

**COMBINATION OF AQUEOUS EXTRACTS OF *CURCUMA LONGA* (TURMERIC) AND SOME CALCIUM CHANNEL BLOCKERS SYNERGISTICALLY IMPROVES CCl<sub>4</sub>-INDUCED HEPATOTOXICITY IN ALBINO RATS**Uchendu Ikenna Kingsley<sup>1</sup>, Nnedu Ebuka Bitrus<sup>2</sup>, Orji Oliver Chukwuma<sup>1</sup><sup>1</sup> Division of Clinical Chemistry, Department of Medical Laboratory Sciences, Faculty of Health Sciences and Technology, University of Nigeria, Enugu Campus, Enugu State, Nigeria<sup>2</sup> Division of Immunology, Department of Medical Laboratory Sciences, Faculty of Health Sciences and Technology, University of Nigeria, Enugu Campus, Enugu State, Nigeria.Email address: [ikenna.uchendu@unn.edu.ng](mailto:ikenna.uchendu@unn.edu.ng)**Abstract**

Several liver diseases are caused by a variety of factors and affecting everyone, from infants to older adults. Drugs or alcohol has been implicated as the main cause of some liver diseases. In this study, special attention was drawn to CCl<sub>4</sub>-induced liver damage and how the damaged liver could be treated or prevented by turmeric aqueous extracts alone or in combination with some calcium channel blockers. We investigated the antihepatotoxic effects of aqueous extract of *curcuma longa* (AECL) alone or in combination with nifedipine or amlodipine against CCl<sub>4</sub>-induced acute liver injury in male albino wister rats. Thirty (30) male albino wister rats were randomly grouped into six groups: A, B, C, D, E and F of five animals per group. Rats in groups A-D received CCl<sub>4</sub> (0.4ml/kg b.wt, i.p) for 3 days. Group B received AECL (200mg/kg, oral), Group C received AECL and nifedipine (1mg/100g of rat, i.p), Group D received AECL and amlodipine (1mg/100g of rat, i.p), and group E received AECL alone for 3 days. No treatment was administered to group F (Normal control). CCl<sub>4</sub> administration to the rats resulted in acute liver injury with significantly increased ALT, AST, ALP and total bilirubin levels; 46.00 ± 6.35 IU/L, 46.37 ± 5.88 IU/L, 178.30 ± 19.22 IU/L and 1.45 ± 0.08 mg/dl respectively. The 3 days daily administration of AECL alone or plus nifedipine or amlodipine resulted in the mitigation of the CCl<sub>4</sub>-induced liver damage with significantly decreased ALT, AST, ALP and total bilirubin levels; (p<0.05. p<0.01 or p<0.001). Histopathological results showed a concomitant association with the biochemical findings. This study shows that the combination of the extract and some calcium channel blockers is synergistically hepatoprotective, and can be used to prevent hepatotoxicity.

**Keywords:** *ethnopharmacology, curcuma longa, Calcium channel blockers, hepatoprotection*

## Introduction

Turmeric (*curcuma longa*) is an annual plant that belongs to the ginger family of herbs, zingiberaceae. It has been used in folklore medicine to treat numerous diseases (1). Nutrition and the liver interrelate in many ways and the possible effect of slowing down liver damage has been considered. Several studies have emphasized on the importance of dietary composition in the treatment of liver diseases (2-6).

Several biochemical parameters are used as measures or indicators of liver function and any damage to the liver can be diagnosed by analyzing these liver parameters. These include: Bilirubin, Aspartate aminotransaminase (AST), Alanine aminotransaminase (ALT) and Alkaline phosphatase (ALP) etc. Increased plasma level of these transaminase enzymes is suggestive of liver injury. Metabolism in the liver protects tissues in higher organisms from potentially harmful blood-borne environmental chemicals. Ironically, the metabolic products of detoxification reactions that protect other tissues from effects of the primary toxicant can be destructive to the liver when in excess or chronically present. The function of detoxification is mainly through the cytochrome P450 group of isoenzyme, therefore the liver is mostly affected by toxic level of harmful xenobiotics, including carbon tetrachloride (CCl<sub>4</sub>).

Carbon tetrachloride is one of the many chemical substances implicated in liver damage in recent times. It is known that lytic liver injury monitored by blood plasma ALT level occurs twelve hour after a single injection of CCl<sub>4</sub>. Studies have also shown that exposure to CCl<sub>4</sub> is metabolized by CYP2E1 to the highly reactive trichloromethyl free radical which causes hepatocellular damage through lipid peroxidation (7).

The aims of this research were to evaluate the phytochemicals in *Curcuma longa* and investigate the combined effects of aqueous extract of *Curcuma longa* and some calcium channel blockers on function and histological structure of the liver in albino rats treated with carbon tetrachloride (CCl<sub>4</sub>).

## Methods

### *Collection and authentication of Tumeric*

Fresh samples of tumeric (*Curcuma longa*) were purchased from Akwatta-Ogbete, a local main market in Enugu, Nigeria. The plant material was authenticated by a consultant taxonomist at the herbarium section of the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka, and a voucher specimen was deposited at the herbarium for future reference.

### *Preparation of aqueous extract of Tumeric*

Preparation as described by Al-Tae et al. (8) was done, with slight modification. Briefly, water extraction of turmeric was prepared by boiling 100gm in 1000ml distilled water over low flame for 15 minutes, using a heat-stable flask. The flask was then plugged and removed from the heat and allowed to cool. After cooling, the content of the flask was sieved using clean muslin cloth and filtered with Whatman No.1 filter paper. The filtrate was used to prepare the required concentration.

### *Phytochemical Analysis of tumeric:*

Preliminary phytochemical screening of tumeric (*Curcuma longa*) for the presence of glycosides, flavonoids, saponins, steroids, tannins, carbohydrates, proteins and terpenoids was carried out at Department of Pharmacognosy, Faculty of Pharmaceutical Science, University of Nigeria Nsukka. Procedures outlined by Trease and Evans (9) were employed for the analyses.

### *Reagents and solutions*

The CCl<sub>4</sub> was of analytical grade and was purchased from Ogbete main market, Enugu

### *Preparation of calcium channel blocker solutions*

32 tablets of 10 mg (i.e. 320 mg) Amlodipine and 20 tablets of 20 mg Nifedipine (400 mg) obtained from a Pharmacist at Ogbete main market, Enugu, were grinded to powder, dissolved in 100ml distilled water to give a stock solution of 3.2mg/ml and 4mg/ml respectively.

### *Induction of Acute Liver Injury*

Hepatic injury was induced in each experimental animal by intraperitoneal injection with CCl<sub>4</sub> (0.4ml/kg of body weight), daily for three days.

### *Experimental animals and maintenance*

A total of thirty (30) adult male albino wistar rats, with average weight of (120±20g) were used in this study. They were obtained from the animal house of the College of Veterinary Medicine, University of Nigeria, Nsukka, Enugu state, Nigeria. The animals were housed in metallic cages in the animal house under ambient temperature (25±3°C) and 12-hour light and dark periodicity. They were adequately fed with commercial rat pellets (Neimeth Livestock Feeds Ltd., Ikeja) and water *ad libitum*. The animals were kept under observation for about 14 days before the onset of the experiment for acclimatization. All the animals used in this study were handled according to Institutional guidelines describing the use of rats and in accordance with the American Physiological Society guiding principles for research involving animals and human beings (10). In addition, proper care was taken as per the ethical rule and regulation of the concerned committee of the University of Nigeria, Nsukka, Enugu State, Nigeria.

#### *Experimental Design.*

A total of 30 male albino wister rats were used. The rats were randomly allocated to six (6) groups (A–F) of five (5) animals per group in well ventilated cages. The experimental animals received the following treatments on a daily basis for three days, together with stipulated feed and water.

- Group A: (Negative Control) received intraperitoneal administration of carbon tetrachloride CCl<sub>4</sub> (0.4ml/kg body weight) only for 3 days.
- Group B: received CCl<sub>4</sub> and turmeric extract (200mg/kg, oral) for 3 days.
- Group C: received CCl<sub>4</sub>; turmeric extract and Nifedipine (1mg/100g of rat, i.p) for 3 days.
- Group D: received CCl<sub>4</sub>, turmeric extract and Amlodipine (1mg/100g of rat, i.p) for 3 days.
- Group E: received turmeric extract only for 3 days.
- Group F: (Normal Control): No treatment was administered to this group.

#### *Sacrificing of animals and sample collection*

Blood samples for the determination of liver biomarkers were taken by cardiac puncture of the left ventricle of the heart under chloroform

anaesthesia and the liver harvested for histological analysis.

#### *Biochemical analysis*

The serum obtained was used for the analysis of serum total bilirubin concentration and the liver enzyme markers [aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP)] were analysed using Rx Monza Analyzer and standard laboratory kit from Randox Laboratories Ltd, UK. Measurement of Bilirubin (Total) was by Colorimetric method as described by Malloy and Evelyn (11). Measurement of ALT and AST were by colorimetric method as described by Reitman and Frankel (12). Measurement of ALP was by colorimetric method as described by Kind and King (13).

#### *Histopathological analysis*

The excised livers were fixed in 10% formal saline for 24 hours and further processed using the conventional paraffin wax embedding technique for light microscopic examination. The paraffin-embedded liver tissues were sectioned at 5microns and stained using the Haematoxylin and Eosin [H and E] staining procedure by Baker *et al.*, (14). The histological sections were examined using an Olympus™ light microscope.

#### *Statistical analysis*

Data analysis was done using GraphPad prism version 6.0 (GraphPad, San Diego, CA, USA). The results of the biochemical assays were reported as mean±SEM (standard error of mean). The level of

## **Results**

#### *Phytochemical results*

Phytochemical analysis indicated the presence of alkaloids, flavonoids, tannins, saponins, terpenoids, phenolic compounds and phytosterols in the plant extract (Table 1).

*Combined effects of aqueous extract of curcuma longa (Turmeric) and some calcium channel blockers on body weight.*

Combined effects of aqueous extract of curcuma longa (Turmeric) and some calcium channel blockers on body weight of rats treated with carbon tetrachloride (CCl<sub>4</sub>) is represented in figure 1. It was

observed that rats in the CCl<sub>4</sub> group were sluggish, lose appetite and response to stimulus was slow. The mean decrease in body weight was highest in the CCl<sub>4</sub>-alone group (negative control) in comparison with other groups.

#### Biochemical results

Table 2 shows the results of liver biochemical parameters ALT, AST, ALP (enzymes markers of hepatic injury) and total bilirubin levels in six (6) groups of five (5) animals which received intraperitoneal administration of carbon tetrachloride CCl<sub>4</sub> (0.4ml/kg body weight) and/or aqueous extracts of *curcuma longa* (AECL) or in combination with some calcium channel blocker (nifedipine or amlodipine) for 3 days. From the results, CCl<sub>4</sub> to the rats induced a highly significant acute liver injury when compared with normal control rats. However, AECL showed significant hepatoprotection (\*P<0.05) in comparison with negative control (CCl<sub>4</sub> alone). Furthermore, in combination with the calcium channel blockers, nifedipine or amlodipine, there was highly significant hepatoprotection against the hepatotoxicant, CCl<sub>4</sub> (\*\*P<0.01 or \*P<0.05). There was better protection against CCl<sub>4</sub>, when the extract was coadministered with a calcium channel blocker.

#### Histopathological results

In figure 2, microscopic examination of the liver isolated from the rats at sacrifice revealed no histopathological alteration in AECL alone and the normal control rats (Figure 2E and 2F respectively). Presence of significant vacuolation and necrosis of the hepatocytes were observed in the liver of rats treated with intraperitoneal administration of CCl<sub>4</sub> alone for 3 days (Figure 2A); however non-significant degenerations were observed in rats administered with AECL alone or in combination with nifedipine or amlodipine separately (Figure 2B and 2C and 2D respectively), in the presence of CCl<sub>4</sub> challenge. The livers of rats in group B, C and D showed no significant histological alterations when compared with the normal control group.

#### Discussion

Drug hepatotoxicity leads to filling up of cytochrome P450 binding sites. The formation of reactive metabolites which is associated with

covalent binding and oxidative stress (as a result of decrease Glutathione) will either cause sensitization to TNF or induce intracellular stress and these would lead to apoptosis (15). (See Figure 3)

CCl<sub>4</sub>-induced liver injury is the best-characterized model of xenobiotic-induced hepatotoxicity, and is commonly employed for evaluating antihepatotoxic/hepatoprotective activities of drugs or bioactive substances (16). The bio-activation of CCl<sub>4</sub>, primarily through the activity of CYP2E1, generates the free radicals CCl<sub>3</sub> and CCl<sub>3</sub>OO which result in hepatic damage. These free radicals initiate lipid peroxidation by abstracting a hydrogen atom from the polyunsaturated fatty acid of a phospholipid (17). The CCl<sub>4</sub>-induced lipid peroxidation in turn increases the permeability of plasma membrane to Ca<sup>2+</sup>, leading to severe disruption of calcium homeostasis and necrotic cell death (17). The extent of hepatic damage is assessed by the increase in serum levels of the cytoplasmic enzymes AST, ALT, ALP, and GGT, and by histopathological examination.

Hiroki et al. (18) report that hypocalcaemia has an ameliorating effect on CCl<sub>4</sub>-induced hepatotoxicity. This again suggests that calcium influx is involved in CCl<sub>4</sub>-induced hepatotoxicity. In this study, the effects of calcium channel blockers (nifedipine and amlodipine) in combination with aqueous extract of turmeric on CCl<sub>4</sub>-induced liver damage were determined and the liver showed a significant reestablishment of several of the biochemical parameters: a considerable decrease in AST, ALT, ALP and total bilirubin levels was observed. These calcium channel blockers act by preventing the influx of calcium ions into hepatocytes, examples are: verapamil, nifedipine, amlodipine and diltiazem (19).

The histological and biochemical changes indicate that combination of turmeric extract with nifedipine or amlodipine synergistically protects liver from CCl<sub>4</sub>-induced damage. This may be attributed to the bioactive antioxidant phytochemicals present in the extract; or alteration in extracellular and intracellular Ca<sup>2+</sup> concentration, general vasodilator action or to antilipoperoxidative properties of calcium channel blockers. Nifedipine and amlodipine are well known selective Ca<sup>2+</sup> influx blockers in the myocardium, vascular smooth muscles and various parts of the brain (20).

It is possible that nifedipine and amlodipine inhibit  $\text{Ca}^{2+}$  influx and modulate intracellular calcium which helps in preventing  $\text{Ca}^{2+}$  accumulation in liver cells, since it was demonstrated that cytosolic  $\text{Ca}^{2+}$  is elevated 100 folds in rat hepatocytes exposed to  $\text{CCl}_4$  which is capable in initiating irreversible liver cell injury (16). General vasodilator effect of these calcium antagonists improves hepatic blood flow which may be useful in preventing  $\text{CCl}_4$ -induced centrilobular hypoxia, since reduced hepatic blood flow and associated centrilobular hypoxia account for the centrilobular necrosis in  $\text{CCl}_4$ -poisoning. Mak and Weglicki (21) have reported antiperoxidant effect of calcium antagonists in sarcolemmal membrane. Nayler and Britnell (22) have suggested that  $\text{Ca}^{2+}$  antagonists provide cellular protection by an unknown mechanism.

Many factors may be involved including not only the salvage of the ATP and creatine phosphate reserve, an ability to protect membrane against damage caused by lipid peroxidation may be major contributory factor. If the antilipoperoxidative effect of  $\text{Ca}^{2+}$  blockers occurs in liver cells also, it may be beneficial for preventing  $\text{CCl}_4$ -induced liver damage, since increased lipoperoxidation in membrane is also a strong pathogenic factor in aetiology of  $\text{CCl}_4$ -induced liver damage (20).

In the present study, biochemical results show that AECL alone, in the presence of  $\text{CCl}_4$  challenge, showed a significant hepatoprotection. Interestingly, when combined with nifedipine or amlodipine separately, a positively significant synergy in hepatoprotection was observed.

In the  $\text{CCl}_4$  group, animals were sluggish, lose appetite and response to stimulus was slow. In this group, there was significant elevation in serum AST, ALT, ALP and total bilirubin levels when compared to the normal control rats (table 2). The above results show that  $\text{CCl}_4$  hepatotoxicity was effectively produced in the negative control group. The results of our study show that  $\text{CCl}_4$  hepatotoxicity was effectively produced in all cases. Similar observations have been earlier reported by Mir et al. (23).

The group of rats which was administered plant aqueous extract alone or in combination with a calcium channel blocker, serum AST, ALT, ALP and total bilirubin values were significantly decreased. These results show that the extent of the liver damage is reduced as the enzyme values are lower

than  $\text{CCl}_4$  (negative control) group. On histological examinations, there were minimal lesions on the hepatocytes and vacuolation was almost absent which reveals that turmeric extract contain the active ingredient(s) which protects the liver. Although there was decrease in body weight but mean decrease of absolute body weight was lower than negative control group (figure 1). It appeared likely that hepato-protective phytochemicals are readily available in the aqueous extract to produce their protective action against the injury. Obviously, it is also possible that bioactive phytochemicals are soluble in aqueous extract. The present study shows that when rats were administered with aqueous extract of *C. longa*, the AST, ALT, ALP and total bilirubin levels significantly decreased ( $P < 0.05$  or  $P < 0.01$ ) when compared with negative control group. Increase in rat body weight could be due to recovery of the rats, and thus better feeding and general well being.

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**Table 1:** Qualitative phytochemical analysis of aqueous extract of *Curcuma longa*

Constituent	Indication
Carbohydrate	-
Reducing Sugar	-
Alkaloids	+
Glycosides	+
Saponins	+
Tannins	+
Flavonoids	++
Resins	-
Proteins	-
Oils	-
Phenolic Compounds	++
Terpenoids	++
Phytosterols	+

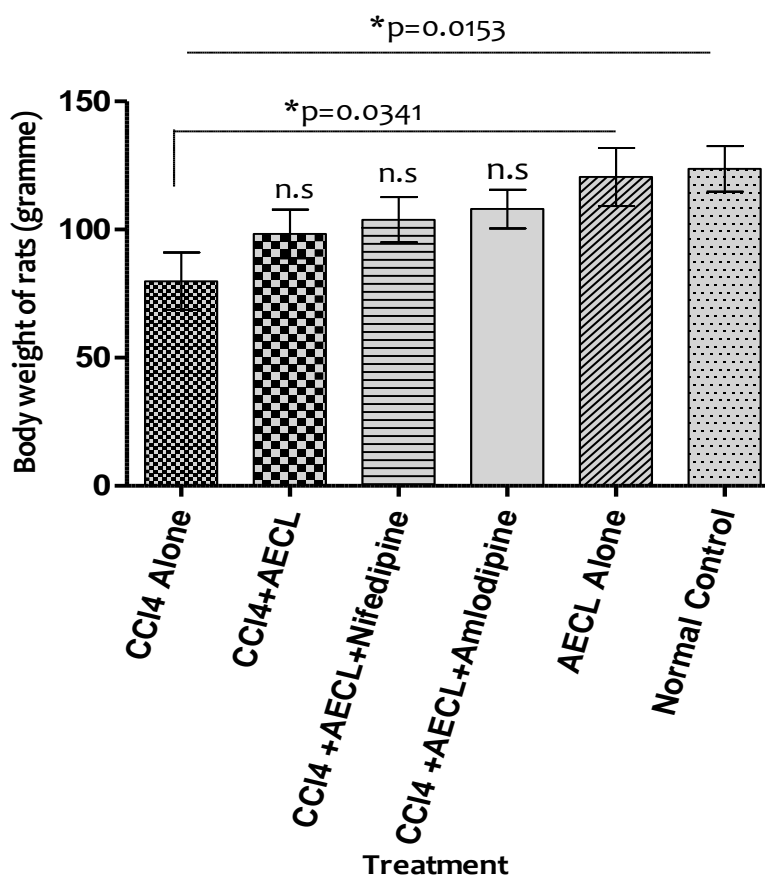
**Key:** ++ = present; + = present (in trace amount); - = absent

**Table 2:** Statistical comparison of liver biochemical concentrations in different experimental animal groups.

Groups	ALT (IU/L)	AST (IU/L)	ALP (IU/L)	Total bilirubin (mg/dL)
CCl <sub>4</sub> Alone	46.00 ± 6.35	46.37 ± 5.88	178.30 ± 19.22	1.45 ± 0.08
CCl <sub>4</sub> + AECL	28.33 ± 2.03*	30.27 ± 1.67*	131.30 ± 7.45*	1.24 ± 0.19
CCl <sub>4</sub> + AECL + Nifedipine	24.00 ± 1.16**	25.50 ± 0.65**	124.05 ± 1.53**	0.92 ± 0.17*
CCl <sub>4</sub> + AECL + Amlodipine	23.00 ± 2.08**	23.00 ± 2.08**	122.85 ± 2.52**	0.90 ± 0.15*
AECL Alone	17.50 ± 2.16***	17.00 ± 2.08***	121.70 ± 3.28**	0.77 ± 0.05**
Normal Control	16.470 ± 1.24***	16.00 ± 1.16***	121.30 ± 3.28**	0.70 ± 0.08**

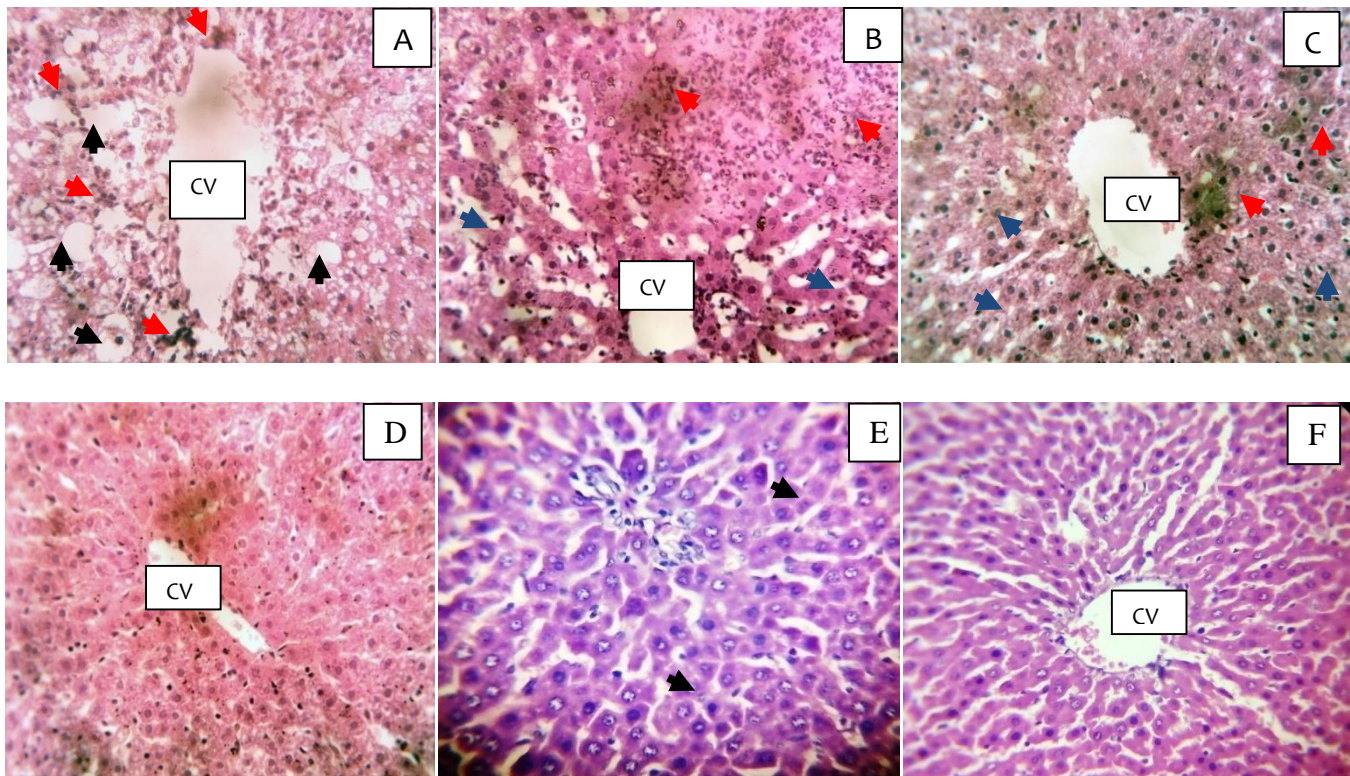
Values given as Mean ± SEM. \*\*\*P < 0.001, \*\*P < 0.01 or \*P < 0.05 is significant when CCl<sub>4</sub> alone (negative control) is compared with all other groups.

**Figure 1:** Combined effects of aqueous extract of *Curcuma longa* (Turmeric) and some calcium channel blockers on body weight of the rats.



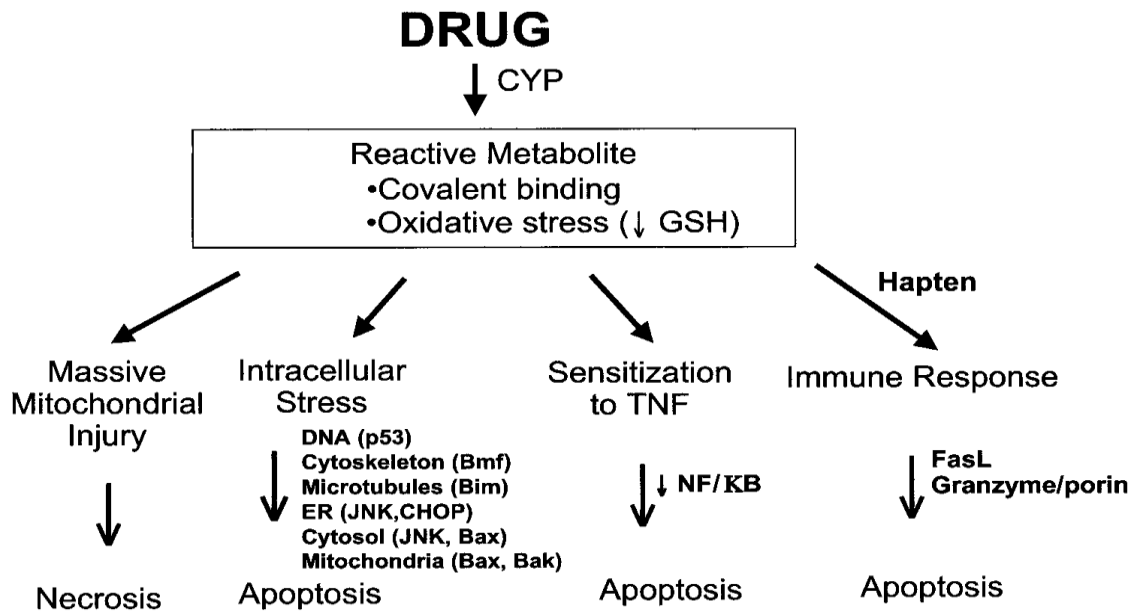
Histogram show the body weight of rats in the experimental groups. The preliminary data show intraperitoneal administration of CCl<sub>4</sub> induced a significant weight loss. However, oral administration of *Curcuma longa* (Turmeric) alone or in combination of Nifedipine and Amlodipine separately nonsignificantly induced higher body weight when compared with CCl<sub>4</sub> group (negative control). The data are presented as mean±SEM of body weight (gramme) for individual treatment. See *Materials and Methods* for experimental details. Statistical analyses were performed using ANOVA (\* p < 0.05).



**Figure 2:** Histopathology and photomicrograph of liver.

**(A)**  $\text{CCl}_4$  alone- treated rats. There are extensive necrosis of the hepatocytes (red arrow head) and vacuolations (black arrows) around the central veins (CV). **(B)**  $\text{CCl}_4$  + AECL- normal hepatocytes (blue arrows) seen. A patch of necrotic cells with inflammatory cells is also seen (red arrows). CV is a central vein. **(C)**  $\text{CCl}_4$ +AECL+nifedipine-treated rats. Normal hepatocytes (blue arrows) seen with some necrotic cells (red arrows). CV is a central vein. **(D)**  $\text{CCl}_4$ +AECL+amlodipine-treated rats. Hepatocytes appear normal. **(E)** AECL alone- treated rats. Hepatocytes appear normal (black arrow heads). **(F)** Normal control rats. No pathological lesions in the liver. CV is a central vein [Stain: H and E;  $\times 400$ ].

Figure 3: Cellular mechanisms of drug hepatotoxicity.



Bmf, Bim, Bax, and Bak are proapoptotic members of the B cell lymphoma-2 protein family; CHOP, c/EBP homologous protein-10; GSH, glutathione; JNK, c-jun-N-terminal kinase; f, inhibition. Metabolism of chemicals takes place in the liver. Drug hepatotoxicity leads to filling up of cytochrome P450 binding sites. The formation of Reactive metabolites which is associated with covalent binding and oxidative stress (as a result of decrease Glutathione) will either cause sensitization to TNF or induce intracellular stress and these would lead to apoptosis. If a drug metabolite produced by cytochrome P450 is able to act as a hapten, it would covalently bind to a liver protein and, subsequently, alter that protein. This altered protein would then be perceived as foreign substance by the immune system, resulting in an autoimmune attack on normal hepatocellular constituents eventually leading to apoptosis. On the other hand, this reactive metabolite may cause massive mitochondrion injury thereby leading to necrosis of the liver tissues. Adopted from kaplowitz (15).