The Intervention of the Pirfenidone with Pericyst Layer Building of the Hydatid'' Cyst

Yousif Tawfeeq*[1], Ahmed A. Mohammed[2], Yassir Mustafa Kamal[1]

[1]Branch of Pharmacology & Toxicology, [2] Branch of Clinical Laboratory Sciences, College of Pharmacy, Mustansiriyah University, Baghdad-Iraq
Correspondence Author: Darkknight_1945@yahoo.com

ABSTRACT

Hydatid disease is a zoonosis caused by *Echinococcus granulosus* larval stage, characterized by developing a fluid-filled hydatid cyst in any part in the body, with a particular liver and lung localization. The released hydatid cyst fluid (HCF) antigens actively induce epithelial to mesenchymal transition (EMT) in the epithelial cells surrounding the mass of the hydatid cyst, result in pericyst layer construction, which plays a vital role in preventing the antiparasitic medications from being reached to the parasite. Therefore, the current study attempted to employ the antifibrotic effect of the Pirfenidone to hinder the induced EMT by HCF antigens. For this reason, the adenocarcinomic human alveolar basal epithelial cells (A549 cell line) was used as a model system to human alveolar epithelial cells type II (AEC II) to assess the effect of the Pirfenidone on the induced EMT by HCF antigens. Different assays used to investigate the EMT induction, then Pirfenidone effect assessed using immunofluorescence assay to detect cytoskeletal markers expression prior to and after treatment. The results reached that the Pirfenidone interrupting the induced EMT in A549 cells, and thus, it may interrupt the EMT and pericyst fibrosis in the same way in the infected tissues, which would facilitate the antiparasitic drugs delivery across the cyst wall and help in parasite killing.

INTRODUCTION

Unilocular hydatid disease or Cystic *Echinococcosis* (CE) is a chronic and complex zoonotic infection caused by the metacestode (the hydatid cyst) of the tapeworm *Echinococcus granulosus*. Although CE has global importance due to the social and economic loss associated with human treatment, it has been recognized as one of 18 neglected tropical diseases as recorded by the WHO [1-3]. Hydatid cysts can infect any organ in the intermediate host (domestic livestock or humans), with particular liver and lung localization. Human ingestion of parasite eggs contaminating his food is the prime source of infection, where following their hatching, they release the oncospheres which develop to hydatid cysts in the infected internal organs [4].

The hydatid cyst is a unilocular chamber filled with a complex fluid called hydatid cyst fluid (HCF), composed of many soluble substances such as proteins, carbohydrates and salts. In addition, HCF contains both host and parasite macromolecules such as human albumin, antigen 5 (Ag5) and antigen B (AgB) [5]. HCF is enclosed by the cyst wall, which composed of an inner thin germinal layer (GL) surrounded by acellular laminated layer (LL) with variable thickness and surrounded by a fibrous outer layer (pericyst), with the later represents the first mechanical defense line of the metacestode against the host immune elements [5-8]. The proteins secreted by the protoscoleces which are develop inside the cyst are thought to play fundamental roles in the complex host-parasite relationship, especially in the fibrous layer formation around the cyst, which plays an important role in this relationship [9-12].

The fibrous pericyst layer is clearly a defense mechanism by which the host is trying to control the parasitic infection and restrict its development. However, it could be a protective mechanism to the parasite, which will act as an additional barrier to counteract the damaging effect of the host immune mechanisms [5]. Additionally, it prevents the medications from the penetration via the cyst wall, which unfortunately reduces the effectiveness of these medicines and force a longer duration for the treatment when using them.

Fibrogenesis and collagen accumulation can be initiated and regulated in tissues by different mechanisms. In lungs, for example, fibrosis could involve the alveolar epithelial cells type II (AECII), which, under specific conditions, can undergo an epithelial to mesenchymal transition (EMT) [13-15]. Although the underlying mechanisms remain unclear, transforming growth factor- β (TGF- β) and fibroblast growth factor- 2 (FGF- 2) constitute the main EMT- inducing factors in many types of fibrosis [16,17].

It has been found that EMT plays an important role in fibrous layer formation around the hydatid cyst. The EMT-like changes resulting from the exposure to the parasite-derived molecules present in the HCF have been studied in the lung epithelial cell line A549[5,18], where previous studies showed that these cells exhibiting EMT-like changes, similar to those observed in normal alveolar epithelial cells type II [5].

Pirfenidone is an antifibrotic agent that has been approved by many medical organizations for the treatment of idiopathic pulmonary fibrosis (IPF) [19]. Randomized control of clinical trials has demonstrated that the Pirfenidone suppresses the deterioration of the percentage forced vital capacity (FVC), with manageable toxicity in the patients with IPF [20]. In addition, the Pirfenidone has been found suppresses the lung fibrosis by downregulating the TGF-β, platelets- derived growth factor (PDGF) and collagen synthesis in a hamster model. Moreover, the Pirfenidone found to be suppressing the liver fibrosis by downregulating the TGF - β and may be useful to suppress or revert the EMT too [19-22]. Therefore, this study aimed to assess the antifibrotic properties of the Pirfenidone in the corruption of the induced EMT by

HCF antigens *in vitro*, as this method would help in the prevention of pericyst layer building around the hydatid cyst and facilitate the penetration of the suitable scolicidal medicines to the inside of the hydatid cyst and avoid the surgical choice.

MATERIAL AND METHODS
Collection of HCF samples
Hydatid cysts were directly collected from the patients after cystectomy from Baghdad general hospital, Baghdad-Iraq. All HCF samples were collected aseptically, filtered and stored at -20°C until use. Samples antigenicity were assessed by ELISA against positive human sera, the highest reacting samples (9 samples) were pooled together to be used in the cell culture work to induce the EMT. The total proteins concentration of the pooled samples was quantified with the Bio-Rad colourimetric assay according to the manufacturer's instructions. All samples were checked for the presence of bacterial endotoxins using the ToxinSensorTM Chromogenic LAL Endotoxin Assay Kit (GenScript/ Netherland).

Cells culture
The A549 cells (ATCC, Manassas, VA, USA) were seeded at a density of 4×10^4 cell/well in six well plate (JETBIOFIL/ China), maintained in RPMI-1640 medium (Euroclone SPA/ Italy) supplemented with 10% fetal bovine serum (FBS Biowest/ USA), 1% L-glutamine and 1% penicillin-streptomycin-Fungizone (Gibco/ ThermoFisher/ UK) which described as a complete medium (CM). Cells incubated in 37°C incubator (Memmert/ Germany) in a humidified atmosphere with 5% CO_2. In the experiments, the CM changed when necessary to serum-limited medium termed as (SLM) which containing 0.5% FBS instead.

Investigating the effect of the Pirfenidone and HCF on cell proliferation
To investigate the effect of the Pirfenidone on A549 cell proliferation in the presence of HCF effect, cells were seeded at a density of 4×10^4 cells/well in six well plates, maintained in RPMI-1640 SLM and incubated in 37°C in a humidified 5% CO_2 environment to allow cells adherence. Seeded cells then divided into four sets of triplicates. The old medium replaced with a new one where the first group treated with 2mM Pirfenidone alone, the second group treated with 2mM Pirfenidone plus 10% pooled HCF (protein concentration 32µg/mL), the third group treated with 10% pooled HCF alone (the same protein concentration) whereas the fourth group left without treatment as a control and provided with only SLM. All the groups then incubated for 120hr, and their medium changed each 24hr with exactly the same medium components in each group. Cell proliferation was monitored each 24hr using the direct microscopic examination and the growth curve based on crystal violet staining colorimetry according to (Chiba *et al*,1998) with minor modification.

Prior to using the Pirfenidone in this experiment, the Pirfenidone (Cipla) was firstly dissolved in a deionized distilled water at 1M concentration. The toxic concentration on cells was determined in a separate experiment using the MTT assay (data not shown) according to Al-Sudani *et al*. (2019). The suitable Pirfenidone concentration for use was 2mM which was prepared by diluting the stock solution in RPMI-1640 SLM.

Investigating the effect of the Pirfenidone and HCF on cell migration
Migration of A549 cells was followed up using wound healing assay according to Mohammed (2017). When seeded cells confluence reached approximately 80%, all the wells washed up with SLM and a longitudinal scratch was made in the middle of each well using a 100µL micropipette tip, then cells were washed once more and divided into four groups treated as mentioned in section 2.3 above and incubated for 48hr. The wound area was monitored using an inverted microscope after 24 and 48 hrs and wound closure was assessed. The distance between the wound edges was measured at 5 points along the wound with equal distance between each two points (6mm).

Investigating the Pirfenidone effect on the induced EMT by the HCF
To induce the EMT in the A549 cells, cells were initially seeded at a density of 1×10^3 cell/well in 24 well plates and maintained in RPMI-1640 SLM. Four plates were used to investigate the expression of the epithelial markers (E-cadherin and Cytokeratin) and mesenchymal markers (Fibronectin and Vimentin) according to Mohammed *et al.* (2018). Each plate divided into four groups each with three wells, the first group treated with 5ng/mL TGF-β1 (Sigma-Aldrich/USA) which is known as EMT stimulator, the second group treated with 10% pooled HCF (protein concentration 3.2×10^4 ng/mL), the third group treated with 10% pooled HCF alone (the same protein concentration), but after 120hr incubation, a 2mM Pirfenidone was added to the old medium contents of this group until the end of the experiment (extra 120 hr), whereas the fourth group left untreated, used as a control and provided with only SLM. The medium in each group replaced with a new identical medium each 24hr. All the four groups were treated with the above described medium until the end of the experiment.

Evaluating the induced EMT and Pirfenidone effect by the immunocytochemistry
The induced EMT assessed using the immunocytochemistry (ICC) and immunofluorescence microscopy to detect the expression of the cytoskeletal markers E-cadherin, cytokeratin, fibronectin and vimentin according to Mohammed (2017) protocols. Cells were initially washed in PBS 2 times for 3 min, then fixed with 4% formaldehyde (Cell Path/ USA) for 5 minutes, followed by 2 washes with PBS (Euroclone SPA/ Italy). In order to increase the marker penetration to cells, 1% Triton X-100 (Bio Basic Inc/ Canada) was added for 5 min followed by 3 washes with PBS for 5 minutes each time. To block the unspecific binding sites, plates were incubated with 3% BSA for 1 hr at room temperature, then 1:1000 diluted mouse anti-human E-cadherin (Clone HECD-1, R&D, USA) produced in mouse was added to the plate and incubated in the fridge overnight in a wet tray. In the next day, the plate was washed 3 times with PBS, then incubated with 1:2000 Alexa Fluor 546 goat anti-mouse IgG (Invitrogen/ Thermo Fisher, UK) for 2 hr in the dark, followed by last wash with PBS, 3 times 5 min each, and finally mounted in 10µl mounting medium containing DAPI (Invitrogen/ Thermo Fisher/ UK) for 1 hr to stain cell nuclei then wells covered with coverslips. The plate was examined using a Leica M165 FC microscope system (Leica Ltd) and Image software (Leica Application Suite (LAS) X).

The rest groups were treated similarly, with exception of using 0.5% triton, and the marker used were either mouse anti-human pan cytokeratin (clone C-11, Invitrogen/ ThermoFisher, UK), or mouse anti-human

fibronectin (clone IST-9, Invitrogen/ ThermoFisher, UK), or mouse anti-human vimentin (Invitrogen/ ThermoFisher, UK). The EMT markers expression was quantified by the analysis of images at 6 fields in each well under the same image exposure. The position of fields was chosen from a predefined grid pattern determined for all slides using the x-y coordinates on the microscope stage. Cells were counted within each spot area, then the percentage of the average total number of positively labeled cells (cells expressing a certain marker) were calculated in each group. The counts were performed at 6 points in each well for 3 replicate wells.

statistical analysis

A one-way ANOVA (IBM SPSS Statistics 20) was carried out for all treatments and all markers and also to compare all markers between HCF-treated and control wells, and between HCF treated and TGF-β1 treated cells. In all cases, values of ($P < 0.05$) were considered statistically significant.

RESULTS

The effect of the Pirfenidone and HCF on cell proliferation

The results disclosed that the Pirfenidone inhibiting the inducing effect of the HCF on A549 cell proliferation. After 48 hr treatment with Pirfenidone, cell proliferation was markedly reduced in the group treated with the Pirfenidone plus HCF compared to the group treated with HCF alone. Interestingly, the inhibitory effect of the Pirfenidone on the inducing effect of the HCF on cell proliferation was increased remarkably after 120hr incubation in the same group compared to the group

treated with HCF alone, where cell proliferation highly significantly reduced ($p \leq 0.01$) after 120 hr incubation with 2mM Pirfenidone. Whereas after 72hr incubation, there was insignificant difference ($p \geq 0.05$) in cell proliferation between the group of the Pirfenidone plus HCF and the control group, and also with the group treated with the Pirfenidone alone, and the same results observed after 120 hr incubation in the same groups. Moreover, there was a highly significant difference between the group treated with HCF alone and the control ($P \leq 0.01$), and insignificant difference ($p \geq 0.05$) between the control group and the group treated with Pirfenidone alone as shown in Figure 1.

The effect of the Pirfenidone and HCF on cell migration

The results of this test showed that there is a significant difference ($p \leq 0.05$) in cell migration between the group treated with the Pirfenidone plus HCF and the group treated with HCF alone after 24 hr incubation, and it was highly significant ($p \leq 0.001$) after 48 hr incubation. On the other hand, there was insignificant difference ($p \geq 0.05$) in cell migration in the group treated with Pirfenidone plus HCF compared to the control after 24hr and 48hr incubation. Additionally, a significantly reduced cell migration ($p \leq 0.05$) observed in the group treated with the Pirfenidone alone compared to the group treated with HCF alone. Moreover, it has been found that the gap between the wound edges in the group treated with the Pirfenidone alone was insignificantly different from the control during the 48hr incubation ($p \geq 0.05$), as it is clear in Figure 2.
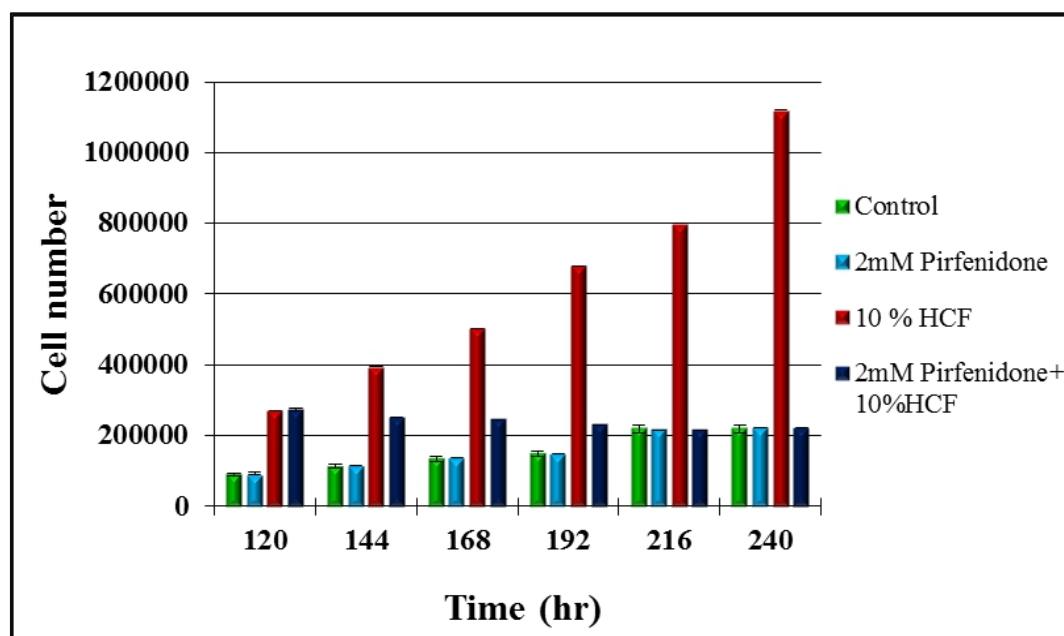

Figure 1: Illustrates the difference in cell proliferation in all treated groups after 120hr incubation followed by Pirfenidone treatment. The results represent the mean ± SEM of three replicates.

The Pirfenidone effect on the EMT induced by the HCF

The result of this experiment disclosed the prominent role of the Pirfenidone in the interruption of the EMT changes induced by the HCF. Evident elongation has been observed in the cells treated with the HCF and TGF-β1 compared to the control cells, whereas cells treated with the Pirfenidone plus HCF showed unremarkable morphological changes compared to the control group, Figure 3.

The ICC work clarified the projected differences in cytoskeletal markers expression associated with the EMT changes in the cells of the four groups of treatments. Interestingly, these markers expression were parallel with the morphological changes in these cells. The E-cadherin expressions was significantly different ($p \leq 0.05$) between the group of cells treated with the Pirfenidone plus HCF and the group trated with HCF alone, with markedly reduced expression in the marker has been

observed in the cells treated with HCF alone. In addition, the cells of the control group and the group treated with Pirfenidone plus HCF exhibited a similar high percentage of E-cadhrin marker expression in their cells, 83% and 80% respectively, with insignifacnt difference statistically ($p \geq 0.05$) between the two groups. While the group treated with the TGF-β1 showed 12% percentage of E-cadhrin expression in its cells, which was the lowest level among the groups. However, this level of expression was insignficantly different ($p \geq 0.05$) compared to the cells treated with HCF alone which was 15%, Figure 4.
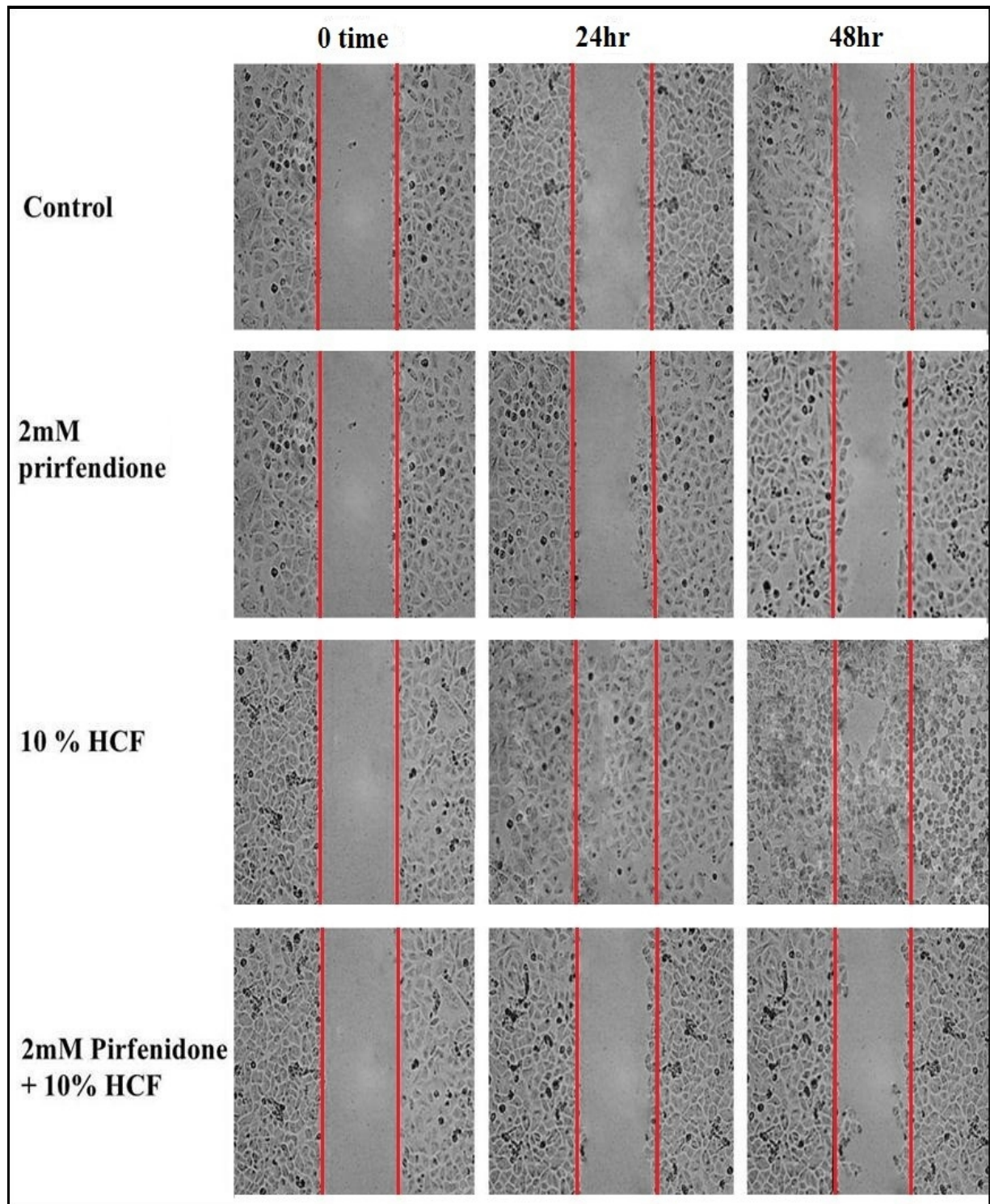

Figure 2: Shows the results of wound healing assay and the effect of the Pirfenidone in the inhibition of the inducing effect of HCF on cell migration after 0, 24 and 48 hours of incubation. The lines represent the wound edges (×100). (a) the control group, untreated with neither Pirfenidone nor HCF. (b) the group treated with 2mM Pirfenidone alone. (c) the group treated with (18 µg/mL) pooled HCF alone, and (d) the group treated with 2mM Pirfenidone plus pooled HCF, (X100).
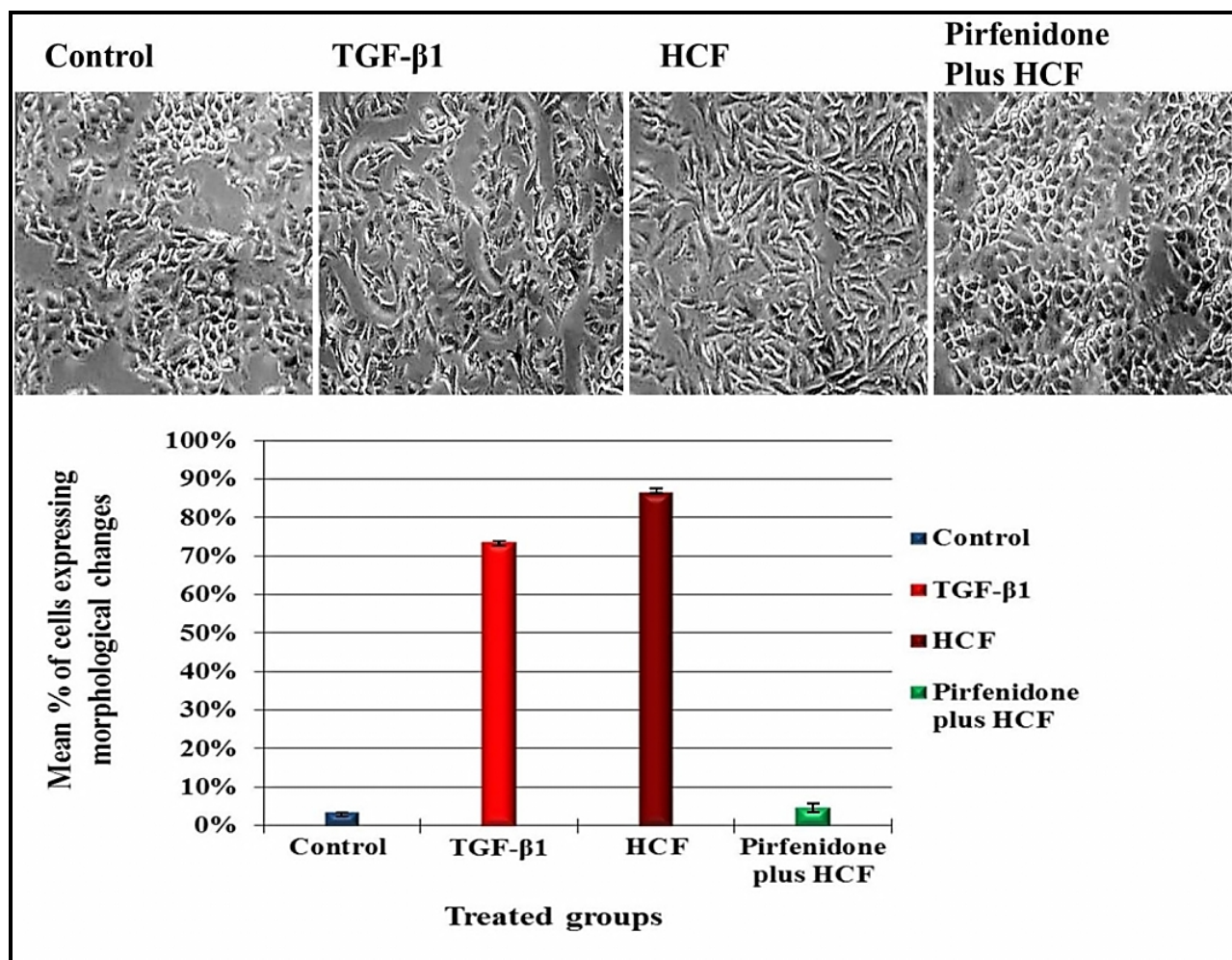
Figure 3: Shows the inhibiting effect of the Pirfenidone to the EMT changes induced by the HCF and the morphological changes of A549 cells under the inverted microscope. (a) the control group, untreated with neither Pirfenidone nor HCF. (b) the group treated with 5ng/ mL TGF-β1. (c) the group treated with 10% pooled HCF alone (protein concentration 5.7×10^4 ng/mL), and (d) the group treated with 2mM Pirfenidone plus 10% pooled HCF, (X200).

The percentages of cytokeratin expression was nearly the same in the cells of all the four groups. Statistically, there was insignificant difference ($p \geq 0.05$) in the percentage of this marker expression between the cells of the control group 97.5% and the cells treated with HCF 83% alone, and also between the cells treated with TGF-β1 78.5% and cells treated with Pirfenidone plus HCF 97.8%. However, the marker expression was perinuclear in position, yet, a little extensive expression has been observed in some cells in the group treated with HCF and TGF-β1, whereas the cells in the other two groups showed a more extensive pattern of expression, as shown in Figure 5.

On the other hand, the expression of fibronectin was significantly down regulated ($p \leq 0.05$) in the cells treated with the Pirfenidone plus HCF where the percentage of this marker expression was 15% in this group compared to its percentage in the cells treated with the HCF 79%, and the cells treated with TGF-β1 72%. Nevertheless, there was insignificant difference ($p \geq 0.05$) in the percentage of matker expression between the cells treated with the Pirfendone plus HCF and the control group which was 10%. Additionally, the difference between the cells treated with HCF alone and cells treated with TGF-β1 was insignificantly different ($p \geq 0.05$), Figure 6.

Regarding the vimentin expression, all the groups showed nearly the same percentage of marker expression. However, different patterns of marker expression have been observed in the cells of all the groups, where marker expression was different in the intensity and distribution manner as shown in Figure 7. The group treated with the Pirfenidone plus HCF and the control group had the same manner of expression and intensity of vimentin expression which was concentrated in the perinuclear area. While HCF treated cells showed a scattered pattern of expression in many cells similar to the pattern of TGF-β1 treated cells, and sometimes in perinuclear vesicles similar to the control group. Statistically, there was insignificant difference ($p \geq 0.05$) in marker expression between the group treated with the Pirfenidone plus HCF and the control group, and insignificant differences ($p \geq 0.05$) among the group treated with the Pirfenidone plus HCF, HCF alone and TGF-β1.
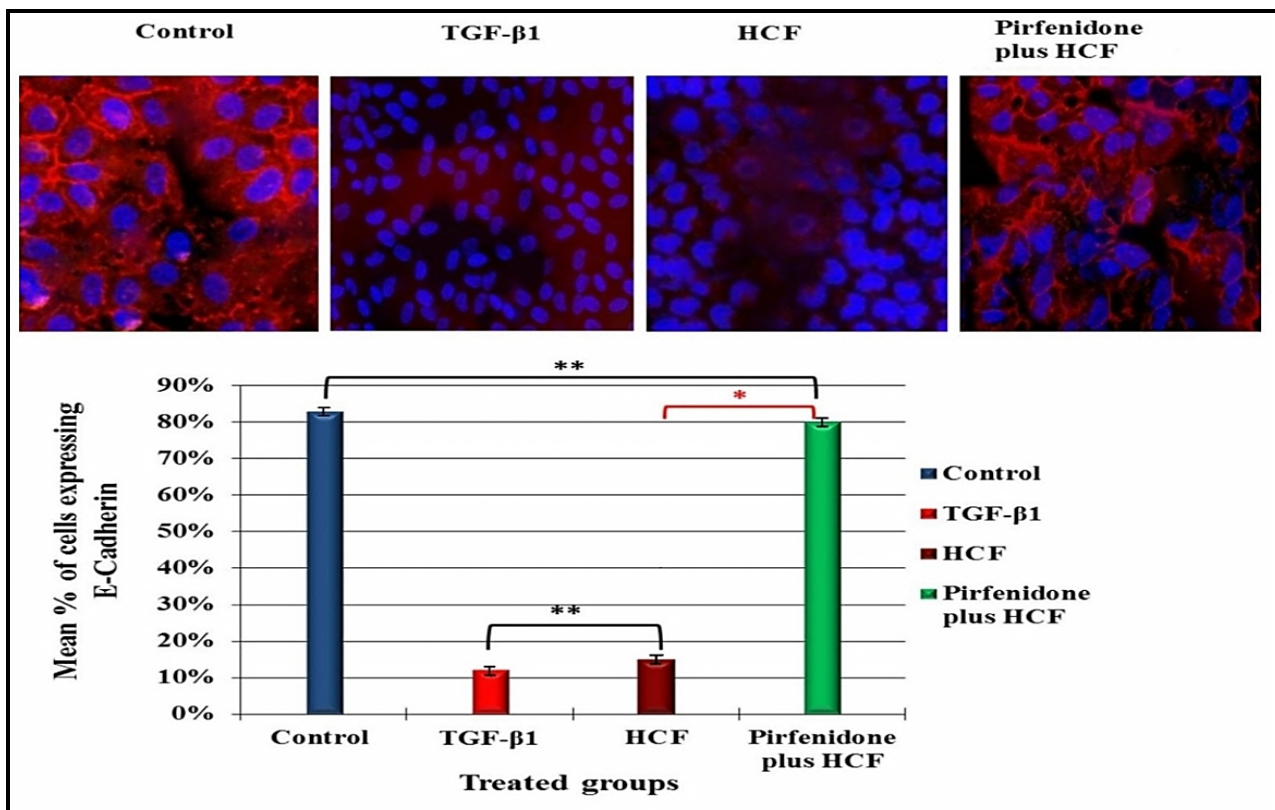
Figure 4: Merged DAPI/ immunofluorescence, E-Cadherin expression in A549 cells treated with HCF, TGF-β1, Pirfenidone plus HCF and controls (SLM alone), original magnification ×400. Cell counts represent the mean ± SEM of 3 replicate wells. Cell counts indicate significantly less cells expressing E-cadherin in the group treated with HCF alone.

*significant differences ($p \leq 0.05$) between HCF treated cells compared to controls.
**insignificant differences ($p \geq 0.05$) between Pirfenidone plus HCF treated cells and controls.
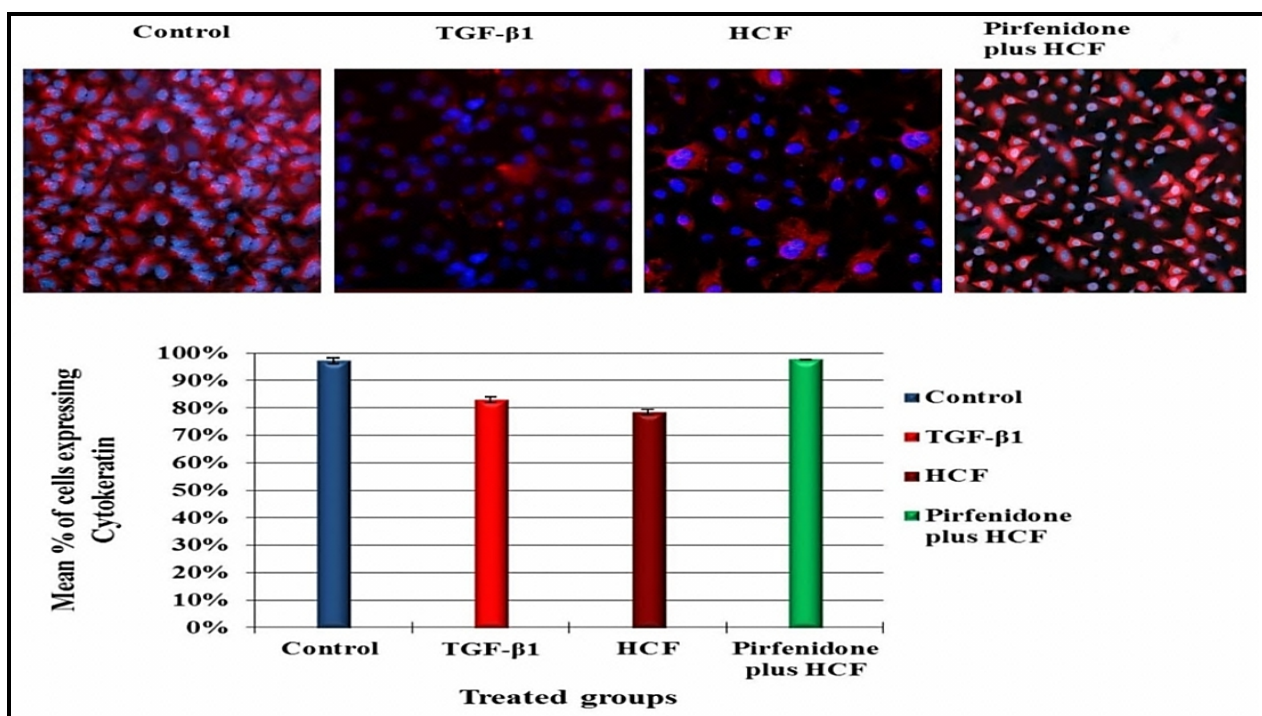

Figure 5: Merged DAPI/ immunofluorescence, Cytokeratin expression in A549 cells treated with HCF, TGF-β1, Pirfenidone plus HCF and controls (SLM alone), original magnification ×400. Cell counts represent the mean ± SEM of 3 replicate wells. Cell counts indicate insignificant difference ($P \geq 0.05$) in the percentage of cells expressing cytokeratin in all groups. The marker expression was perinuclear in position with a little extensive expression observed in some cells treated with HCF and TGF-β1, whereas cells treated with Pirfenidone plus HCF and controls showed more extensive pattern of expression.
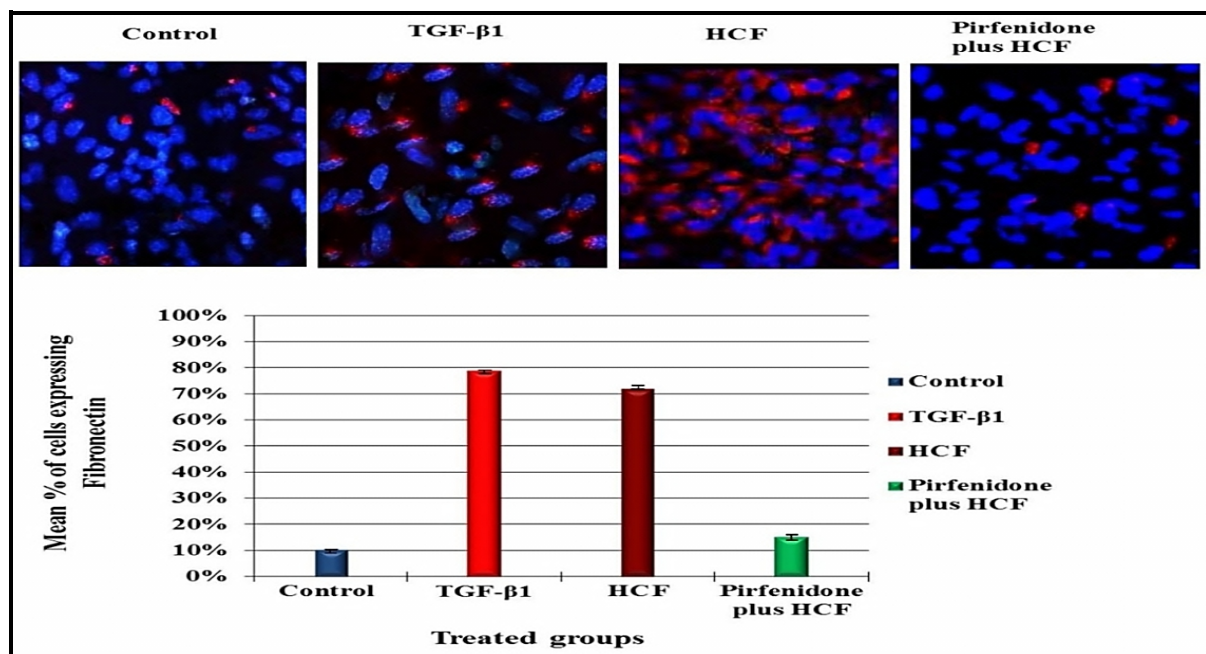
Figure 6: Shows fibronectin expression in A549 cells in the groups treated with TGF-β1, HCF, Pirfenidone plus HCF and controls (SLM alone). The expression of fibronectin was significantly down regulated ($p \leq 0.05$) in the cells treated with the Pirfenidone plus HCF and the controls compared to the cells treated with HCF alone or with TGF-β1. Whereas the difference in marker expression in the cells treated with HCF alone and cells treated with TGF-β1 was statistically insignificant ($p \geq 0.05$), (X400).
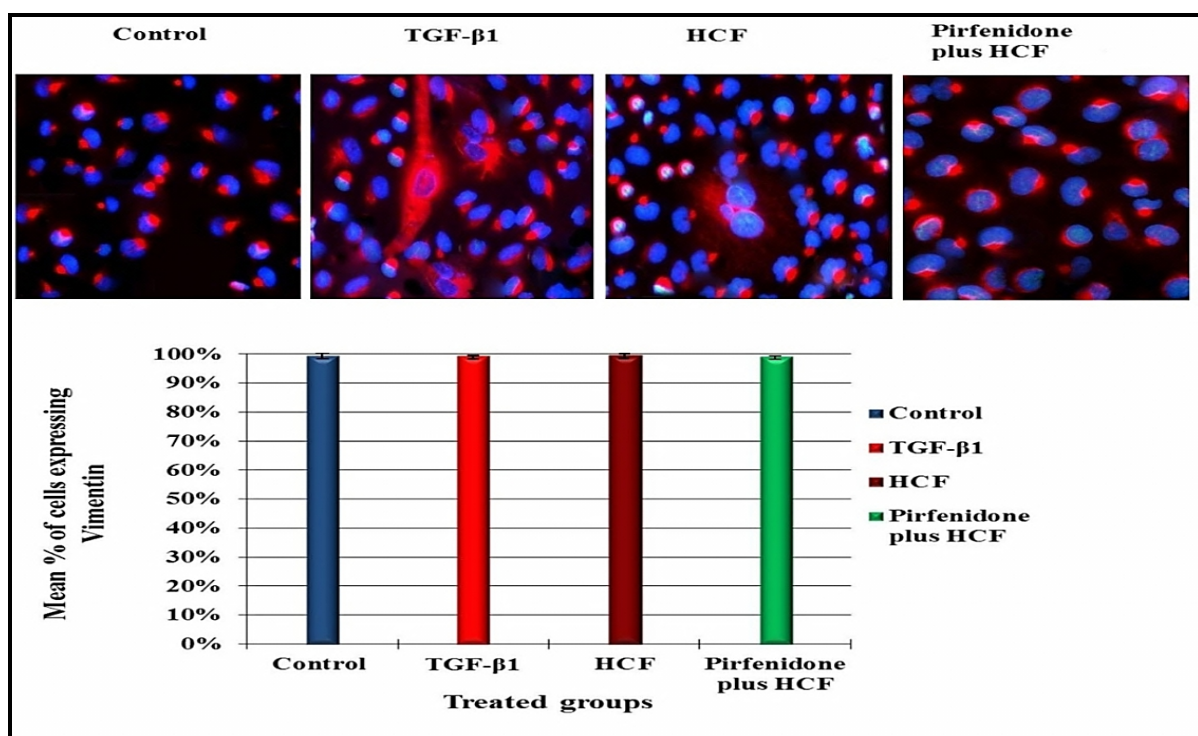

Figure 7: Shows vimentin expression in cells treated with TGF-β1, HCF, Pirfenidone plus HCF and controls (SLM alone). All groups showed the same level of marker expression but in different intensity and manner in each group. The marker expression in cells treated with Pirfenidone plus HCF was in perinuclear vesicle as in the control group, while in the cells treated with HCF alone and TGF-β1 many cells showed more disperse vimentin expression in addition to the perinuclear expression, X400.

DISCUSSION

According to the previous studies, the Pirfenidone proved to be useful in the interfering with fibrosis and EMT changes in different ways. For instance, it has been reported that the Pirfenidone suppresses the EMT of cells in the IPF and lung cancer, where it is inhibiting the TGF-β signals which in turn, inhibiting the myofibroblast differentiation in the lung fibroblast cells [24-26], and suppress the differentiation of several other cell types [27,28]. In addition, it has been found that it also reduces the fibroblast proliferation and attenuate the induced α-smooth muscle actin by the TGF-β [27]. Moreover, the

Pirfenidone has been evaluated in the suppression of the differentiation of nasal polyp-derived fibroblasts [28] and found to be suppresses the invasiveness and migration of the pancreatic cancer cells [29]. For all the above Pirfenidone characteristics, it has been used for the first time in this study to investigate its possible interrupting effect to the induced EMT by the HCF of *E. granulosus* metacestode, and whether it can interfere with the associated changes induced by the parasite antigens as it happens normally in the infected tissues of the lung. The study designed to use the A549 cells as a model system to the human alveolar epithelial cells type II.

Prior to starting the experimentation, it was important to investigate the optimum Pirfenidone concentration, which can act on the EMT changes in the A549 cells without triggering cytotoxic effects may affect the viability of the cells. The results of this trial reached that 2mM concentration is the suitable concentration to be used for the next experiments, which is consistent with the finding of [30].

The results of this study proved that the Pirfenidone actively reduce the A549 cell proliferation and migration induced by the HCF antigens and inhibiting the associated transformation of the epithelial cells to mesenchymal cells, which have a key role in the process of fibrosis. Additionally, the Pirfenidone successfully interrupted the morphological changes associating the EMT in cells where treated cells conserved their morphology and epithelial characteristics as it has been verified by examining their cytoskeletal markers expression.

The designed experiments aimed to mimic the induced changes resulted from the infection with a hydatid cyst in the lungs tissue. As it is previously known, the pericyst layer is a protective layer to both host tissue and the parasite. Yet, it is playing a significant role in the limitation of antiparasitic drug delivery to the inside of the cyst [5-12]. Therefore, the study aimed to use the Pirfenidone to interrupt the pericyst building if the infection diagnosed in the early stages, or at least destroy part of the built layer in the later stages, to facilitate the transmission of the antiparasitic drugs to the inside of the cyst and help in parasite killing. In addition, this method will render the cyst exposed to the immune system of the infected patient, which would also help in the parasite eradication.

Since hydatidosis is a chronic disease and may be discovered in the late stages after the fibrous layer around the cyst was formed partially or completely, the performed experiment in this study was designed to induce EMT changes in the cells by cell exposure to the HCF for a period of 120hr before starting the treatment with the Pirfenidone. Although this is a relatively short time compared with the time consumed by the parasite to build the pericyst layer in the patient's body, the experiment in the current study tried to mimic the scenario that naturally happens in the infected tissues in the human body when the parasite starts the induction of the EMT in preparation to build the pericyst layer.

Interestingly, the results of this experiment may raise a suggestion that the Pirfenidone may not only stop the induced proliferation and migration effects of the HCF on A549 cells, but it may also revert the effect of the HCF and bring the cells back to their normal function as it confirmed by the analysis of the cytoskeletal markers, Figures 3-7. It is noteworthy that the significant effect of the Pirfenidone on the A549 cells started from 72 hr following the treatment and continued to the end of the experiment, which may suggest that it might be possible to use the Pirfenidone for a period of 3-5 days prior to start the treatment with the specific antiparasitic drugs. In sum, this method is an attempt to increase the effectiveness of the treatment using the chemotherapy and help the patients to benefit from the medicine and avoiding them the risk of surgery, especially those who can't pass the surgical removal of cysts.

CONCLUSION

The Pirfenidone possess the ability to interrupt the EMT induced by the HCF antigens and may interrupt the pericyst layer building around the hydatid cyst, as it is interfering with the induced EMT changes that precedes the fibrosis and prevent the cells' transformation. Accordingly, it can be suggesting that using the Pirfenidone before to start the use of the antiparasitic chemotherapy against the hydatid cyst may increase the effectiveness of these medicines, as it would open a way to these medicines to reach the parasite more easily than when it surrounded with a heavy fibrous layer, as well as render the cyst exposed to the immune system of the patient which would also help in the parasite eradication.

ORCID
A. A. Mohammed http://orcid.org/0000-0001-5147-4861
Yassir Al-Mulla Hummadi https://orcid.org/0000-0002-7543-2807

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