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Evaluation of Anticancer Activity of Apigenin Alone and combination with Imatinib in SCID Mice Bearing K562 Disseminated Leukemic Model

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ABSTRACT

The effects of Apigenin alone and combination with Imatinib were investigated on the CML disseminated model *in-vivo*. A SCID mouse disseminated CML model was set up by inoculation with K562 cells and used to study whether leukemic cells can be inhibited by Apigenin alone and combination with Imatinib.

After being inoculated with K562 cells, peripheral blood smear and BM aspiration expressed with tumour cells in the blood stream indicating that the CML model is successfully established.

The mice treated with Apigenin + Imatinib drugs exhibited a significant attenuation of splenomegaly than Imatinib and Apigenin alone treated animals as compared with the disease control group. The Apigenin + Imatinib treated group was 100% free from hind limb paralysis and mortality than Apigenin and Imatinib treated groups, which was found to be 67% and 83% respectively. The BM smear results showed that Apigenin + Imatinib, Apigenin alone and Imatinib treated group showed normal M:E ratio in range of 2:1 - 4:1.

It is concluded that the natural dietary flavonoid compound Apigenin possess anticancer activity. Apigenin + Imatinib have high potential to inhibit the dissemination of leukemic cells than Apigenin alone and is promising anticancer drug.

KEY WORDS: Chronic Myeloid Leukemia, Bone marrow, Myeloid, Erythroid ratio.

1. INTRODUCTION

Cancer is a major public health burden in India and many other developed countries. It is a generic term for a group of more than 100 diseases that can affect any part of the body. In 2010, globally around 281,500 people died of leukemia. Leukemia is cancer of the blood or bone marrow. A person with leukemia suffers from an abnormal production of blood cells, usually leukocytes. Chronic myelogenous leukemia (CML), accounts for about 20% to 35% of all adult leukemias. It typically occurs at ages 40 to 60 years, with about 20% to 40% of patients asymptomatic and diagnosis having been suggested by hepatosplenomegaly on examination or abnormal results like leukocytosis, anemia, or thrombocytosis on routine hematologic testing (Sokal, 1987).

CML is defined as the myeloproliferative disorder resulting from the clonal expansion of a transformed mulitpotent hematopoietic stem cell. It is characterized by the Philadelphia chromosome resulting from a balance translocation between chromosome 9 and 22, leads to the formation of the Bcr-Abl fusion oncogene. The dysregulated Bcr-Abl on coprotein interacts with other cytoplasmic molecules and leads to activation of downstream signaling pathways, Rase-ERK, PI3K and STAT5, thereby driving cancer cell survival and proliferation (Cilloni, 2012).

Various remedies like stem cell transplantation, chemotherapy, radiation therapy, targeted therapy, immunotherapy etc., have been reported for the treatment of CML. However, they have major drawbacks. Allogeneic stem-cell transplantation complications involving donor compatibility and treatment tolerance limit the feasibility of this therapy. IFN-α therapy results in a high possibility of neurotoxicity as well as some other intolerable side effects in older patient. The alternative first-line therapies are tyrosine kinase inhibitors and chemotherapy. Their high systemic toxicity and drug resistance limits the successful outcomes in most cases. Thus, there is urgent need to look for alternative ways to develop novel therapeutic approaches that target signaling pathways other than Bcr-Abl drug candidates with fewer side effects and less cost. The practical goals of this approach should be to lower the incidence of invasive cancer and deaths from cancer at an early age through pharmacological interventions relying on prevention rather than cure.

Among various natural compounds, recently researchers have focused on flavonoids, since these compounds are found to be broadly distributed in fruits, vegetables and plants, which proved the way for its effectiveness in dietary based cancer prevention. Flavonoids has been reported for various biological effects such as inhibiting platelet aggregation, reducing cell proliferation etc and the average daily intake of flavonoids in a normal diet is around 1-2g (Havsteen, 2002).

Apigenin is a flavonoid belonging to the flavone structural class and chemically known as 4',5,7,-trihydroxyflavone. Apigenin is richly present in common fruits and vegetables such as parsley, onions, oranges, tea,

chamomile, wheat sprouts and in some seasonings. Evidence of previous research reported that Apigenin ability to interfere with the process leading to tumor spreading and cancer cell dissemination (Caltagirone, 2000).
Apigenin had been reported to possess anticancer properties in several cancer lines including breast, cervical, lung, overian, skin, thyroid, gastric, hepatocellular, neuroblastoma, adrenocortical, colon, leukemia and pancreatic cancer (Yin, 2001). But there is lack of research related with the effect of Apigenin in CML. This had driven an interest for the present study to investigate the *In-vivo* effect of Apigenin for the very first time on K562 cell lines (human chronic myeloid leukemia) in disseminated leukemic model using SCID mouse.

2. MATERIALS AND METHODS

All procedures of this study were performed in the laboratories of Department of Oncology at Syngene International Pvt. Ltd, Bangalore in the period from December 2015 to May 2016.

Test system: Justification: The procedure relies on immunodeficient mice to provide a host for the establishment of human xenografts. SCID mouse implanted with human tumor is a suitable model for evaluating anti-cancer activity of test compounds.

Test Species	*Mus musculus*	Cancer cell line	K562 (Human Chronic Myelogenous Leukemia)
Strain	C.B-17/IcrHsd-Prkdc^{scid}Lyst^{bg-J}	Cell concentration	2 x 10^6 cells/animal
Sex	Male	Test Compound	Apigenin
Source	Harlan, USA.	Reference Compound	Imatinib
Age at the start of experiment	5-6 weeks	Dose and Dosing schedule	Apigenin: 40 mg/kg, i.p.; QD for 21 days
Body Weight of animals	20-23g	Imatinib: 50 mg/kg, p.o.	QD for 21 days
No. of animals/group	n= 6	Duration of the study	60 days

Safety of Apigenin dose: Apigenin at 40 mpk dose was administered i.p, once daily for 14 days in mice group (n=6). All animals were free adverse effects and the selected dose was found to be safe to use in experiment.

Animal Care:

Animal Welfare: Animals were taken care as per the regulations of Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India and Association for Assessment and Accreditation of Laboratory Animal Care guidelines. The 'Form B' for carrying out animal experimentation was reviewed and approved by the Institutional Animal Ethics Committee (IAEC Protocol Approval No: SYNGENE/IAEC/538/08-2014).

Housing and Feeding: Animals were maintained in a controlled environment with 22 ± 3°C temperature, 50 ± 20% humidity, a light/dark cycle of 12 hours each and 15-20 fresh air changes per hour. Animals were housed group wise and autoclaved corncob was used as a bedding material. The animals were fed, *ad libitum*, with certified irradiated laboratory rodent diet during the study period. Fresh, potable drinking water, filtered through RO, was provided *ad libitum* after autoclaving to all animals via bottle fitted with nozzle.

Preparation of Animals: The animals were kept under acclimatization in the experimental room for a period of at least 5 days. A thorough physical examination was performed before selecting the animals and only animals that were apparently healthy were used for the study.

Animal Identification: Animals were individually numbered and the cage cards indicating the experiment, study number, date of tumor implantation, date of randomization, tumor type, mouse strain and gender were displayed on corresponding cages. After randomization, group identity, test compound, dosage, schedule and route of administration information was also incorporated on cage labels.

Formulation and Preparation:

Preparation of test item and dosing solutions:

Apigenin Preparation: Apigenin test compounds and Imatinib were freshly prepared daily prior to administration. The required amount of Apigenin was weighed, transferred into glass vials. Required amount of aqueous suspension containing DMSO, Cremophor, KOH and Saline (ratio of 1:1:0.8:7.2) was added to the drug powder and pH was adjusted to 7 using Hcl. The dose volume was maintained at 10 ml/kg body weight. The dose of the Apigenin 40mpk was selected based on the previously published research articles (Amit, 2012)

Imatinib Preparation: The required amount of Imatinib was weighed, transferred into mortar and triturated gently using pestle to get a uniform powder. Required amount of aqueous suspension containing 0.5% hydroxypropyl methyl cellulose (HPMC) and 0.1% Tween 80 (ratio of 98:2) was added to the drug powder and triturated well to get a homogenous suspension. The dose volume was maintained at 10 ml/kg body weight. The dose of Imatinib 50 mpk was selected based on the previously published research articles (Jia, 2013).

Study Schedule: The study schedule of K562 disseminated leukemic model is shown in table.1.

Table.1. Study schedule of K562 Disseminated Leukemia Model

Groups	Treatment	Treatment regimen			
		No. of Animals	Dose [mg/kg]	Application route	Dosing schedule
I	Normal Control	6	-	-	-
II	Vehicle Control	6	10 ml/kg	I.P	QD for 21 days
III	Apigenin	6	40 mg/kg	I.P	QD for 21 days
IV	Imatinib	6	50 mg/kg	Oral	QD for 21 days
V	Apigenin + Imatinib	6	40 mg/kg + 50 mg/kg	Apigenin –I.P Imatinib - Oral	QD for 21 days

Experimental Procedure:

Preparation of K562 Cells - Human chronic myelogenous leukemia disseminated study: All procedures were performed in laminar flow hood following sterile techniques. K562 (human chronic myelogenous leukemia) cell lines were obtained from Syngene International Pvt Ltd. Procured from ATCC. Human chronic myelogenous leukemia (K562) with a viability of >90 % was chosen for the study. Approximately 2 X 10^6 cells K562 were resuspended in 100 µL of sterile 1 X PBS and were injected intravenously via tail vein of the SCID mice. The concentration of K562 cells for inoculation was selected as per the previously published research article (Jian *et al*., 2011).

Experimental design:

Human chronic myelogenous leukemia: Animals were randomized based on body weight three days post injection of K562 cell line and treatment schedule was initiated as mentioned below in table 03 and preparation of Test Compounds is shown in table 03

Table.2. Experimental Design for CML

Group	Number of animals / group	Cell line	Agent	Dose /application
I	6	-	Control	-
II	6	K562	Vehicle control	DMSO, Cremophor, 0.5% KOH and Saline (ratio of 1:1:0.8:7.2) i.p.; QD for 21 days
III	6	K562	Apigenin	40 mg/kg, i.p.; QD for 21 days
IV	6	K562	Imatinib	50 mg/kg, p.o.; QD for 21 days
V	6	K562	Apigenin + Imatinib	40 mg/kg, i.p.; QD for 21 days + 50 mg/kg, p.o.; QD for 21 days

Table.3. The Preparation of Test Compounds

Group	Dose	Working solution	Dose
I - Normal Control	-	-	-
II - Vehicle Control	-	DMSO, Cremophor, 0.5% KOH and Saline (ratio of 1:1:0.8:7.2) i.p.; QD for 21 days	10 ml/kg
III - Apigenin	40 mg/kg	4 mg/ml	10 ml/kg
IV - Imatinib	50 mg/kg	5 mg/ml	10 ml/kg
V - Apigenin+ Imatinib	Apigenin - 40 mg/kg Imatinib - 50 mg/kg	Apigenin - 4 mg/ml Imatinib - 5 mg/ml	10 ml/kg 10 ml/ml

Observations:

Body Weight: Body weights were measured once every three days during the study period. The % change in body weights of individual mouse was calculated.

Clinical Signs: Animals were observed individually for visible general clinical signs every day during the study period.

Hematological analysis: Peripheral blood cells were counted using microscope and differential leukocyte count were analyzed using ADVIA I20 analyzer at different time points of study to find out disease progression.

Necropsy: On the day of necropsy, blood samples were first collected under light isoflurane anesthesia from all the groups for differential leukocyte count (DLC). Subsequently, animals were euthanized by carbon dioxide asphyxiation and necropsy was done to evaluate the following parameters;

- Bone marrow smear
- Bone marrow biopsy
- Evaluated for gross changes in Spleen, femur and organs were collected for histological sections.

Statistical Analysis: Statistical evaluation was carried out by ANOVA (One way/Two-way) followed by Bonferroni posthoc test using GraphPad Prism5. Values of $p \geq 0.05$ indicate statistically significant differences between groups.

3. RESULTS

Treatment with Apigenin, Imatinib and combination of Apigenin with Imatinib compounds was initiated 3 days post injection of K562 cell line. The percentage change in body weight during the study period is presented in Table 04 and Figure 01. Among the test groups, Apigenin + Imatinib treatment group showed moderate transient body weight loss during the treatment period. However the body weight gradually regained during the post treatment period. The transient weight loss during the treatment with Apigenin+Imatinib may be attributed to toxicity arising from potential drug interaction of the combination therapy. The weight loss during the treatment with Apigenin+Imatinib may be due to toxicity of the combination therapy. Irrespective of test groups, vehicle control group demonstrated severe body weight loss (10%) which may be due to disease progression.

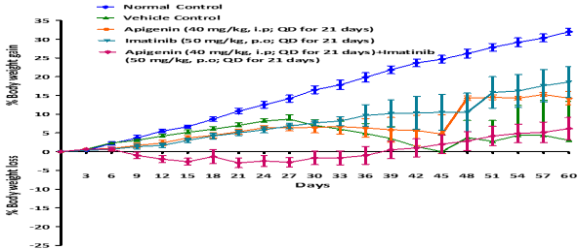


Figure.1. Mean Body Weight Changes in SCID Mice Treated with Apigenin, Imatinib and Apigenin+Imatinib Compounds

Table.4. Mean Body Weight of SCID mice - K562 Chronic Myelogenous Leukemia Disseminated Model

Treatment Groups	Mean Body Weight (g)										
	Days	**0**	**3**	**6**	**9**	**12**	**15**	**18**	**21**	**24**	**27**
Normal Control	Mean	22	22	22	23	23	23	24	24	24	25
	SEM	0.4	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
	% Change	0	1	2	4	5	7	9	11	12	14
Vehicle Control	Mean	22	22	22	23	23	23	23	23	24	24
	SEM	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	% Change	0	1	2	3	4	5	6	7	8	9
Apigenin (40 mg/kg, i.p.) QD for 21 days	Mean	22	22	22	23	23	23	23	23	24	24
	SEM	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.7
	% Change	0	1	1	2	2	4	4	5	6	6
Imatinib (50 mg/kg, p.o.) QD for 21 days	Mean	22	22	22	23	23	23	23	23	24	24
	SEM	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6
	% Change	0	0	1	1	2	3	4	5	6	7
Apigenin (40 mg/kg, i.p.) QD for 21 days + Imatinib (50 mg/kg, p.o.) QD for 21 days	Mean	22	22	22	22	22	22	22	22	22	22
	SEM	0.6	0.6	0.7	0.7	0.7	0.7	0.9	0.7	0.7	0.7
	% Change	0	1	1	-1	-2	-3	-1	-3	-2	-3

Treatment Groups	Mean Body Weight (g)											
	Days	**30**	**33**	**36**	**39**	**42**	**45**	**48**	**51**	**54**	**57**	**60**
Normal Control	Mean	25	26	26	26	27	27	27	28	28	28	29
	SEM	0.2	0.2	0.2	0.2	0.2	0.2	0.3	0.3	0.2	0.3	0.3
	% Change	16	18	20	22	24	25	26	28	29	30	32
Vehicle Control	Mean	23	23	23	23	22	22	23	23	24	24	23
	SEM	0.8	0.9	1.0	1.1	1.2	1.3	1.1	1.2	1.2	1.4	1.5
	% Change	7	6	5	4	2	0	6	5	9	8	7
Apigenin (40 mg/kg, i.p.) QD for 21 days	Mean	24	24	24	24	24	23	26	26	26	26	26
	SEM	0.8	0.9	1.0	1.2	1.3	1.5	0.7	0.7	0.7	0.7	0.6
	% Change	7	7	7	6	6	5	17	17	17	18	17
Imatinib (50 mg/kg, p.o.) QD for 21 days	Mean	24	24	24	24	25	25	25	26	26	26	26
	SEM	0.6	0.6	0.6	0.8	0.9	1.0	1.1	0.4	0.4	0.5	0.5
	% Change	8	8	9	10	10	10	10	16	17	18	19
Apigenin	Mean	22	22	22	22	23	23	23	23	23	23	24

(40 mg/kg, i.p.) QD for 21 days + Imatinib (50 mg/kg, p.o.) QD for 21 days	SEM	0.7	0.7	0.8	0.8	0.8	0.8	0.8	0.8	0.7	0.8	0.8
	% Change	-2	-2	-1	0	1	2	3	4	5	5	6

Values are expressed as Mean ± SEM (n=6)

On Day 0, male SCID mice were injected K562 cells ($2x10^6$ cells/animal) via tail vein. Three days post injection of K562 cells, (On Day 3) treatment was initiated with Apigenin test compound (40 mpk, i.p., QD for 21 days), Imatinib (50 mpk, p.o., QD for 21 days) and combination of Apigenin (40 mpk, i.p., QD for 21 days) + Imatinib (50 mpk, p.o., QD for 21 days) Post dosing, the animals were monitored for incidence of hind limb paralysis. The incidence of hind limb paralysis in vehicle control and treatment group animals is represented in Table 05, Figure 02. In vehicle control group, among six animals, four animals was observed with hind limb paralysis. That is, one animal at day 27, one animal at day 33 and two animals at day 30 post injection of K562 cells. In Apigenin group, two animals out off six was observed with hind limb paralysis at day 27 post injection of K562 cells. In Imatinib group, one animal out off six animals was observed with hind limb paralysis at day 36 post injection of K562 cells. But in combination group, all animals was free from hind limb paralysis. The percentage of animals without hind limb paralysis in vehicle control was only 34%. However, in Apigenin and Imatinib treated groups the percentage of animals free from hind limb paralysis was 67% and 83% respectively (Table 9, Figure 09). Treatment with combination of Apigenin + Imatinib group animals was 100% free from incidence of hind limb paralysis. The order of percentage protection from hind limb paralysis was found to be Apigenin + Imatinib > Imatinib > Apigenin.

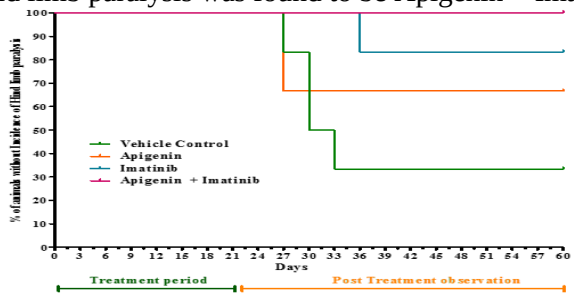


Figure.2. Incidence of Hind Limb Paralysis in SCID mice - K562 Chronic Myelogenous Leukemia Disseminated Model

Table.5. Incidence of Hind Limb Paralysis in SCID mice - K562 Chronic Myelogenous Leukemia Disseminated Model

Groups	**Dose**	**Hind limb paralysis**
I Normal Control	-	-
II Vehicle Control	-	Hind limb paralysis was observed in **4**/6 of animals 27 days post injection of cells (Animal No: 5) 30 days post injection of cells (Animal No: 1 and 3) 33 days post injection of cells (Animal No: 2)
III Apigenin	40 mpk, i.p., QD for 21 days	Hind limb paralysis was observed in **2**/6 of animals 27 days post injection of cells (Animal No: 7 and 9)
IV Imatinib	50 mpk, p.o., QD for 21 days	Hind limb paralysis was observed in **1**/6 of animals 36 days post injection of cells (Animal No: 15)
V Apigenin + Imatinib	(40 mg/kg, i.p.) QD for 21 days (50 mg/kg, p.o.) QD for 21 days	No evidence of hind limb paralysis was observed in **0**/6 of animals

On Day 0, male SCID mice were injected K562 cells ($2x10^6$ cells/animal) via tail vein. Three days post injection of K562 cells, (On Day 3) treatment was initiated with Apigenin test compounds (40 mpk, i.p., QD for 21 days), Imatinib (50 mpk, p.o., QD for 21 days) and combination of Apigenin (40 mpk, i.p., QD for 21 days) + Imatinib (50 mpk, p.o., QD for 21 days) post dosing, the animals were monitored for incidence of mortality. The % survival of animals during the study is represented in Figure 10. In vehicle control group, among six animals, mortality was observed in three animals, two animals at day 48 and one animal at day 54 post injection of K562 cells. In Apigenin group, among six animals, mortality was observed in two animals at day 48 post injection of K562 cells. In Imatinib group, only one animal out off six animals was found dead on day 51 post injection of K562 cells. But in combination group, all animals were free from mortality. Therefore, the % survival of animals was found to be only 50% in vehicle control, 66% in Apigenin test group, 83% in standard Imatinib treated group and 100% in

Apigenin+Imatinib treated group. The order of % survival of animals is found to be Apigenin + Imatinib > Imatinib > Apigenin.

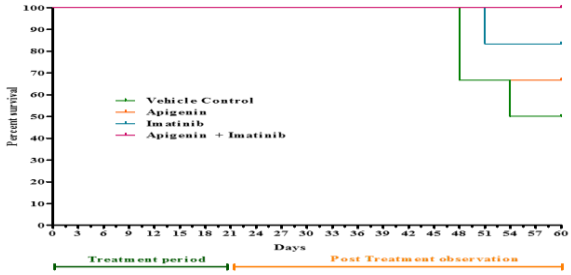


Figure.3. Percent Survival of SCID mice - K562 Chronic Myelogenous Leukemia Disseminated Model

At the end of experiment, at the time of necropsy, spleen was collected and the weight was recorded. It was then preserved in 10% neutral buffered formalin and processed for histopathological analysis. Spleen size ranks among the most important risk factors in chronic myeloid leukemia (CML). Statistically significant Increase in spleen weight was observed in vehicle control group, when compared with normal control group. The increase in the spleen weight may be due to the chronic leukemic progression leading to hyperleukocytosis that resulted in marked splenomegaly. There was no statistically significant decrease in the spleen weight when an Apigenin test group was compared with vehicle control group. Apigenin as standalone therapy was not able to attenuate increase in spleen weight as compared to vehicle control. However, there was statistically significant decrease of spleen weight when Imatinib and combination of Apigenin + Imatinib test groups was compared with vehicle control group (Figures.4, Table.6).

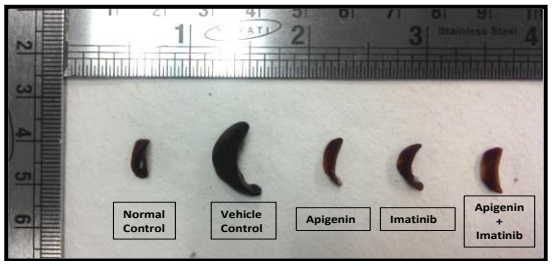


Figure.4. Representative Photos of Spleen in SCID mice - K562 Chronic Myelogenous Leukemia Disseminated Model

Table.6. Mean Spleen Weight in SCID mice - K562 Chronic Myelogenous Leukemia Disseminated Model

Group	Dose	Spleen weight Mean ± SEM
I Normal Control	-	67 ± 4.6
II Vehicle Control	-	148 ± 35.0
III Apigenin	40 mg/kg, i.p., QD for 21 days	106 ± 21.9
IV Imatinib	50 mg/kg, p.o., QD for 21 days	49 ± 3.4
V Apigenin + Imatinib	40 mg/kg, i.p., QD for 21 days 50 mg/kg, p.o., QD for 21 days	47 ± 3.1

Values are expressed as mean SEM (n=6)

Blood samples were analyzed using ADVIA 2120i hematology analyzer for differential leukocyte count (DLC) at day 7, day 32 and day 60. The results are represented in Fig 05 - 09. All hematopoietic cells were found to be increased except basophils in vehicle control group as compared to normal control group at the end of experiment (day 60). But that increase was not statistically significant. Standalone therapy of Apigenin as well as Imatinib and combination therapy was not shown any statistically significant changes in hematopoietic cell counts.

Clinical Pathological Analysis:

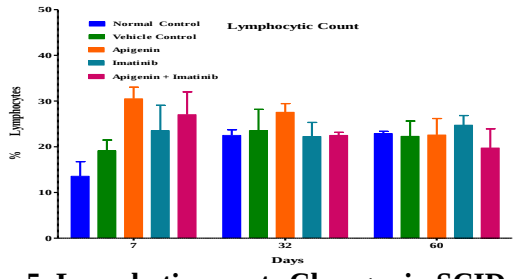


Figure.5. Lymphatic counts Changes in SCID mice - K562 Chronic Myelogenous Leukemia Disseminated Model

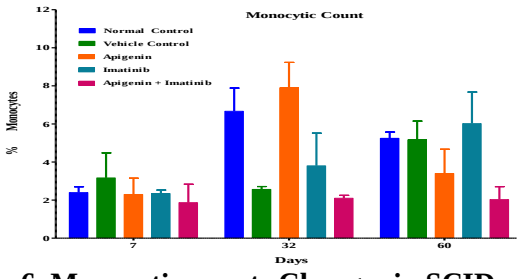


Figure.6. Monocytic counts Changes in SCID mice - K562 Chronic Myelogenous Leukemia Disseminated Model

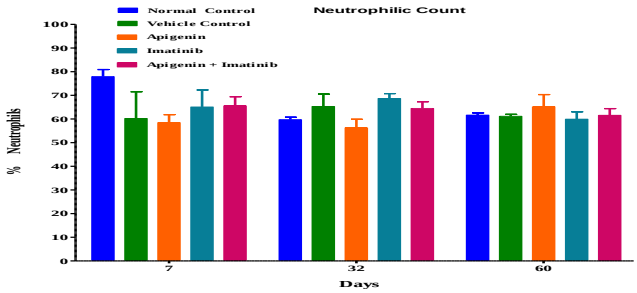


Figure.7. Neutrophillic count Changes in SCID mice - K562 Chronic Myelogenous Leukemia Disseminated Model

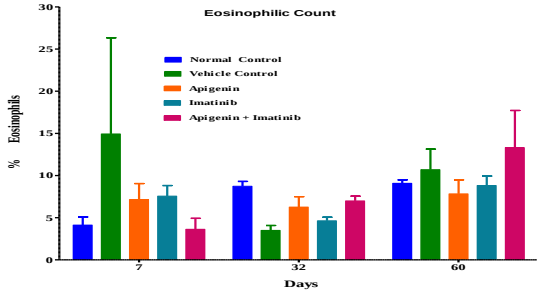


Figure.8. Eosinophilic count Changes in SCID mice - K562 Chronic Myelogenous Leukemia Disseminated Model

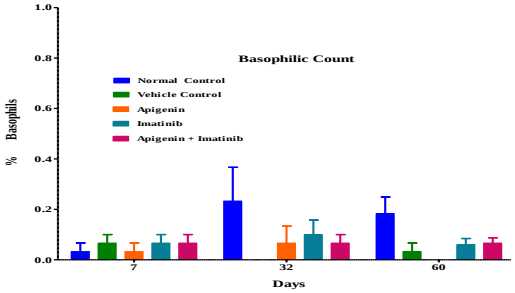


Figure.9. Basophilic count Changes in SCID mice - K562 Chronic Myelogenous Leukemia Disseminated Model

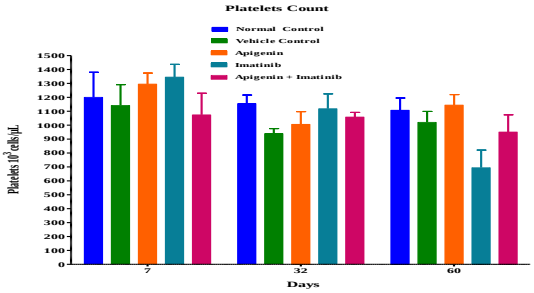
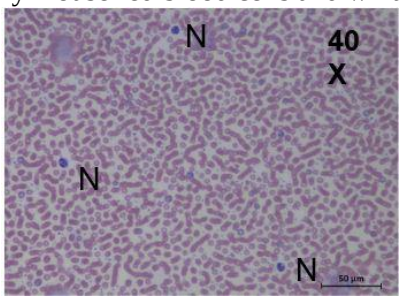
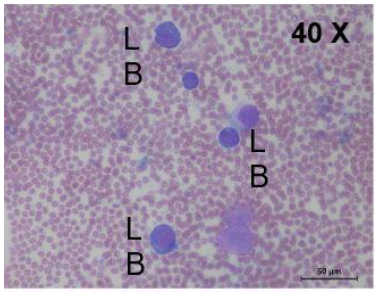


Figure.10. Platelets count Changes in SCID mice - K562 Chronic Myelogenous Leukemia Disseminated Model

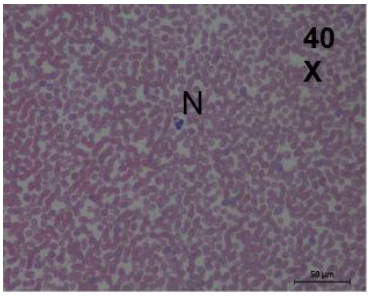
Photomicrographs of blood smears stained with Wright's stain shows multiple tumor cells in the vehicle control group. Note the variability in the size of the cells. All the cells were at least four times bigger than mice RBCs and WBCs. At least one tumor cell in three fields (40 x magnifications) showed signs of division, indicating the high mitotic potential of the tumor cells. N- Neutrophil, LB- Lymphoblast. However the photomicrographs of blood smears from various treatment groups showed a complete absence of tumor cells in circulation and the presence of only mouse red blood cells and white blood cells.



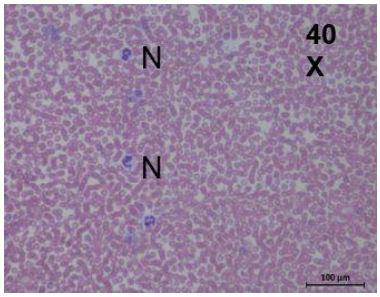
Normal Control



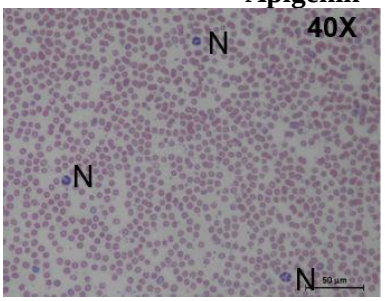
Vehicle Control



Apigenin



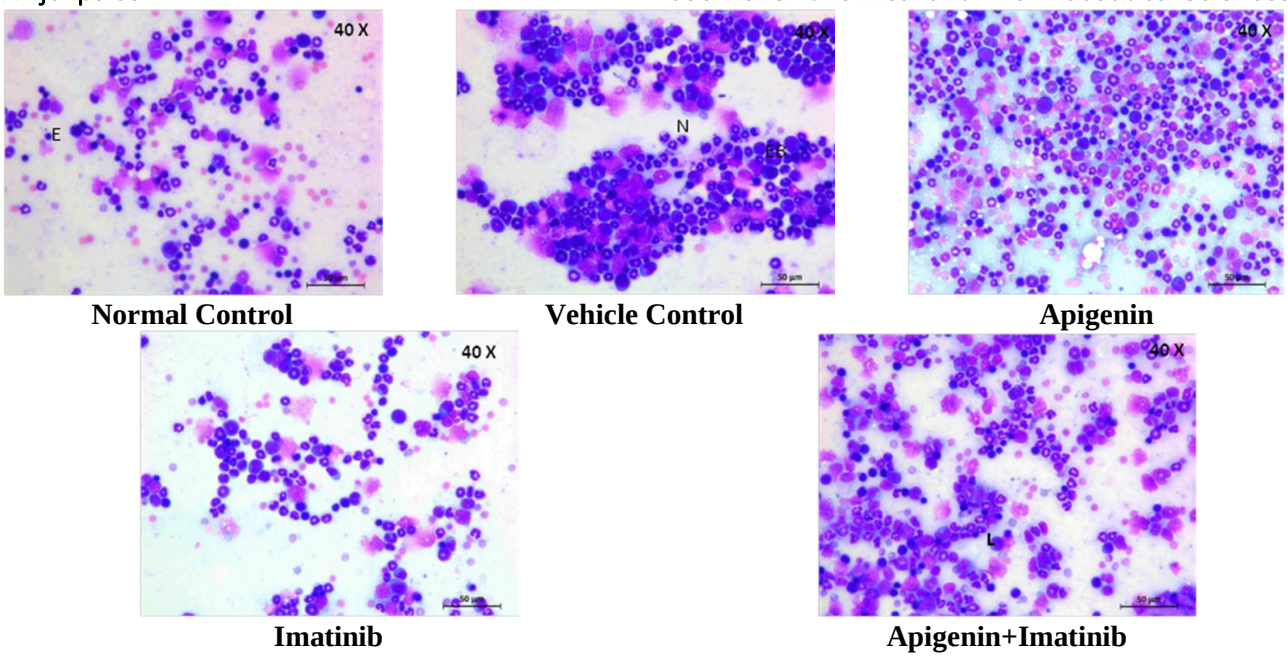
Imatinib



Apigenin+Imatinib

Figure.11. Representative Photomicrographs of Blood Smears in SCID mice - K562 Chronic Myelogenous Leukemia Disseminated Model

Photomicrographs of blood smears stained with Wright's stain shows multiple tumor cells in the vehicle control group. Note the variability in the size of the cells. All the cells were at least four times bigger than mice RBCs and WBCs. At least one tumor cell in three fields (40 x magnifications) showed signs of division, indicating the high mitotic potential of the tumor cells. N- Neutrophil, LB- Lymphoblast. However the photomicrographs of blood smears from various treatment groups showed a complete absence of tumor cells in circulation and the presence of only mouse red blood cells and white blood cells (Figure.11).



Normal Control **Vehicle Control** **Apigenin**

Imatinib **Apigenin+Imatinib**

Figure.12. Representative Photomicrographs of Bone Marrow Smear in SCID mice - K562 Chronic Myelogenous Leukemia Disseminated Model

E- Erythroid cells, N- Neutrophil, EB- Erythroblast, MB- Myeloblast, L- Lymphocyte

Femur was isolated and was made a longitudinal cut so as to expose the marrow. The tip of brush is rolled gently in the exposed marrow and several strips of marrow are made on glass slides and it is stained with Wright Giemsa stain. The bone marrow smear was evaluated for myeloid/erythroid lineage ratio by counting 500 cells at three different fields in the slides. The normal range of M:E ratios is 2:1. The representative photomicrographs of bone marrow smears were shown in figure.12 and table.7.

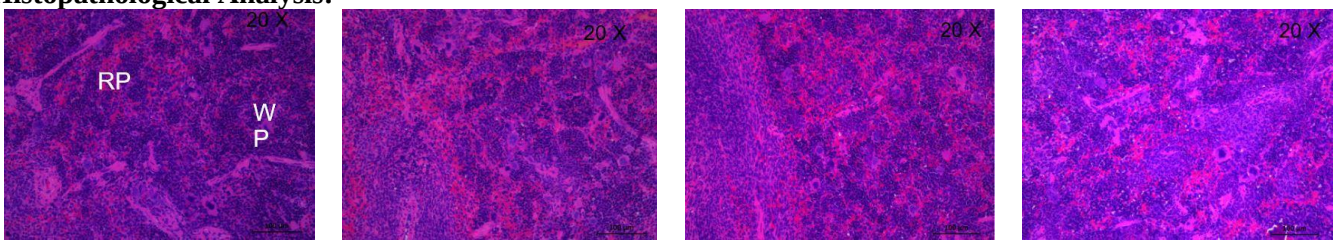
In vehicle control group, the M:E ratio was in the range of 8:1 – 16:1, which indicates that there were a increased myeloid series such as myeloblast, promyelocyte, metamyelocyte, band neutrophils, segmented neutrophils, basophils, band eosinophils when compared to erythroid series such as rubriblast, lymphocyte, plasma cells in circulating bone marrow. In test groups, the M:E ratio were in the normal range in between 1.7:1 – 4:1.

Standalone therapy of Apigenin and Imatinib as well as combination therapy was able to restore the myeloid:erythroid ratio.

Table.7. Mean Ratio of Myeloid:Erythroid Series in Bone Marrow Smear

Groups	Mean ratio of Myeloid:Erythroid
Vehicle control	9.2:1
Apigenin	2.7:1
Imatinib	02:01
Apigenin+Imatinib	2.1:1

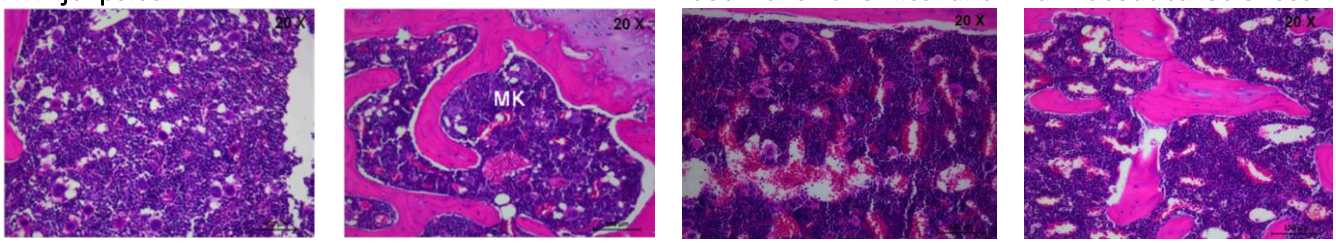
Histopathological Analysis:



Vehicle Control **Apigenin** **Imatinib** **Apigenin +Imatinib**

Figure.13. Representative Photomicrographs of Spleen in SCID mice - K562 Chronic Myelogenous Leukemia Disseminated Model

Representative photomicrographs of Spleen in SCID mice - K562 Chronic myelogenous leukemia disseminated model shown in the figure 13. In vehicle control group, effacement of architecture of spleen with high infiltration of myeloid cells (white pulp) and as well as infiltration by neoplastic cells with scanty cytoplasm was observed. In test groups, normal histology with splenic parenchyma composed of normal red pulp and white pulp was observed.



Vehicle Control **Apigenin** **Imatinib** **Apigenin+Imatinib**

Figure.14. Representative Photomicrographs of Femur in SCID mice - K562 Chronic Myelogenous Leukemia Disseminated Model

MK-Megakaryocytes

Decalcified, paraffin embedded, H and E stained sections of bone marrow. In vehicle control group, typical leukoerythroblastic cells with circulating immature cells in bone marrow and also presence of hypercellular precursor cells of myeloid lineage was observed. Increased prominent megakaryocytes are present.

Hyper cellular cells were due to an increase of neutrophils and their precursors. Blasts may be modestly increased in number. Normal myeloid erythroid and megakaryocytic cells were markedly reduced. Representative photomicrographs of Spleen in SCID mice - K562 myelogenous leukemia disseminated model shown in the figure.14.

In test groups, immature myeloid cells were located along the bone trabeculae, with maturing neutrophils, eosinophils, etc., located more centrally, or away from bone. Erythroid and megakaryocytic elements were found in the central areas, often near marrow sinuses. Erythroid precursors tend to cluster in red cell islands. Megakaryocytes were relatively few in number.

DISCUSSION

Chronic myelogenous leukemia (CML) represents leukemia of hematopoietic stem cells (HSC). One of the most widely used disseminated tumor models is K562 chronic myelogenous leukemia in SCID mice. The current study was carried out to evaluate the efficacy of Apigenin at dose of 40 mg/kg i.p. and Apigenin+Imatinib test compounds at the dose of 40 mg/kg i.p. + 50 mg/kg p.o., QD dosing for 21 days in K562 disseminated leukemic model. Imatinib was used as reference standard at a dose of 50 mg/kg, p.o., QD dosing for 21 days.

The Apigenin and Apigenin+Imatinib test compounds at the tested dose were relatively well tolerated during the study period. There was progressive body weight loss (mild to moderate) in all animals in combination group during the treatment period, which may be due to the toxicity of the combination therapy. But, this decrease in the body weight was recovered in the post observation period.

The Mice with disseminated K562 leukemia developed hind-limb paralysis within 27 days in vehicle control. The order of percentage protection from onset of hind limb paralysis was found to be Apigenin + Imatinib > Imatinib > Apigenin.

Treatment with Apigenin+Imatinib test compound, Imatinib reference compound and Apigenin test compound attenuated the increase of spleen weight and the occurrence of splenomegaly when compared to the vehicle control group, which may be due to inhibition of immature red blood cell production. This is also evident from the improved spleen histopathology of Apigenin+Imatinib and Apigenin compound treated groups.

The peripheral blood smears from the vehicle control group revealed the presence of multiple tumor cells, indicating the signs of disease progression. However, blood smears from Apigenin+Imatinib and Apigenin compound treatment groups uniformly showed the complete absence of tumor cells in circulation and the presence of only mouse RBCs and WBCs similarly like a standard Imatinib treatment group. The bone marrow smear revealed the presence of increased myeloid series in vehicle control group. However this increased myeloid series was restored with Apigenin+Imatinib and Apigenin treatment groups similar to the Imatinib treated group.

The histology of spleen revealed with high infiltration of neoplastic and white pulp in vehicle control. However, Apigenin+Imatinib and Apigenin treatment group showed a normal histology comprising of normal red pulp and white pulp as similar to Imatinib group. The histology of femur showed increased blasts cells in vehicle control group. However the test groups showed normal myeloid erythroid cells with few megakaryocytic cells.

4. CONCLUSIONS

This present investigation, mice with disseminated K562 leukemia in vehicle control developed severe body weight loss, hind-limb paralysis within 33 days, spleenomegaly, and smear of peripheral blood and bone marrow expressed neoplastic cells, high myeloid precursors respectively, indicating that the CML model was successfully established. The combinatorial therapy of Apigenin with Imatinib and Apigenin as standalone therapy clearly describes the beneficial effects in prevention and treatment of CML in animal model. More detailed investigations are needed in future to fully identify with its mechanisms of action against Apigenin + Imatinib combination therapy.

Moreover, lack of data for its pharmacokinetic and pharmacodynamic profiles in humans. The above mentioned evidence regarding Apigenin is interesting and warrants greater attention. Future studies in this direction will hopefully bring this fascinating flavone to the forefront of cancer therapeutics. However, the consumption of foods and drinks which are rich sources of Apigenin on a daily basis in limited doses could certainly have positive impacts on the body.

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