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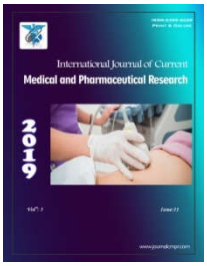

Research Article

IN VITRO ANTICANCER ACTIVITY OF SILVER NANOPARTICLE SYNTHESISED FROM LEAVES OF MURRAYA KOENIGII AGAINST CANCER CELL LINES

Padmaa Paarakh M[1]* and Preethy Ani Jose[2]

[1]Department of Pharmacognosy, The Oxford College of Pharmacy, Bangalore 560 068, Karnataka, India
[2]Department of Pharmaceutics, The Oxford College of Pharmacy, Bangalore 560 068, Karnataka, India

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ABSTRACT

Objective: The primary point of the investigation was to screen the silver nanoparticle synthesized from the methanol concentrate of *Murraya koenigii for* its *invitro* anticancer activity against MCF 2, PC3, A-549, HeLa and HepG2 Cell lines. **Methods:** Silver nanoparticles were prepared from methanol concentrate of *Murraya koenigii* and nanoparticlesynthesised was analysed by UV, SEM and TEM analysis. The impact of nanoparticles synthesized on MCF-2, PC3, A-549, HeLa and HepG2 disease cell lines were assessed by MTT colorimetric assay. After assessing the cytotoxicity effect, the impact of cell cycle and apoptosis was assessed by flow cytometric method. **Results:** TEM analysis showed the particle size of 25.56 nm for silver nanoparticle. The adequacy of silver nanoparticle synthesised from *Murraya koenigii* against MCF-2, PC3, A-549, HeLa and HepG2 cell line demonstrated that the hatching of malignancy cells decreased the suitability of PC-3 and A 549 cancer cell lines only with IC_{50} values as 103.2 and 117.8µg/ml respectively. Cell cycle analysis and apoptosis study showed that Silver nanoparticles are effective in controlling the cell cycle and blocking the apoptosis in all the cell lines used. **Conclusion:** All in all, *Murraya koenigii* has critical cell reinforcement movement and anticancer action against all cell lines used.

INTRODUCTION

Cancer is six letter dreadful diseases which are affecting number of individuals in the world with significant morbidity and mortality rates. Cancers may be caused in one of three ways, namely incorrect diet, genetic predisposition, and via the environment which may take 20–30 years to develop. The present therapies are radiotherapy and chemotherapy which makes their health re-established. But both treatment and immune system are severely affected as treatment methods are not cells selective. As per American Cancer Society 24 million cases of cancer will bediagnosed, with 14 million deaths worldwide by 2030.

As per World Health Organization, world's populations in developed countries rely on traditional medicine and folklore for their health care. Number of drugs has been isolated from medicinal plants which are found to have potential uses against various cancers eg. Paclitaxel, docetaxel, podophyllotoxin, camptothecin, vincristine, vinblastine, vindesine, vinorelbine and others.

Coming to Nanotechnology which is mainly concerned with the application of nanoparticles in various fields of medicine, chemistry, physics, materials science, and engineering. Nanoparticles are synthesized by various physical and chemical methods which are not ecofriendly. The newer studies are focused towards greener or biosynthesis of nanoparticles due to the use of mild conditions such as temperature, pH and pressure [1, 2].

There is need for search for newer drugs from plant which are utilized regularly for food and which is also affordable and easy available. We have chosen one such plant.

Murraya koenigii Linn (Rutaceae) commonly known as curry leaves, growing 4– 6 m tall, up to 40 cm thick. It is used as antiemetic, antidiarrhoeal, dysentery, febrifuge, blood purifier, tonic, stomachic, flavoring agent in curries and chetneys as per traditional system of Medicine. The oil is used externally for bruises, eruption, in soap and perfume industry [3-4]

This plant has been reported to have antibacterial, antifungal, hypoglycemic, anti-oxidative, hypolipidemic, cytotoxic, antihypertensive, larvicidal, antiprotozoal, anti lipid peroxidative, respiratory disorder, trypsin inhibitor, anticancer activities [5, 6].

Accordingly, in the present investigation, we green synthesised silver nanoparticles and furthermore assessed *invitro* anticancer movement against MCF-2, PC3, A-549, HeLa and HepG2 cell lines, cell cycle impact and apoptosis.

Corresponding author:* **Padmaa Paarakh M
Department of Pharmacognosy, the Oxford College of Pharmacy, Bangalore 560 068, Karnataka, India

MATERIALS AND METHODS

Accumulation of Sample *Murraya koenigii* fruit was gathered from Bangalore, Karnataka (India) and verified. The leaves wereair dried.

Preparation of Extract

The leaves was air dried and controlled with a mechanical processor, going through a strainer and put away in a sealed shut compartment. At that point, 50 gms of air-dried powder were constantly refluxed with 50 % methanol at 55˚C for 30 minutes on water bath. The solution is filtered and made up to 100 ml.

Drugs and Chemicals

Silver nitrate, Goldchloride was purchased from SD Fine chemicals Ltd, Mumbai. Propidium iodide: Cat # P4864, Sigma, RNase A: Cat # 109169, Bochringer Mannhein GmbH was purchased. All chemicals and reagents used in this study were at least of analytical grade.

Synthesis of Nanoparticles

A set of 1 mM, 2 mM and 3 mM aqueous solution of silver nitratewere prepared for synthesis for silvernanoparticles [7]. Exactly 9 mL of each 1 mM, 2 mM and 3 mM silvernitrate solutionwas added to 0. 1 mL; 0.2 mL; 0.3 mL; 0.4 mL and 0.5 mL methanol extract of the dried peel fruit of *M.koenigii* to obtain silver nanoparticles. The different concentrations were used to standardize the concentration of silver nitrate and extract needed for synthesis of silver nanoparticles. 1 mM solution was found to give best yield. The nanoparticles were synthesized at room temperature and formation of nanoparticles was confirmed by checking λmax using UV spectrophotscopy.

Lyophilisation Procedure for the Reluctant Sample Mixture

After the formation of nanoparticles, the solution containing silver nanoparticles were lyophilized. The reluctant samples were centrifuged 10,000 rpm for 15 minutes. Discard the supernatant and collected the pellet and freeze dried. The lyophilized samples were kept at 4 °C for further analysis.

Characterization of Silver Nanoparticles [MLSSN]

Ultra Violetvisible spectroscopy analyses were carried out by Shimadzu UV–visible spectrophotometer in the range of 200 nm – 800 nm, with the scanning speed of 100 nm/min. The morphology examination of dried powder samples were analysed by SEM and TEM analysis giving information about the surface morphology and particle size.

In vitro anticancer activity

Preparation of test solutions

32mg/ml sample stock was prepared in sterile DMSO. For cytotoxicity studies, serial two fold dilutions from 320µg/mL to 10µg/mL were prepared which is then used for treatment.

Cell lines and culture medium

All the cell lines were procured from ATCC, stock cells was cultured in DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) in a humidified atmosphere of 5% CO_2 at 37^{o}C until confluent. The cell was dissociated with cell dissociating solution (0.2 % trypsin, 0.02 % EDTA, 0.05 % glucose in PBS).

The Cytotoxic Assay screening was performed using MTT assay as per Mosmann[8] method to test the cytotoxicity of *M.koenigii* synthesised silver nanoparticle against MCF 2, PC3, A-549, HeLa and HepG2 cell lines. In brief, the monolayer cell culture was trypsinized and the 100µl of 50,000 cells were added to each well of the 96 well microtiterplate. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different test concentrations of test drugs were added on to the partial monolayer in microtiter plates. The plates were then incubated at 37^{o}C for 24hrs in 5% CO_2 atmosphere. After incubation the test solutions in the wells were discarded and 100 µl of MTT (5 mg/10 ml of MTT in PBS) was added to each well. The plates were incubated for 4 h at 37^o C in 5% CO_2 atmosphere. The supernatant was removed and 100 µl 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 590 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 cell lines.

- **% Inhibition** = 100 – (OD of sample/OD of Control) x 100.

Cell Cycle Analysis

Silver nanoparticle induced changes in cell cycle were measured using the protocol [9]. In brief, MCF 2, PC3, A-549, HeLa and HepG2 cell lines were exposed for 24 h at 10–50 µg/ml MLSSN. After the treatment, cells were fixed in chilled 70% ethanol for 1 h. Then, cells were washed twice by centrifugation, and cells were stained with propidium iodide for 60 min in dark. Stained cells were acquired by flow cytometer.

In brief, 1 x10^6 cells were seeded and cultured for 24hrs in a 6-well plate containing 2 ml of serum free media. Cells were then treated with desired concentrations of given samples were prepared in complete media and incubated for another 24hrs. Cells were then harvested and centrifuged at 2000 rpm for 5 minutes at room temperature and supernatant was discarded carefully retaining the cell pellet. Cell pellet was washed by resuspending in 2mL of 1XPBS. The washing was repeated another time with the same conditions. Supernatant was discarded retaining the pellet. Cells were fixed by resuspending in 300 µl of Sheath fluid followed by addition of 1mL of chilled 70% EtOH drop by drop with continuous gentle shaking and another 1mL of chilled 70% EtOH added at once. The cells were then stored at 4 °C for or overnight. Post fixing, the cells were centrifuged at 2000rpm for 5mins. The cell pellet was washed twice with 2 ml of cold 1XPBS. Cell pellet was then resuspended in 500µl of sheath fluid containing 0.05 mg/ml PI and 0.05 mg/ml RNase A and incubated for 15mins in dark. The percentage of cells in various stages of cell cycle in compounds treated and un-treated populations were determined using FACS Caliber (BD Biosciences, San Jose, CA).

Apoptosis assay

Apoptosis/necrosis induced by silver nanoparticle in cancer cell lines was analysed using Annexin-V and 7- AAD Kit (Beckman Coulter). The amount of apoptosis/necrosis in the

treated MCF 2, PC3, A-549, HeLa and HepG2 cell lines was analysed by flow cytometry following the protocol [10].

In brief, The day before induction of apoptosis, plated 1 X 10^6 MCF-2, PC3, A-549, HeLa and HepG2 cell linesper well for a 6-well plate using DMEM media with 10% FBS and 1% PenStrep, incubated overnight at 37˚C at 5% CO_2.The media was replaced with test solutions of different concentrations in the media containing 10% FBS. The treated cells were incubated for 24hrs at normal culture conditions. The cells were harvested and well contents were completely transferred to the sterile FACS tubes. The cell contents were centrifuged at 2000 rpm for 5mins and supernatant was discarded. Washed the cells twice with cold PBS following the centrifugation and then resuspended the cells in 1 mL 1X Binding Buffer at a concentration of ~1 x 10^6 cells/mL. Transfer 500 µL of the cell suspension (~5 x 10^5 cells) to a new FACS tube. 5 µL Annexin V and 10 µL PI was added to the tubes, cells were gently mixed and incubated for 20 minutes at RT in the dark. The cells were analysed by flow cytometry as soon as possible (within 1 hour).

RESULTS AND DISCUSSION

The UV spectrum showed that nanoparticles had max at 480 nmand as the concentration increases there is increase in the formation of nanoparticles [Fig 1]

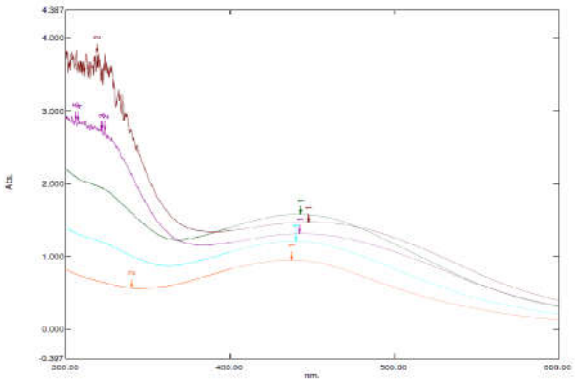

Fig 1 UVspectrum of Silver nanoparticles synthesised from *Murraya koenigii*

Red line-0.1ml; blue line-0.2 ml; black line-0.3 ml; violet line-0.4 ml and brown line -0.5 ml extract of *M.koenigii*

From SEM analysis and TEM analysis, it was very clear that nanoparticles are synthesized and the average particle size was found to be 25.56 nm for silver nanoparticle[Fig 2; Fig 3].

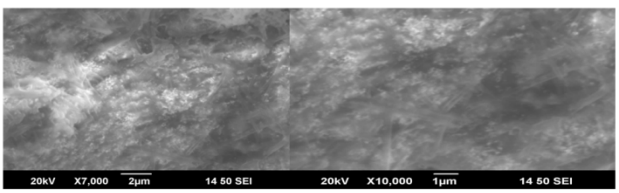

Fig 2 SEM analysis [different magnification] of silver nanoparticles synthesised from *Murraya koenigii*

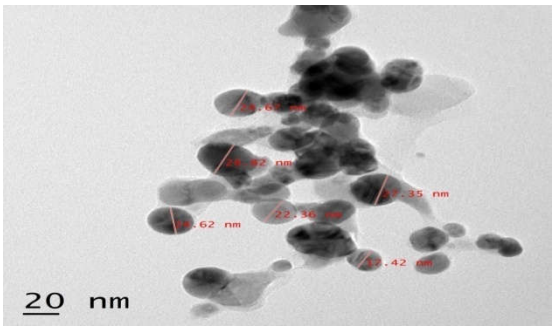

Fig 3 TEM analysis of silver nanoparticles synthesised from *Murraya koenigii*

It is seen that the administration of malignancy and irresistible sicknesses dependably require the quest for new medications. Albeit various medications are at present being used for malignant growth chemotherapy, they display cell poisonous quality, actuates genotoxic, cancer-causing, and teratogenic impacts in non-tumor cells. These reactions limit the utilization of chemotherapeutic specialists regardless of their high viability in treating target threatening cells. Along these lines, the quest for novel medications that are both viable and non-harmful bioactive plant items has been expanded. As of late, ethno botanical and customary employments of normal mixes, particularly of plant inception got much consideration as they are very much tried for their viability and for the most part accepted to be alright for human use.

Traditionally, the *in vitro* determinations of toxic effects of unknown compounds have been performed by counting viable cells after staining with a vital dye like MTT system which measures the activity of living cells via mitochondrial dehydrogenases. The key component is (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) or MTT, is a water soluble tetrazolium salt yielding a yellowish solution which is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by mitochondrial dehydrogenase enzymes of viable cells. The obtained formazanis solubilized by using DMSO and purple solution is spectrophotometricallymeasured[8]. An increase or decrease in cell number results in a concomitant change in the amount of formazan formed, indicating the viability of the cells by the test material.

The effect of silver nanoparticle synthesised from *Murraya koenigii* against MCF-2, PC3, A-549, HeLa and HepG2 cell line demonstrated that the hatching of malignancy cells decreased the suitability of PC-3 and A 549 cancer cells lines only with IC_{50} values as 103.2 and 117.8 µg/ml respectively. The IC_{50} values for MCF 2, HeLa and HEPG2 was found to be 177.2, 231.1 and 224.9 µg/ml respectively [Fig 4].

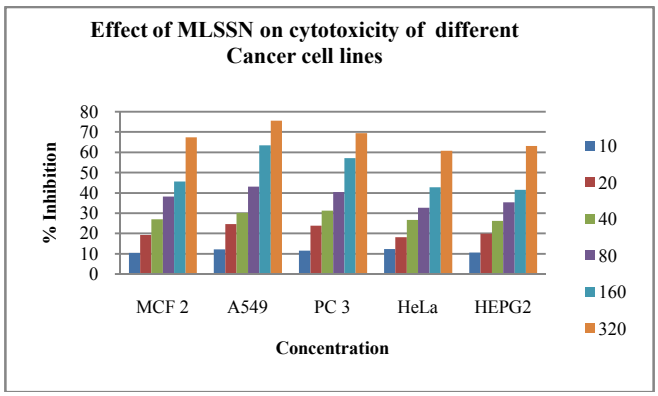

Fig 4 Effect of Cytotoxicity of different concentration of silver nanoparticles against cancers cell lines

The cycle of increase in components (growth) and division, followed by growth and division of these daughter cells, etc., is called the cell cycle. The two most obvious features of the cell cycle are the synthesis and duplication of nuclear DNA before division, and the process of cellular division itself -mitosis. These two components of the cell cycle are usually indicated in shorthand as the "S phase" and "mitosis" or "M". One of the earliest applications of flow cytometry was the measurement of DNA content in cells. This analysis is based on the ability to stain the cellular DNA in a stoichiometric manner. The location to which these dyes bind on the DNA molecule varies with the type of dye used. The most common DNA binding dye in use today is the blue-excited dye Propidium Iodide (PI) which binds to DNA and double stranded RNA (and is thus almost always used in conjunction with RNaseA to remove RNA). When diploid cells which have been stained with a dye that stoichiometrically binds to DNA are analysed by flow cytometry, a "narrow" distribution of fluorescent intensities is obtained. The study suggests that the cells treated with the samples have shown marked increase in % of cells in S phase & G2M phase compared to control; as the check points at these phases play a major role in DNA replication & cell division [11-13].

The result of silver nanoparticle MLSSN showed that G0/G1, S and G2M phase are 73.1,17.21,8.68;59.91,20.68,19.02; 36.4,44.6,19.48; 39.98,12.96,8.7and 81.18,3.48,14.96for MCF 2, PC3, A 549, HEPG2, HeLa cells when compared to control as 84.04,3.10,9.96; 73.18,12.8, 13.88; 79.63, 4.93, 10.19; 68.92, 10.99,18.16 and 82.14, 5.8, 11.56 respectively[Fig 5]. From the cell cycle analysis, it is clear that MCF2, PC 3,A 549 and HEPG2 causes increase in S and G2M stage and HeLa causes increase only in G2M stage suggesting that MLSSN has control on the cell replication and cell division.

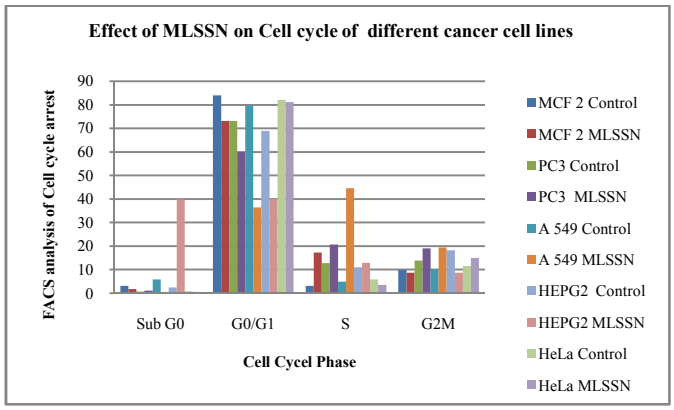

Fig 5 Effect of silver nanoparticles against cancers cell lines on cell cycle

Apoptosis is a cell death process characterized by morphological and biochemical features occurring at different stages. Once triggered apoptosis proceeds with different kinetics depending on cell types and culminates with cell disruption and formation of apoptotic bodies. Recently, it was shown that cells undergoing apoptosis break up the phospholipid asymmetry of their plasma membrane and expose PS which is translocated to the outer layer of the membrane. This occurs in the early phases of apoptotic cell death during which the cell membrane remains intact. This PS exposure may represent a hallmark (early and widespread) in detecting dying cells. Changes in PS asymmetry, which is analysed by measuring Annexin V binding to the cell membrane, were detected before morphological changes associated with apoptosis have occurred and before membrane integrity has been lost. By conjugating FITC to Annexin V it is possible to identify and quantitate apoptotic cells on a single cell basis by flow cytometry. Staining cells simultaneously with FITC-Annexin V (green fluorescence) and the propidium iodide (red fluorescence) allows the discrimination of intact cells, early apoptotic and late apoptotic or necrotic cells [10].

The result of silver nanoparticle MLSSN showed that viable cell, early apoptotic cell, late apoptotic cell and necrotic cell are 46.56,14.06,35.04,4.34;72.22,6.64,17.02,4.12; 73.44,1.5, 17.09,7.97; 63.18,4.7,19.08,13.04 and 74.6,3.88,8.9,12.62 for MCF 2, PC3, A 549, HEPG2, HeLa cells when compared to control as 95.42, 0.38, 0.40,3.80; 92.422.84,3,1.74; 92.76, 0.19,6.09,0.96; 96.86, 1.28,0.46,1.4 and 96.6, 0.14, 1.68, 1.58 respectively [Fig 6]. MLSSN caused decrease in viable cancer cells, increase in the number of late apoptotic and necrotic cells suggesting possessing apoptotic activity.

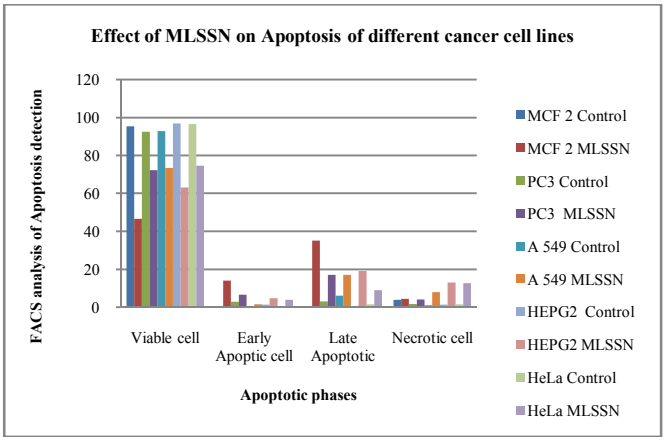

Fig 6 Effect of silver nanoparticles against cancers cell lines on Apoptosis

Curry leaves extract and their constituents are known to exert their activity by diverse mechanisms and are known to inhibit angiogenesis, proliferation, invasiveness, growth, and also to induce apoptosis [2]. As suggested from the result and previous studies on *M. koenigii* has shown that silver nanoparticle has very good activity against cell lines.

CONCLUSION

The cytotoxicity study, inhibition of cell division and increase in number of late apoptotic cell and necrotic cell clearly suggest the role of silver nanoparticle in prevention of proliferation of cancer cells. In future we have planned to target the silver nanoparticle at the site of cancer cell itself. Thus, silver nanoparticles synthesised using the dried peel of *Murraya koenigii* MLSSN is promising agent for Nano chemoprevention of various cancer cell used for the study.

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Ethical Issues

There is none to be applied.

Conflict of Interest

None to be declared.

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