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In-Vitro Immunomodulatory Effects of Pure Andrographolide On Chicken Spleenocytes

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ABSTRACT

Andrographolide is a diterpene lactone present in aerial parts of *Andrographis paniculata* especially in the leaves. It was reported to possess a wide variety of bioactivities. In the present research immunomodulatory effects of pure andrographolide have been studied on chicken spleenocytes *in vitro*. The immunological intervention of andrographolide was assayed using Nitro Blue Tetrazolium dye reduction assay, Inducible Nitric Oxide Synthase assay and Macrophage function test. Cytotoxic effects of andrographolide on spleenocytes were tested using MTT assay. Andrographolide suppressed Nitro Blue Tetrazolium dye reduction assay response at all experimental concentrations. 45% reduction in immune response was observed when spleenocytes were treated with andrographolide at a concentration of 40µg/ml. The overall Inducible Nitric Oxide Synthase assay response of spleenocytes get decreased with incorporation of andrographolide in media. It was observed that when spleenocytes were mitogenically stimulated in presence of Covcanavalin A and Phytohaemagglutinin ,the suppression of Inducible Nitric Oxide Synthase assay response was observed. Andrographolide was found to enhance the phagocytic ability of spleenocytes upto 10 folds. It was found to be nontoxic at the concentration of 40µg/ml and 96% of spleenocytes remained viable as estimated by Trypan blue dye exclusion method. It was found to have immunosuppressive properties and it can be used to treat inflammatory disorders such as allergies, rheumatoid arthritis etc.

Keywords: Andrographolide, Cytotoxicity, Spleenocytes, Mitogens, Immunosuppression.

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INTRODUCTION

Indian vegetation is very rich in medicinal plants as mentioned in ancient literature. Ashawgandha *(Withania somnifera)* was used as a remedy of diabetes and hypercholesterolemia[1]. *Coriandrum sativum* is a herb cultivated widely around the world and it is commonly used to cure nausea, vomiting, rheumatism and indigestion. It has antelmintic, anti-microbial, antioxidant and hepatoprotective action. Its parts are used treatment of gastric ulcers[2]. Various kinds of herbal plant products are used to modulate the immune system. Z*izyphus mauritiana* was observed to be an immunorestorer *in-vivo* by counteracting the immunosuppression induced by hydrocortisone[3]. *Aegle marmelos* is used for the treatment of HIV/AIDS, fungal infections secondary to HIV, hyperglycemia and gastric ulcer. It has antidiabetic, antioxidant, radioprotective and antimalarial activities[4].Various kinds of herbal plant products are used to modulate the immune system.

Andrographis paniculata is one of the medicinal plants belonging to family Acanthaceae and have a wide range of the physiological effects. It is also known as *Kalmegha* and is commonly used as a remedy of inflammatory lung diseases. Traditionally, its extract is used as anti-inflammatory, immunomodulatory, anti-microbial and anti-viral drug. Roots of *Andrographis paniculata* mainly contain flavonoids, alkanes, ketones but aerial parts of *Andrographis paniculata* are rich in aldehydes. Main chemical constituents found in aerial parts include Andrographolide(AG), Neoandrographolide and Didehydroandrographolide[5]. Its formulations are used for treating cancer, autoimmune diseases, Alzheimer's disease and improvement of liver function[6]. AG was reported to have anti-cancerous property and is widely used as an anti-cancer drug. Encapsulation of AG into SLN improved bio-pharmaceutical properties[7]. It was found to downregulate tumour growth and invasion of Non-small-cell-lung cancer (NSCLC) by enhancing activity of HLJ 1 promoter[8]. AG was reported to induce programmed cell death in platelets by activating caspase-8 dependent extrinsic apoptotic pathway[9]. It was also observed to suppress the invasion ability of colon cancer cells by inhibiting matrix metalloproteinase-2 activity but had no effect on its expression[10]. AG was reported to be a chemotherapeutic agent. It was observed to inhibit the proliferation of glioblastoma cells and may be a potential candidate for the treatment of glioblastomas[11].

AG was observed to increase transcription factor Nrf-2 accumulation and promoted its binding to anti-oxidant response element and hence may have protective role against Chronic Obstructive Pulmonary Disease COPD[12]. AG has been reported to have hepatoprotective property against

rifampicin induced liver damage in male albino rats[13]. AG was observed to act synergistically with curcumin as anti-malarial agent *in-vivo*[14]. AG possesses an anti-diabetic activity by inhibiting action of α-glucosidase and it can be an efficient herbal molecule for management of diabetes[15]. It was reported to have anti-inflammatory potential against cerebral ischaemia in *in-vivo* rat model having Permanent Middle Cerebral Artery Occlusion (pMCAO). Andrographolide was observed to decrease the cerebral infarct volume and neurological deficits induced by pMCAO. Andrographolide also reduced the activation of microglia, TNF-α, IL- 1β and prostaglandin in ischaemic brain areas[16] . It was hypothesized that AG could reduce the joint pain and other symptoms of rheumatoid arithritis[17]. The present study was conducted to investigate the immunological potential of AG to exploit its therapeutic potential against disorders of immune system.

MATERIALS AND METHODS

Materials

Endotoxin free Minimal Essential Media (MEM) and Andrographolide was purchased from *Sigma Aldrich*. Nitroblue tetrazolium dye, 1-Naphthyl Ethylenediamine Dihydrochioride (NEDD), Sulphanilamide, L - Arginine, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide), PHA,Con-A, RPMI 1640 and Fetal Bovine serum (FBS) was purchased from *Himedia*. All the materials were stored according to the manufacturer instructions.

Isolation of spleenocytes:

Spleenocytes were isolated from chicken spleen aseptically by syringe perfusion method using RPMI 1640 supplemented with 10% FBS. Cell suspension was centrifuged at 400g for 3-4 minutes at a temperature of 4˚C. Supernatant was discarded and pellet was reconstituted and incubated in ACK buffer (8.02% Ammonium chloride, 1% Potassium hydrogen chloride, 0.003% Di-sodium EDTA in 500 ml autoclaved water) for 5 minutes at 37˚C to lyse RBCs. The debris (lysed RBCs) was removed by centrifugation at 200g for 2 min. Repeated washing and centrifugation was done with ACK buffer until white coloured pellet was obtained. Pellet was washed with RPMI 1640 to remove residual ACK buffer and reconstituted in same media. Cell viability was tested with the help of Trypan blue dye exclusion assay[18]. Cell number was estimated using haemocytometer and it was adjusted to 2×10^9 viable cells/ml by dilution with MEM. It was labeled as cell suspension and used in later studies.

Immunological Effects of AG On Chicken Spleenocytes

All the experiments were performed in triplicates and statistically significant with P-Value below 0.05

Cytotoxic Effect of Andrographolide On Spleenocytes by MTT Assay[19]

1:1 of spleenocytes and RPMI 1640 (supplemented with 10% FBS) were incubated at 37°C for 48h in humidified CO_2 chamber for pre conditioning of cells. Con A and PHA were supplemented to the cell suspension for induction of blastogenesis at a concentration of 10µg/ml and 40µg/ml respectively. All the samples were treated with different concentrations of AG ranging from 10µg/ml to 40µg/ml. Negative control was also taken. All the samples were incubated at 37°C for 24h in humidified CO_2 chamber.150 µl of MTT (It was prepared in 0.1 M PBS at a concentration of 5mg/ ml. It was sensitive in light hence it was stored at 4˚C in amber coloured tubes) was added to all the samples and incubation was done at 37°C for 4 h in humidified CO_2 chamber. Dimethylsulphoxide (DMSO) was added to solublize formazon and absorbance was taken at 590nm.

Nitroblue Tetrazolium Reduction (NBT) Assay[20]

Spleenocytes were taken in different test tubes. Incubated at 37°C for 30 minutes with NBT dye (0.0023% NBT and 0.116% sucrose in 0.1 M PBS. It was filter sterilized using 0.22 micron filter and stored in dark amber coloured tube at 4˚C) and different concentrations of AG ranging from 10µg/ml-40µg/ml. Negative control was also taken. Centrifugation was undertaken at 200g to remove cells and supernatant was discarded. Cells were washed with 0.1 M PBS (pH 7.2).The reaction was stopped by adding 1% HCl. Dioxan was added to extract formazon from the cells by incubating them at 70°C for 20 min. Absorbance (OD) was taken at 520nm with dioxan as reference.

Percent reduction in NBT response = {(OD of negative control - OD of test samples)/OD of negative control} × 100

Inducible Nitric Oxide Synthase (INOS) Assay[21]

Spleenocytes were taken in different test tubes. PHA and Con-A were added to stimulate the mitogenic activity of spleenocytes at a concentration of 40µg/ml and 10µg/ml respectively. One culture tube was also taken without the addition of mitogens. Different concentrations of AG were added to the test tubes and incubation was done at 37°C in humidified CO_2 atmosphere for 2 hours. 20 µl of 0.2 M fresh Arginine solution was added to all the test tubes. Final volume was made to 2ml by adding RPMI 1640 supplemented with 10% FBS. All the test tubes were incubated for 24h at 37°C in CO_2 atmosphere. 1.5ml of Griess reagent (made by mixing equal quantity of 0.1% NEDD in water and 0.1% sulphanilamide solution in 2.5% Orthophosphoric acid) was added and the tubes were kept in dark for 10min. Absorbance was noted immediately at 540nm taking Griess reagent as reference.

Effect Of AG on Phagocytic Capacity of Spleenocytes[22]

Harvested overnight grown 1ml *E.coli* culture by centrifugation at 8000g. Cell pellet was washed the with Kreb Ringer's phosphate (KRP) buffer to remove traces of growth media. Re-suspended the culture in 5ml KRP buffer. Incubated 100µl of Bacterial cells with 200µl Spleenocytes in presence of 40µg/ml of AG on agar plates. Plates were incubated for 24 hours at 37°C. Colony forming units were counted and compared with control samples

Phagocytosis (%) = {Bacterial count in control - Bacterial count in test samples}/ Bacterial count in control

Lymphocyte Proliferation Assay[23] (Mixed Lymphocyte Reaction)

Spleenocyte suspension was prepared with final viable cell count of 2×10^9 cell/ml. 100 µl spleenocyte suspension of goat and chicken was taken into different culture tubes. One culture tube containing 100 µl mixed spleenocytes (50 µl goat Spleenocytes + 50 µl chicken Spleenocytes) was also taken as shown in table 1. Test sample (AG) of concentration ranging from 10-40 µg/ml was added each culture tube. The final volume of the reaction mixture was made to 500µl by adding RPMI 1640 fortified with 10% FBS. Cells were incubated for 24 hours in atmosphere of CO_2 at 37˚C in a humidified chamber. Culture tubes were centrifuged at 300g for 3-4 minutes at 4˚C and supernatant was discarded. 500µl fresh RPMI 1640 and 5µL of MTT was added to the pellet and gently mixed by spooling. Culture tubes were incubated for 4 hours in humid CO_2 chamber at 37˚C. 100µl DMSO was added to each culture tubes to extract formazon completely from cells. Absorbance was taken 540 nm taking DMSO as reference. Percentage reduction in proliferation of allogenically different spleenocytes was calculated as follows:

% Reduction in MLR response = {(O.D. of control- O.D. of test) / O.D. of control} × 100

Table 1: Experimental setup of Lymphocyte Proliferation Assay.

	Concentration of AG (µg/ml)				
	10	20	30	40	Control
Goat Spleenocytes	100	100	100	100	100
Chicken Spleenocytes	100	100	100	100	100
Mixed Spleenocytes	50+50	100	100	100	100
MEM	390	380	370	360	400

RESULTS AND DISCUSSION.

Isolation of Spleenocytes from Chicken Spleen

The Spleenocytes were isolated from chicken spleen by syringe perfusion method mentioned elsewhere under aseptic conditions and cell viability was analyzed using Trypan blue dye

exclusion assay. The cell fractions that were more than 85 % viable were used for further studies and less viable samples were discarded. The viable cells were pre-conditioned in RPMI 1640 supplemented with10% FBS at 37˚C in humidified CO_2 chamber for 2 hours.

Cytotoxic Effects of AG On Chicken Spleenocytes (MTT Assay)

The cytotoxicity of AG was studied using MTT assay. There was very less effect of AG on viability of spleenocytes. 96 % of the cells remained viable at its low concentration and 94 % of total spleenocytes remained viable at its 40 µg/ml concentration as shown in the figure 1. The compound was observed to be non toxic to the cells and can be used safely for further studies. The results of MTT assay were found in accordance with Jiang and coworkers[24].

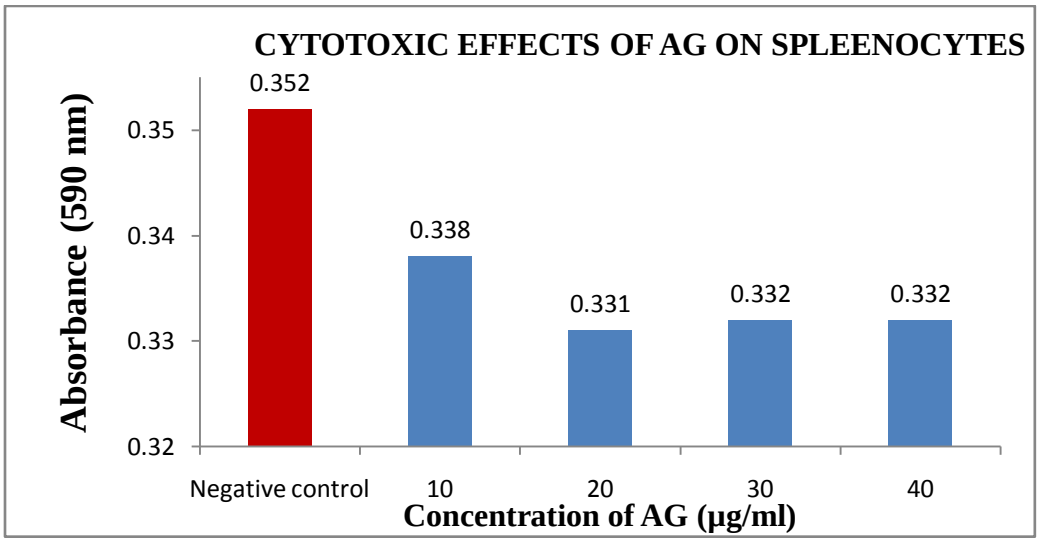

Figure 1 The graph shows the Cytotoxicity of different concentrations of Andrographolide to Spleenocytes *in-vitro*

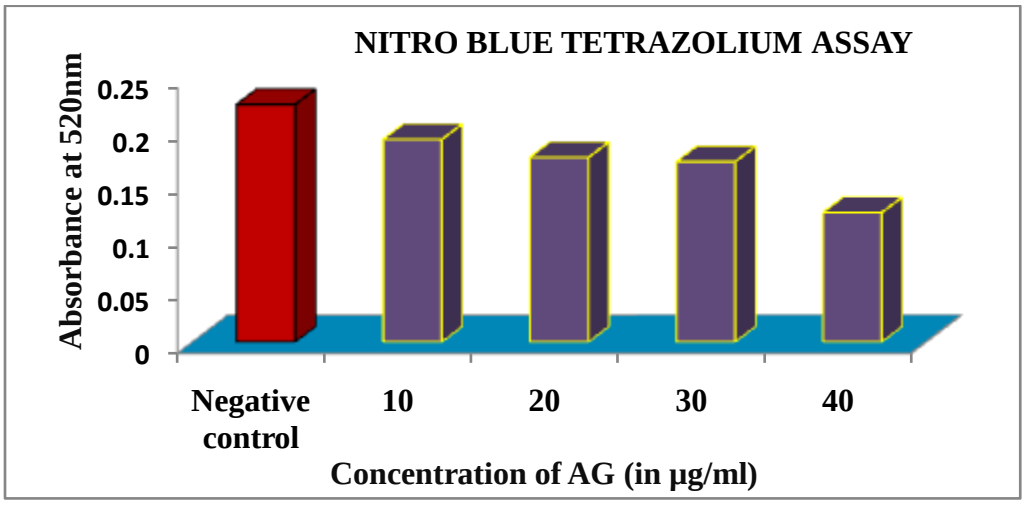

Figure 2: The graph shows the NBT response of Spleenocytes to different concentrations of Andrographolide *in-vitro.*

Nitroblue Tetrazolium Reduction (NBT) Assay

AG was found to suppress the NBT response *in-vitro*. It inhibited formazon formation at all the experimental concentrations. AG potently diminishes the NBT response to 45 % at a concentration of 40µg/ml as shown in figure 2 This reduction in NBT response may be due to the suppression of activity of cellular Dehydrogenases and Reductases. The results of NBT assay were found to be parallel with that of Saranya and coworkers [25]

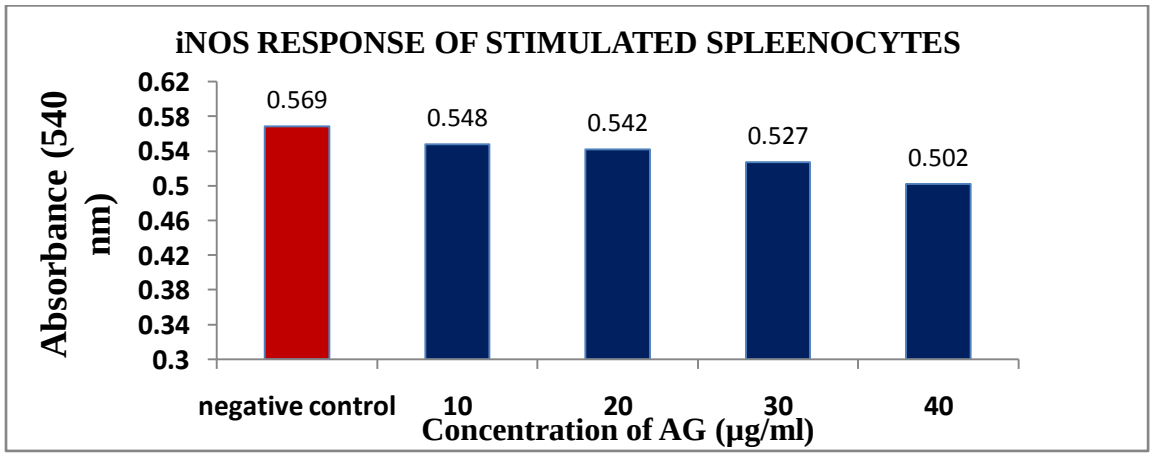

Figure 3.: The graph shows the iNOS response of Spleenocytes to different concentrations of AG in-vitro.

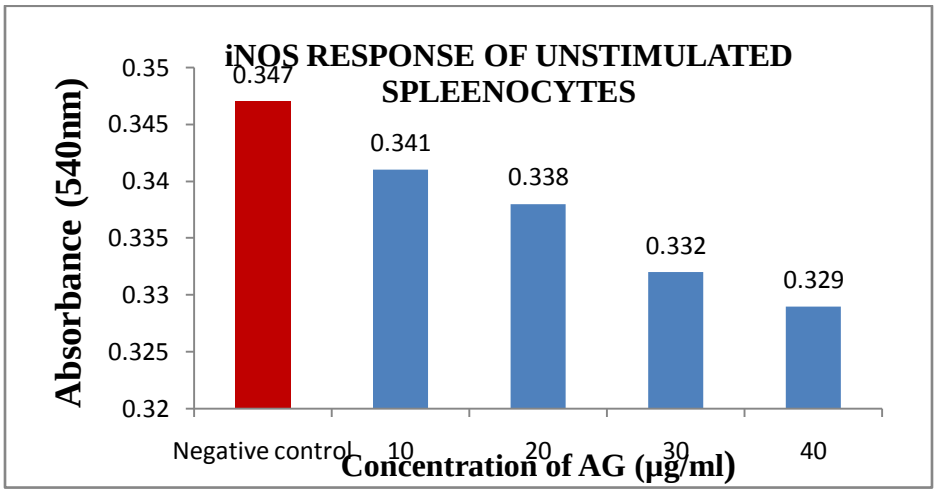

Figure 4 : The graph shows the iNOS response of unstimulated spleenocytes to different concentrations of AG *in-vitro*.

Inducible Nitric Oxide Synthase (INOS) Assay

Andrographolide was found to be effective in down regulating the enzyme activity of *Nitric Oxide Synthase* of mitogenically stimulated cells as depicted by figure 3 However unstimulated cells did not show any impressive decrease in iNOS response as shown in figure 4. This may be due to the reason that actively proliferating cells are more prone to inhibition by AG. It might be

the inhibitor of the *Nitric Oxide Synthase* at molecular level by switching off the gene or by under expression of enzyme at translation level. The results were found in accordance with Chiou and coworkers [26]

Effect of AG On Phagocytic Ability Of Spleenocytes

Phagocytosis is one of the methods to assay the immunological activity of immune cells. The incorporation of AG in the media upregulates the process of phagocytosis. 10 folds increased Phagocytosis was observed at a concentration of 40µg/ml as depicted in Table 2 but at lower concentration showed no significant effect. The results were found to be in compliance with Al-Bayaty and coworkers[27].

Table 2: Effect of Andrographolide on Phagocytic ability of spleenocytes

Group Name	Spleenocytes Suspension	E.coli Suspension (2×10^5 cells/ml)	Concentration of AG	CFU/ml
Negative Control	200µl	100µl	**Nil**	**2.2×10^6**
AG (40µg/ml)	200µl	100µl	40µg/ml	**2×10^5**

Lymphocyte Proliferation Assay (Mixed Lymphocyte Reaction)

Mixed lymphocyte reaction (MLR) is an assay to estimate the proliferation of allogenically different immune cells against each other. Andrographolide down regulates the proliferation efficiency of spleenocytes in dose dependent manner. Andrographolide was effective in 13 % reduction in proliferation activity of spleen cells at a concentration of 10µg/ml which increases to 33 % when its concentration of was increased to 40µg/ml as shown in figure 5. This decrease in proliferation may be due to the fact that the compound is influencing cell cycle at molecular level but how it acts at cellular level is yet to be confirmed. The results were observed to be in equivalence with that of Pannosian and coworkers[28]

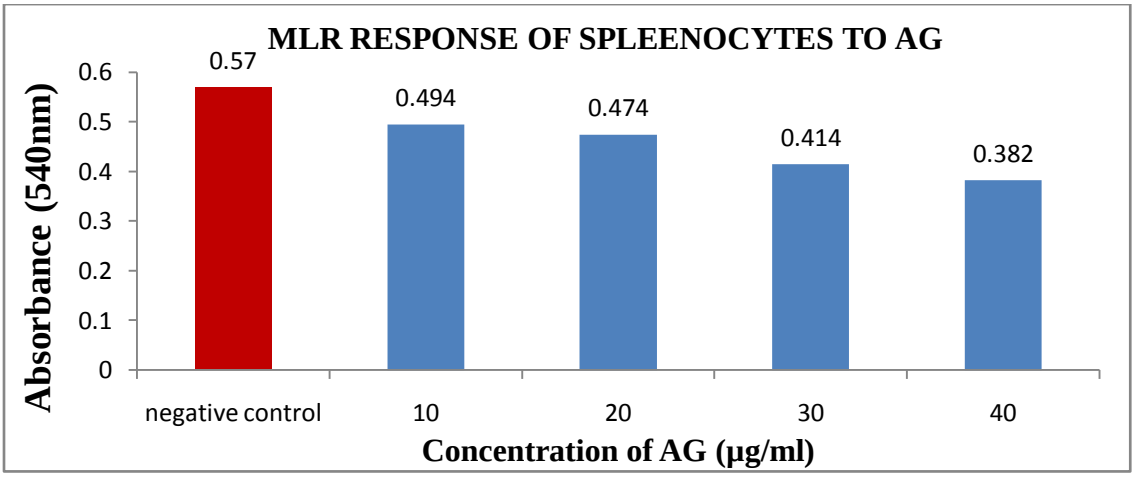

Figure 5: The graph showing MLR response of Spleenocytes to different concentrations of AG *in-vitro*.

CONCLUSION

Andrographolide is one of the bioactive compounds present in aerial parts of *Andrographis paniculata*. The immunological effects of pure AG were studied *in-vitro*. AG was found to be non toxic and it downregulated NBT, iNOS and MLR response in dose dependent manner. It was efficient in enhancing Phagocytosis response of Spleenocytes at higher concentrations. Andrographolide was found to be immunosuppressive in nature and it can be an effective agent in treatment of Allergies and various autoimmune diseases. It can also be used as non-steroidal drug for production of generalized immunosuppression. It was also found to inhibit the proliferation of fast dividing cells and hence it can be a potent molecule to manage and treat cancers.

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