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Oluwadunni Joan Okwulu
Department of Veterinary Pharmacology and Toxicology, University of Ibadan, Ibadan, Nigeria

Matthew Oluwole Abatan
Department of Veterinary Pharmacology and Toxicology, University of Ibadan, Ibadan, Nigeria

Adeolu Alex Adedapo
Department of Veterinary Pharmacology and Toxicology, University of Ibadan, Ibadan, Nigeria

Corresponding Author:
Adeolu Alex Adedapo
Department of Veterinary Pharmacology and Toxicology, University of Ibadan, Ibadan, Nigeria

Hepatoprotective and hepatoregenerative effects of ascorbic acid, selenium and allopurinol in acetaminophen-induced liver damage in rats

Oluwadunni Joan Okwulu, Matthew Oluwole Abatan and Adeolu Alex Adedapo

Abstract
The hepatoprotective and hepatoregenerative properties of Ascorbic acid, Selenium and Allopurinol were evaluated in Wistar rats because they are antioxidants and are to possess antioxidant properties due to their ability to scavenge free radicals and oxidized them thereby reducing stress caused by toxicants in the system and thus help in ameliorating the damage caused by toxicants. The effects of these antioxidants were also evaluated in the kidney to determine their effectiveness in protecting the kidney and ameliorate the damage caused in the kidney.

Albino rats (Wistar) were used in this study and were grouped into 9 of five animals per group for both hepatoprotective and hepatoregenerative studies. Acetaminophen at 800mg/kg dose was used to induce hepatotoxicity and nephrotoxicity while different doses of ascorbic acid, selenium and allopurinol were used to mitigate these effects in a 9-day study. Biochemical assays and histopathology were used to evaluate the *in vivo* study.

Results showed that there was significance difference ($p<0.01$) in AST, ALT, ALP, BUN and T.BIL with significance difference ($p<0.05$) when Ascorbic acid was compared with Acetaminophen. Selenium showed significance difference ($p<0.01$) in AST and ALP, (p=0.01) in ALT and BUN. Allopurinol showed significant difference ($p<0.01$) in AST, ALP and BUN, p=0.01 in ALT in the hepatoprotective group. In the hepatoregenerative group, Ascorbic acid showed significance difference ($p\leq0.01$) in ALP and BUN while the Selenium group ($p\leq0.01$) in AST, ALT, ALP and GGT; in Allopurinol group ($p<0.01$) in AST, ALP and GGT and $p<0.05$ in ALT, BUN and Total Bilirubin. There was no significance difference in all the parameters when Ascorbic acid was compared with Silymarin group. The histopathological analyses also indicate this.

It could thus be concluded that that Ascorbic acid, selenium and allopurinol has hepatoprotective effect hence their use in liver damage may be justified.

Keywords: antioxidants, hepatoregenerative, hepatoprotective, histopathology

Introduction
Liver is a highly specialized tissue in the body that is very vital because it is responsible for a large number of biological activities that are needed for the normal functioning of the body which include production of clotting factors, metabolism of fatty acids, proteins and carbohydrate; it maintain glucose level in the body by the storage of glucose as glycogen (Lavoie *et al*., 2005), it is also involve in absorption and metabolism of bilirubin, it filters the blood for the removal of xenobiotic and it is also involve in vitamin storage (Cotran *et al*., 2005) [3]. It is the only organ in the body that can regenerate if about 25% of the tissue remains (Uhlen *et al*., 2015) [25]. Damage to the liver can be as a result of parasitic invasion as in Facioliasis (Maton *et al*., 1993) [16], viral infections, intake of alcohol, smoking, inhalation of harmful substances and intake of drugs either at normal dosage or at overdose (Zakim *et al*., 2002) [28].

Drug-induced liver injury (DILI) is not an uncommon adverse event encountered in clinical practice, since a vast number of compounds, including herbs and alternative medications, are metabolized in the liver microsomes (Laura *et al*., 2003) [12]. *N*-acetylcysteine (NAC) therapy is considered as the mainstay therapy, but liver transplantation might represent a life-saving procedure for selected patients (Laura *et al*., 2003) [12].

Overdoses of the analgesic and antipyretic acetaminophen represent one of the most common pharmaceutical product poisonings in the United States today (Litovitz *et al*., 2002). Although considered safe at therapeutic doses, in overdose, acetaminophen produces a centrilobular hepatic necrosis that can be fatal (Jaeschke *et al*., 2015) [8].

It was shown that acetaminophen is metabolically activated by cytochrome P450 to form a reactive metabolite that covalently binds to protein (Mc Gill *et al*., 2012) [17]. The reactive metabolite was found to be N-acetyl-p-benzoquinone imine (NAPQI1), which is formed by a direct two-electron oxidation (Yoon *et al*., 2016) [27]. More recently, the hepatic injury and subsequent hepatic failure due to both intentional and non-intentional overdose of acetaminophen (APAP) has affected patients for decades, and involves the cornerstone metabolic pathways which take place in the microsomes within hepatocytes (Papay *et al.,* 2009) [19]. APAP hepatotoxicity remains a global issue to chromes 2E1, 1A2, 3A4, and 2A6 (Patten *et al.*, 1993; Thummel *et al.*, 1993) [20, 24] which have been reported to oxidize acetaminophen to the reactive metabolite. Also, Dr. Gillette's laboratory showed that NAPQI is detoxified by glutathione (GSH) to form an acetaminophen GSH conjugate. After a toxic dose of acetaminophen, total hepatic GSH is depleted by as much as 90%, and as a result, the metabolite covalently binds to cysteine groups on protein, forming acetaminophen-protein adducts (Blieden *et al.*,2014) [1]. One possible mechanism of cell death is that covalent binding to critical cellular proteins results in subsequent loss of activity or function and eventual cell death and lysis (Navarro *et al.,* 2006) [18].

Ascorbic acid (AA), commonly known as vitamin C plays an important role in the human body, although its function at the cellular level is not yet clear. Vitamin C can both act as a strong, efficient, and cheap antioxidant agent and, at the same time, behave as a radical promoter. Vitamin C is an electron donor, and this property accounts for all its known functions. As an electron donor, vitamin C is a potent water-soluble antioxidant in humans (Jose *et al.*, 2013) [10]. It is necessary for the synthesis of collagen, a protein that has many connective functions in the body. It also requires (AA) for the synthesis of hormones, neurotransmitters and in the metabolism of certain amino acids and vitamins. Participate in the liver for detoxification of toxic substances and blood level for immunity (Fadime *et al.,* 2017) [4]. As an antioxidant, Vitamin C has an effect antihistaminic as it reacts with histamine and peroxide for reducing inflammatory symptoms. Its antioxidant capacity is associated with reduced incidence of cancer; Vitamin C or ascorbic acid (AsA) is a naturally occurring organic compound with antioxidant properties, found in both animals and plants (Ji *et al*., 2014) [19]. It functions as a redox buffer which can reduce, and thereby neutralize reactive oxygen species. It is a cofactor for enzymes involved in regulating photosynthesis, hormone biosynthesis, and regenerating other antioxidants; which also regulates cell division and growth, it is involved in signal transduction, and has roles in several physiological processes, such as immune stimulation, neurotransmitters and iron absorption, has also roles in detoxifying the body of heavy metals (Sebastian *et al.*, 2003) [22]. Severe deficiency of vitamin C causes scurvy, whereas limited vitamin C intake causes symptoms, such as increased susceptibility to infections, loosening of teeth, dryness of the mouth and eyes, loss of hair, dry itchy skin, fatigue, and insomnia. In contrast, vitamin C can also act as a prooxidant, especially in the presence of transition metals, such as iron and copper, starting different hazardous radical reactions (Marija *et al.,* 2018) [15].

Selenium is a powerful antioxidant that fights oxidative stress and helps defend your body from chronic conditions, such as heart disease and cancer (Ju *et al.*, 2017) [11].

They work by neutralizing excess free radicals and protecting cells from damage caused by oxidative stress. Selenium may also lower markers of inflammation in your body one of the main risk factors for heart disease (Tabrizi *et al.,* 2017) [23]. It increased levels of glutathione peroxidase, a powerful antioxidant; Selenium is a crucial mineral with antioxidant and immune functions, and its deficiency may increase the risk of coronary heart disease (CHD); Selenium is an essential mineral that plays a key role in plenty of major metabolic processes. A growing body of literature has shown that selenium deficiency leads to an increase in plasma total cholesterol (TC) and triglyceride (TG) levels (Hasani *et al*., 2018) [6].

In addition to the inhibition of xanthine oxidase, Allopurinol is known to act, dependent on the dose, as a free radical scavenger, an antioxidant, and a "scavenger" of hypochlorous acid (Butler *et al.,* 2000, Farquhason *et al.,* 2002, Jacob *et al.*, 2006,) [2, 5, 7]. The prototypical xanthine oxidase inhibitor allopurinol has been applied in different models of tissue injury, based on its reported ability to inhibit the production of reactive oxygen species (ROS), and the release of inflammatory mediators such as TNF-α (Rachmat *et al.,* 2013, Prieto-Moure *et al.,* 2014) [21].

This study evaluates the hepatoprotective and hepatoregenerative effects of Ascorbic acid, Selenium and Allopurinol against acetaminophen induced liver damage.

Material and Methods

Experimental animals and care

A total of 40 Wistar rats of both sexes weighing between 180-220g were purchase from the Faculty of Veterinary Medicine, University of Ibadan and the study was carried out in Experimental animals Unit of the Department of Veterinary Pharmacology and Toxicology of the same university. The rats were handling in accordance with international principles guiding the use and Handling of experimental animals (United States National Institutes for Health, 1985) after an ethical approval was obtained from the animal and use research ethics committee of University of Ibadan. The approval number is UI-ACUREC/19/0121. The rats were maintained on standard rat feed (vital feeds from Grand cereals Limited, Jos (a subsidiary of UAC Nigeria) and potable water were made available ad libitum during the period of acclimatization and throughout the experimental period. The rats were also maintained at ambient temperature between 28-30 ^{0}C, humidity of 55±5% and standard (natural) photoperiod of approximately 12 hours of light (06:30 hours-18:30 hours) alternating with approximately 12 hours of darkness (18:30 hours- 06:30 hours).

Acetaminophen-induced hepatotoxicity in animals

The animals were grouped into 8 with 5 Albino rats in each group for both hepatoprotective and hepatoregenerative study. Group 1 received distilled water for ten days; Group 2 received Acetaminophen 800mg/kg for the last two days while group 3-5 are the hepatoprotective group. Group 3 animals received Sylimarin 100mg/kg for 8 days before Acetaminophen 800mg/kg on day 9, Group 4 were administered Ascorbic acid 200mg/kg for 8 days before Acetaminophen 800mg/kg on day 9, and Group 5 were given Selenium 1mg/kg for 8 days before Acetaminophen 800mg/kg on day 9, Group 6 were given Allopurinol 50mg/kg for 8 days before Acetaminophen 800mg/kg on day 9.

Groups 7-8 are the hepatoregenerative groups

Group 7 animals were given 800mg/kg of Acetaminophen on day 1 and 2 before given Ascorbic acid 100mg/kg till day 9, Group 8 were given Acetaminophen 800mg/kg on day 1 and 2

before given Selenium 1mg/kg till day 9, while Group 9 were administered Acetaminophen 800mg/kg on day 1 and 2 before given Allopurinol 50mg/kg till day 9

Biochemical assays

On day 10, animals were anaesthesized with diethyl ether and blood samples were collected from retro-orbital vein into plain bottles for the determination of biochemical parameters such as Alkaline phosphatase (ALP), Aspartate transaminase (AST), Alanine transaminase (ALT), Blood urea (BUN), Creatinine (CRT), Total bilirubin (T.Bil), Total protein (T.PRO), Albumin (Alb), Globulin (Glo) and Gamma-glutamy tranferase (GGT). Well-labeled plain bottles were used to collect 10 mL of blood sample which was allowed to clot for 4 hours before centrifuging using Uniscope Laboratory Centrifuge (Model SM 112, Surgifriend Medicals, England) at 2000 revolution per minute for 15minutes to separate the sera from clotted blood cells. Each serum was carefully separated in the plain bottles that were well label accordingly at room temperature of 23-26 ^{o}C. The activities of AST and ALT were estimated as described by Reitman and Frankel 1957; While Rec (1972) method was used to estimate ALP. The activities of T. Bil were determined by colorimetric method using a kit supplied by Randox test kit (UK). The blood urea was determine by using urease-Berthelot (enzymatic) colorimetric method and serum total protein was evaluated based on the Gornall *et al* (1949) method. Louis (1944) method was used in the determination of Alb and Glo. Gamma-glutamy transferase was determined by following the standard procedures; increase in the absorbance was read using semi auto analyzer by kinetic method.

Gross and histopathology

The organs were identified (kidney and liver), sectioned and rinsed in normal saline. The tissue was fixed in 10% formal-saline, dehydrated with 100% ethanol solution and embedded in paraffin and sectioned to 5µm, stained using H&E method, cleared in xylene and mounted in a mountant (Akanbi & Taiwo 2014).

Data Analysis

Data analysis was using GraphPad Prism version 6. Results expressed as mean ± SD were analysed using one-way analysis of variance (ANOVA) and Students t-test. Values of probability less than 0.05 were considered statistically significant (O' Neil *et al.*, 2013).

Result

Table 1: Showing the hepatoprotective effects of Ascorbic acid, Selenium and Allopurinol

Parameters	Group					
	1	2	3	4	5	6
TP	7.47±0.67	6.67±0.15	6.95±0.35	6.6±0.36	7.13±0.28	7.2±0.52
ALB	3.27±0.38	2.9±0.1	2.85±0.21	3.0±0.2	2.86±0.38	3.03±0.30
GLO	4.67±0.78	3.7±0.05	4.1±0.14	3.6±0.26	4.26±0.20	4.16±0.30
A/G RATIO	0.7±0.22	0.7±1.36	0.65±0.07	0.8±0.1	0.63±0.05	0.66±0.05
AST	47±1.41	37.3±1.53	41±2.82	38.6±2.88b	43±1.73b	42.3±0.57b
ALT	35.25±0.95	26±1.00	31±2.82	30±0b	33±1a	32.6±1.15a
ALP	127.75±0.5	80±6.08	101.5±6.36	112±7.02b	124±1.15b	123±2.5b
BUN	19.05±0.19	15.63±0.15	18±0.42	18.0±0.72	18.0±0.72a	18.1±0.32b
CREAT	0.75±0.05	0.533±0.05	0.6±0	0.76±0.05	0.76±0.05	0.76±0.05
TB	0.475±0.05	0.23±0.05	0.35±0.07	0.33±0.05b	0.4±6.79	0.36±0.11
GGT	0.375±0.019	0.23±0.02	0.26±0.02	0.29±0.05a	0.31±0.05	0.34±0.06

I-Acetaminophen group; II- Normal group; III- Silymarin 100mg/kg; IV- Ascorbic acid 200mg/kg group; V- Selenium 1mg/kg group; VI-Allopurinol 50mg/kg group

There was decreased in the level of the biomarkers in the hepatoprotective group of Ascorbic acid. AST, ALT, ALP, BUN, and T.BIL lower than the Acetaminophen group (p<0.01) and GGT level was decreased (p<0.05).

There was also decreased in the level of AST and ALP p<0.01 in the Selenium group. ALT and BUN level was reduced (p=0.01)

The level of AST, ALP, BUN in the Allopurinol group were decreased (p<0.01) and ALT level (p=0.01)

There was no significance difference in all the parameters expect for ALP (≤0.0001) when compared with the Silymarin group (Table 1).

Table 2. Showing the Hepatoregenerative effects of Ascorbic acid, Selenium and Allopurinol

Parameters	Groups					
	1	2	3	4	5	6
TP	7.47±0.67	6.67±0.15	6.15±0.49	7.25±0.63	6.96±0.28	6.85±0.07
ALB	3.27±0.38	2.9±0.1	2.55±0.07	3.3±0.42	3.2±0.15	3.03±0.30
GLO	4.67±0.78	3.7±0.05	3.6±0.42	3.95±0.21	3.73±0.15	4.16±0.3
A/G RATIO	0.7±0.22	0.7±1.36	0.75±0.07	0.75±0.07	0.83±0.05	0.85±0.07
AST	47±1.41	37.3±1.53	35.5±0.07	43±5.65	40.6±2.08b	39.5±0.70c
ALT	35.25±0.95	26±1.00	26±1.41	33±4.24	30±2b	30±0b
ALP	127.75±0.5	80±6.08	77.5±2.12	118.5±3.5b	119±3.05b	111±11.31c
BUN	19.05±0.19	15.63±0.15	16.1±0.70	18.3±0.42a	17.63±0.77b	18.2±0.84a
CREAT	0.75±0.05	0.533±0.05	0.5±0	0.75±0.07	0.73±0.05	0.75±0.07
TBIL	0.475±0.05	0.23±0.05	0.15±0.07	0.35±0.07	0.4±6.79	0.4±0a
GGT	0.375±0.019	0.23±0.02	0.22±0.02	0.32±0.08	0.30±0.06a	0.27±0.007c

I-Acetaminophen group; II- Normal group; III- Silymarin 100mk/kg; IV- Ascorbic acid 200mg/kg group; V- Selenium 1mg/kg group; VI-Allopurinol 50mg/kg group.

In the hepatoregenerative group, Ascorbic acid decreased the level of ALP and BUN ($p \leq 0.01$), there was decreased in the level of AST, ALT, ALP, BUN and GGT ($p \leq 0.01$) in the Selenium group. Allopurinol group showed decreased in the level of AST, ALP, GGT ($p < 0.01$) and ALT, BUN, T.BIL ($p < 0.05$) when the groups are compared with the Acetaminophen group.

There was no significant difference in all the parameters except for AST, ALP and ALT ($p \geq 0.0001$) in Selenium and Allopurinol group when compared with Sylimarin group (Table 2).

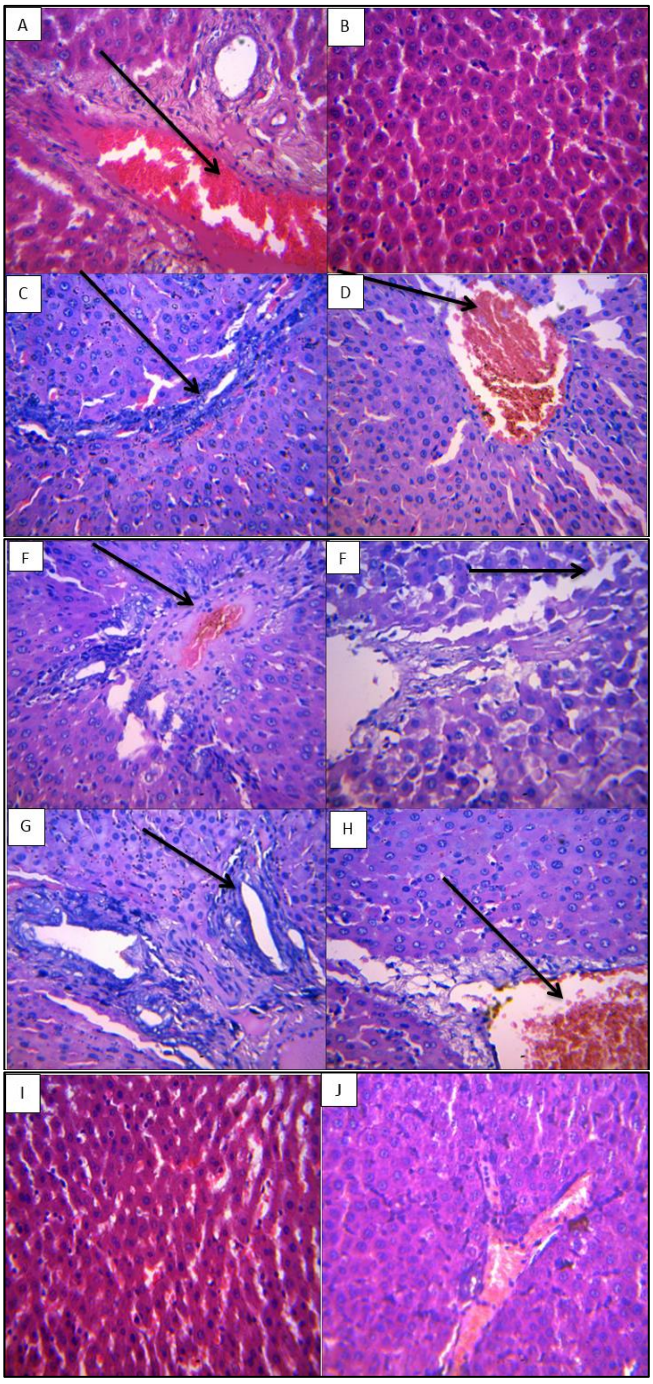

Fig 1: Liver of hepatoprotective and hepatoregenerative groups

a) In the Acetaminophen treated group, there is moderate portal congestion with mild periportal cellular infiltration and a moderate to severe portal fibrosis in the liver. Stained with H&E with the magnification of x400
b) There is no visible lesion seen in the liver of the negative control group. Stained with H&E with the magnification of x400
c) In the Ascorbic acid hepatoprotective group, there is a mild periportal cellular infiltration in the liver. Stained with H&E with the magnification of x400
d) In the Selenium hepatoprotective group, there is a moderate periportal cellular infiltration. Stained with H&E with the magnification of x400
e) There is a moderate to severe portal congestion in the Allopurinol hepatoprotective group. Stained with H&E with the magnification of x400
f) Some hepatocytes at the periportal area are distorted and swollen in the Ascorbic acid hepatoregenerated group. Stained with H&E with the magnification of x400
g) The bile ducts are hyperplastic and there is mild fibrous hyperplasia in the Selenium hepatoregenerated group. Stained with H&E with the magnification of x400
h) In the Allopurinol hepatoregenerated group, there is a moderate periportal cellular infiltration. Stained with H&E with the magnification of x400
i) Liver of Sylimarin 100mg/kg group showing no visible lesion in hepatoprotective group
j) Liver of Sylimarin 100mg/kg group showing very mild portal congestion in the hepatoregenerative group. Stained with H&E with the magnification of x400

Discussion

This study investigated the ameliorating effects of three antioxidants (Ascorbic acid, Selenium and Allopurinol) on liver damage caused by Acetaminophen and the protective effects of the antioxidants on the liver as well as the kidney. Antioxidants are known to neutralize the effects of free radicals on organs in the system and to protect the body system from damage caused by stress generated by these radicals. Acetaminophen in this study induced stress on the liver and kidney thereby causing damage as indicated in table 1 and figure 1 above. Acetaminophen causes increased expression of all the liver biomarkers such as the Aspartate transaminase, Alanine transaminase, alkaline phosphate, creatinine, blood urea and total protein; the histopathology of the liver shows there is moderate portal congestion with mild periportal cellular infiltration and a moderate to severe portal fibrosis. The kidney also shows severe diffuse tubular degeneration and necrosis.

The hepatoprotective effect was demonstrated in Table 1 where the Ascorbic acid was able to reduce all the parameters that were increased by the hepatotoxicant with the liver showing only mild to periportal infiltration and the kidney showing no visible lesion. This may be because it is an electron donor and can always donate electron to free radical in the system as a result of the oxidative stress generated by the toxicant thereby inactivating it and neutralizing the effect (Sabetian *et al.*, 2003). Its ability to participate in the liver for detoxification of toxic substances and boost blood level for immunity (Fadime *et al.*, 2017) [4] may be responsible for the hepatoprotective effects shown in this work.

In the hepatoregenerative group, Table 2 shows that Ascorbic acid was able to reduce the level of ALP and BUN significantly and also the other parameters, the histopathology in figure 6 show there were hepatocytes at the periportal area are distorted and swollen and kidney show no visible lesion; Ascorbic acid was able to reduce the effects of

Acetaminophen induced stress on both organs, this may be because of its ability to participate in liver detoxification of toxic substances (Yurika *et al*., 2018).

Selenium

In the hepatoprotective group, Selenium was able to reduce the biochemical parameters (AST, ALP, ALT and BUN), thereby reducing the effect of the toxicant to some level. The histopathological results show few tubular degenerated and tubular casts. Selenium increases the level of glutathione peroxidase, a powerful antioxidant that is very effective in the reduction of oxidative stress (Tabrizi *et al.*, 2017) [23].

Selenium was able to also reduce AST, ALT, ALP, BUN and GGT in already damage liver, though there was moderate congestion of the renal cortex in the kidney and hyperplastic bile duct with mild fibrous hyperplasia in the liver. Selenium is an antioxidant and has reduced the effect of Acetaminophen in this work but not to a very minimal level compared to Ascorbic acid at the dose used in this work.

Allopurinol was able to reduce AST, ALP, BUN and ALT in the hepatoprotective group, histologically there was mild cortical congestion in the kidney and moderate portal congestion in the liver; this show it has ability to protect the toxic effect of Acetaminophen but not as Ascorbic acid. It was able to reduce all the biochemical parameters in the hepatoregenerative group but the histology show very mild renal cortical congestion in the kidney and moderate periportal cellular infiltration in the liver ability. Allopurinol inhibits the production of reactive oxygen species (ROS), and the release of inflammatory mediators such as TNF-α (Rachmat *et al.*, 2013, Prieto-Moure *et al.*, 2014) [21]. In this work Allopurinol was able to protect the liver from severe damage by Acetaminophen and treat the liver of damage cause by the toxicant but in a very minimal level thus may not be recommended for these purposes because the effect is not efficient enough compared to Ascorbic acid.

In this work, Ascorbic acid was able to protect the liver and kidney damage and also treat the damage caused by Acetaminophen at high dose on both organs compared to Selenium and Allopurinol.

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