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COMPARATIVE STUDY OF CYTOCHROME P450 INHIBITORS ON CULTURED MOUSE HEPATOCYTES

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Abstract

Liver is a vital organ and plays a major role in metabolism. Many liver diseases such as Wilson's disease, hepatitis (B and C), liver cancer or genetic defects leads to liver injury and ultimately liver fibrosis or cirrhosis. Cytochrome P450 inhibitors (Ketoconazole, Bifonazole, Clotrimazole) are potential pharmacological agents which can initiate the apoptosis, inhibitors of cytochrome P450, inhibit the metabolism of all-trans-retinoic acid (RA) and have anti inflammatory effects. This study is focused on using of these pharmacological agents in pre-treatment and treatment strategies to investigate the effect the CCl_4 induced liver injury in cultured mouse heptocytes. Heptocytes were isolated from mouse liver and cultured in 6-well collagen coated plates. On the next day, they were divided into 6 groups; vehicle control, CCl_4 control, Drug control, CCl_4 + Drug group, Pretreated group and Treated group. After 4 hours, cell viability was analyzed. All drugs 1µM (Ketoconazole, Bifonazole, Clotrimazole) were used and all the groups were in triplicate. The cell viability was assessed by trypan blue and % LDH release. In protecting heptocytes from CCl_4, Ketoconazole and bifonazole showed ($p<0.001$) highly significant results as compared to clotrimazole in CCl_4 + Drug group while clotrimazole have ($p<0.01$) significant higher effect on cell viability than ketoconazole, bifonazole (pretreatment) and ketoconazole showed ($p<0.001$) highly significant effects on cell viability when compared with bifonazole, clotrimazole (post-treatment) respectively. In conclusion, this study demonstrated that ketoconazole, bifonazole was more effective than clotrimazole in preventing CCl_4 injury on heptocytes while clotrimazole showed effective in case of pre treatment and ketoconazole showed more effective in case of post treatment respectively. We suggest that ketoconazole have the potential to treat liver injury or effective treatment in liver diseases.

Keywords: Ketoconazole, Bifonazole, Clotrimazole, Cytochrome P450, Cultured Hepatocytes

Introduction

Different pathways or strategies are used to prevent cells from any harmful effects of toxins, drugs, carcinogens by decreasing or inhibiting metabolic enzymes responsible for their activation into reactive species (phase 1) and at the same time induce phase 2 enzymes that can deactivate reactive radicals and electrophiles (1).

Targeting cytochrome P450 enzymes is also a new approach for development of anti-cancer drug therapy (2). Cytochrome P450 blockers can be used to prevent liver injury because of inhibition of cytochrome P450 in the liver which is required by the carcinogen, pollutant, drugs for their bioactivation into reactive species which can damage liver resultantly (3). Cytochrome P450 can induce or inhibit the metabolism of drugs, pollutants, carcinogens. So, it is of great interest to inhibit the metabolism of toxic/carcinogens which are converted into active and reactive radical species which then cause liver damage (4). It is not a big deal to get benefit from Cytochrome P450 inhibitors and to use them as therapeutics agents in different diseases. They can inhibit the metabolism of carcinogen and carcinogens are not converted into their reactive species which can cause liver damage and lipid peroxidation (5).

For in vivo studies carbon tetrachloride (CCl_4) has been widely used to study liver fibrosis in experimental rodents (6). CCl_4 or any toxic carcinogen is first metabolized or bio-activated to form active species which can cause liver damage. Cytochrome P450 is the enzyme which convert CCl_4 into reactive CCl_3^- species which can cause membrane damage and lipid peroxidation. However, CCl_4 administration twice a week by intraperitoneal injection to rodents for four weeks can cause liver fibrosis (7).

Anti-fungal drugs are classified into two groups, Imidazoles and triazoles (8). The basic mechanism of action of these azoles is inhibition of ergosterol synthesis by acting on fungal cytochrome P450 enzymes. The azoles have great specificity for fungal cytochrome P450 than human P450 enzymes but Imidazoles have lesser degree of specificity than triazoles and cause drug interaction and inhibition of human cytochrome P450 enzyme system (9). Imidazoles include ketoconazole, bifonazole, clotrimazole, econazole, sulconazole (8). Ketoconazole is first orally available anti fungal drug for various types of fungal infection. It is a strong inhibitor of mammalian cytochrome P450 (10). It induces apoptosis in different carcinoma cell lines (11).

The aim of the current study is to demonstrate the effect of different Imidazoles which are synthetic in nature and which may have more potential to prevent hepatocyte injury.

Materials and Methods

Drugs Used:

Ketoconazole, Bifonazole, Clotrimazole was gifted from Mass Pharma (Pvt) Limited Lahore and other chemicals were purchased from Sigma Aldrich, USA.

Animals:

The research conforms to the Guide for the care and use of Laboratory Animals by the US National institutes of Health (NIH publication No. 85-23, revised 1985). All animals were treated according to the procedures approved by the Institutional Review Board (IRB) at the National Centre of Excellence in Molecular Biology, Lahore, Pakistan.

Hepatocyte Isolation:

Hepatocytes were isolated from C57BL/6 mice (n=3) according to the two step perfusion method as described previously (12). Isolated hepatocytes were plated at a concentration of 2.5×10^3 cells/well in collagen coated plates (Becton Dickinson, USA) in RPMI 1640 medium (Sigma Aldrich, USA) supplemented with 100 ug/ml streptomycin (MP Biomedicals, USA), 100 units/ml penicillin (MP Biomedicals, USA) and 10% fetal bovine serum (Sigma Aldrich, USA) in a humidified incubator at 5% CO_2 and 37°C temperature. Medium was replaced after 24 hrs followed by various treatments.

Treatment of Hepatocytes:

Hepatocytes were plated in 6 well collagen coated plates at a density of 2.5×10^3 cells/well and allowed to attach for 24 hour before treatment, incubated at 37°C in 5% CO_2 atmosphere.

- *Ketoconazole treated*

Six groups were made and each group was in triplicate. Following groups were made: Normal, CCl_4(8mM), Ketoconazole(1µM), CCl_4(8mM) + Ketoconazole (1µM), Ketoconazole (1µM) 30 min before CCl_4(8mM) (Pre-treated) *****, CCl_4(8mM) then last 30 min Ketoconazole(1µM) (Treated) ******.

- *Bifonazole treated*

Six groups were made and each group was in triplicate. Following groups were made: Normal,CCl_4(8mM), Bifonazole(1µM), CCl_4(8mM) + Bifonazole(1µM), Bifonazole(1µM) 30 min before

CCl_4(8mM) (Pre-treated)*, CCl_4(8mM) then last 30 min Bifonazole(1µM) (Treated)**.

- *Clotrimazole treated*

Six groups were made and each group was in triplicate. Following groups were made: Normal, CCl_4(8mM), Clotrimazole(1µM), CCl_4(8mM) + Clotrimazole (1µM), Clotrimazole(1µM) 30 min before CCl_4(8mM) (Pre-treated)*, CCl_4(8mM) then last 30 min Clotrimazole(1µM) (Treated)**.

*Groups were first treated with the drug for 30min and incubated at 37°C in 5% CO2 atmosphere then CCl_4(8mM) was introduced in the well for 4 hour .

**Groups were first treated with CCl_4(8mM) for 4 hour then treated with 30 min with the drug.

All the groups were incubated for 4 hour, incubated at 37°C in 5% CO_2 atmosphere.

Cell Viability Assay:

Trypan blue exclusion method was used to check the cell viability after treating cultured cells in 6-well plates for 4 hours. The medium was transferred from the culture plate to the eppendrrof.Cells were washed with PBS and then trypan blue solution (Sigma Aldrich, USA) was added. The plates were incubated at 37°C for 8-10 minutes. Cells were again washed with PBS and analyzed under phase contrast microscope (13).Six high power fields of each well were selected. Total number of cells were counted. The number of trypan blue positive cells were divided by total number of cells examined and then multiplied by 100 for calculating dead cells in each well.

Lactate Dehydrogenase Assay:

Lactate dehydrogenase was measured according to manufacturer protocol (Sigma Aldrich, USA). Medium was removed from each well and centrifuge at 250 × g for 4 minutes to pellet cells. Aliquot was transferred to a new ependdroff. Lactate dehydrogenase assay mixture was prepared by mixing equal volumes of LDH assay substrate solution, LDH assay dye solution and LDH assay cofactor preparation. 96-well plate was used and each sample was added in triplicate with twice the volume of lactate dehydrogenase assay mixture. 96-well plate was covered with aluminum foil to protect it from light and incubated at room temperature for 30 minutes. After 30 minutes, 1/10 volume of 1N HCl was added to each well for termination of reaction. Then plate reader was used to measure absorbance at a wavelength of 490 nm and 690 nm and subtract 690 nm value from 490 nm to calculate LDH release.

Statistical analysis:

Analysis between different treatment groups vs control was performed by one-way ANOVA with bonferroni post-hoc test. P-value of less than 0.05 was considered statistically significant.

Results

Trypan Blue Assay & % LDH Release:

Ketoconazole Treatment:

The cells were stained with trypan blue (figure 1) and the number of dead cells per field was counted. Hepatocytes showed highly significant ($p<0.001$) loss of viability in CCl_4 control group as compared to vehicle control. Ketoconazole did not show any significant loss of viability as compared to vehicle control. CCl_4 control group showed highly significant ($p<0.001$), very significant ($p<0.01$), significant ($p<0.05$) loss of viability as compared to CCl_4+ketoconazole group, pretreated group and treated group respectively. Pretreated group showed significant ($p<0.05$) loss of viability as compared to CCl_4+ketoconazole. Treated group showed very significant loss of viability as compared to CCl_4+ketoconazole. Graphical representation of these groups is shown in figure 2.

Percentage LDH was measured from the media. Hepatocytes showed highly significant ($p<0.001$) increase in % LDH release in CCl_4 control when compared to vehicle control.CCl_4 control showed very significant ($p<0.01$) increase in % LDH release when compared to ketoconazole control and CCl_4+Ketoconazole. CCl_4 control showed significant ($p<0.05$) increase in % LDH release when compared to pretreated and treated groups. Graphical representation of these groups was shown in figure 3.

Bifonazole Treatment:

The cells were stained with trypan blue (figure 4) and the number of dead cells per field was counted. Hepatocytes showed highly significant ($p<0.001$) loss of viability in CCl_4 control group as compared to vehicle control. Bifonazole did not show any significant loss of viability as compared to vehicle control. CCl_4control group showed highly significant ($p<0.001$), significant ($p<0.05$) loss of viability as compared to CCl_4+ bifonazole group, pretreated group respectively. Pretreated group showed significant ($p<0.05$) loss of viability as compared to CCl_4+ bifonazole. Treated group showed highly significant ($p<0.001$) loss of

viability as compared to CCl_4+ bifonazole. Graphical representation of these groups is shown in figure 5.
Hepatocytes showed highly significant increase (p<0.001) in % LDH release in CCl_4 control when compared to vehicle control.CCl_4 control showed highly significant (p<0.001) increase in % LDH release when compared to CCl_4+bifonazole. Preteated group showed significant (p<0.05) increase in % LDH release when compared to CCl_4+bifonazole. Treated group showed very significant (p<0.01) increase in %LDH release as compared to CCl_4+bifonazole (figure 6).

Clotrimazole Treatment:
The cells were stained with trypan blue (figure 7) and the number of dead cells per field was counted. Hepatocytes showed highly significant (p<0.001) loss of viability in CCl_4 control group, CCl_4+Clotrimazole and treated group as compared to vehicle control. Clotrimazole did not show any significant loss of viability as compared to vehicle control. CCl_4 control group showed very significant (p<0.01) loss of viability as compared to pretreated group. Graphical representation of these groups is shown in figure 8.
Hepatocytes showed highly significant increase (p<0.001) in % LDH release in CCl_4 control as compared to vehicle control.CCl_4 control showed highly significant (p<0.001), very significant (p<0.01) increase in % LDH release as compared to CCl_4+clotrimazole and pretreated group, respectively. Treated group showed highly significant (p<0.001) increase in %LDH release as compared to CCl_4+clotrimazole and pretreated group as shown in figure 9.

Comparison Of Ketoconazole, Bifonazole, Clotrimazole In-Vitro
Trypan Blue Assay & % LDH Release:

- **CCl_4 + Drug (Ketoconazole, Bifonazole, Clotrimazole) Group**

Hepatocytes showed highly significant (p<0.001) loss of viability in CCl_4 as compared to vehicle control. CCl_4 control showed highly significant (p<0.001) loss of viability as compared to CCl_4+ketoconazole and CCl_4+bifonazole. CCl_4+clotrimazole showed highly significant (p<0.005) loss of viability as compared to CCl_4+ketoconazole andCCl_4+bifonazole (figure 10).
CCl_4 control group showed very significant (p<0.01) increase in %LDH when compared to CCl_4+ketoconazole, CCl_4+bifonazole, CCl_4+clotrimazole as shown in figure 11.

- **Pretreated Group**

CCl_4 control showed significant loss (p<0.05) of viability as compared to pretreated with ketoconazole and bifonazole group but highly significant (p<0.001) loss of viability was observed as compared to pretreated with clotrimazole as shown in figure 12.
CCl_4 control group showed highly significant (p<0.001) %LDH release as compared to pretreated ketoconazole and pretreated clotrimazole groups. Pretreated bifonazole group showed very significant (p<0.01) %LDH release when compared to pretreated ketoconazole and pretreated clotrimazole (figure 13).

- **Treated Group**

CCl_4 control showed very significant (p<0.01) loss of viability as compared to treated with ketoconazole while non-significant loss of viability as compared to treated with bifonazole and clotrimazole as shown in figure 14.
CCl_4control group showed highly significant (p<0.001) % LDH increase when compared to treated ketoconazole. Treated bifonazole and treated clotrimazole showed highly significant (p<0.001) %LDH increase as compared to treated ketoconazole as shown in figure 15.

Discussion

In vitro model of heptocytes injury induced by carbon tetrachloride was established to study heptoprotective effect of cytochrome P450 inhibitors (ketoconazole, bifonazole, clotrimazole). It has been known recently that carbon tetrachloride is metabolized by the cytochrome P450 in the liver to produce trichloromethyl radicals and then these radicals react with oxygen to form trichloromethyl peroxyl radicals, which causes cell damage by covalently binding to cellular macro-molecules and lipid peroxidation (14).
To measure the injury by CCl_4, cell viability was determined by trypan blue assay and percentage LDH release. This study showed that heptocytes treated with CCl_4 8mM raised highly significant levels of LDH as also reported by Yin, Cao (15) while in this study we have used three azoles treatment with CCl_4. Ketoconazole, bifonazole and clotrimazole (p<0.01) significantly decreased the level of LDH release while pretreated with ketoconazole and clotrimazole highly significantly (p<0.001) reduce LDH release and treated group showed that ketoconazole was highly significant among all these three azoles while trypan blue assay showed (p<0.001) highly significant decreased percentage of dead cell in ketoconazole and bifonazole groups as compared to CCl_4 and clotrimazole group however pretreated group showed that clotrimazole was highly significant (p<0.001) among all these azoles and treated group showed that ketoconazole was (p<0.01) bit significant percentage of

dead cells as compared to all other groups. Dresser, Spence (16) also reported that ketoconaozle inhibited cytochrome P450 enzyme system and as studied by Wiseman, Smith (17) anti oxidant effect of ketoconazole which was stronger than other azoles miconazole and clotrimazole.

Korashy, Brocks (18) studied the inducing effect of ketoconazole on Nqo1 gene expression which is a phase II enzyme to detoxify xenobiotic and maintain endogenous anti-oxidants in their reduced and active forms to protect tissue from oxidative stress , carcinogens etc.

In our study, comparative analysis of protective effect of ketoconazole and bifonazole against CCl_4 induced heptocytes injury showed highly significant viability as compared to clotrimazole, while pretreatment effect of these three drugs showed that clotrimazole has highly significant cell viability as compared to ketoconazole and bifonazole. However, after CCl_4 injury, post treatment showed that ketoconazole has highly significant cell viability as compared to bifonazole and clotrimazole.

Lin, Huang (19) demonstrated the role of ketoconazole on apoptosis, viability, mitogen activated protein kinases (MAPKs) and Ca^{2+} levels in osteosarcoma cells. As, demonstrated by propidium iodide staining and activation of caspase 3, decreased cell viability via apoptosis at 20-200 µM of ketoconazole. In our study ketoconazole, bifonazole and clotrimazole 1µM showed protective effect on heptocytes as assessed by trypan blue and LDH release assay that ketoconazole prevent normal cells from CCl_4 injury and pre- and post- treatment showed significant and very significant increase in cell viability respectively. Hence, it is proved that azoles can only induce apoptosis in cancerous cells.

The result of this study showed that ketoconazole have the potential to treat cultured heptocytes as compared to bifonazole and clotrimazole and we suggest that ketoconazole have the potential to treat chronic liver injuries.

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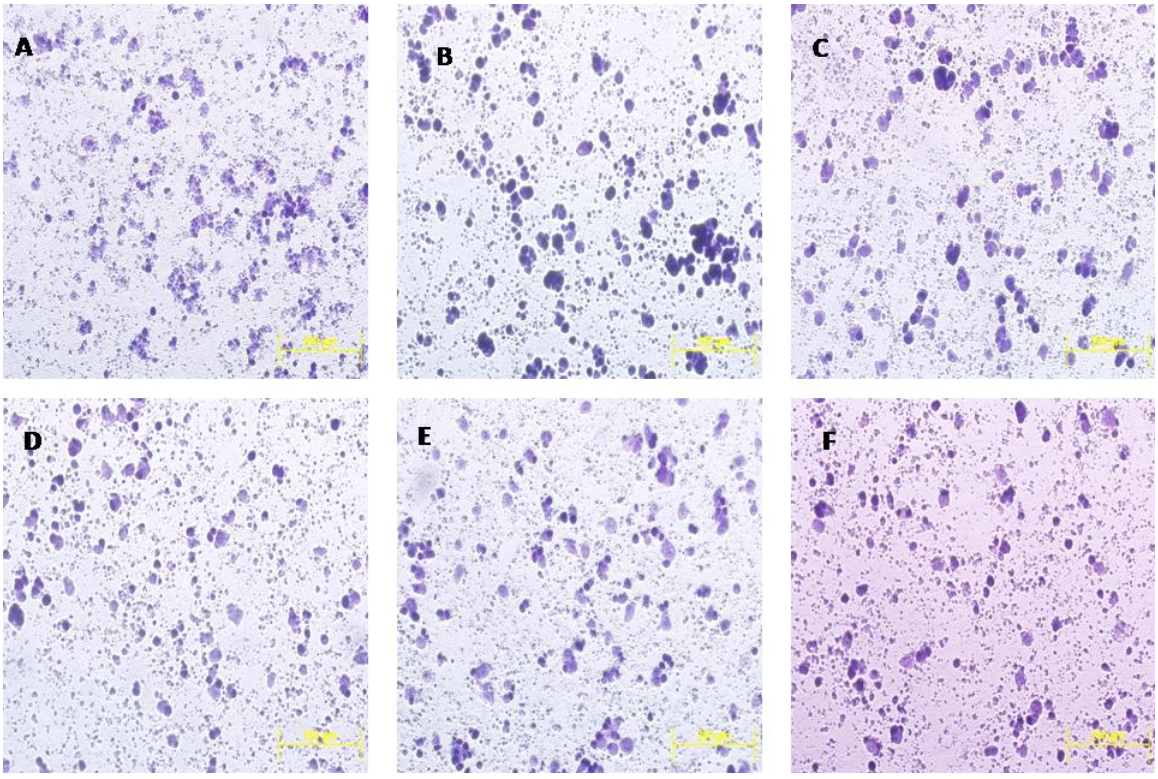

Fig.1 Microscopic examination of trypan blue assay of ketoconazole
A:Normal group B:CCl_4Control group C:Ketoconazole control group D: CCl_4+ketoconazole group
E:Pretreated ketoconazole group F: Treated ketoconazole group

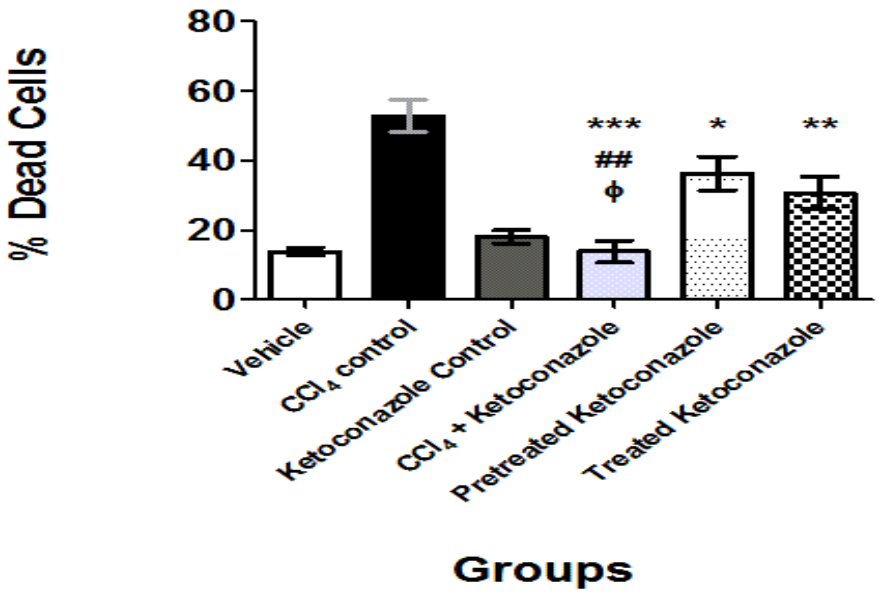

Fig. 2 Graphical representation of different groups of ketoconazole on hepatocytes
Data was mean ± SEM ***p<0.001 CCl_4 control vs CCl_4+ketoconazole, *p<0.05 CCl_4 control vs Pretreated ketoconazole, **p<0.01 CCl_4 control vs Treated ketoconazole, $^{\#\#}$p<0.01 CCl_4+ketoconazole vs Pretreated ketoconazole, $^{\Phi}$p<0.05 CCl_4+ketoconazole vs Treated ketoconazole.

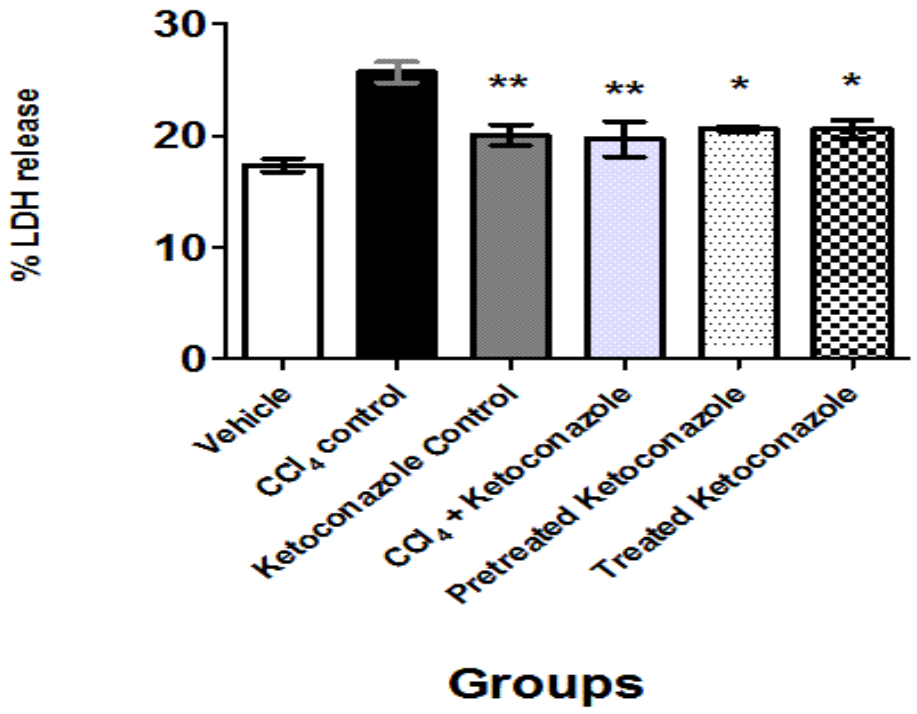

Fig. 3 *Graphical representation of % LDH release in groups of ketoconazole*
Data was mean ± SEM, $^{**}p<0.01$ CCl_4 control vs ketoconazole control and CCl_4+ketoconazole, $^{*}p<0.05$ CCl_4 control vs Pretreated ketoconazole and Treated ketoconazole

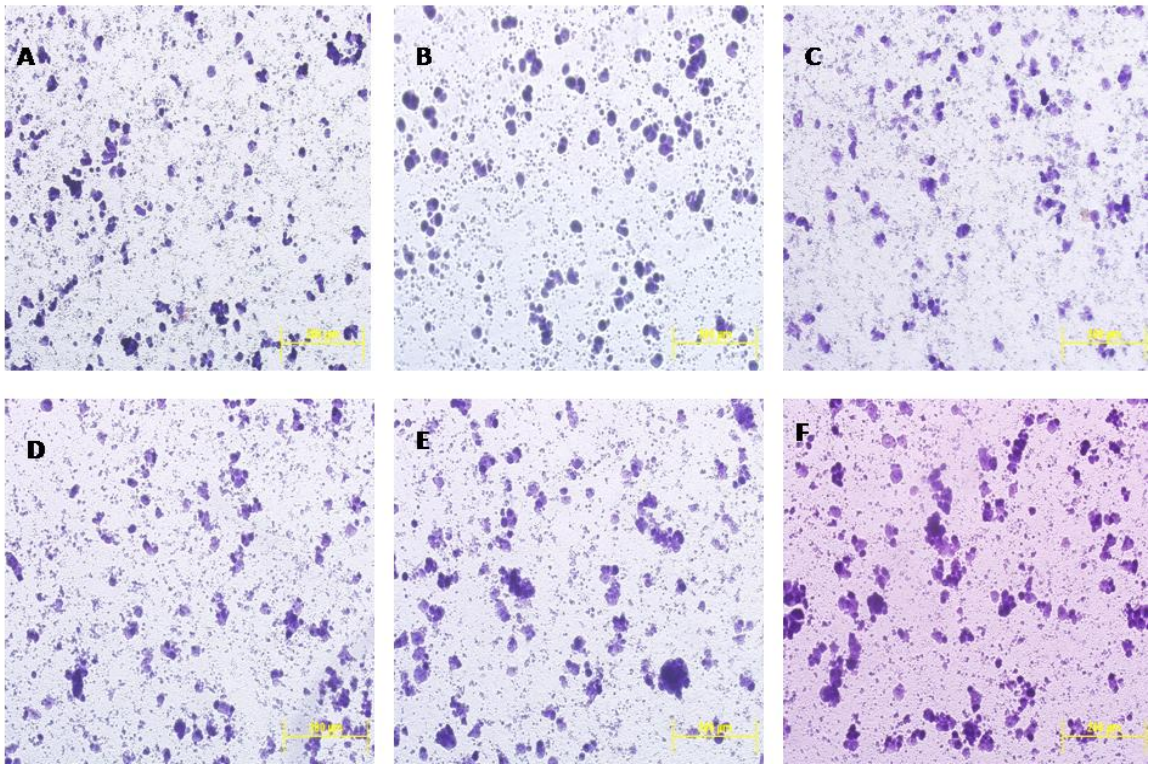

Fig. 4 Microscopic examination of trypan blue assay of bifonazole

A:Normal group B:CCl_4 control group C:Bifonazole control group D: CCl_4+ bifonazole group E:Pretreated bifonazole group F: Treated bifonazole group

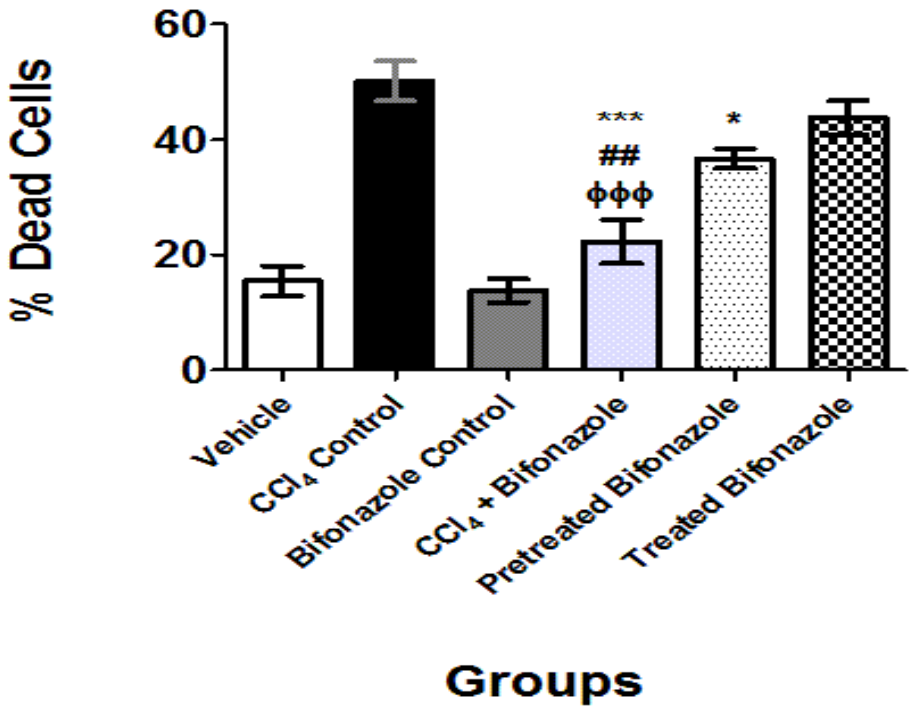

Fig. 5 *Graphical examination of different groups of bifonazole on hepatocytes*
Data was mean ± SEM $^{***}p<0.001$ CCl_4 control vs CCl_4+bifonazole, $^{*}p<0.05$ CCl_4 control vs Pretreated ketoconazole, $^{\#\#}p<0.01$ CCl_4+ketoconazole vs Pretreated ketoconazole, $^{\Phi\Phi\Phi}p<0.001CCl_4$+ketoconazole vs Treated ketoconazole.

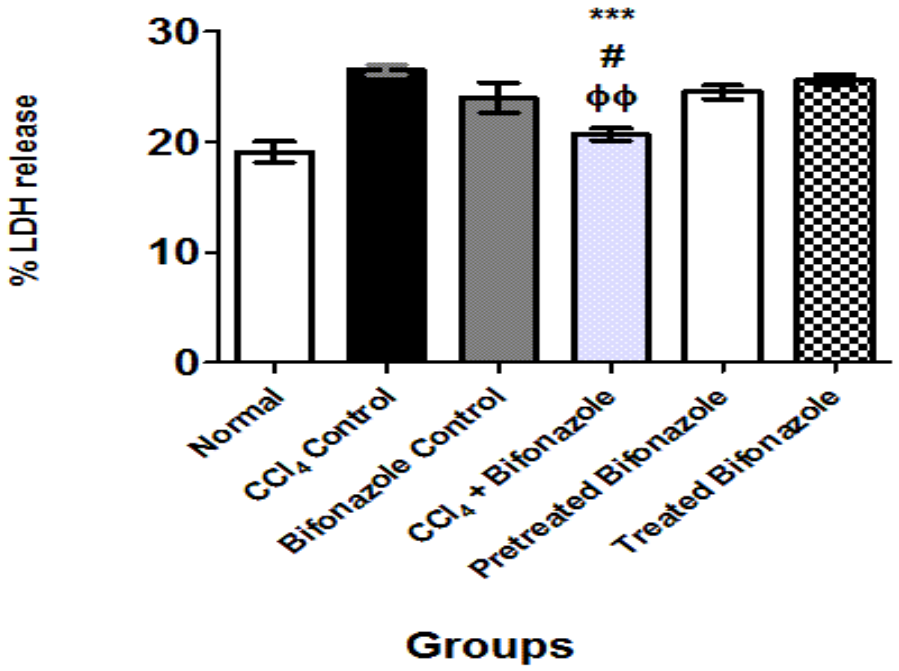

Fig.6 *Graphical representation of % LDH release in groups of bifonazole*
Data was mean ± SEM $^{***}p<0.001$ *CCl_4 control vs CCl_4+bifonazole,* $^{\#}p<0.05$ CCl_4+ bifonazole vs Pretreated bifonazole, $^{\Phi\Phi}p<0.01$ CCl_4+ bifonazole vs Treated bifonazole

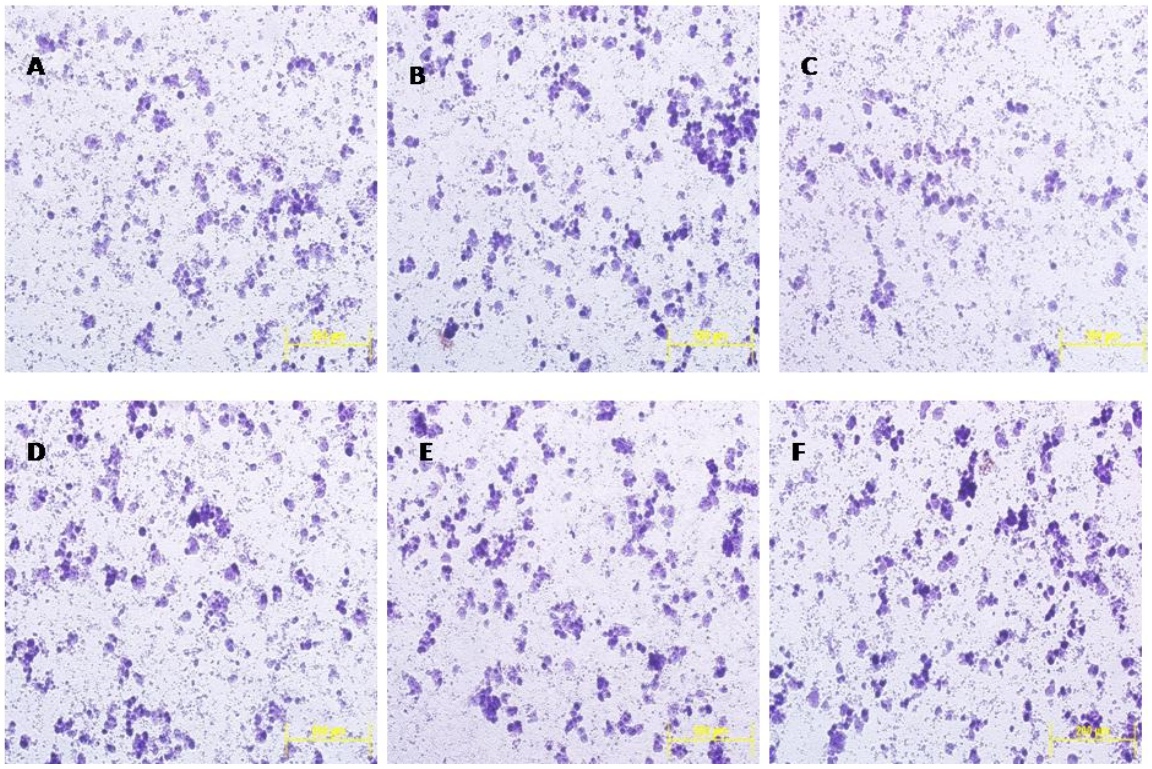

Fig.7 *Microscopic examination of trypan blue assay of clotrimazole*
A:Normal group B:CCl_4 control group C:Clotrimazole control group D: CCl_4+ clotrimazole group E:Pretreated clotrimazole group F: Treated clotrimazole group

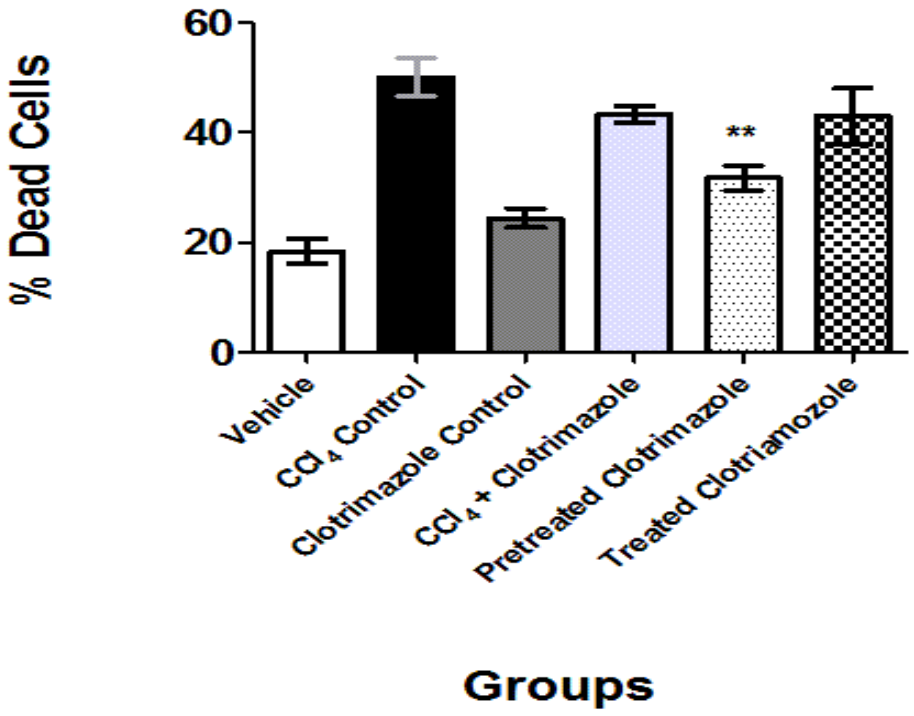

Fig.8 *Graphical representation of different groups of clotrimazole on hepatocytes*
Data was mean ± SEM $^{**}p<0.01$ CCl_4 control vs Pretreated clotrimazole

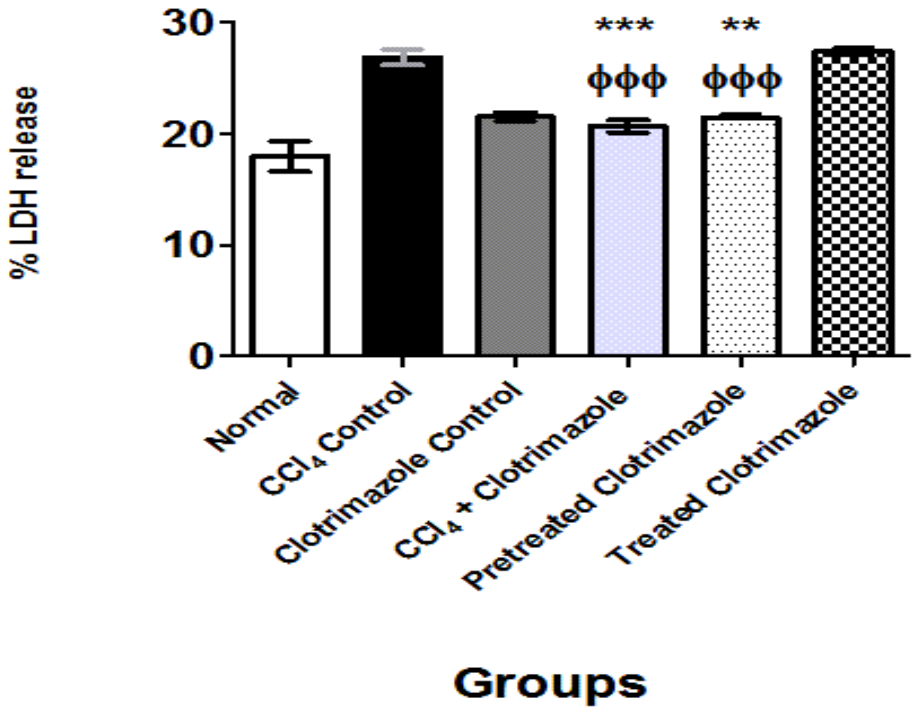

Fig. 9 *Graphical representation % LDH release in groups of clotrimazole*

Data was mean ± SEM ***p<0.001 CCl_4 control vs CCl_4+ clotrimazole, **p<0.01 CCl_4 control vs Pretreated clotrimazole ,$^{\Phi\Phi\Phi}$p<0.001 Treated clotrimazole vs CCl_4+ clotrimazole and Pretreated clotrimazole

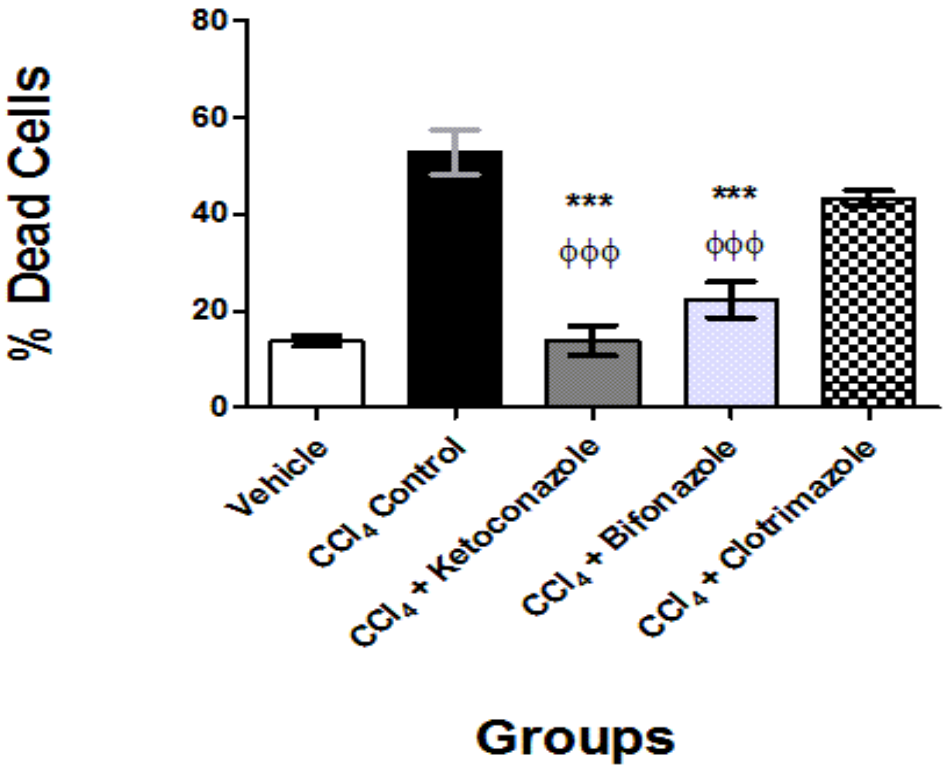

Fig. 10 *Graphical representation of comparison of trypan blue assay of CCl_4+Drugs (ketoconazole, bifonazole, clotrimazole)*
Data was mean ± SEM, ***p<0.001 CCl_4 control vs CCl_4+ketoconazole and CCl_4+bifonazole, $^{\Phi\Phi\Phi}$p<0.001 CCl_4+clotrimazole vs CCl_4+ ketoconazole and CCl_4 + bifonazole.

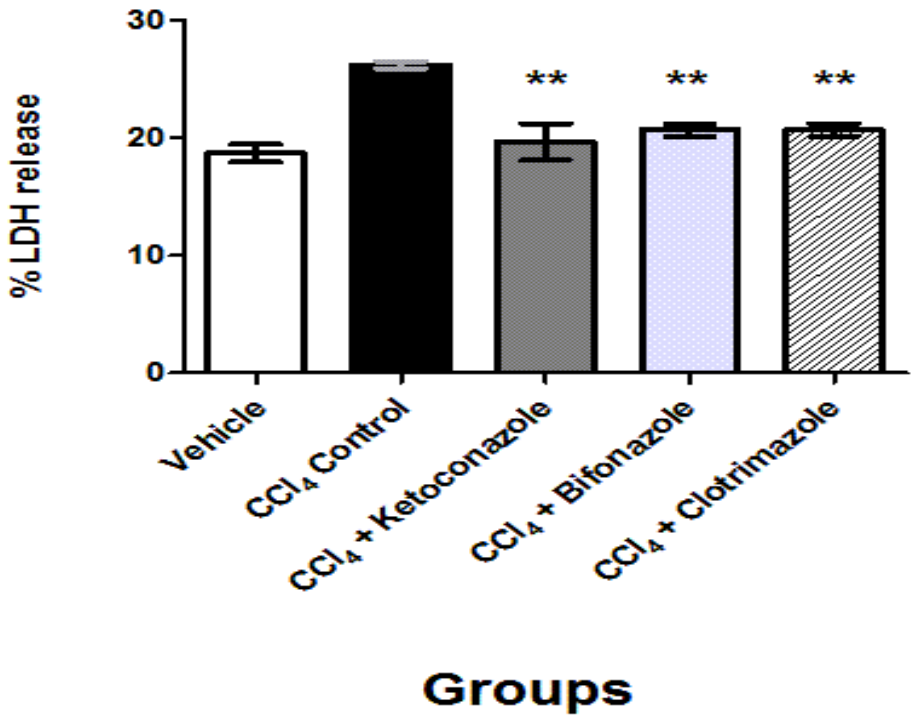

Fig.11 *Graphical representation of comparison of % LDH release of CCl_4 + Drugs (ketoconazole, bifonazole, clotrimazole)* Data was mean ± SEM. $^{**}p<0.01$ CCl_4 control vs CCl_4+ketoconazole, CCl_4+ bifonazole and CCl_4+clotrimazole.

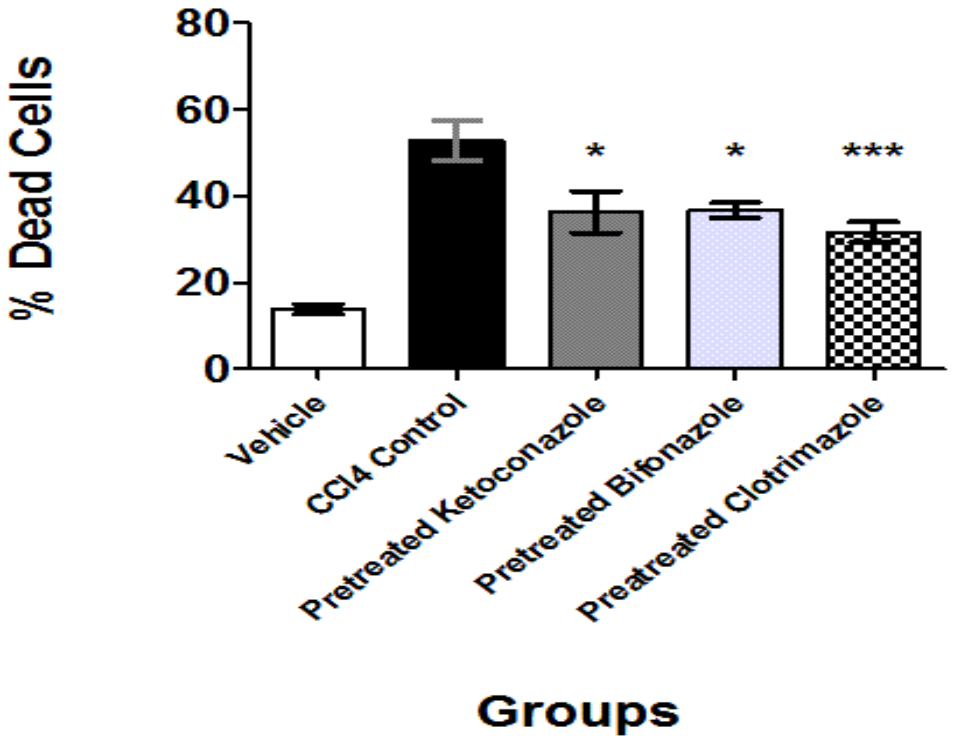

Fig.12 *Graphical representation of comparison of trypan blue assay of pretreated groups (ketoconazole, bifonazole, clotrimazole)*
Data was mean ± SEM.*p<0.05 CCl_4 control vs Pretreated ketoconazole and Pretreated bifonazole, ***p<0.001 CCl_4 control vs Pretreated clotrimazole

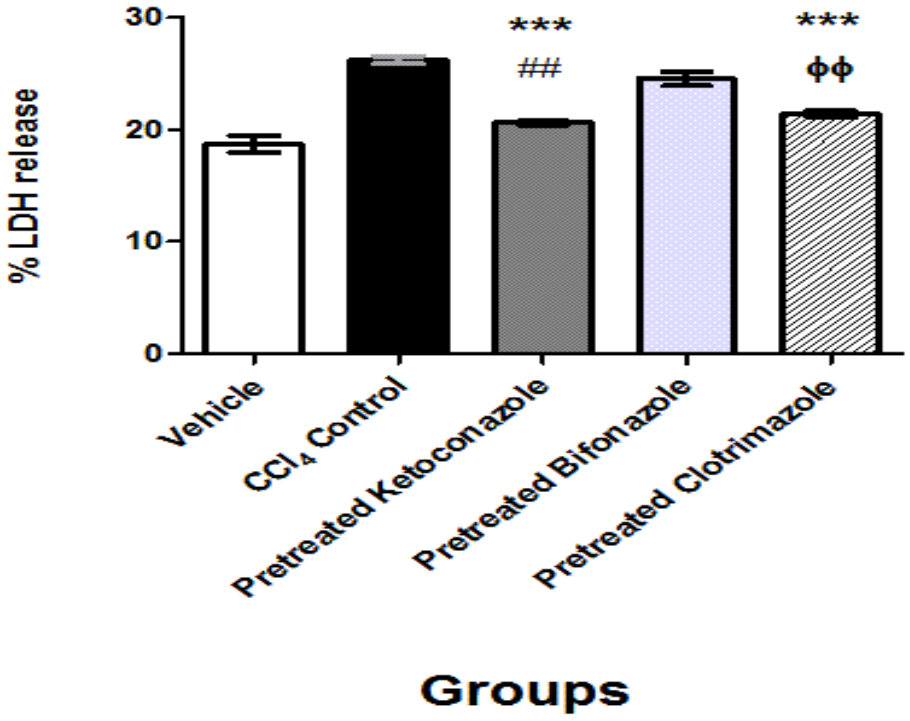

Fig.13 *Graphical representation of comparison of % LDH release of pretreated groups (ketoconazole, bifonazole, clotrimazole)*

Data was mean ± SEM, ***p<0.001 CCl_4 control vs Pretreated ketoconazole and Pretreated clotrimazole, $^{\#\#}$p<0.01 Pretreated bifonazole vs Pretreated ketoconazole, $^{\Phi\Phi}$p<0.01 Pretreated bifonazole vs Pretreated clotrimazole.

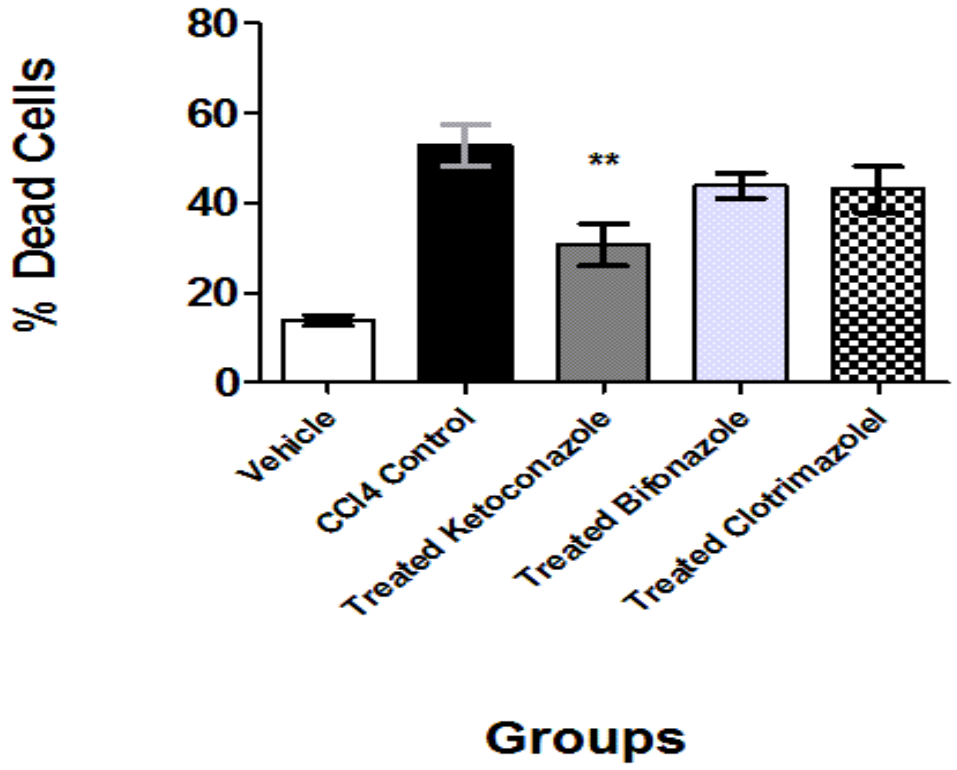

Fig.14 *Graphical representation of comparison of trypan blue assay of treated groups (ketoconazole, bifonazole, clotrimazole)*
Data was mean ± SEM. $^{**}p<0.01$ CCl_4 control vs Treated ketoconazole.

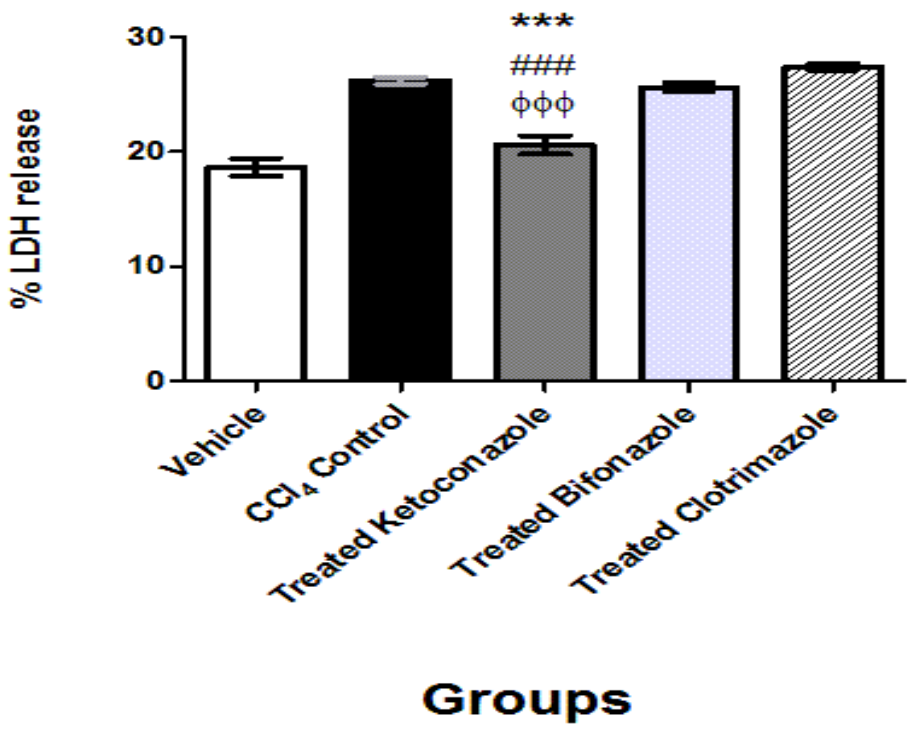

Fig.15 *Graphical representation of comparison of % LDH release of treated groups (ketoconazole, bifonazole, clotrimazole)*

Data was mean ± SEM. ***p<0.001 CCl_4 control vs Treated ketoconazole, $^{###}$p<0.001 Treated bifonazole vs Treated ketoconazole, $^{\Phi\Phi\Phi}$p<0.001 Treated clotrimazole vs Treated ketoconazole