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Toxic Effects of Plant Mediated Silver Nanoparticles on Human Cancer Cell Lines and Bacteria

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Abstract: In pharmaceutical industry, plant mediated nanoparticles, predominantly silver nanoparticles (SNPs) are extensively under consideration for the production of novel drugs. These plant mediated nanoparticles do not harm the nature and therefore, their synthesis is called "Green Synthesis". In the current study, toxic effects of plant mediated SNPs were appraised against human cancer cell lines and bacteria.

Synthesis of stable silver nanoparticles was done by mixing $AgNO_3$ solution (aq) with *Salix nigra* extract as a reducing agent. The extract was intermixed with reagent $AgNO_3$, incubated and studied for the biosynthesis of nanoparticles. Confirmation of synthesis was done by observing color change of plant extracts (SNPs) and UV-Vis spectroscopy. Characterization was done by FTIR, DLS, XRD and SEM. Furthermore, these nanoparticles were tested for their toxic effects on human cancer cell lines and bacteria.

Results against cancer cell lines presented meaningful anti-cell proliferation outcome in smaller concentrations (nano-molar). The antibacterial potential of nanoparticles was investigated by determining the inhibition zone.

Our findings showed that the extract of *Salix nigra* was a very righteous bioreductant and capping agent for the synthesis of silver nanoparticles which, in turn, showed a good toxic activity against human cancer cell lines and clinically inaccessible human pathogens. Thus, the results of this study would be helpful in formulating value added herbal based drugs in biomedical industries.

Key words: UV-Vis, FTIR, XRD, SEM, Human cancer cell line.

INTRODUCTION

Nano based science and technology is generally referred as nanotechnology. In the scientific literature, 10^{-3}, 10^{-6}, 10^{-9}, 10^{-12} and 10^{-15} are referred as mili, micro, nano, pico, and femto. Nanotechnology is one of the most commercially practicable technologies of the 21st century, due to the fact that it helps in creating materials and devices, ranging from 0.1 to 100 nanometer, in any dimension. By the year 2015, nanotechnology acquired $1 trillion market share worldwide. The idea of the nanotechnology was 1st presented in 1960 by Richard Feynman, however, researchers' interest accelerated in the year 1985, with the discovery of fullerene C60 by Kroto *et al* (1985). Plant synthesized nanomaterial, including nanoparticles, nanotubes, nanowires, fullerenes, nanorods, dendrimers, quantum dots, nanoclusters, nanocoatings, nanocrystals and nanocomposites, etc., display meaningfully better and inimitable properties over their corresponding bulk resources (Nalwa, 1999). Some reports shows that silver nanoparticles (SNPs) are non-toxic to humans and are most effective against viruses, prokaryotic and eukaryotic microorganism at low concentrations. They mostly do not have any side effects (Jeong *et al*., 2005). Moreover, numerous salts of silver and their offshoots are commercially manufactured as antimicrobial agents (Krutyakov *et al*., 2005). In small concentrations, silver is benign for human cells, but disastrous for microorganisms (Sharma *et al*., 2009). Nanotechnology refers to a field of applied science and technology, amalgamating theme of which is the control of matter on the atomic and molecular scale (Nanda and Saravanan, 2009). The field of nanotechnology is one of the most dynamic areas of research in modern material science. The most significant application of silver nanoparticles is in medical business, such as, typical ointments, which are made to avert infection against burnt and open injuries (Ip *et al*., 2006). Silver nanoparticles have

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miscellaneous applications both *in vitro* and *in vivo* conditions (Haes and Duyne, 2002). A number of tactics, such as, reduction of solutions (Goia and Matijević, 1998), chemical and photochemical reactions in reverse micelles (Taleb *et al*., 1997), thermal decomposition of silver compounds (Esumi *et al*., 1990), radiation assisted (Henglein, 2001), electrochemical (Rodríguez-Sánchez *et al.,* 2000), sonochemical (Zhu *et al.*, 2000), microwave assisted processes (Pastoriza and Liz-Marzan, 2000) and recently via green chemistry route (Bar *et al*., 2009; Begum *et al*., 2009; Song and Kim, 2009) are available for the production of silver nanoparticles. Although, there are many methods, existing for the synthesis of silver nanoparticles, biological synthesis, using plant sources, offers several advantages, such as, best in cost-effectiveness, non-toxic and eco-friendly (Aymonier *et al*., 2002; Sun and Xia, 2002).

Synthesis of nanoparticles, using plant extracts, is currently under misuse. This traditional synthesis method is more suitable for pharmaceuticals and biomedical applications (Goodsell, 2004). Biosynthetic processes of nanoparticles would be more useful, if nanoparticles are produced extracellularly using plants or their extracts in a controlled style according to their size, shape and dispersity (Kumar and Yadav, 2009).

The current study was aimed to investigate the toxic effects of synthesized silver nanoparticles from the leaf extract of *S. nigra* on human cancer cell lines and various bacterial strains.

MATERIALS AND METHODS

Plant extract preparation: The mature, undamaged and healthy leaves of *S. nigra* (Black Willow) were selected (collection was made from District Bannu, Khyber Pakhtunkhwa, Pakistan), washed carefully with double distilled water (DDW) and shade dried for two weeks. Leaves were then kept in hot air oven at 60°C for a period of 24-48 hours. The dried leaves were mechanically crushed to get fine powder. The leaf powder was decontaminated at 121°C for 15 minutes. 10gm of powder was taken and intermixed with 100ml of distilled water. It was then kept in boiling water bath at 60°C for 10 minutes. The extract was filtered and stored in refrigerator at 4°C for further studies.

Synthesis of Ag nanoparticles: To synthesize silver nanoparticles, 10 ml (10%) of plant extract was intermixed with 90 ml of $AgNO_3$ (99.99%) solution (1 mM/ml) and incubated at room temperature for 1hr. The solution mixture was thereafter put into a shaker (150rpm) at 30ºC for a period of 48hrs to facilitate reaction. The color change in reaction mixture (metal ion solution + leaf extract) was noted through naked eye observation.

Characterization of nanoparticles

UV-visible spectroscopy analysis: Synthesis of Ag NPs by reducing the respective metal ion solution by methanolic leaf extract may be easily perceived by UV-Vis spectroscopy (Shimadzu; UV 1700 Double Beam Spectrophotometer). The absorption spectra of both extract and metal ion concentration were measured in 300-700nm range. Synthesized Ag NPs presented peaks in visible region of the electromagnetic spectrum around 420nm, which confirmed the formation of nanoparticles.

Measurement by FT-IR: For FT-IR, the reaction mixture was centrifuged at 6000rpm for 15 minutes. To get rid of other compounds, the pellets were washed for three times with 20ml of distilled water. The sample was dried and grinded with KBr and then analyzed.

Shimadzu 8400S was used for the measurement of the samples and using a spectral range of 500-4000 m^{-1} with a resolution of 4 cm^{-1}. The FTIR spectra of leaf extract measured before and after the synthesis of NPs were then analyzed for the study of probable functional groups for the synthesis of Ag NPs.

Dynamic light scattering (DLS) or Quasi-elastic light scattering (QELS): DLS is sometimes also known as Quasi elastic light scattering. It is a non-invasive method used to determine the size and size distribution in a sub-micron area. Zeta potential and size distribution of reduced silver nanoparticles was measured using Zetasizer WT, Malvern. Zeta potential and the mean size of the particles in the sample were successfully attained.

X-Ray diffraction measurements: Structural scrutiny of synthesized nanoparticles was carried out using XRD. Nanoparticles were loaded in PANalytical X-Ray diffractometer, which was operating at a voltage of 4000V and current of 20mA. The scanning was carried out with 2θ angle from 20° to 80° at 0.02°/min, with 2θ time constant. To get precise position of atoms, crystal structures of all materials were refined.

Scanning electron microscope analysis: Scanning electron microscopes (SEM) are based on the principle of the reflecting light microscopes. In scanning electron microscope, the beam of the electrons is reflected after colliding the surface of the desired sample and noted by the detector and transformed into an image. In this study, nanoparticles were synthesized, using plant extract as a capping agent. SEM analysis was done using JEOL, Japan: Model MJSM5910 machine (CRL, Peshawar-Pakistan).

Toxicity of silver nanoparticles on human cancer cell lines: MTT assay was used to check the cell sustainability and extract IC_{50} value. Different oncogenic cell line models, i.e., MCF-7 (Human breast cancer cells), Hep-2 (human laryngeal cancer), HT-29 (human colon cancer) cell line were inoculated into 96-well plates and incubated at moistened condition at a temperature 37°C with 5% CO_2. Cells were treated with varying concentration of silver nanoparticles immediately after 24hrs. Incubation

chamber was removed after 48hrs and 12µL MTT dye and 88µL serum free media was added and let it incubate at 37°C. DMSO was replaced instead of MTT media after 4hrs and incubated for 30 minutes. Elisa reader was used for measuring the absorbance at 570nm. By taking absorbance and making calculations of the silver nanoparticle's concentration, the anticancer activity data was standardized. From this data, IC_{50} was calculated.

Cell viability (%) = Mean OD / control OD × 100

Toxicity on bacteria: Toxicity of synthesized silver nanoparticles was carried out using well diffusion method against clinically isolated human pathogens, *E coli*, *K pneumoniae* and *S. aureus*. Streptomycin was used as positive control. For antibacterial assay, the pathogenic cultures were brought into broth medium. More or less 7-mm diameter of well were punctured into the agar plates with the help of sterile cork borer. The cultures were mopped on test media with sterile cotton pad. 30µl of synthesized nanoparticles having various concentrations (25µl, 50µl, 70µl and 100µl) were injected into the wells and then the plates were incubated at 37°C for 24h and the zone of inhibition was recorded after this time period.

Statistical analysis: All data were recorded as mean ± SE of 3 replicates and repetition of experiments in duplicate. Statistical variances among control and samples for all trials were determined using Student's t-test with two-way Anova, which was set at $p \leq 0.05$.

RESULTS AND DISCUSSION

Biosynthesis of Ag nanoparticles: Biosynthesis of nanoparticles started just after few minutes of *S. nigra's* extract was subjected to $AgNO_3$. Color reaction was perceived at regular interval of time in which clear silver nitrate solution was turned into brown color confirming the formation of silver nanoparticles. Results are shown in Fig. 1.

Fig. 1: Color change indicating biosynthesis of silver nanoparticles by *Salix nigra.*

UV-Vis spectroscopy: The formation of silver nanoparticles from 1mM solution of silver nitrate was confirmed, using UV-Vis spectral analysis, as it is one of the popular characterization techniques to determine nanoparticle formation and its properties. The UV–Vis spectroscopy of nanoparticles exhibited characteristic peaks which indicated the formation of nanoparticles (Fig. 2).

Ag NPs having free electrons give rise to a SPR or surface plasmon resonance absorption band. It is due to the combined vibration of electrons of metal nanoparticles in resonance with the light wave. SPR spectra for Ag NPs were obtained at 430nm approx. with brown color. The plasmon resonance band broadens with the decrease in particle size in accordance with the quantum size theories. Increasing time for reaction and extract's concentration plays a vital role in the rapid synthesis of NPs. In green synthesis, various reports are there, which shows that the plant extracts work as a capping/reducing agent for the development of metal nanoparticles.

Fourier transform infrared spectroscopy: The FTIR spectra of leaf extract measured before and after the synthesis of Ag NPs were analyzed for the study of possible functional groups for the formation of Ag NPs. The main purpose of the FTIR measurement was to identify the molecules in the leaf extract, which were responsible for ion's reduction and the capping agents stood accountable for the nanoparticles solution's stability. The FTIR spectrum of *Salix nigra* extract showed broad band at 3400, 3000 and 1600 cm^{-1}, which corresponded to OH group, CH unsaturated and carbonyl groups. In the NPs, a slight shift was in the hydroxyl stretching broad band from 3500 to 3375, and band from 1600 to 1632 cm^{-1}. The band at 3000 was very weak. From FTIR, it's clear that CH unsaturated, hydroxyl groups and carbonyl moiety present in plant extract are responsible for reduction and stabilization of NPs (Figs. 2-3)

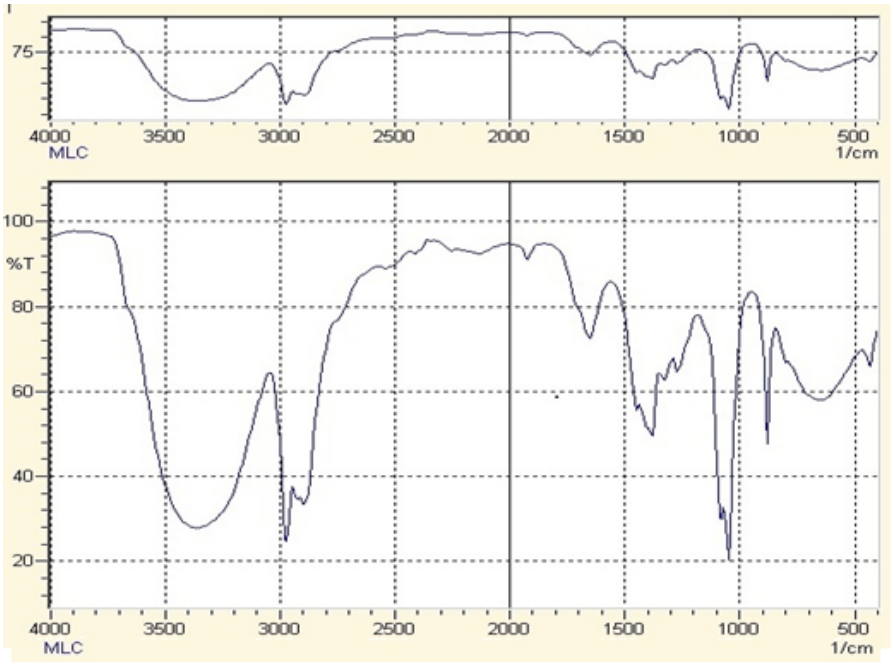

Fig. 2: FTIR spectra of crude extract.

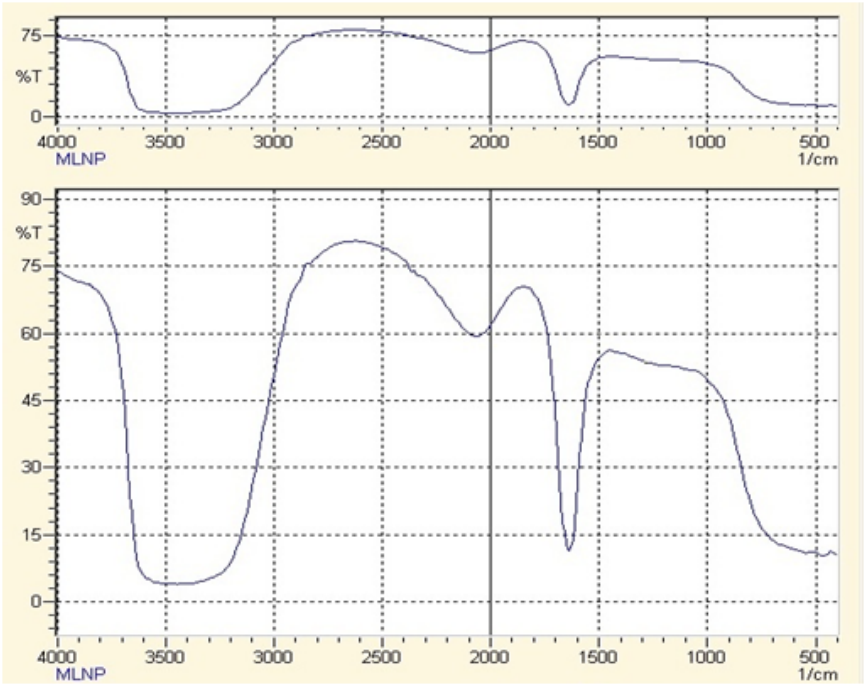

Fig. 3: F TIR spectra of synthesized silver nanoparticles.

DLS analysis and zeta potential: The DLS scrutiny mentioned that the produced Ag\NPs were broadly dispersed in the solution. The size of the nanoparticles was more or less 62 nm, whereas, zeta potential noted was 28 mv and it displayed decent stability. DLS recorded size as somewhat larger than measured by the SEM and it could be explicated by the dynamic light scattering method, which measured the hydrodynamic radii of the particles.

Particle-size distribution was assessed by computerized study of SEM pictures. This was done, using JMicroVision code (provides basic image processing functions). The code is able to calculate the average diameter of the particles in an image from any one of their geometrical characteristics. The calculated average diameters obtained by the three geometrical characteristics are shown in Table 1.

Table 1: Average diameters of Ag particles, as calculated by three geometrical characteristics in the digital processing of scanning electron microscopy images.

Extract source	Average diameter (nm)			Mean (nm)
	by area	by perimeter	by dimensions	
S.nigra	40 ± 4	70 ± 8	52 ± 6	54 ± 1

XRD analysis: The X-ray diffraction form of Ag-NPs synthesized by *S. nigra* is shown in Fig. 4. The *S. nigra* pattern displays no peak assigned to crystal structure. Extensive peak which was centered at 17.52° could be assigned to organic materials in *S. nigra* extract. When $AgNO_3$ was added, the peak shifted to 22.90°. The plant mediated silver nanoparticles pattern presented powerful peaks at 37.80°, 44.20°, 64.40°, 77.30° and 81.70° that could be accredited to 111, 200, 220, 311, and 222 crystallographic planes of the face-centered cubic silver crystals, respectively. It is also anticipated that the wideness in the peaks may also arise from the local crystal defects (elongation strain/compression stress) in the nano-crystals (Fig. 4).

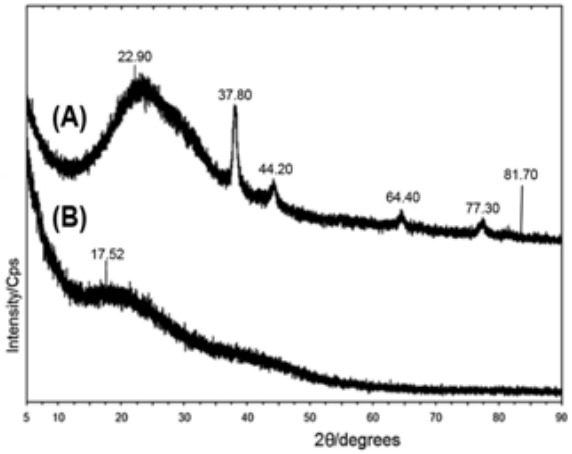

Fig. 4: XRD spectra of nanoparticles.

SEM imaging: SEM technique was used in order to notice the size of silver nanoparticles and their images are shown in Fig. 5. SEM utilized SEM grids, which were made, using sample powder, on a grid caked with copper. Grid was dried using a lamp. The size of the silver nanoparticles obtained was 54.05 nm (Fig. 5).

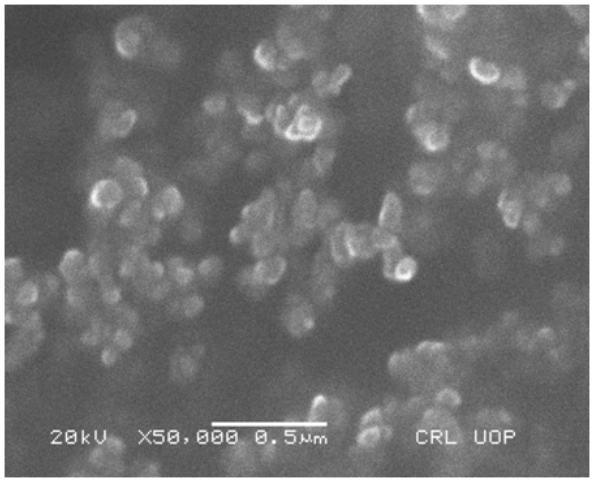

Fig. 5: SEM image of synthesized silver nanoparticles.

Toxic effects against MCF-7, Hep-2, HT-29 cell line: Different concentrations were used to assess *in vitro* toxic effects against MCF-7 (human breast cancer), Hep-2 (human laryngeal cancer) and HT-29 (human colon cancer) cell line. 5-fluorouracil was used as standard drug. Potential cytotoxic results were obtained against Hep-2 cell line, MCF-7 cell line and HT-29 cell line. A microscope was used to screen the plates in order to perceive morphological variations. The result showed a potent cell propagation inhibition of MCF-7 cells by the synthesized SNPs with an IC_{50} value of 12.5µg/ml of the concentration, HT-29 cells with an IC_{50} value of 25 µg/ml of the concentration and Hep-2 cells with an IC_{50} value of 48 µg/ml of the concentration. In this way, the produced SNPs were proved to be active and toxic against MCF-7 cell lines, Hep-2 and HT-29 cell lines. Our study displayed significance of bio-reduced SNPs for inhibiting human cancer cell lines. These results are in agreement with the reports of Ivask *et al.*, 2014 (Table 2).

Table. 2: Cell viability of different human cancer cell lines.

Concentration (µg/ml)	Dilutions	Cell viability (in percentage)					
		MCF-7 cell lines		HT-29 cell lines		Hep-2 cell lines	
		Sample	STD	Sample	STD	Sample	STD
250	Neat	12.40 ± 0.60	4.74 ± 0.30	12.78 ± 0.98	6.58 ± 0.44	10.40 ± 0.88	5.10 ± 1.30
125	1:1	19.07 ± 1.00	14 ± 0.20	17.04 ± 1.32	10.78 ± 0.52	17.65 ± 1.10	8.61 ± 1.90
100	1:2	24.20 ± 1.15	18.30 ± 0.45	24.12 ± 0.89	12.20 ± 0.10	25.24 ± 0.45	10.28± 1.56
75	1:4	31.00 ± 1.11	24.22 ± 1.00	30.60 ± 0.76	18.870± 1.30	30.60 ± 1.00	16.32 ± 1.45
50	1:8	34.35 ± 1.01	27.52 ± 1.20	46.52 ± 1.30	24.00 ± 1.00	**51.30 ± 1.45**	18.70 ± 1.18
25	1:16	42.51 ± 1.00	32.20 ± 1.15	**50.52± 0.26**	33.20 ± 1.08	60.70 ± 1.62	24.88 ± 1.52
12.5	1:32	**50.44 ± 1.50**	38.20 ± 0.60	60.40 ± 0.20	44.80 ± 0.11	69.11 ± 1.32	49.41 ± 1.56
10	1:64	58.45 ± 1.21	52.00 ± 1.30	64.60 ± 1.50	56.85 ± 1.08	80.88 ± 1.40	58.16 ± 1.80
Cell control	-	100	100	100	100	100	100

The cytotoxic potential of Ag-nanoparticles might be due to their interference with the functioning of cellular proteins, which, in turn, brings changes in cellular chemistry. Cytotoxic effects of silver

nanoparticles help in reducing the disease progression as it has a major role in anti-tumor activity. The cytotoxic effects may also be due to active physiochemical interaction of silver atoms with the nitrogen bases and phosphate groups in DNA.

Antibacterial activity of synthesized nanoparticles: The exact mechanism through which nanoparticles exert their antibacterial action is still not understood however, various theories are there that present the action of silver nanoparticles on numerous microbes. In this study, the antibacterial activity of synthesized silver nanoparticles against *E. coli* and *K. pneumoniae* was higher than that against *S. aureus* and it was due to the disparity in the cell wall composition between gram positive and gram negative bacteria (Table 3). Toxicity of nanoparticles to bacteria is due their large surface area which facilitates the contact with the micro-organisms resulting in change in their metabolic activities. The nanoparticle's penetration takes place once they attach their selves to the microbial cell membrane. Bacterial membrane, having sulphur and their DNA with phosphorus, may interact with the silver ions and results in the disturbance in respiratory chain, cell propagation and ultimately in cell death. The silver ions, which enter into the bacterial cell wall, may increase the bactericidal effects of the SNPs (Mittal *et al*., 2013; Aruoja *et al*., 2015).

Table 3: Zone of inhibition of different bacterial strains.

	Zone of inhibition (mm)				
	25 µl	**50 µl**	**75 µl**	**100 µl**	**Control**
Escherichia coli	9 ± 1.0	11 ± 0.9	13 ± 1.0	15 ± 1.2	17 ± 0.9
K. pneumoniae	8 ± 1.0	9 ± 0.8	11 ± 0.5	13 ± 1.4	17 ± 0.9
Staphylococcus aureus	5 ± 1.2	7 ± 0.9	8 ± 0.6	10 ± 1.2	15 ± 1.1

CONCLUSION

Ag nanoparticles act as an antitumor mediator by slowing the progressive development of tumor cells. Current result showed that Ag nanoparticles induced cytotoxicity on various cancer cell lines that caused tumor advancement, thus efficiently governing the progression without toxic effects to normal cells. This may be due to their inhibitory potential involving different signaling cascade, which are accountable for the progress and pathogenesis of illnesses which are not yet investigated.

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