.*,162.2u/l SGPT and 250.17u/l ALP, that was caused by paracetamol induced toxicity *(table.1 & 2)*. It is evident from the results of statistical analysis that the orthocetamol detoxify the paracetamol-induced hepatotoxicity in post-treated group animals. The graphical figure of animals that were post-treated with orthocetamol represent their biochemical parameters decreases a hepatotoxicity level caused by Paracetamol but a better result is observed on low dose *i.e.*, 10mg/kg *(fig.2).* In post treated histo-morphological analysis, it was found that hepatocytes show minor to moderate inflammation as the dose range increased *(fig 4, E-G).*

The above data shows that the orthocetamol is therapeutically active at low doses, and the therapeutic activity decreases with the increase of dose of orthocetamol.

3. ***Effect of lethal dose (LD_{50}) of orthocetamol***

In lethal dose toxicological studies involving biochemical analysis, showed that orthocetamol in high dose ranging from 200mg/kg-2000mg/kg showed increased SGPT level and decreased ALP level *(table.1 & 2)*; same as graphical representation results *(fig.3).* The histomorphological analysis of lethal dose toxicological studies *(LD_{50})* showed that hepatocytes were normal to minor inflamed; that is non-toxic range *(fig.4, H-J).* All the biochemical results demonstrated that the levels of both SGPT and ALP were within normal levels *(table 1 & 2)*. On finding lethal dose toxicity *(LD_{50})* for orthocetamol; the graphical statistical analysis shows no such

hepatotoxicity on a high dose range *(fig.3)*. The histomorphological analysis showed decline in toxicity as compared to paracetamol that's toxicity has been reported at 800mg/kg in preclinical studies *(Fig.4, H-J)*.

DISCUSSION

Orthocetamol is an ortho-positional isomer of paracetamol. Toxicities and pharmacological activities of this isomer is rarely documented in the available literature [12]. In analog drug research, it has been observed that the possibility in modification in structure activity relationship *(SAR)* through modification in functional group position which result in change of physiochemical, biochemical, and pharmacological properties of drug. Paracetamol is a well-known antipyretic and analgesic drug but it causes liver damage when used above the therapeutic range. Liver damage occur by paracetamol is caused by the generation of reactive oxygen species *(ROS)*, induced oxidative stress, depletion of glutathione and over production of cytokines such as *TNF α* that damage the liver and produce necrosis [13]. Research has found that there are many alternates to prevent paracetamol toxicity.

Antioxidants have very crucial role in prevent oxidative stress and to inhibit liver toxicity. Such as non-enzymatic antioxidants are ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), glutathione *(GSH)*, carotenoids, flavonoids, and other antioxidants [14]. Similarly, natural antioxidant compounds with anti-inflammatory effects include resveratrol present in grapes [15], quercetin in apples [16], Bisoprolol in Chamomile [17] and Epigallocatechin Gallate is present in green tea, a flavonoid in various fruits and vegetables [18, 19]. Phenol and monophenol have also a prominent role to reduce liver toxicity. Phenols is particularly attractive and prophylactic agents and have pharmacological effects, these are integral part of the diet.

Many of these biological functions have been attributed to their antioxidant activity and inhibit free radicals [20]. Monophenolic compounds have low molecular weight and have less toxicity, Orthocetamol (2-AMP) found natural in wheat, it is less toxic, low molecular weight and have lipophilic in nature [15, 16, 18].

To avoid the paracetamol induced liver toxicity a well-known documented derivative and antidote is N-acetyl cysteine *(NAC)* which is glutathione precursor. It inhibits paracetamol inflammation and toxicity, but N-acetyl cysteine has many adverse effects as it is dose dependent and hydrophilic. It has short half-life so repeated doses are required. Long term use causes anaphylactoid reactions, neurotoxicity, and hepatotoxicity. The prolonged use reverses the mechanism which include severe oxidative stress occur due to reactive oxygen species *(ROS)* thus causing necrosis of liver [9]. Therefore, the search of an efficacious and non-toxic alternate to N-Acetylcysteine has remained a goal and challenge for researchers and medical scientists. In this connection orthocetamol was found to be an effective hepato-protective agent in pretreated experiment, competitive inhibitor in post treated experiment and high therapeutic potential in lethal dose toxicity *(LD_{50})*.

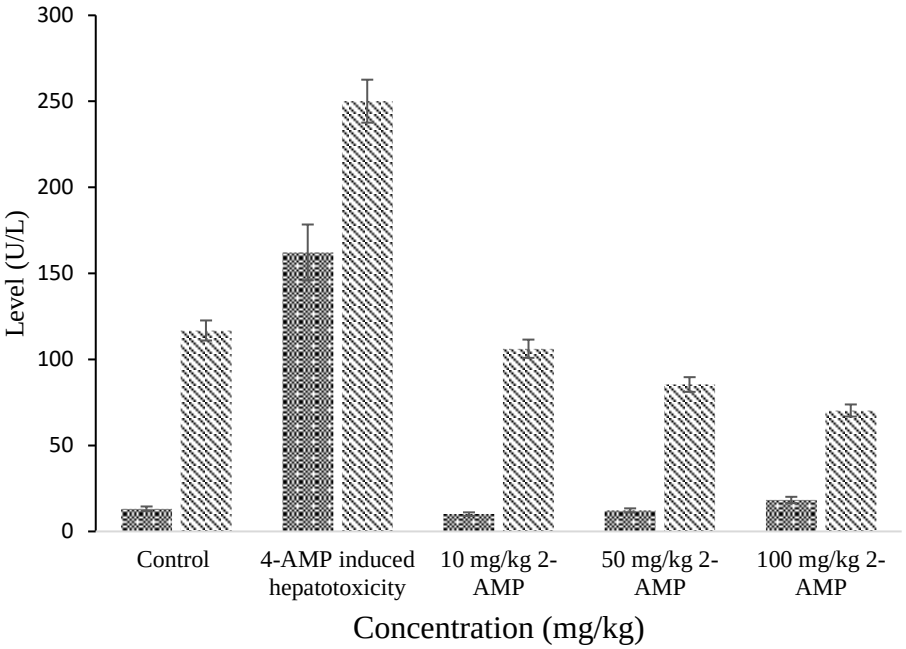


Fig.1 Column graph of pretreated group animals with orthocetamol (2-AMP), showed mimic of hepato-toxicity, induced by paracetamol (4-AMP)

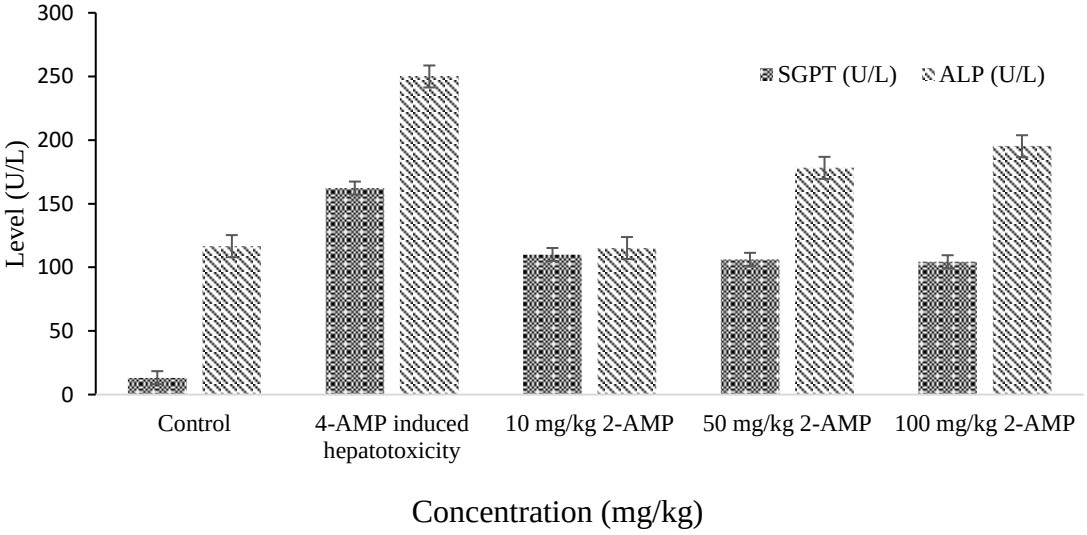


Fig. 2 Column graph of post-treated group animals with orthocetamol (2-AMP) showed detoxify hepatotoxicity, induced by paracetamol (4-AMP)

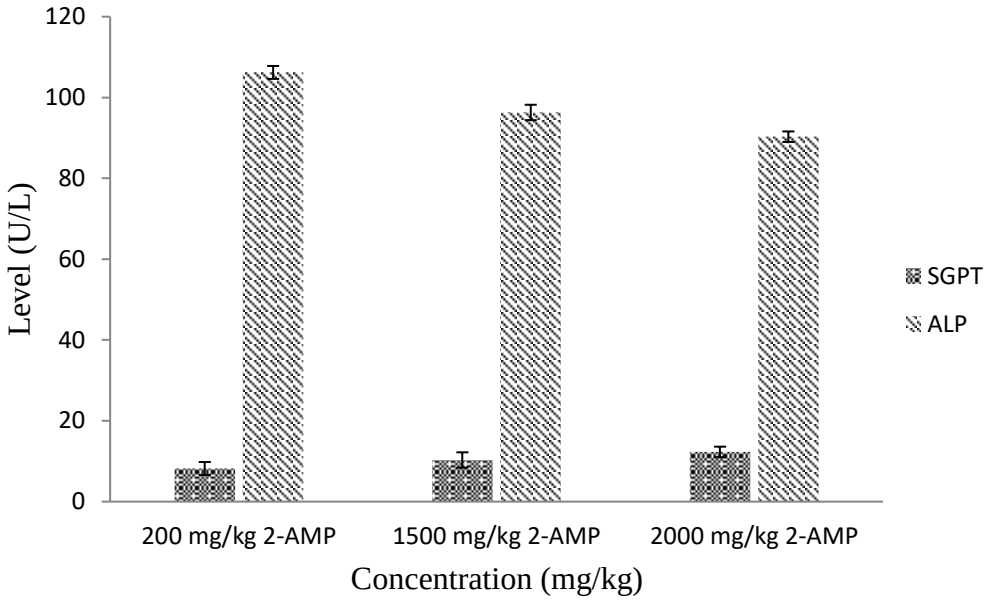
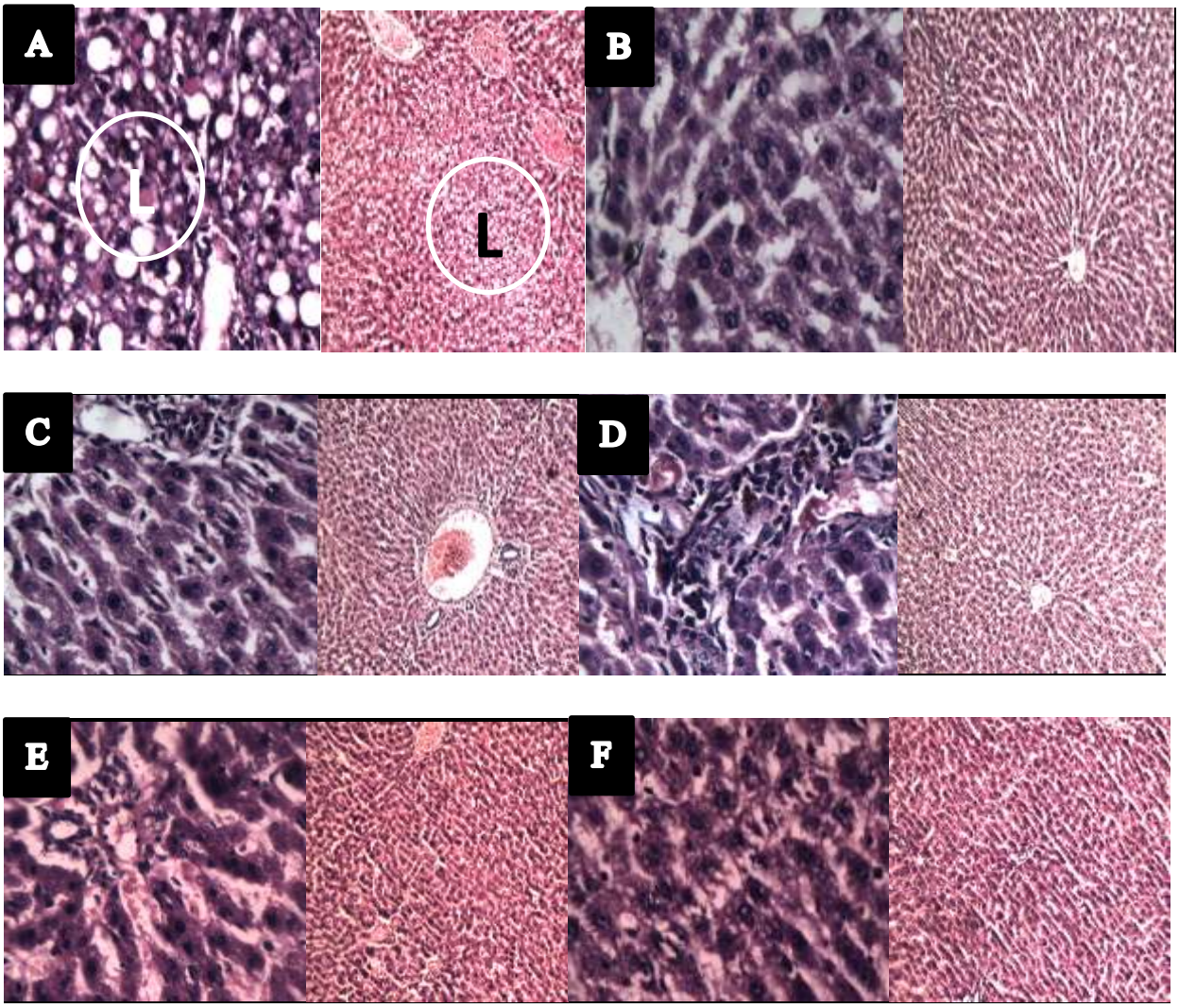


Fig.3 Column graph of lethal dose toxicity (LD_{50}) group animal with orthocetamol (2-AMP); showed high dose to mimic hepatotoxicity, induced by paracetamol (4-AMP)

Liver acute toxicological studies were performed to evaluate the orthocetamol hepato-safety. The Intra peritoneal (i.p), single doses of 800mg/kg of paracetamol develop significant liver damage in rats [21], *(table.1, 2 and fig.4, A)*. The results found through SGPT & ALP showed high hepatotoxicity by paracetamol [22] *(table.1 & 2)*. The pre-treated and post-treated drug administration of orthocetamol modulates the biochemical and histomorphological parameters of paracetamol induced oxidative stress in individual group of rats. Large doses or long-term use of paracetamol have been associated with impaired liver function, possibly due to induced oxidative stress [23]. A histomorphological study of rat hepatocytes was performed for paracetamol isomer. At the lower, pretreated orthocetamol doses of 10mg/kg-100mg/kg; orthocetamol inhibits and prevent significant liver toxicity *(table.1 & 2)*. The statistical analysis showed orthocetamol increase in SGPT and decrease in ALP thus it inhibits the SGPT & ALP toxic level; caused by paracetamol *(fig. 1)*. Histomorphological figures showed that orthocetamol resist paracetamol induced liver toxicity *(fig.4, B-D)*. Pretreated biochemical assay demonstrates the orthocetamol inhibitory potential and effects which revealed that after the orthocetamol

administration there were no paracetamol induced hepatotoxicity appeared in pretreated group animals. In post treated group animals at the dose range 10mg/kg-100mg/kg, orthocetamol showed decrease in SGPT level and increase in the ALP level; and both the biochemical parameters were within limits and no toxicity caused by paracetamol *(table. 1 & 2)*. The histomorphological evaluation showed that orthocetamol has antagonistic effects and act as competitive inhibitor, to antagonize paracetamol induced liver toxicity *(fig.4, E-G)*. The statistical analysis showed a gradual decrease in SGPT & ALP from toxic range which was caused by paracetamol induced hepatotoxicity *(fig.2)*. These observations supported the statement that the orthocetamol can be employed as a better alternate to detoxify the paracetamol induced hepatotoxicity. Such therapeutic effect to reduce paracetamol induced liver toxicity and inflammation from toxic lethal dose range, is most beneficial at low doses *i.e.*,10mg/kg but the therapeutic window gets narrower with the increase in strength and concentration of drug *(fig.2)*. The toxicity of orthocetamol at such high concentration *i.e.*,100mg/kg is because of severs oxidative stress. These results demonstrated that at pretreated concentration range, the orthocetamol shows hepatoprotective effect and was also found that using in post treated concentration range, orthocetamol has therapeutic action in counter-acting paracetamol induced toxicity in albino rats. All these hepato-protective and hepato-therapeutic effects come closer on lower concentration range of pretreated and post treated group animals, respectively. Furthermore, using orthocetamol at higher concentrations *i.e.,* at lethal dose toxicity it showed a therapeutic potential as similar to previously describe in lower doses, *(table.1 & 2)*. The statistical analysis represents no hepatotoxicity on higher concentrations *(table.1 & 2 and fig.3)*.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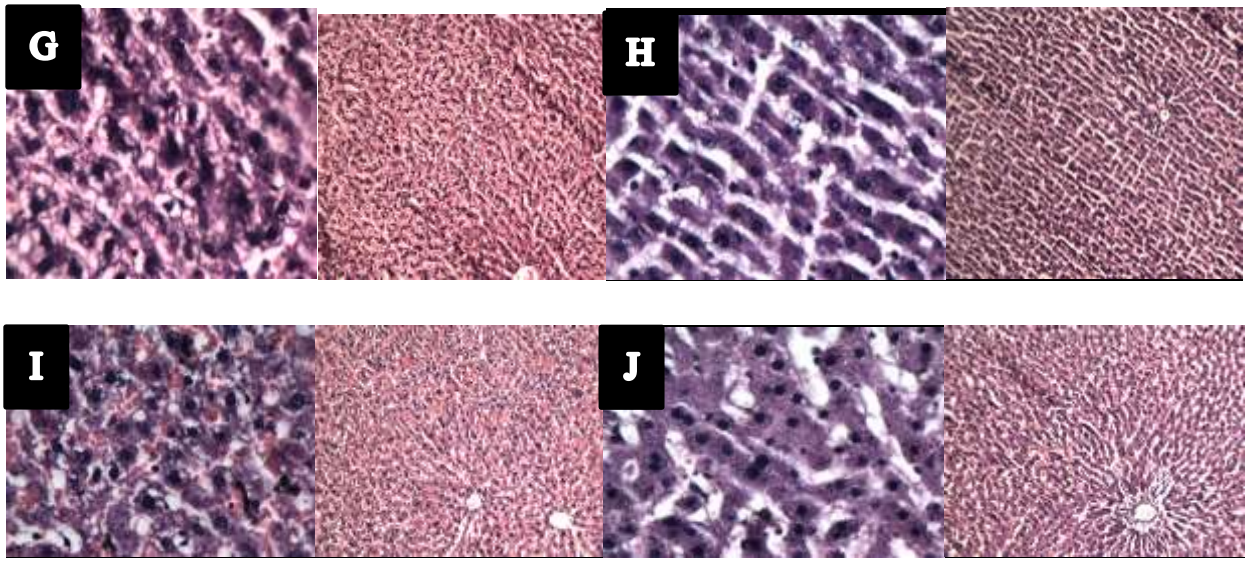

Fig.4 Hematoxylin & eosin staining figures indicate the histopathological change in rat hepatocytes. *Picture: A* is a histopathological view of hepatocytes on paracetamol induced hepato-toxicity; the encircled portion indicates necrosis, inflamed cells; while *"L"* denote lymphocytes comes out in blood. *Picture: B-C* showed the morphological view of hepatocytes on pretreated with orthocetamol at 10mg/kg-50mg/kg; indicate the hepatocytes that were normal and are in non-pathological shape. *Picture: D* showed morphological changes indicate mild inflame hepatocytes on pretreated with orthocetamol at 100mg/kg. *Picture: E-G* showed morphological changes in hepatocytes on post treated with orthocetamol at 10mg/kg-100mg/kg; indicates therapeutic effects of orthocetamol on paracetamol induced hepatotoxicity. *Picture: G* indicates inflammation by minimizing therapeutic effect with increased of concentration. On determine lethal dose toxicity *(LD_{50}) Picture: H-J* showed normal hepatocytes at 200mg/kg-2000mg/kg.

The histomorphological figures showed no such inflammation as compared to paracetamol induced hepatotoxicity reported at 800mg/kg *(fig.4, H-J)*. The non-hepatotoxic nature of orthocetamol has been reported in mice [24]. The non-hepatotoxicity nature of orthocetamol may be due to the phenolic as well as antioxidant nature of the compound [14, 20].

In acute toxicological studies of paracetamol ortho-isomer, we found that orthocetamol is comparatively less or non-toxic in comparison to paracetamol. Orthocetamol has wide therapeutic index and therapeutic window. It shows effect on very low doses. Low dose effect might be lipophilic nature [13]. It may easily cross cell membrane by passive transport.

CONCLUSION

Orthocetamol has a therapeutic potential in minimizing the toxic effects of paracetamol in both pretreated and post treated group animals. This hepato-protective nature of orthocetamol is believed to be due to its ability to retard the generation of reactive oxygen species (ROS) thus showing an antioxidant potential. The antioxidant potential is almost comparable to other lipophilic antioxidant, such as Vitamin E. Usually orthocetamol is effective at very lower concentrations and rarely significant at higher concentrations. Conclusively speaking, orthocetamol may be used as a suitable alternative antidote of paracetamol in paracetamol induced hepatotoxicity. The other pharmacological actions and their possible mechanisms need to be identified.

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Ethics Statement

The animal study was reviewed and approved by Ethical committee, Pakistan Institute of Professional Studies, College of Pharmacy Abbottabad Pakistan

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