



EVALUATION OF ANTICANCER, ANTIVIRAL AND HEPATIC PROTECTIVE ACTIVITIES OF PMF DERIVED FROM CAMEL URINE

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Abstract

Over years antiviral and anticancer drugs are continuously produced, however, no cure can be produced yet. Different studies were conducted to produce protective medicine using various natural and artificial sources but none has succeeded. This study aimed to investigate the *in vitro* anticancer and antiviral activities and the *in vivo* hepatoprotective effect of PMF derived from camel urine. The *in vitro* results showed that toxicity inducing by PMF concentration to Vero cells was significantly lower than that of hepatic cancer cell line model (HEPG-2) ($P < 0.05$). PMF had promising virucide effect as vesicular stomatitis virus (VSV) activity was completely inhibited by 75 minutes post treatment with PMF. *In vivo* study revealed that oral PMF administration for 90 days led to hepatic protective activity against carbon tetrachloride (CCl₄) induced liver damage in rats. In conclusions, PMF is a promising bio-product from camel urine showed antiviral and anticancer potentials *in vitro* on cell lines and hepatic protective effects *in vivo* in CCl₄ treated rats.

Keywords: Anticancer drug; antiviral activity, camel urine, HEPG-2, PMF, VSV.

1. Introduction

Natural products play an important role in our healthcare system (Schwartzmann, 2000; Gerhauser et al., 1997). They offer a valuable source of potent compounds with a wide variety of biological activities and novel chemical structures, many of which are important for novel antimicrobial drug development (Goldman et al., 2010; Rancovic et al., 2010). Camel urine is a physiological catabolic product; it has been used traditionally in the treatment of many diseases in Arabic countries (Alhaider et al., 2012). Amer and Al-Hendi (1996) analyzed urine of mature camels (5 - 10 years old) revealed that its relative density ranged from 1.02 to 1.07 gm/dL, pH varied between acidic and alkaline. Levels of urea ranged from 18-36 gm/dL and creatinine from 0.2 - 0.5 gm/L. Microscopic analysis proved the presence of phosphorus, calcium oxalate, ammonium urate and some epithelial and granular cells. Al-Attas and his colleagues (2008), using neutron activation analysis, estimated the amount of some essential elements within milk and urine of camels and discovered that they contain large amounts of Na and K, which can substitute for the loss of such elements in human suffering from diarrhea. PMF is a new pharmaceutical drug prepared by extracting lyophilized camel urine and was prove to have selective toxicity and anticancer substrates and to exert negligible effects on vital organs (Khorshid, 2008). The extracted fraction of PMF was found in 150 mg /g of PM 701 (lyophilized camel urine) and it was able to inhibit the proliferation of cancer cells significantly without affecting the normal cells at cell culture level (El-Shahawy et al., 2010; Khorshid, 2009; Khorshid, 2008; Moshref et al., 2006; Khorshid et al., 2005).

Cell culture and animal models play a crucial role in the development of new therapies. A novel treatment cannot be applied directly to humans. Treatments (both pharmacological and non-pharmacological) can be potentially dangerous. Cell culture can verify the mechanism of the new therapy and aid in establishing a safe human dose range (Giaever and Keese, 1986). A reliable and predictive animal model must be employed to assess the safety and efficacy of treatment

prior to its use on humans. In addition, cell culture and animal models are far less expensive than clinical studies ([Giaever and Keese, 1986](#)).

The present work aimed to evaluate both cytotoxicity and antiviral activity of PMF extract from camel urine using hepatic cancer cell line model (HEPG-2) as a cancer model and vesicular stomatitis virus (VSV) as a virus model in the *in vitro* part of the study. Also to evaluate the hepatic protective effect of PMF administration on rats treated with CCl₄ as a rat model of hepatotoxicity in the *in vivo* part of the study.

2. Material and methods

2. *In vitro* study

2.1 *Vero and Human hepatocellular carcinoma cells propagation onto cell culture*

Both African green monkey cells (Vero) and human hepatocellular carcinoma cell line (HEPG-2) (ATCC-HB-8065) were cultured in 75-cm² tissue culture flasks using EMEM and medium 199E (GIBCO-USA) supplemented with 10% fetal bovine serum (GIBCO-USA). Cell lines were maintained according to the method described by [Thomson and his colleagues \(1998\)](#) in which the cells were grown until they reached confluent monolayer. Cells were incubated with trace trypsin-EDTA in the incubator at 37°C until the cells detached from the surface. Cells were re-suspended in growth medium to the desired concentration according to cell count. The cell suspension was incubated in new culture growth media in new flasks and incubated at 37°C until confluence. The cell number in the suspension was calculated using Trypan blue exclusion assay according to [Altman and his coworkers \(1993\)](#) method.

2.2 *Vesicular stomatitis virus propagation onto cell culture*

Sterile VSV was inoculated into pre-cultured Vero cells tissue culture flask. Infected flasks were incubated at 37°C (Jouan-France) for an adsorption period of 1-1.5 hrs with gentle mixing at timed intervals of 15 min. Maintenance medium was added to infected flasks. Non-infected cell culture control was included. Cultures were incubated at 37°C and examined daily under the inverted microscope (Hund, Germany) until the initial detection of a cytopathic effect. Flasks that showed 90% cytopathic effects (CPE) were frozen and thawed three successive times for virus extraction according to [Nee and his researchers \(2011\)](#) method.

2.3 *Virus infectivity titer assessment*

The virus infectivity titer was assessed according to [Reed and Muench study \(1938\)](#) method to determine the highest dilution of the virus that produced CPE in 50% of the infected cells (TCID₅₀). VSV was 10 fold diluted in culture medium. Vero cells pre-cultured on 96 well plates (10⁴ cells/ well) were infected using the serially diluted VSV on the reciprocal wells. On confluence, the medium was decanted 100 µl/well of each dilution of the virus suspension into 8 wells (using separate tips for each dilution). Two successive columns of non-inoculated wells were maintained as a negative control. Plates were incubated at 37°C; seven days post incubation, the number of wells per each dilution showed CPE were recorded. The 50% endpoint

was determined according to the method of **Reed and Muench (1938)** as follows: 50% endpoint = (percentage of CPE of >50% – 50%) / (percentage of CPE of >50 % – percentage with CPE of <50%) X log dilution.

2.4 Evaluation of PMF cytotoxicity using MTT assay

The MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide) assay is based on the conversion of MTT into Formosan crystals by living cells that determined mitochondrial activity. Since for most cell populations, the total mitochondrial activity is related to the number of viable cells (**VanMeerloo and Kaspers, 2011**). Cellular cytotoxicity was determined using MTT assay as described by **Ueda et al. (1989)**. PMF was dissolved in EMEM, at the concentrations of 24 and 120 µg/ml. Double fold dilutions were prepared of both concentrations. Cell lines were counted as 10⁵ cells/ ml and cultured in 96-well tissue culture plates. On confluence, growth media were discarded and 100 µl of each dilution of PMF was inoculated into 8 wells of the plates. The plates were incubated at 37°C for 24 hrs. Media containing PMF of different dilutions were decanted and plates were washed with phosphate buffered saline three times. Plates were stained using MTT (0.05 ml/well) for 4 hrs. at 37° C. Developed MTT-Formazan complex was dissolved using 0.05 ml of dimethyl sulfoxide. Plates were incubated for 30 minutes at 37° C for cell lysis. Optical density was measured using ELISA reader at a wavelength of 550-570 nm. The percentage of viability was determined according to the method described by **Ho-Joon et al. (2000)** as follows:

Number of residual living cells = (OD of treated cells/ OD of untreated cells) X number of negative control cells (10⁴ cells/ 0.1ml)

Percentage viability = (Number of residual living cells / number of negative control cells) X 100.

2.5 Evaluation of anti-viral activity of PMF against VSV

The antiviral activity of PMF against VSV was determined according to the method described by **Shinji (2005)** where non-toxic concentrations of PMF was prepared in EMEM to contain 60, 30, 15 µg/ml, respectively. Vero cells were counted as 10⁵ cells/ ml and cultured in 96-well tissue culture plates. On confluence, growth media were discarded. PMF (under test for antiviral activity) were dispensed to the reciprocal wells of the precultured plates 100 µl/well. One PMF non treated plate was maintained for viral control titration. All plates were incubated at 37°C for 24 hrs. VSV was 10 fold serially diluted in EMEM (6 dilutions 10⁻² - 10⁻⁸). Growth/ treatment media were decanted; each virus dilution was inoculated at 100 µl /well. Plates were incubated at 37°C and examined daily under the inverted microscope. Three days post incubation, the virus titer was calculated in each plate. The antiviral activities were calculated by determining VSV mean titer in treated and untreated cells. The difference between both titers refers to the antiviral activity.

2.6 Virucidal activity

Virucidal activity was monitored based on the evaluation of direct contact of VSV with PMF follow by collecting samples from 37°C incubated PMF–virus mix. Collected samples which titrated the treatment test were repeated in triplicates and the mean depletion rate was calculated according to **Reed and Muench (1938)** method.

2.7. *In vivo* study

In this study 180 adult male Albino rats (weights 200- 230 grams) were used. The animals were purchased from the animal house at King Fahd medical research center (KFMRC) in King Abdul-Aziz University (KAU). "All applicable international, national, and/or institutional guidelines for the care and use of animals were followed during all experimental procedures." They were maintained on a standard pellet diet and tap water and each group were kept in a single metallic cage under a 12 hour light/dark cycle and room temperature 22-24°C in the animal house at KFMRC, KAU. Rats were acclimatized to the environment for one week prior to experimental use. Rats were divided into four equal groups (45 rats each). These groups were G1: the healthy negative control group, G2: control group of carbon tetrachloride (CCl₄) that received intra-peritoneal injection of CCl₄ (1 ml/kg b.w.) (Merck-Germany) for 2 days to induced hepatotoxicity, G3: healthy positive control group that received PMF orally (0.35 g /200 g) for 90 days, and G4: the experimental group which injected intraperitoneal with CCl₄ (1 ml/kg b.w.) in the first two days of the experiment and received PMF orally (0.35 g /200 g) for 90 days. Hepatotoxicity was induced by the intra-peritoneal injection of CCl₄ 1:1 diluted with paraffin oil in the first two days of the experiment following the method of [Khan and Alzohairy \(2011\)](#). PMF was extracted and prepared according to the method of [Khorshid et al. \(2009\)](#). The animals (control and experimental animals) were sacrificed at the end of the experiment under light diethyl ether anesthesia at fasting state.

2.8 Histopathology Examination

Light microscopic examination: The liver was excised from the animals and washed with the normal saline. About one cm piece was cut and fixed in 10% neutral formalin for 12-24 hours. It was then dehydrated and cleared in ethanol and xylene respectively followed by embedding in paraffin wax from which blocks were prepared. Sections of 5 µm thickness were prepared from the blocks using a microtome ([Talib et al., 2007](#)). These were processed in alcohol-xylene series and were stained with haematoxylin and eosin stain (H and E) ([Clayden et al., 1971](#)) using standard techniques and subjected to histopathology examination.

2.9 Statistical analysis

The data analysis was made using SPSS software for statistical analysis version 20. Data were represented as mean +/- SD. Statistical significance between treated and untreated groups was determined using one way ANOVA (LSD test). Differences at *P* values less than 0.05 were considered significant

3. Results

The toxicity of PMF was dependent upon PMF concentration. It was clear that toxicity inducing concentration of PMF to Vero cells was significantly lower than that of hepatic cancer cell line toxicity (*P*<0.05). Our data showed that the inhibitory concentration for 50% of treated cells [IC₅₀] was 144 µg for Vero cells and 90 µg for HEPG-2 cell lines (Figures 1, 2 & 3) using Masterplex Reader fit (2010) software.

Virucidal activity of PMF was monitored based on an evaluation of safe concentration that affects the virus epitopes relatively to time. Samples were collected at one hour interval but shortly after 2 hours the virus was completely inactivated. The test was repeated and samples

were collected at 15 minutes intervals where the virus was confirmed the prim test, after 2 hours the virus was completely inhibited and virus titer was significantly depleted compared to the control at the same thermal conditions ($P < 0.05$). The virus titer showed a somewhat stationary phase / plateau throughout the 15-60 minutes post treatment followed by obvious depletion of viral infectivity titer in the 2nd hour and no virus activity could be detected by 3 hours post treatment. The depletion rate of VSV relative 0times was plotted (Figure 4).

The histopathology examination of rats' livers indicated that under experimental conditions, PMF showed hepatic protective effects against CCl_4 induced liver damage in rats. Where the liver of normal control rats (Figure 5A) appeared normal compared with the infected animals with CCl_4 that treated with PMF for only 7 days (Figure 5B), which showed significant pathological changes and inflammation around central vein. However, the infected group which treated with PMF for a long time, 90 days (Figure 5D) showed light normal micrograph of rat liver compared to controls (Figure 5C).

4. Discussion

Cytotoxicity of natural products may be based on the effect of toxic materials on cell organelles including mitochondrial, nuclear and cellular membrane damage. This damage leads to breakage of cellular and mitochondrial membranes enhancing cell death and viral protein assembly ceasing in cases of viral infection. Cancer is a disease characterized by uncontrolled cellular proliferation and differentiation. Nearly all new cancer treatments have impacts on viruses; which would be advantageous. Data obtained from this study revealed that the toxicity of PMF was relative to PMF concentration. It was clear that toxicity inducing concentration of PMF to Vero cells was significantly less than that of hepatic cancer cell line toxicity. The cytotoxic effect may be attributed to HEPG-2 cells apoptotic enhancing activity under the effect of camel urine derived fraction, PMF that may enhance cell and mitochondrial membrane rupture. In our previous work ([El-Shahawy et al., 2010](#)), we recorded that lyophilized camel urine, which coded PM701, is a natural product that has anticancer, antimicrobial and antifungal effects.

The PMF has been fractionated from a PM701 sample and this fractionated sample has *in vitro* anti-carcinogenic effect on lung cancer, hepatic cancer, glioma cancer, breast cancer, colon cancer and leukemic cells ([Khorshid et al., 2011](#)). These data coincided with [Nujoud et al. \(2011\)](#) despite the use of different cell lines and their attention to immune reactivity. They also recorded that camel urine showed cytotoxicity against various human cancer cell lines, with only marginal effects on non-tumorigenic epithelial and fibroblast cells. Interestingly, 216 mg/ml of lyophilized camel urine (CU) inhibited cell proliferation and triggered more than 80% of apoptosis in different cancer cells, including breast carcinomas and medulloblastomas ([Nujoud et al., 2011](#)). Apoptosis was induced in these cells through the intrinsic pathway via a decrease in Bcl-2. Furthermore, CU down-regulated the cancer-promoting proteins surviving, β -catenin and cyclin D1 and increased cyclin-dependent kinase inhibitor p21 level. In addition, [Nujoud et al. \(2011\)](#) had shown that CU has no cytotoxic effects against peripheral blood mononuclear cells and had strong immune-inducer activity by inducing interferon (IFN)- γ and inhibiting the Th2 cytokines interleukin (IL)-4, IL-6, and IL-10. The results obtained by this study were also consistent with [Al-Harbi et al. \(1996\)](#) who showed that CU treatment caused a significant cytotoxic effect in the bone marrow cells of mice and that cytotoxicity appeared at higher doses comparable with cyclophosphamide (CP), standard drug. Meanwhile, unlike CP, the camel urine treatment failed

to induce any clastogenicity. Also, they reported that the cytotoxicity induced by camel urine treatment was substantiated by the reduction of liver nucleic acids and glutathione levels and increased malondialdehyde (MDA) contents in the same animals. CP treatment was found to have high clastogenic, cytotoxic and able to reduce the levels of nucleic acids, proteins, and glutathione and increased MDA concentration due to its pro-oxidant activity. [Al-Harbi et al. \(1996\)](#) claimed the non-clastogenic nature of camel urine to the anti-oxidant and anti-mutagenic compounds present in it. They stated that the pre-treatment with camel urine increased the cytotoxicity of CP and intensified CP induced reduction of liver nucleic acids, and glutathione and increased MDA concentration. The increase of CP induced cytotoxicity appeared to be partly due to the additive effect of the two treatments on cellular lipid peroxidation. All these studies may also apply to our work here as antiviral activity of camel urine fraction, PMF.

Urine constituents showed a variable reactivity to cancer cell lines / apoptotic activity relative to age and sex of camels ([Alghamdi and Khorshid, 2012](#)). At the same time, [Alhaider et al. \(2012\)](#) recorded that all types of camel urine differentially inhibited the induction of Cyp1a1 gene expression by TCDD, the most potent Cyp1a1 inducer known as a carcinogenic chemical. They found that virgin camel urine showed the highest degree of inhibition at the activity level, followed by lactating and pregnant camel urines. Furthermore, they have shown that virgin camel urine significantly inhibited TCDD-mediated induction of Cyp1a1 at mRNA and protein expression levels. The ability of virgin camel urine to inhibit Cyp1a1 was strongly correlated with its ability to inhibit AhR-dependent luciferase activity and DNA binding as determined by EMSA, suggesting that AhR-dependent mechanism is involved. [Alhaider et al. \(2012\)](#) study provided the first evidence that camel urine inhibited the TCDD-mediated toxic effect by inhibiting the expression of Cyp1a1, at both transcriptional and post-transcriptional levels through an AhR-dependent mechanism. This agrees with our data that camel urine proved its anticancer activity based on its toxic effect on human hepatoma cell line and on VSV. Safe concentrations of PMF on VSV could not be proven where virus infectivity titer showed no change 24 hours after on pretreated cells infection compared to virus control infectivity titer.

Our results revealed that VSV showed a significant depletion rate in virus infectivity titer 15 minutes post-treatment with PMF while an almost stationary phase was detected through 30 - 45 minutes mark followed by a sharp declining phase of virus infectivity titer. VSV could not be detected at 75 minutes post-treatment recording depletion rate of VSV in the order of 0.49 log₁₀/15 minutes. Finally, 75 minutes post-treatment, the virus could not be detected compared to the control. Antiviral activity may be attributed to the lysozyme enzyme found in urine that enhances its antimicrobial activity in general and it may work on the viral epitopes denaturation preventing the adhesion potential of the virus to cell receptors. Our data were in agreement with [El-Shahawy et al. \(2010\)](#) results who reported that the PMF has an antimicrobial activity which in the present study showed significantly inhibit VSV virus infectivity titer at 60 minutes. PMF may affect viral epitopes integration due to its chemical structure which affects virus replication enzymes.

The histopathology examination of rats' liver indicated that under present experimental conditions, PMF had hepatic protective effects against CCl₄ induced liver damage in rats. The infected group which treated with PMF for 90 days showed almost normal micrograph of rats' liver compared to controls.

At last, the promising fast antiviral activity must be enhanced using a wide range of virus models with DNA, RNA, and cancer inducing viruses. We need to trace the antiviral activity expressed by gene profiles namely MX and 2, 5 oligoadenylatesynthetase, as marker genes for antiviral activity, *In-vitro* and *in-vivo* studies for intensified evaluation of PMF must be done.

5. Conclusion

PMF is a promising bio product from camel urine that has anticancer and antiviral potentials. Moreover, our *in vivo* study results revealed hepato-protective effects of PMF of rats' liver treated with carbon tetrachloride as a model of hepatotoxicity which increased the demand for further investigations and more studies concerning gene expression profile to prove the anti apoptotic activity of PMF to explain the mechanism of its anti-cancer action.

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Competing interests:

The authors declare that no competing interests at this moment.

Authors contributions:

FatenKhorshid: The principle investigator that was conducted this project conceived and designed the study and revised manuscript for important intellectual content. She acts as guarantor of the study; SnaaGhazy Al-Attas and NagwaTawfik: shared in design the study and collection of data; Yossery A Elsourojysupplied the virus and share in writing the manuscript, Samah Noor: revised inputs regarding the design and drafted the manuscript and RandaAlshehri: made the *in vivo* part of the experiment. The final manuscript was approved by all authors.

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Figure legends:

Figure 1 Evaluation of viability of Vero cell line post treatment with PMF relatively to concentration using MTT assay (IC₅₀ = 144 μgm).

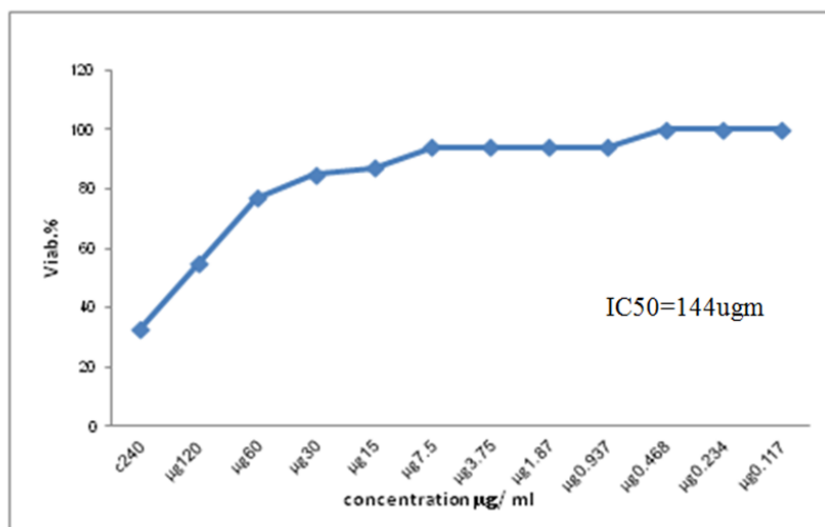


Figure 1

Figure 2 Evaluation of viability of hepatocellular carcinoma cell line (HEPG-2) post treatment with PMF relatively to concentration using MTT assay (IC₅₀ = 90 μgm).

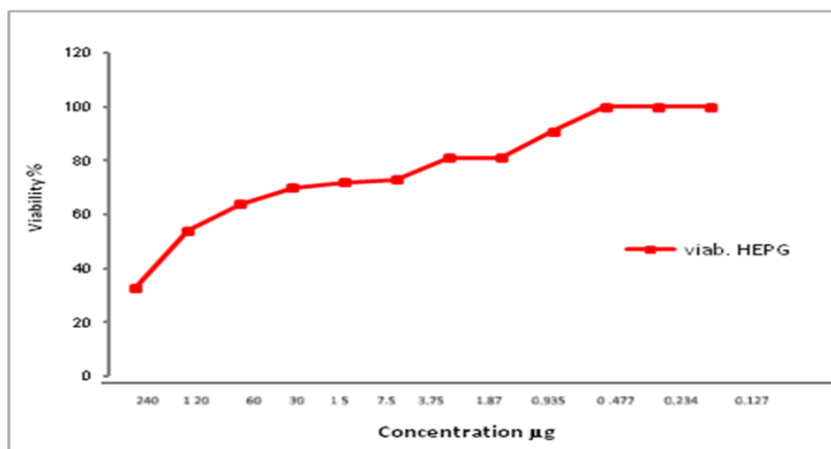


Figure 2

Figure 3 Evaluation of vesicular stomatitis virus (VSV) titer post treatment at 37° C comparing with negative control.

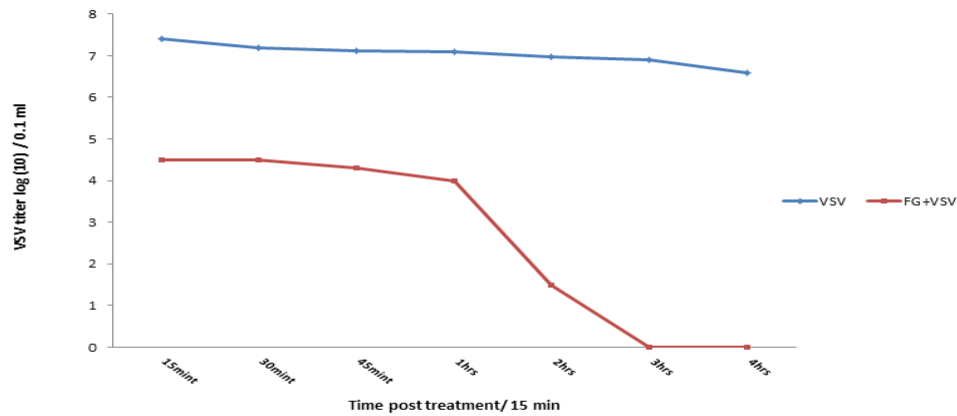


Figure 3

Figure 4 Comparative evaluation of virucidal activity of PMF to vesicular stomatitis virus (VSV) relatively to time.

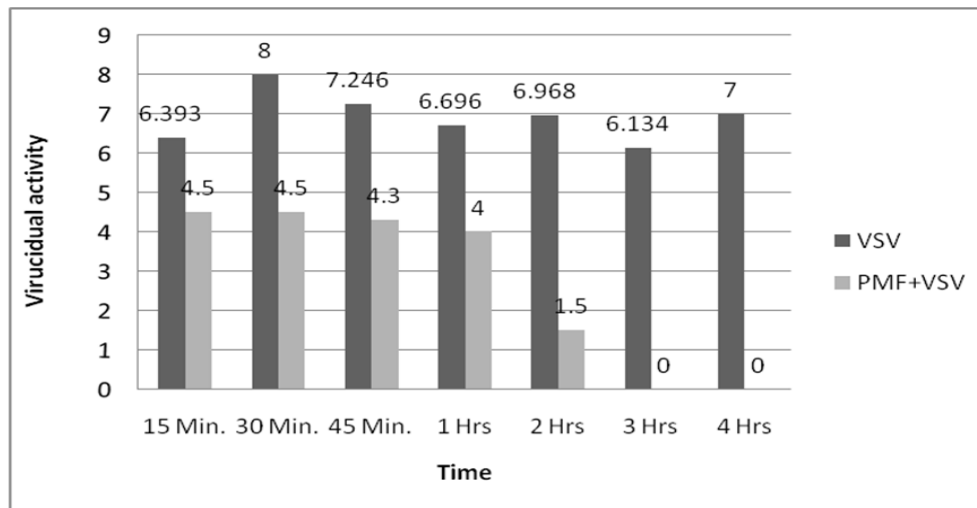


Figure 4

Figure 5 Light microscope images of haematoxyline-eosin stained photographs of Rat liver. (A) Negative control rats after 7 days showed normal central vein (C.V), (B) Infected group treated with PMF (after 7 days) showed change of C.V, (C) Negative control rats after 90 days showed normal C.V, (D) Infected group treated with PMF after 7 days showed fewer changes in C.V area.

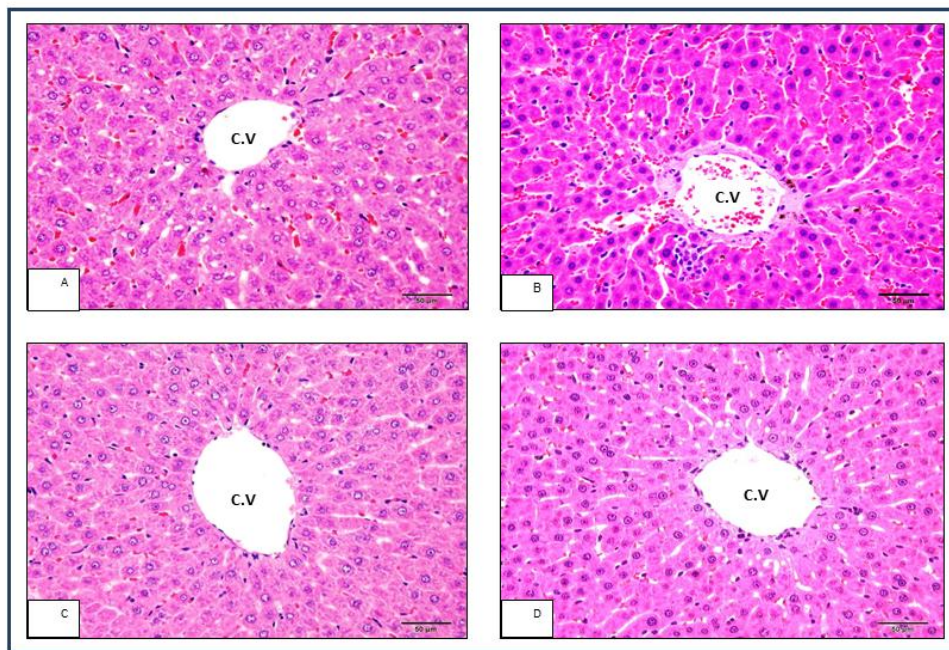


Figure 5