TOXICOLOGICAL STUDIES OF PARACETAMOL POSITIONAL ISOMERS

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ABSTRACT

Paracetamol is a well-known analgesic and anti-pyretic drug. Its hepatotoxicity is still a big challenge for scientific and clinical community. There is rich data available regarding its benefits and hazards. However very rear data is available to their positional isomers i.e., metacetamol and orthocetamol. Metacetamol is previously reported as non-hepatotoxic isomer with analgesic and antipyretic potential. The preclinical acute toxicological activities of Paracetamol (4-acetamidophenol) positional isomers; 2-acetamidophenol and 3-acetamidophenol was aimed in this research work. In acute toxicological studies, paracetamol isomers were analyzed for lethal dose toxicity in 50% rats population. LD_{50} of 2-acetamidophenol, 3-acetamidophenol and 4-acetamidophenol (Paracetamol) were found 1.30g/kg,1.15g/kg and1.25g/kg, respectively. The results showed that all the isomers were safer and having wide therapeutic window. 2-acetamidophenol was found to be non-hepatotoxic, non-nephrotoxic and non- hemotoxic when 100mg/kg dose (i.p) administered to female Sprague dawley rats. At 500 and 1000mg/kg doses of 2-acetamidophenol, it was found to be hepatotoxic and mild nephrotoxic. 3-acetamidophenol showed mild hepatotoxicity but severe nephrotoxicity when 1000mg/kg (i.p) dose administered to female Sprague dawley rats. No haematotoxicity was observed in 3-acetamidophenol at toxic dose range.

Keywords: Paracetamol, Metacetamol, Orthocetamol, Toxicity studies, Hepatotoxicity, Haematology, Nephrotoxicity

INTRODUCTION

Drug analogs and isomers are widely used in the drug development process to improve drug safety, efficacy, enhance material stability and reduce adverse or toxic effects [1]. This strategy is useful to improve the drug quality for the remedy of various diseases. To this time, the use of some promising drugs has been limited clinically because of their adverse effects [2]. These drugs may become widely used in a clinical setting if they are substituted with their appropriate isomer. During the past few decades, isomeric research has succeeded in minimizing the adverse effects in several clinical and preclinical practice. Modification in the position of functional group, results in the origination of novel positional isomers with the new set of possible physicochemical and / or pharmacological potentials. Structure activity relationship (SAR) plays a pivotal role in fortitude of specific properties of an individual drug molecule [3]. Slight modification in either orientation or position of functional group may dramatically bring new set of drug safety, efficacy, and stability. Therefore, the medicinal chemist continuously plays with the drug molecule, make derivatives and analogs of the existing drug to improve the dark bands of old drug in the treatment of various diseases. These isomeric drugs bring new outcomes and reveals the hidden pharmacological potential of unknown compounds, these compounds might be a better addition in the existing drugs. There are several examples of isomeric drugs such as S (+) ibuprofen, which has less gastric mucosal toxicity than racemic ibuprofen in the rat model [2]. These isomeric drugs are clinically use and easily available in the market with their specific identity and are different from their parent drug. Due to relatively minimal cost of drug research, more and more pharmaceutical industry tends to develop new drugs and prefers to work on isomeric compounds to shorten the time and cost of research as many isomeric drugs has shown better efficacy, safety, and stability [3]. In the current work we tested the paracetamol positional isomers for acute toxicological evaluation.

MATERIAL AND METHODS

Animal Used

Female Sprague Dawley (SD) rats weighing 215-230 g (8-10 weeks) were used. 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. Rats were randomly distributed to each treatment group of 6 animals each.

Material

Paracetamol, 2-acetamidophenol and 3-acetamidophenon purchased from sigma Aldrich. All chemicals and solvents were of analytical grade.

Methods

Lethal dose toxicity test in rats (LD_{50})

LD_{50} is defined as the dose of testing material, which produces death in 50% of the animals. The LD-50 of paracetamol isomers has been measured by Lorke's method [4] for the analysis of acute toxicity tests.

Acute liver, kidney, and blood toxicity

Paracetamol and their isomers were analyzed for liver function tests, kidney function tests and blood safety profile by standard methods and protocols described for biochemical assays [5].

Histopathological studies of rat liver

Liver tissues of control and drug-treated mice were obtained by killing the animal, cutting liver sections, and storing in 10% formalin, and then processed into paraffin-embedded blocks, and then sectioning was performed using a microtone cutting machine. Finally, they were stained with hematoxylin and eosin [H&E] [5] to observe the morphological change and compare with the control.

RESULTS

Lethality Test (LD_{50})

2-acetamidophenol and 3-acetamidophenol, which cause death in 50% of rats within 24 hours (LD50), were calculated using the method described by Lork, 1983 [4]. Briefly, paracetamol isomer at doses of 10, 100, 500, 1000 and 1500 mg/kg were administered (i.p.)

to groups of 3 rats. Based on the mortality results in each group at 24 hours, 4 additional rats were given different doses of paracetamol isomers to obtain the lowest and most toxic values. The LD50 was calculated by the geometric mean of these values. The LD_{50} of 2-acetamidophenol, 3-acetamidophenol and paracetamol is found to be 1.3 g/kg, 1.15 g/kg and 1,250g/kg respectively/24 hour in male rats as shown in **Table 1**.

Hepatotoxicity

Hepatotoxicity studies of 2-acetamidophenol and 3-acetamidophenol were evaluated using biochemical parameters, including transaminase levels in rat blood. Individual groups of animals were given 1000 mg/kg, 500 mg/kg and 100 mg/kg of paracetamol isomers. Rats treated with acetaminophen at doses of 1000 mg/kg and 500 mg/kg developed significant hepatocellular injury as evidenced by significant ($P<0.05$) increases in serum aspartate transaminase (AST) and alkaline phosphatase (ALP) activities compared to control as shown in **Table 7**. However, at the dose of 500mg/kg and 1000mg/kg 2-acetamidophenol toxicity was almost equivalent to paracetamol while the 3-acetamidophenol at the same dose range showed no such type of hepatotoxicity and nephrotoxicity regarding biochemical parameters of liver enzymes. A dose of 100 mg/kg (which is 10-20 times its therapeutic dose range in rats) was found to be non-hepatotoxic and nephrotoxic as assessed biochemically (and histologically) and a major comparative difference was observed is that 2-acetamidophenol did not increase the ALP level and neutrophils count at any dose range of the compound administered as commonly observed in the paracetamol as well as non-hemotoxic 3-acetamidophenol. Furthermore, we studied the effects of 2-acetamidophenol and 3-acetamidophenol isomers of paracetamol on rat hepatocytes using a histopathological model. In this model, we used hematoxylin and eosin (H&E) staining. 2-acetamidophenol and 3-acetanidophenol in the dose range of 100 mg/kg to 500 mg/kg did not show significant hepatotoxicity, hepatocellular damage, necrosis of ventricular veins etc. However more than 500mg/kg-1000mg/kg-1000mg/kg. It was found to be almost as toxic to hepatocytes as paracetamol while 3-acetamidophenol was mildly toxic as expected **Table 2,3, 4 and histological plates 1-10**.

Nephrotoxicity

Nephrotoxicity of 2-acetamidophenol and 3-acetamidophenol and Paracetamol were assessed. 2-acetamidophenol and 3-acetanidophenol, kidney function tests revealed that 3-acetanidophenol is nephrotoxic at the dose range of 500mg/kg-1000mg/kg. The creatinine level was significantly high while 2-acetanidophenol showed elevated creatinine level that indicates nephrotoxicity only at the dose of 1000mg/kg. While paracetamol did not show nephrotoxicity at any dose range (**Table 5, 6 & 7**).

Haematotoxicity

2-acetanidophenol, and 3-acetanidophenol was tested in dose range 0f 500mg/kg to 1000 mg/kg, Intra peritoneal administered dose to the individual groups of rats. Biochemical parameters were assessed to identify the possible hepatotoxicity. Blood hemoglobin, red blood cells count, hematocrits and neutrophils count were observed. Slight hematocrits and neutrophils elevation was seen in 500mg/kg dose of 2-acetanidophenol while the significant elevation was seen at the same dose when 3-acetamidophenol and 4-acetamidophenol (Paracetamol) was used at the same dose. This finding clearly shows that at 500mg/kg dose 2-acetamidophenol is safer than rest of the isomers.

Furthermore, it was found that paracetamol and all isomers of paracetamol significantly elevated the neutrophils count at the dose of 1000mg/kg. This similarity shows that at higher doses paracetamol and their isomer produce toxicity in almost with same pattern. The elevation in hematocrits was only seen in 2-acetanidophenol administration at the dose range of 500mg/kg-1000mg/kg (**Table 8, 9 and10**).

Table.1 LD_{50} of Paracetamol isomers in Sprague Dawley rats

Compound	**Route of Administration**	**LD_{50}(g/kg)**
2-acetamidophenol	I.P	1.3
3-acetamidophenol	I.P	1.15
4-acetamidophenol	I.P	1.25

Table.2 2-acetamidophenol Hepatotoxicity

Treatment (mg/kg)	ALT (IU/L)	AST (IU/L)	ALP (IU/L)
Control/Non treated rat	48.63 ± 0.32	146.3 ± 0.83	116.8 ± 1.11
2-acetamidophenol (100)	48.88 ± 0.95	148.1 ± 0.87	112.9 ± 3.34
2-acetamidophenol (500)	72.00 ±0.77**	138.3 ±0.88***	108.5 ± 0.84
2-acetamidophenol- (1000)	105.1 ±0.87***	165.1 ± 0.89 **	59.88 ± 0.85

Table.3 3-acetamidophenol Hepatotoxicity

Treatment (mg/kg)	ALT (IU/L)	AST (IU/L)	ALP (IU/L)
Control/ Non treated rat	48.63 ± 0.32	146.3 ± 0.83	116.8 ± 1.11
3-cetamidophenol- (500)	45.13 ± 0.81*	448.0 ± 2.38***	95.00±1.10***
3-cetamidophenol (1000)	91.38 ± 0.84***	580.3 ± 1.35***	144.8±0.77***

Table.4 4-acetamidophenol/paracetamol Hepatotoxicity

Treatment (mg/kg)	ALT (IU/L)	AST (IU/L)	ALP (IU/L)
Control/ Non treated rat	48.63 ± 0.32	146.3 ± 0.83	116.8 ± 1.11
4-cetamidophenol (500)	92.63 ± 0.99***	448.0 ± 2.38***	135.8±1.11***
4-cetamidophenol (1000)	110.5 ± 0.73***	580.3 ± 1.35***	132.8±1.55***

Table.5 2-acetamidophenol Nephrotoxicity

Treatment (mg/kg)	CREATININ (mg/dl)	BLOOD UREA NITROGEN (BUN)(mg/dl)
Control/ Non treated rat	0.2743 ± 0.006	24.60 ± 0.297
2-AMP* (100)	0.2657 ± 0.004	20.01 ± 0.455***
2-AMP (500)	0.2657 ± 0.004**	17.31 ± 0.658***
2-AMP (1000)	0.3529 ± 0.007***	16.99 ± 0.458***

*2-AMP = 2-acetamidophenol

Table.6 3-acetamidophenol Nephrotoxicity

Treatment (mg/kg)	CREATININ (mg/dl)	BLOOD UREA NITROGEN (BUN)(mg/dl)
Control/ Non treated rat	0.2743 ± 0.006	24.60 ± 0.297
3-AMP* (500)	26.77 ± 0.770**	26.77 ± 0.770***
3-AMP (1000)	20.01 ± 0.879**	20.01 ± 0.879***

*3-AMP = 3-acetamidophenol

Table.7 4-acetamidophenol Nephrotoxicity

Treatment (mg/kg)	CREATININ (mg/dl)	BLOOD UREA NITROGEN (BUN)(mg/dl)
Control/ Non treated rat	0.2743 ± 0.006	24.60 ± 0.297
4-AMP (500)	0.3357 ± 0.004**	17.81 ± 1.373
4-AMP (1000)	0.27 ± 0.007	21.19 ± 0.176

*4-AMP = 4-acetamidophenol

Table.8 2-acetamidophenol Haematotoxicity

Treatment (mg/kg)	HEMOGLOBIN (g/dl)	RBCs (million/ul)	HEMATOCRITS (%)	NEUTROPHILS (%)
Control/ Non treated rat	14.20 ± 0.161	7.238 ± 0.046	39.13 ± 0.295	22.63 ± 0.532
2-AMP*(100)	14.85 ± 0.070	7.538 ± 0.056	38.73 ± 0.247	24.13 ± 0.398*
2-AMP - (500)	14.58 ± 0.061	7.294 ± 0.074	39.13 ± 0.295	24.75 ± 1.820

*2-AMP = 2-acetamidophenol

`**Table 9:** 3-acetamidophenol Haematotoxicity

Treatment (mg/kg)	HEMOGLOBIN (g/dl)	RBCs (million/ul)	HEMATOCRITS (%)	NEUTROPHILS (%)
Control/ Non treated rat	14.20 ± 0.161	7.238 ± 0.046	39.13 ± 0.295	22.63 ± 0.532
3-AMP (500)	14.79 ± 0.327	8.028 ± 0.234	39.63 ± 0.460	52.63 ± 1.752
3-AMP (1000)	13.58 ± 0.2177	40.13 ± 0.666	35.38 ± 0.595	65.50 ± 0.925

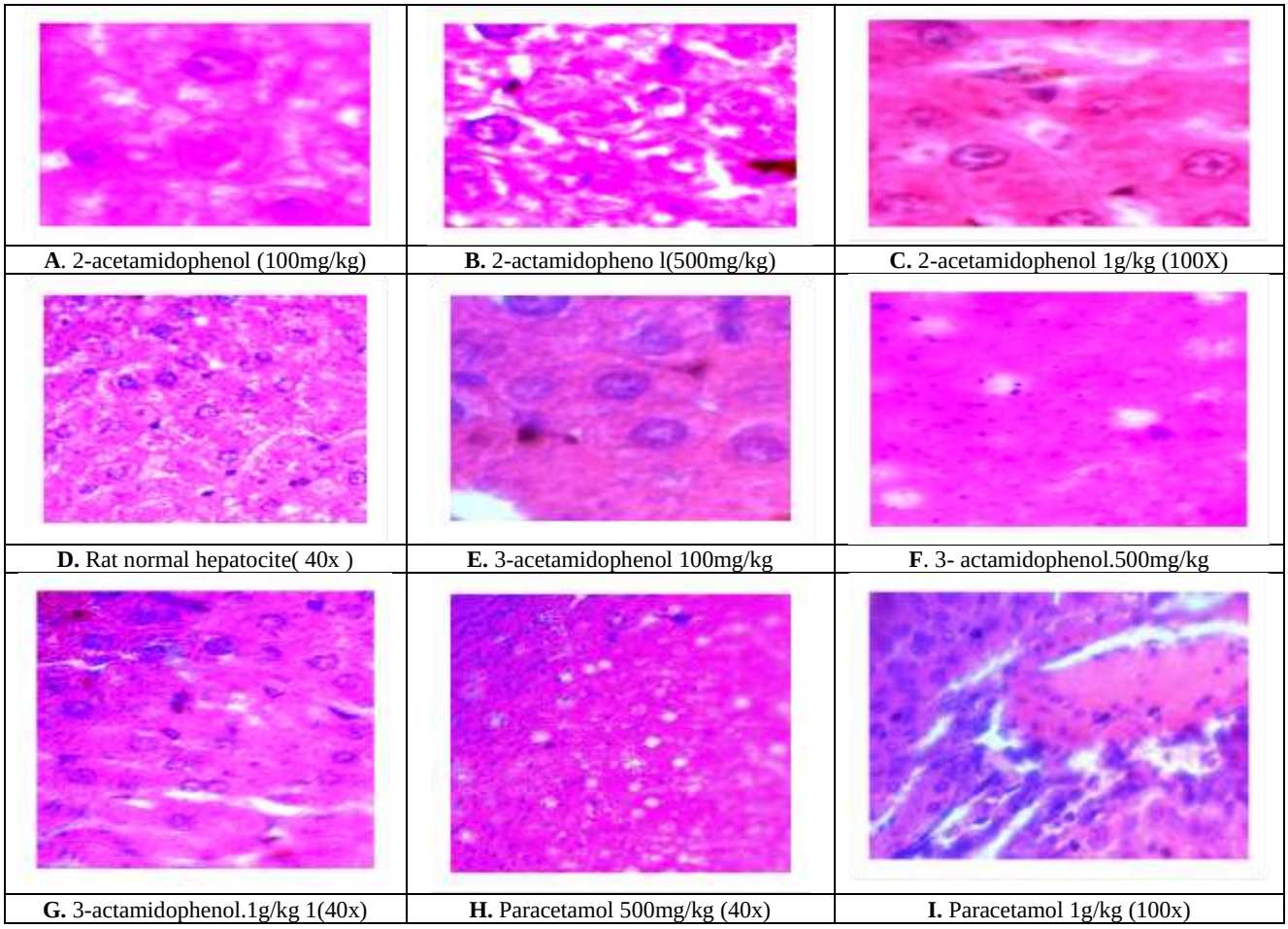
*3-AMP = -3acetamidophenol

Table 10: 4-acetamidophenol Haematotoxicity

Treatment (mg/kg)	HEMOGLOBIN (g/dl)	RBCs (million/ul)	HEMATOCRIT (%)	NEUTROPHIL (%)
Control/ Non treated rat	14.20 ± 0.161	7.238 ± 0.046	39.13 ± 0.295	22.63 ± 0.532
4-AMP - (500)	14.48 ± 0.160	6.790 ± 0.019	39.40 ± 0.473	26.55 ± 0.581
4-AMP (1000)	12.39 ± 0.159	6.348 ± 0.009	34.58 ± 0.235	42.88± 0.497

*4-AMP = 4-acetamidophenol

Histomorphological Studies of 2, 3 and 4-acetamidophenol



A. 2-acetamidophenol (100mg/kg) | **B.** 2-actamidopheno l(500mg/kg) | **C.** 2-acetamidophenol 1g/kg (100X)

D. Rat normal hepatocite(40x) | **E.** 3-acetamidophenol 100mg/kg | **F**. 3- actamidophenol.500mg/kg

G. 3-actamidophenol.1g/kg 1(40x) | **H.** Paracetamol 500mg/kg (40x) | **I.** Paracetamol 1g/kg (100x)

Figure 1. Histopathological Studies **A.** Shows very mild vesicular steatosis, **B.** Shows micro vesicular steatosis and mild inflammation, **C.** Shows ventricular vein necrosis or mild steatosis in lobule area, **D.** Shows normal liver on control or non-treated group, **E.** Shows general normal or very mild vesicular steatosis, **F.** Shows very low inflammation or very mild micro vesicular steatosis, **G.** Shows mild steatosis or micro vascular necrosis in lobule area, **H.** Shows inflammation mild to moderate vascular necrosis in lobule area, and **I.** Shows inflammation in centrilobular vein and moderate vascular necrosis in lobule area.

DISCUSSION

2-acetamidophenol and 3-acetamidophenol are positional isomers of paracetamol. The toxicity of these isomers is rarely documented in the available literature. Paracetamol is an alternative to non-steroidal anti-inflammatory drugs (NSAIDs) because most of pharmacological activities are almost identical to each other. Unfortunately, both the NSAIDs and Paracetamol have their specific set of adverse effects if used in higher doses or prolonged administration, which might be lethal and life threatening.

The present study is the first to demonstrate the $LD_{50,}$ hepatotoxicity, nephrotoxicity, and hemotoxic studies profile of 2-acetamidophenol and 3-acetamidophenol in female Sprague dawley rats using intra peritoneal route of administration.

Toxicological studies

In acute toxicological studies (LD_{50}) we analyzed the Paracetamol positional isomers for LD_{50} toxicity by using previously described method by Lorke D[4]. The LD_{50} of 2-acetamidophenol, 3-acetamidophenol and 4-acetamidophenol were determined. In this single dose acute toxicity test. It was found that 3-acetamidophenol has maximum LD_{50} i.e., 1500 mg/kg while the minimum LD_{50} was recorded for 2-acetamidophenol i.e., 1150 mg/kg. Paracetamol was kept as control and its LD_{50} was found to be 1250 mg/kg. At the doses below 1000mg/Kg, no unwanted clinical sign was observed in the surviving rats. There were no changes in stool, urine, and eye colors of all the animals. No mortality was observed in this dose range within 24hours. However, at 1000mg/kg drowsiness was observed in all isomers treated experimental rats. Previous reports regarding the LD_{50} of the Paracetamol was reported as 1205mg/kg (i.p*)* while 1944mg/Kg per oral in rats according to safety data MSDS for 4-acetamidophenol or Paracetamol. 3-acetamidophenol LD_{50} was observed in mice [6]. In our knowledge, the current data is the first where LD_{50} of 2-acetamidophenol is being documented. The current observations showed that all isomers of Paracetamol have almost nearer LD_{50} which might be due to identical molecular mass, structure and slight variation is due to changes in position of functional group moiety in each isomer.

Liver, kidney, and blood acute toxicological studies

Liver, kidney, and blood acute toxicology studies were conducted to evaluate the safety of 2-acetamidophenol and 3-acetamidophenol. The Intra peritoneal, single doses of 100mg/kg, 500mg/kg, and 1000mg/kg of 2-acetamidophenol, 3-acetamidophenol and 4-acetamidophenol were administered to individual groups of rats. At 500mg/kg dose of 3-acetamidophenol showed no hepatotoxicity, nephrotoxicity and haematotoxicity evaluated by liver transaminases, alkaline phosphatase, creatinine, or blood urea nitrogen and haemoglobin.

In the available literature, the lack of hepatotoxicity of 3-acetamidophenol is often reported in rats [7]. As high or long-term use of paracetamol has been associated with liver dysfunction, possibly due to paracetamol-induced oxidative stress [8-11], 3-acetamidophenol has been reported as a mild or non-hepatic isomer of paracetamol [12]. No hepatotoxic or 3-aminophenol properties have been previously reported [13].

Histopathological studies of rat hepatocytes were performed for Paracetamol isomers. At the doses of 1000mg/kg and 500mg/kg, 2-acetamidophenol produced significant liver toxicity. Hepatocytes infiltration, centrilobular vein necrosis, eosinophilic changes, accumulation of neutrophils at the site of injury was prominent. No fatty liver is observed in 2-acetanidophenol treated rats at any dose. 2-acetamidophenol at the dose of 100mg/ kg did not produce any sort of toxicity. 3-acetamidophenol at the dose of 1000mg/kg produced toxicity but was not as severe as seen in 2-acetamidophenol. The severity of 2-acetanidophenol at such high dose (1000 mg/kg) might be because such toxic dose was 100 to 1000 time greater than therapeutic dose (1mg/kg-10mg/kg). Usually, therapeutic dose verses toxic dose ratio is kept 1:10 while in case of 2-acetamidophenol at1000mg/kg dose, this ratio was 1:100 to 1:1000. 2-acetamidophenol at the dose of 100mg/kg was found to be non-hepatotoxic. The above findings clearly indicate that 2-acetamidophenol and 3-acetamidophenol are less hepatotoxic or non-hepatotoxic than paracetamol. No hepatotoxicity of 2-acetamidophenol has been reported in rats [14]. The non-hepatotoxicity may be due to the phenolic antioxidant nature of this compound. Phenols are well-known for their unique antioxidant strength [14-18].

In contrast, at the dose of 1000mg/kg 2 and 3-acetamidophenol showed nephrotoxicity, evaluated by significant elevation in creatinine level. Creatinine level was increased in 500mg/kg dose of 3-acetamidophenol which showed its adverse effects on renal tubular system.

In haematotoxicity tests, red blood cells and neutrophils count were significantly elevated in 3-acetamidophenol treated group at the dose of 1000mg/kg dose but not in 500mg/kg, which may be due to acute inflammatory response due to toxic dose. Hematological parameter reveals that 2-acetamidophenol was hemotoxic only at dose of 1000mg/kg. Hematocrits and neutrophils were significantly raised while haemoglobin level was not affected. Paracetamol does not significantly affect the blood or do not cause blood toxicity.

Paracetamol in higher doses decrees the haemoglobin level [19]. None of Paracetamol positional isomer i.e., 2-acetamidophenol and 3-acetamidophenol showed haematotoxicity. Acute toxicity studies of paracetamol isomers showed relatively low or no toxicity for 3- and 2-acetamidophenol compared to paracetamol. Both isomers had wide therapeutic indices and therapeutic windows that made them relatively safe.

CONCLUSION

In this study we analyzed the toxicity profile of 2-acetamidophenol and 3-acetamidophenol. We compared these isomers with Paracetamol. Among these drugs and test compounds, 2-acetamidophenol was found to be most safe in a wide doses range. Furthermore, it is found to be non-hepatotoxic, non-nephrotoxic, and non-hemotoxic. Due to its phenolic nature. Our results indicate that 2-acetamidophenol might be best drug candidate and hepatoprotective alternative to Paracetamol.

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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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