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In vitro Hematinic and Hepatoprotective activity profiling by Anti sickling method and MTT assay for Siddha formulation drug – Paandu Kudineer (PK)

Vijayalakshmi V[1], Victoria S[2], Shanmugapriya C[3], Manju Hemamalini N[4]

[1]Post Graduate, Department of Kuzhanthai Maruthuvam, Govt. Siddha Medical College, Chennai – 600106, Tamil Nadu, India.

[2]HOD, Department of Kuzhanthai Maruthuvam, Govt. Siddha Medical College, Chennai – 600106, Tamil Nadu, India.

[3]Lecturer, Department of Kuzhanthai Maruthuvam, Govt. Siddha Medical College, Chennai – 600106, Tamil Nadu, India.

[4]Lecturer, Department of Kuzhanthai Maruthuvam, Govt. Siddha Medical College, Chennai – 600106, Tamil Nadu, India.

Abstract

Anaemia constitutes serious health problem. It is widespread health problem, and the fourth leading cause of hospital admission and the second factor contributing to death. Anaemia is characterized by low count of haemoglobin. The empirical use of herbal preparations in the treatment of anaemia dates from ancient times. Despite the obvious effectiveness and efficacy of iron supplementation, there are certain limitations which include gastrointestinal side effects like nausea, vomiting, constipation and stained teeth. The present study is aimed to evaluate the hematinic and hepatoprotective effects of aqueous extract of polyherbal traditional siddha medicine Paandu Kudineer (PDK) against antisickling activity method and human hepatocytes (chang cell) cell line.

Keywords: Anaemia, PDK, Siddha medicine, Haemoglobin, Red blood cells.

Introduction

Anaemia is characterized by low haemoglobin count. WHO defines anaemia as Haemoglobin levels less than 13 g/dl in males and less than 12 g/dl in females. In adults, the lower extreme of the normal haemoglobin is taken as 14-16 g/dl for males and 12-14 g/dl for females. Newborn infants have higher haemoglobin level and, therefore 15 g/dl is taken as the lower limit at birth.

Although haemoglobin value is employed as the major parameter for determination of anaemia. The low Hb levels results in a corresponding decrease in the oxygen carrying capacity of blood(1) and other parameters such as total number of RBCs, PCV, MCV, MCH and MCHC.

Anaemia is a condition commonly seen in developing countries because of lack of nutrition and frequent use of drugs to treat diseases. Despite the obvious effectiveness and efficacy of iron supplementation, there are certain limitations. The main limitation is the lack of compliance, especially when long-term daily administration is required[3]. Gastrointestional side effects associated with oral iron therapy included nausea, vomiting, constipstion, anorexia, heartburn and diarrhea. In addition, stools may appear darker in colour in patients taking products containing iron. Other side effects associated with oral iron products included stained teeth and iron overload (hemosiderosis). Secondary hemochromatosis due to prolonged iron ingestion has been reported rarely. Stained teeth have primarily occurred following ingestion of iron liquid preparation. Iron overload (hemosiderosis) has been reported in patients genetically predisposed, or have underlying disorders, that augment the absorption of iron[2,4].

From ancient time, medicinal plants in the form of Kudineer formulations are believed to be useful in strengthening the hematopoitic and hepatoprotective of an individual. Various researchers successfully evaluated the potential of several medicinal plants in the treatment of anaemia using *In Vitro* methods.

Siddha physicians suggested various formulations for the treatment of haematological disorders as a source of iron and minerals.

PDK is a polyherbal traditional siddha formulation mentioned in siddha literature whisch is being used for the treatment of Paandu (Anaemia), Sobai (Generalisedodema), Manjalkamalai (Jaundice). But the above trial drug has not so far been scientifically evaluated for its haematanic and hepatoprotective activity. Hence an attempt has been made to evaluate the hematinic activity of PDK in In Vitro anti-sickling activitymethod and evaluate the hepatoprotective activity of PDK in human hepatocytes (chang cell) cell line.

Materials and Methods

Drug Authentication and Preparation

PDK is a polyherbal formulation comprising of 5 types of herbs that is Keezhanelli (*Phyllanthus amarus*), Karisalanganni (*Eclipta prostrata*), Paeipudal (*Trichosanthus lobate*), Venmilagu (*Piper nigrum*), Vilvaver (*Pimpinella anisum*). The drugs were authenticated by medicinal botany department on Government siddha medical college, Arumbakkam, Chennai. The raw drugs purifications are followed by mentioned classical siddha literature. The purified raw drugs are taken in equal quantity. All the raw drugs are crushed into coarse powder, the the coarse powder is taken in mod pot, 120ml of water is added and heated till it is reduced into 30ml.

Methodology

Hematinic activity

In vitro induction of sickling

Blood from the control group rats subjected to toxicity profiling of the drugs were utilised for this study. About 5ml of the blood retrieved from the rats were centrifuged at 5,000 rpm for 10 min in saline thrice to obtain the RBC which were then resuspended in normal saline and used for the analysis. About 100 shear stress (SS) blood cell suspensions were mixed with equal proportion of 2% sodium metabisulphite solution and incubated at 37°C. The time course of the sickling of SS erythrocytes was analyzed microscopically. The number of cells were counted every one hour and the percentage of sickling cells were calculated.

In vitro anti-sickling activity of the Paandu Kudineer

A serial concentrations of test sample PK (100-500 µg/mL) were prepared in the saline solution. For the assay 100 µL of SS-RBC sample pre-incubated with 2% $Na_2S_2O_5$ were added to 100 µl of test sample with different concentration ranges

from 100 to 500 µg/mL. Each mixture was incubated at 37°C for 2 h. After incubation, 10 µL of the mixture was diluted and a drop of each sample was examined under the oil immersion light microscope and both sickled cells and total cells were counted from different fields of view across the slide. For the negative control, the solution containing the test sample were replaced by the saline solution. The percentage of sickling was calculated using the formula:

Percentage of sickling = number of sickling cells /total cells × 100

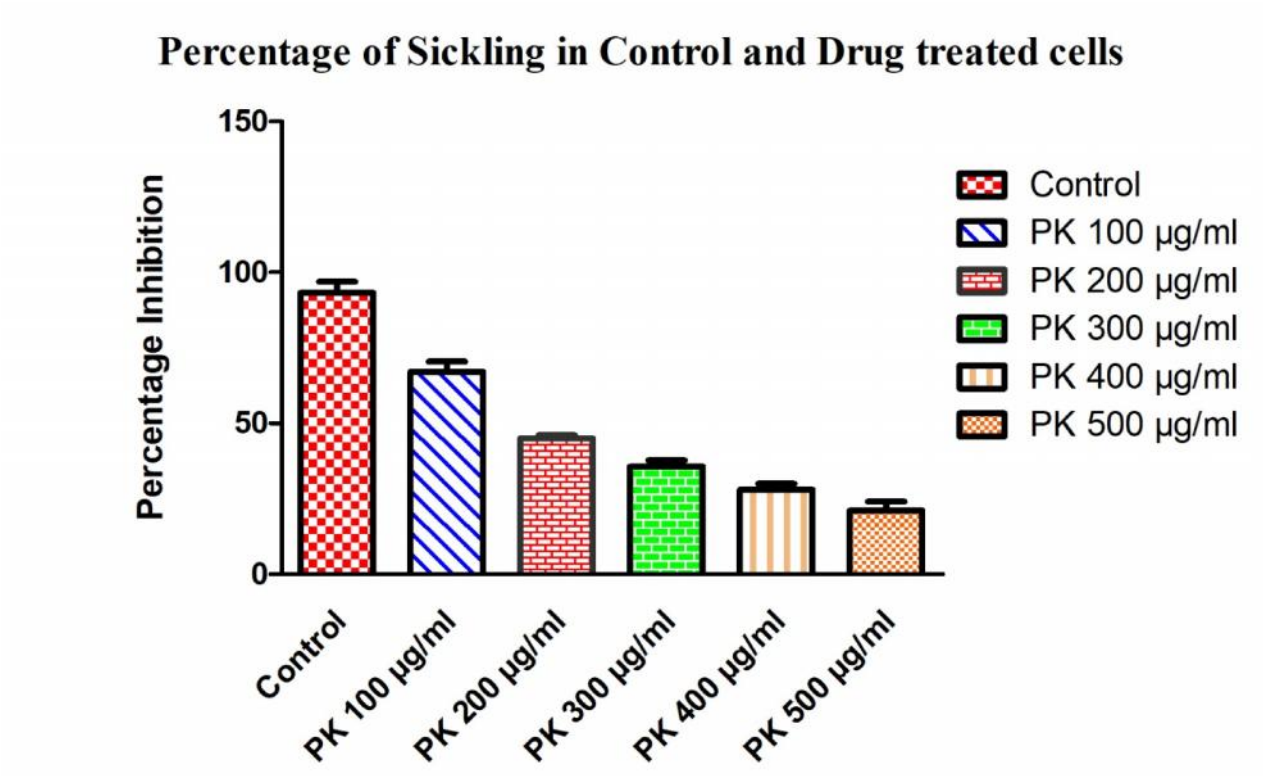
Statistical analysis

Results are expressed as Mean ± SD. The difference between experimental groups was compared by One-Way Analysis of Variance (ANOVA) followed by Dunnet Multiple comparison test.

Final Result

Concentration in µg/ml	Percentage of Sickling
Control	93.27 ± 3.729
PK 100	66.99 ± 3.631
PK 200	45.03 ± 1.056
PK 300	35.7 ± 2.032
PK 400	27.96 ± 2.093
PK 500	20.98 ± 3.359

Each value represents the mean ± SD. N=3



Result Analysis

The result obtained from the present clearly indicates that the test drug PK was effective in inhibiting the sickling of RBC cells. Maximum percentage inhibition of about 27.96 ± 2.093 % was observed at 500 µg/ml when compare to that of the control with the sickling of 93.27 ± 3.729 % .

Conclusion

From the result of the study it was concluded that the test drug PK possess promising anti-inflammatory property in protein denaturation assay.

Hepato-protective Activity

Preparation of test solutions

For hepato protective studies, serial dilutions of test formulation PK (50,100,200 and 400 µg/ml) were prepared using DMSO

Cell culture and maintenance

Chang liver cells, a human hepatocyte cells were obtained from National Centre for Cell Science (NCCS), Pune, India. Cells were maintained in Minimum Essential Media (MEM) supplemented with 10% Fetal Bovine Serum (FBS), with 100units/ml penicillin and 100µg/ml streptomycin. Cells were cultured in $75cm^2$ culture flask and incubated at humidified atmosphere with 5% CO_2 at 37°C.

CCl_4 induced hepatotoxicity in Chang liver cells

Chang liver cells were seeded in 6 well plates at a density of $1X10^5$ cells/well and allowed to grow for a period of 24 h. Test drug was administered at a concentration of 50 µg,100 µg , 200 and 400 µg / ml. Standard silymarin 200 µg / ml for three hour following test drug exposure, 0.1% CCl4 was added to all the wells except control and incubated for a period of 24 h. After incubation the test solutions in the wells were discarded and 100µl of MTT (5 mg/10 ml of MTT in PBS) was added to each well. The plates were incubated for 4h at 37°C in 5% CO_2 atmosphere. The supernatant was removed and 100µl of DMSO was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 570 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (IC_{50}) values is

generated from the dose-response curves for each cell line.

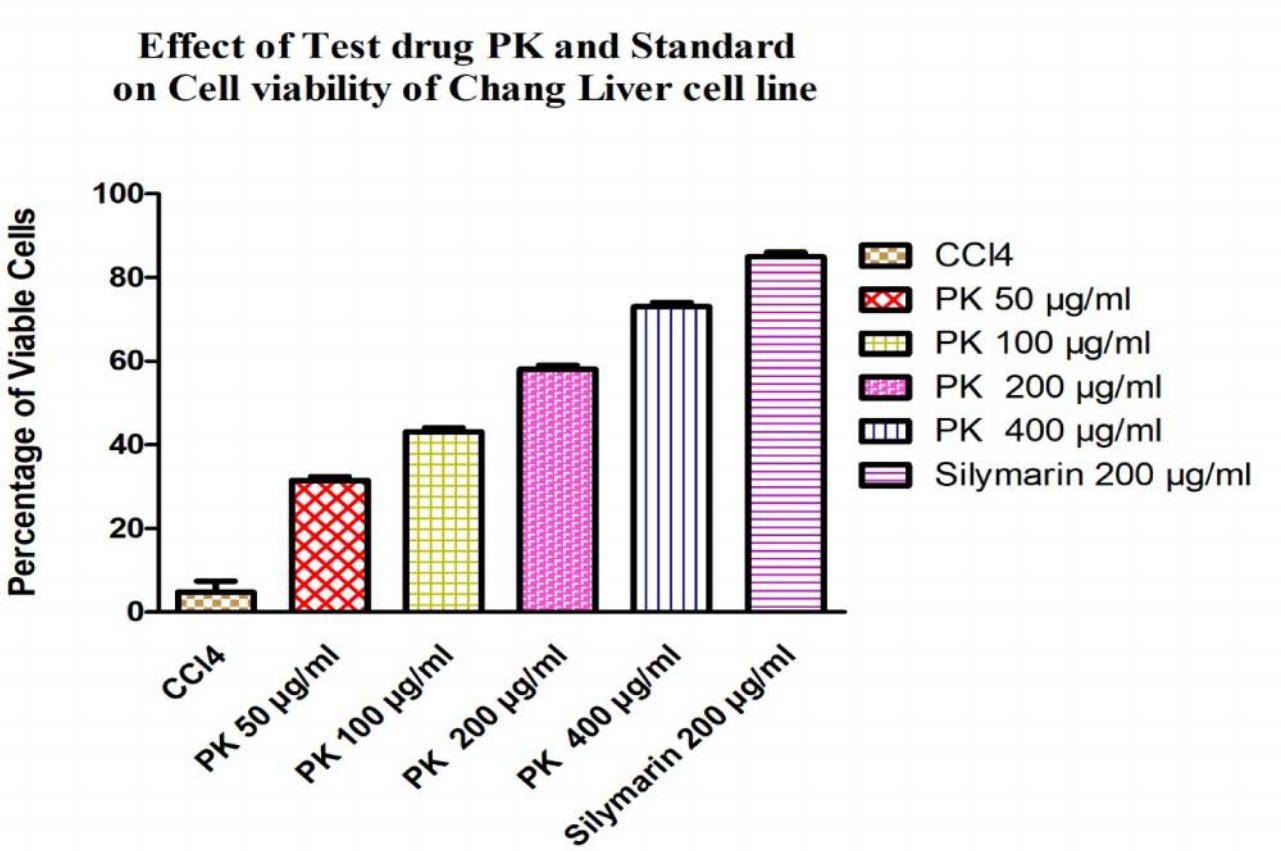
$$\text{Survival rate (\%)} = \frac{A_{sample} - A_b}{A_c - A_b} \times 100$$

MTT Assay

The *in vitro* determination of hepato protective effect of the test formulation have been performed by counting viable cells after staining with a vital dye. The MTT system is a means of measuring the activity of living cells via mitochondrial dehydrogenases. The MTT method is simple, accurate and yields reproducible results. The key component is (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) or MTT, is a water soluble tetrazolium salt upon incubation MTT is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by mitochondrial dehydrogenase enzymes of viable cells. The resulting colored solution is spectrophotometrically measured. An increase or decrease in cell number results in a concomitant change in the amount of formazan formed, indicating the degree of cytotoxicity caused by the test material.

Effect of Test drug PK and Standard on Cell viability of Chang Liver cell line

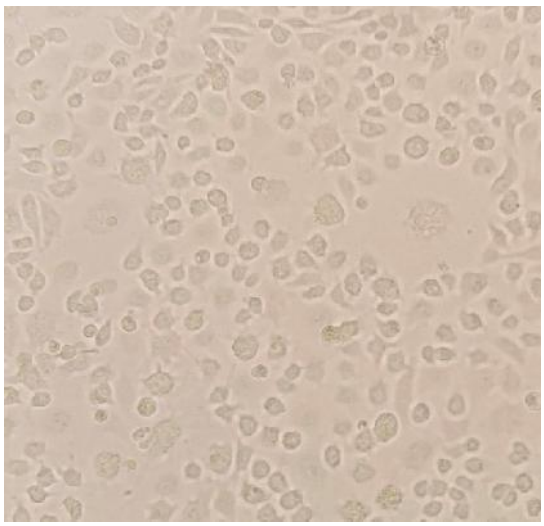
S.No	Concentration in µg/ml	% cell Viability
1	CCl_4 Control	4.75 ± 1.21
2	50	31.56 ± 2.66
3	100	43.05 ± 0.99
4	200	58.12 ± 1.98
5	400	73.3 ± 1.41
6	Silymarin 200 µg	85.27 ± 1.55



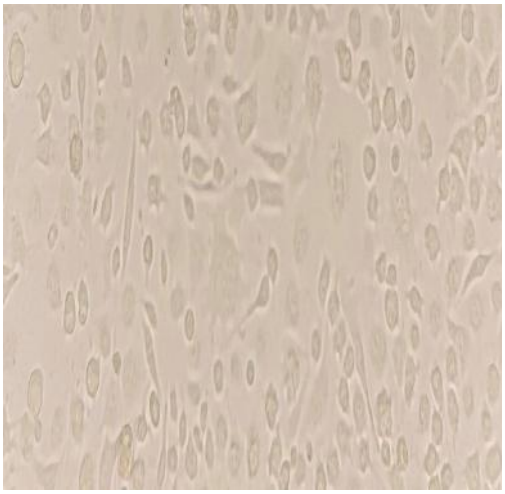
Results and Discussion

In-vitro hepatoprotective activity of test drug on the cell viability of human liver hepatocytes (Chang liver cell line) against CCl4 induced hepatotoxicity was performed at varying concentration ranges from 50 to 400 µg/ml .The reuslt obtained from the study reveals that the percentage of cell viability of chang liver celline increases with increase in concentration of the test drug PK. Highest viability of cell was observed at the concentration of 400µg/ml shows 73.3 ± 1.41%, followed by this 200µg/ml shows 58.12 ± 1.98 similarly 100 and 50 mcg shows 43.05 ± 0.99 and 31.56 ± 2.66% along with standard silymarin with the cell viability of 85.27 ± 1.55 % in MTT assay. It was concluded from the result of the present study that the formulation PK possess promising hepatoprotective activity.

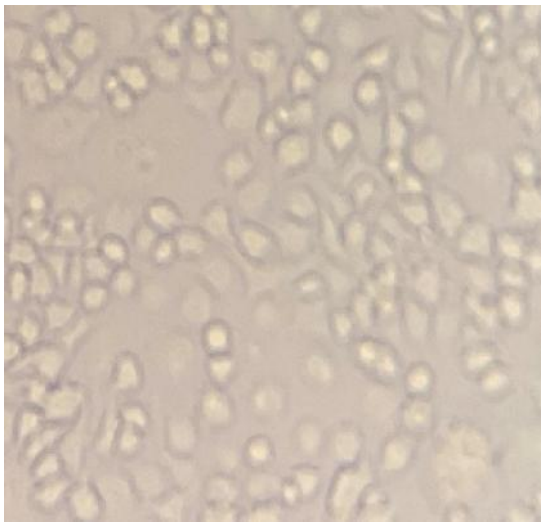
Chang Liver cells – Control group



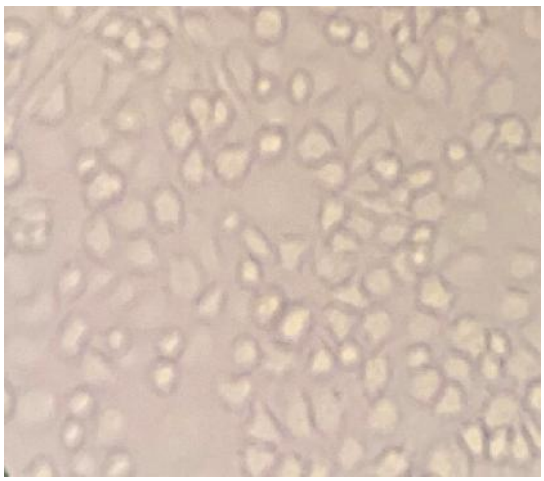
Chang Liver cells Incubated with CCl4



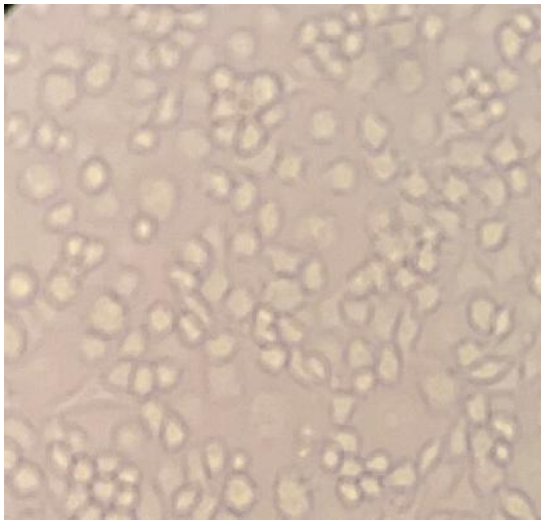
Chang Liver cells Incubated with Test Drug PK - 50 µg



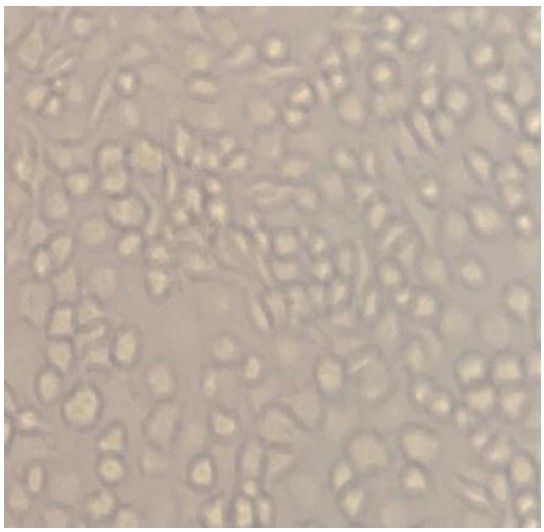
Chang Liver cells Incubated with Test Drug PK - 100 µg



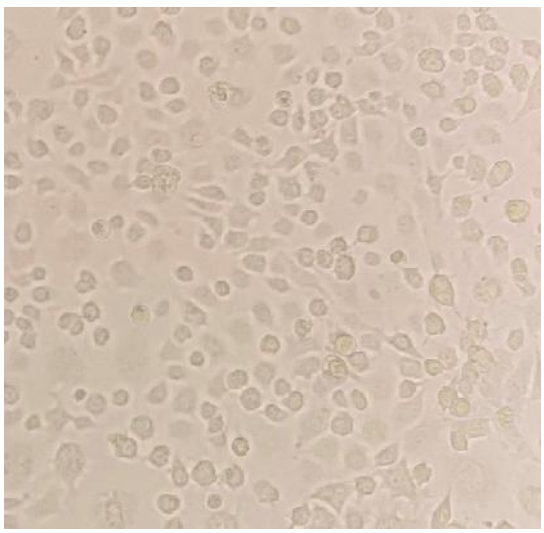
Chang Liver cells Incubated with Test Drug PK - 200 µg



Chang Liver cells Incubated with Test Drug PK - 400 µg



Chang Liver cells Incubated with Standard Silymarin - 200 µg



Conclusion

The collective data of this study revealed that Paandukudineer has considered has considerable hematinic activity as shown in *In Vitro* anti-sickling activity method and hepatoprotective activity of PDK in human hepatocytes (chang cell) cell line indicating the use of this Siddha formulation for the treatment of Paandu (Anaemia). Further studies are required to precisely ensure maximum bioavailability and therapeutic efficacy.

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