Sustainable Nanocrystals of silver using fresh Leaves Extract of Healing Plant Vinca Rosea and Bactericidal activity

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Abstract **- The present manuscript describe sustainable silver nanocrystals using aqueous extracts of fresh leaves of Vinca Rosea, traditional medicine useful in the conservation of traditional cultures, biodiversity, health care, drug development. Vinca Rosea (*Catharanthus roseus*) green plant first originated from islands of Madagascar, flowers may vary in colour from pink to purple and leaves are arranged in opposite pairs, produces nearly 130 alkaloids mainly ajmalcine, vinceine, resperine, vincristine, vinblastine and raubasin. Vincristine, vinblastine are used for the treatment of various types of cancer such as Hodgkin's disease, breast cancer, skin cancer and lymphoblastic leukemia, Ayurveda for antitumour, anti-diabetic, anti-microbial, anti-oxidant, anti-mutagenic effects and has high medicinal values which need to be explored extensively. This method allowed the synthesis of nanocrystals, which was confirmed by UV-Spectra and TEM. UV-Vis spectra and visual observation showed that the color of the fresh leaf extracts of Vinca Rosea turned into brownish yellow, respectively, after treatment with silver. In addition, TEM analysis confirmed that AgNO3 solutions for all concentrations produced Silver Nanocrystals and their average size was less than 30 nm. Moreover, extracts of Vinca Rosea were tested for their bactericidal activity against Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli* bacteria.**

Index Terms **- Nanocrystals, vinceine, resperine, etc.**

INTRODUCTION

Nanocrystals represent crystals with a nanometer size of 1nm–100 nm. The nanocrystals material has new, unique, and superior physical and chemical properties compared to its bulk structure, due to an increase in the ratio of the surface area per volume of the crystals [1-3]. In sadabahar hypoglycemic and antibacterial activities have not been confirmed, although one of the alkaloids isolated from this plant, ajmalicine, has been reported to possess transient depressor action on arterial blood pressure Periwinkle" or *Catharanthus roseus*, commonly known as "Nayantara" or "Sadabahar", the word *Catharanthus* derives from the Greek language meaning "pure flower", *roseus* means red, rose or rosy [3-6].

Table 1-Show Scientific classification and Vernacular names of Vinca Rosea.

Scientific classification	Vernacular names:
Botanical Name(s) : *Vinca Rosea* (*Catharanthus roseus*) Family Name : Apocynaceae Kingdom : Plantae Division : Magnoliophyta (Flowering plants) Class : Magnoliopsida (Dicotyledons) Order : Gentianales Family : Apocynaceae Genus : Catharanthus Species : *C. roseus*	English : cayenne jasmine, old maid, periwinkle Hindi : sada bahar, sadabahar Kannada : batla hoo, bili kaasi kanigalu, ganeshana hoo, kempu kaasi kanigalu Malayalam : banappuvu, nityakalyani, savanari, usamalari Marathi : sadaphool, sadaphul, sadaphuli Sanskrit : nityakalyani, rasna, sadampuspa, sadapushpi Tamil : cutkattu malli, cutukattu malli, cutukattuppu Telugu : billaganneru Gujarati : Barmasi Bengali : noyontara

MORPHOLOGY

Catharanthus roseus is an evergreen subherb or herbaceous plant growing to 1 m. tall. The leaves are oval to oblong, 2.5- 9.0 cm. long and 1- 3.5 cm. broad glossy green hairless with a pale midrib and a short petiole about 1- 1.8 cm. long and they are arranged in the opposite pairs. The flowers are white to dark pink with a dark red center, with a basal tube about 2.5- 3 cm. long and a corolla about 2-5 cm. diameter with five petal like lobes. The fruit is a pair of follicles about 2-4 cm. long and 3 mm broad.

Fig 1- Photo of plant Vinca Rosea with flower.

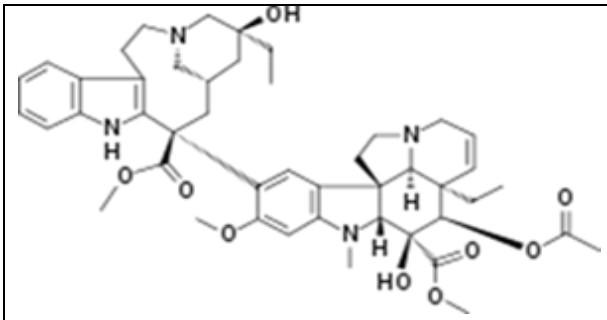
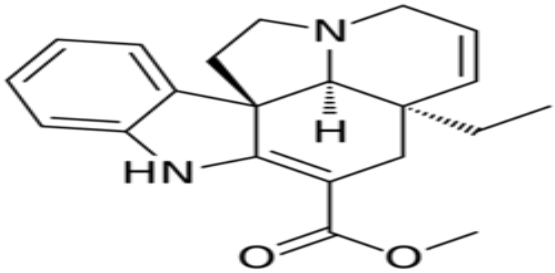
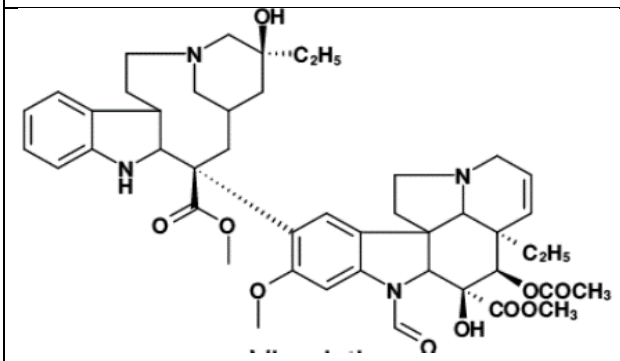
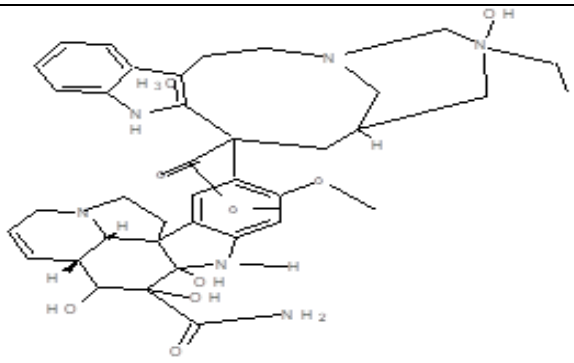
GEOGRAPHICAL DISTRIBUTION

Vinca Rosea is native to the Indian Ocean Island of Madagascar. In the wild, it is found to be an endangered plant and the main cause of their decline is the habitat destruction by the slash and burn agriculture however, it is now common in many tropical and subtropical regions worldwide, including the Southern United states.

POTENTIALLY ACTIVE CHEMICAL CONSTITUENTS

Researchers investigating healing properties of Vinca Rosea that contained a group of toxic alkaloids like actineo plastidemeric, Vinblastine, Vincristine, Vindesine, Vindeline Tabersonine etc. are mainly present in aerial parts whereas ajmalicine, vinceine, vineamine, raubasin, reserpine, catharanthine etc are present in roots and basal stem used in cancer treatment, chemical compounds that are used to perform important biological functions, defend against attack from predators such as insects, fungi and herbivorous mammals, posse's carbohydrate, flavinoid, saponin and alkaloids, pharmaceuticals, agrochemicals, flavor and fragrance, ingredients, food additives and pesticides. The alkaloids Rosindin is an anthocyanin pigment found in the flower of Vinca Rosea [7-10].

Table2- Show the following structure of alkaloids present in Vinca Rosea leaves.

	
1- Vinblastin	2- Tabersonine
	
3- Vincrestine	4- Vindesine

RESULT AND DISCURSION

The results showed that the bacterial growth was inhibited by the extracts containing Ag Nanocrystals. Statistical calculation performed using the Tukey test showed that zones of inhibition for the two bacteria produced by the aqueous leaf extracts of Vinca Rosea containing 4 mM Ag precursors were not significantly

different from that by ciprofloxacin as positive control. A similar result was observed on the zone of inhibition for S. aureus by the extracts of Vinca Rosea leaves containing 4 mM Ag precursor. It was shown that the aqueous extracts of fresh Vinca Rosea leaves containing Ag nanocrystals were comparable to ciprofloxacin in inhibiting bacterial growth.

PHARMACOLOGICAL ACTIVITIES

Anti-cancer activity

The anticancer alkaloids Vinblastine and Vincristine are derived from stem and leaf of Vinca Rosea. These alkaloids have growth inhibition effect to some human tumors. Vinblastine is used experimentally for treatment of neoplasmas and is recommended for Hodgkins disease, chorio carcinoma. Vincristine another alkaloids is used for leukemia in children. Different percentage of the methanolic crude extracts of Vinca Rosea was found to show the significant anticancer activity against numerous cell types in the *in vitro* condition and especially greatest activity was found against the multidrug resistant tumor types. Vinblastine is sold as Velban or Vincristine as on covin [11-14].

Anti-diabetic activity

The extracts of the leaves of Vinca Rosea showed a dose dependent lowering of blood sugar in comparable to the standard drug glibenclamide. The Hypo glycemic effect has appeared due to the result of the increase glucose utilization in the liver, about 20% in diabetic rats when compared to that of the dichloromethane and methanol extracts which lowered the blood glucose level to 49-58%. The hypoglycemic activity of alkaloids isolated from Vinca Rosea has been studied pharmacologically and a remedy derived from the plant has been marketed under the propritery name Vinculin as a treatment for diabetes [15-20].

Anti-microbial activity

Crude extracts from different parts of the plant was tested for anti-bacterial activity. Extract from leaves showed significantly higher efficacy. The anti-bacterial activity of the leaf extract of the plant was checked against microorganism like *Pseudomonas aeruginosa* NCIM2036, *Salmonella typhimuruim* NCIM2501, *Staphylococcus aureus* NCIM5021 and was found that the extracts could be used as the prophylactic agent in the treatment of many of the disease [21-22].

Anti-oxidant property

The anti-oxidant potential of the ethanolic extract of the roots of the two varieties of *C. roseus* namely rosea (pink flower) and alba (white flower) was obtained by using different system of assay such as Hydroxyl radical-scavenging activity, uperoxide radical-scavenging activity, DPPH radical- scavenging activity and nitric oxide radical inhibition method. The result obtained proved that the ethanolic extract of the roots of Periwinkle varieties has exhibited the satisfactory scavenging effect in the entire assay in a concentration dependent manner but *C. roseus* was found to possess more antioxidant activity than that of *C. alba* [23-24].

Anti-helminthic activity

Helminthes infections are the chronic illness, affecting human beings and cattle. *Catharanthus roseus* was found to be used from the traditional period as an anti-helminthic agent. The anti-helminthic property of *C. roseus* has been evaluated by using *Pheretima posthuma* as an experimental model and with Piperazine citrate as the standard reference. The ethanolic extract of the concentration of 250 mg/ml was found to show the significant anti helminthic activity [25-27].

Anti-ulcer property

Vincamine and Vindoline alkaloids of the plant showed anti-ulcer property. The alkaloid vincamine, present in the plant leaves shows cerebrovasodilatory and neuroprotective activity. The plant leaves proved for anti-ulcer activity against experimentally induced gastric damage in rats [28].

Hypotensive property

Extract of leaves of the plant made significant change in hypotensive. The leaves have been known to contain 150 useful alkaloids among other pharmacologically active compounds. Significant antihyperglycemic and hypotensive activity of the leaf extracts have been reported in laboratory animals [29].

Anti-diarrheal property

The anti-diarrheal activity of the plant ethanol leaf extracts as tested in the wistar rats with castor oil as a

experimental diarrhea inducing agent in addition to the pretreatment of the extract. The anti-diarrheal effect of ethanol extracts *C. roseus* showed the dose dependant inhibition of the castor oil induced diarrhea [30].

MATERIALS AND METHODS

Chemicals and Plant Material Collection

All the reagents purchased were of AR grade and used without any further purification. Silver nitrate ($AgNO_3$) was purchased from Chemical Corporation with a ≥99.5% purity. Fresh leaves of Vinca Rosea were collected from Samudrapur region dist-Wardha, Maharashtra, India. Distilled water was used for preparing aqueous solutions all over the experiments.

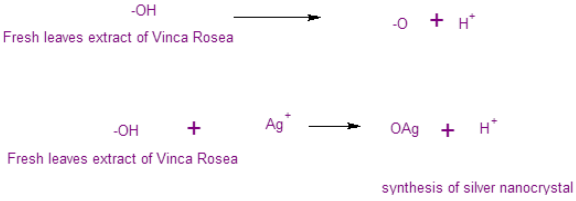


Fig 2- Reaction of $AgNO_3$ with fresh leaves extract

Preparation of Leaf Extract

The fresh leaves of Vinca Rosea were collected and washed with tap water at first, and then the surface was washed under running water with distilled water until no impurities remained. Then, the fresh leaves were cut into small pieces, and 20 g was weighed and put into a beaker with 200 ml of distilled water. The mixture was heated for 40 minutes at 50-60°C while stirring occasionally and then allowed to cool at room temperature.

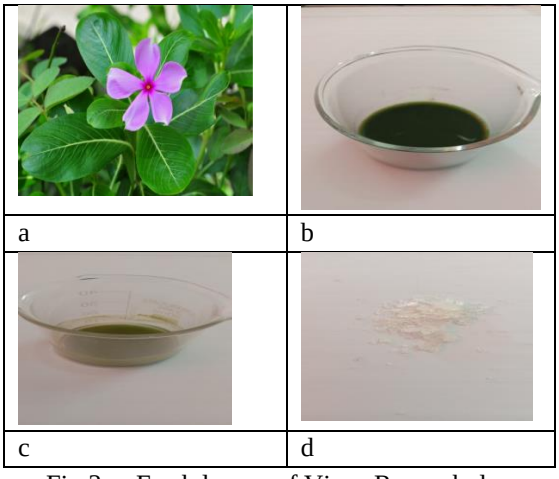


Fig 3-a. Fresh leaves of Vinca Rosea, b. leave extracts, c. After reaction with silver solution leave extracts d. pure $AgNO_3$ powder

SYNTHESIS OF SILVER NANOCRYSTALS

$AgNO_3$ powder was dissolved in distilled water to prepare 10 mM AgNO3 stock solution from which a series of 1 mM, 2 mM, 3 mM, 4 mM, and 5 mM AgNO3 solutions were prepared. The $AgNO_3$ solutions were mixed with the aqueous extract of Vinca Rosea fresh leaves at a ratio of 1: 1 to a volume of 50 mL in a flask. The flask was wrapped with an aluminum foil and was then heated in a water bath at 50-60°C for 2 hours. The mixture was stored in the refrigerator for the antibacterial activity test and further analyzed by using UV-Vis spectrophotometer and TEM.

ANTIMICROBIAL ACTIVITY OF SILVER NANOCRYSTALS AGAINST MICROORGANISMS

All equipment and growing media were sterilized by autoclaving at 115°C and 15 psi for 20 minutes. The antimicrobial activity has been investigated against *S. aureus* as a model for Gram-positive bacteria and *E. coli* as a model for Gram-negative bacteria with disc diffusion method. Preparation of the bacteria stock was done to reproduce and rejuvenate bacteria. This was done by inoculating each one inoculation loop pure culture of *E. coli* and *S. aureus* into 5 ml of nutrient agar solution and then incubated at 40°C for 20 hours in the incubator. Preparation of test bacteria was carried out by inserting one inoculation loop of cultured bacteria into 5 ml of 0.19% NaCl solution. The inhibition method was used in evaluating the antibacterial activity. 20 ml of nutrient agar solution was put into a Petri dish, sterilized for 20 minutes until the nutrient agar solution became solid, and then 0.1 ml of bacterial solution was applied to the nutrient agar growing medium. Thereafter, negative control (distilled water), positive control (Ciprofloxacin) were placed. Next, it was incubated at 37°C for 24 hours before the clear zone diameter was measured using the sliding term. To see the ability of each leaf extract containing silver Nanocrystals in inhibiting bacterial growth compared to ciprofloxacin positive control, a statistical analysis was performed using the Tukey test at the 95% confidence level.

CHARACTERIZATION OF SILVER NANOCRYSTALS

The reduction of pure Ag+ ions was monitored by measuring the UV-Vis spectrum of the reaction medium after diluting a small aliquot of the sample into distilled water. The color change in the reaction mixture (metal ion solution + Vinca Rosea extract) was recorded through visual observation. UV-Vis spectral analysis was done by using UV-Vis spectrophotometer UV-1800 at the wavelength of 200–800 nm. JEOL JEM-1400 Transmission Electron Microscope (TEM), operating at 120 V and an acceleration voltage of 15 kV, was used to analyze the morphology and size of silver nanocrystals. For TEM measurements, extract samples containing silver nanocrystals were dispersed on a copper grid and dried at room temperature. The particle sizes of the silver nanocrystals were measured using Image J software. The histogram of the size distribution was established by Origin software.

RESULTS AND DISCUSSION

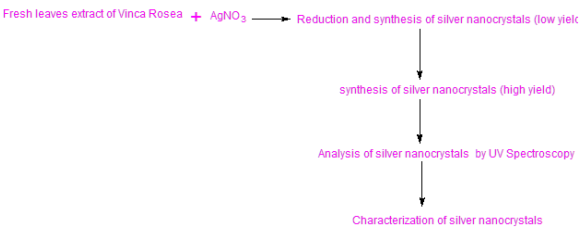


Fig 8- Complete reaction in synthesis and characterization of silver nanoparticles.

UV-VIS SPECTRA ANALYSIS

The aqueous extract of fresh leaves of Vinca Rosea becomes yellowish brown. This warm extract solution changed color again after adding AgNO3 solution. Color changes are possible because some of the Ag ions begin to be reduced due to the effects of heat and produces Ag+ complex. This complex was responsible for changing color from brownish yellow to grayish Vinca Rosea. This color change indicates the formation of silver nanocrystals. UV-Vis spectra of silver nanocrystals synthesized using the Vinca Rosea aqueous extract evince the blue shift of the absorption band with increasing AgNO3 concentration. For 1 mM, 2 mM, 3 mM, 4 mM, and 5 mM samples, the absorption peak is centered on 450–420 nm. This information shows that the silver nanocrystals have formed in the extract, where the Ag+ has been reduced to Ag. Proteins and all secondary metabolites of extract play a critical role in both the reducing and capping mechanism for nanocrystals formation can be seen in Table 1.

Concentration(mM)	Wavelength(nm)	Absorbance
1	451	0.8
2	453	0.9
3	451	1.1
4	448	1.2
5	452	1.6

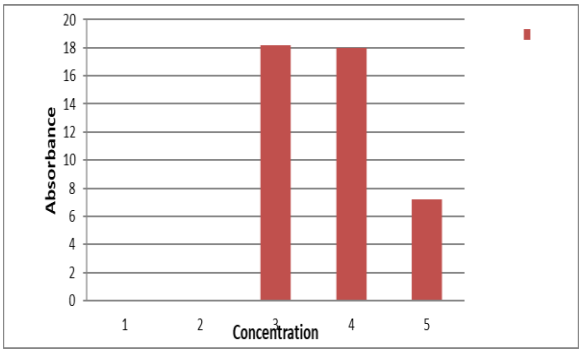


Fig 9 -Peak wavelength and absorbance of silver nanocrystals in aqueous extracts of fresh leaves of Vinca Rosea.

TEM ANALYSIS

The silver nanocrystals are quite poly dispersed and a layer of the organic material surrounding the synthesized silver nanocrystals could explain the good dispersion of these nanocrystals in solution. Generally, the silver nanocrystals using aqueous extracts are well dispersed although some of them were noted to be agglomerated. Notably, the majorities of the particles in the TEM images are not in physical contact with each other but appeared separated by the organic layer. The presence of several poly phenol components including flavonoids and terpenoids facilitated the reduction of Ag ions and also stabilized the surface of the resultant silver nanocrystals.

The Ag ions quantity influenced the size of the particles. When AgNO3 concentration is increased to 5 mM, an obvious change in the size distribution of nanocrystals was observed (Table 2).

Concentration of AgNO3 solution (mM)	Vinca Rosea
1	12 ± 2
2	14 ± 2.1
3	16 ± 2.3
4	19 ± 2.5.
5	120 ± 2.3

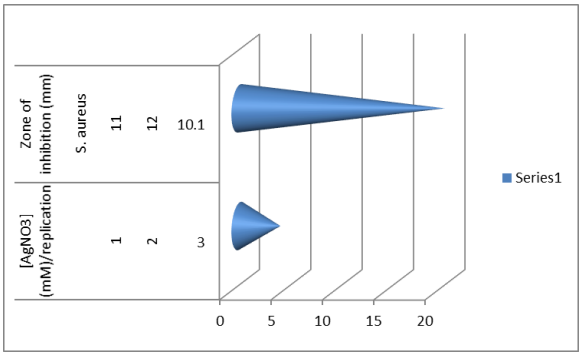


Fig 10-Size of silver nanocrystals produced from various concentrations of AgNO3 using aqueous extracts of fresh leaves of Vinca Rosea.

ANTIBACTERIAL ACTIVITY STUDIES

The present study revealed that the tested leaf extracts of Vinca Rosea plant showed potent antibacterial activity against two bacterial strains: Gram-positive *S. aureus* and Gram-negative *E. coli*. Aqueous extracts of Vinca Rosea containing silver nanocrystals showed activity in all Ag concentrations tested against all bacteria. Antibacterial activity was shown by an inhibition zone which was characterized by a clear zone between the wells and a certain distance. Formation of inhibition zones around the wells shows bacterial sensitivity to antibacterial and antibiotic ingredients. The positive control used in the well was a ciprofloxacin 500 mg solution and functioned as a control of the test solution by comparing the diameter of the inhibition zone formed. On the contrary, distilled water as negative control was used to determine the effect of solvents in the test solution on the growth of *S. aureus* and *E. coli* bacteria. It was clear that it was the extracts containing silver nanocrystals that had the antibacterial activity, not the solvent.

The diameter of inhibition zones formed for each concentration of the $AgNO_3$ precursor added to the aqueous extracts of fresh leaves of Vinca Rosea plants in synthesizing silver nanocrystals presented in Table 3.

Plants	[AgNO3] (mM)/ replication	Zone of inhibition (mm)			
		S. aureus	Positive control	E. coli	Positive control
Vinca Rosea	1	11	18.2	14	17.8
	2	12	18	8.5	2 9.1
	3	10.1	7.2	19	10
	4	20	19	10.5	19
	5	10	19.2	7.8	18.9

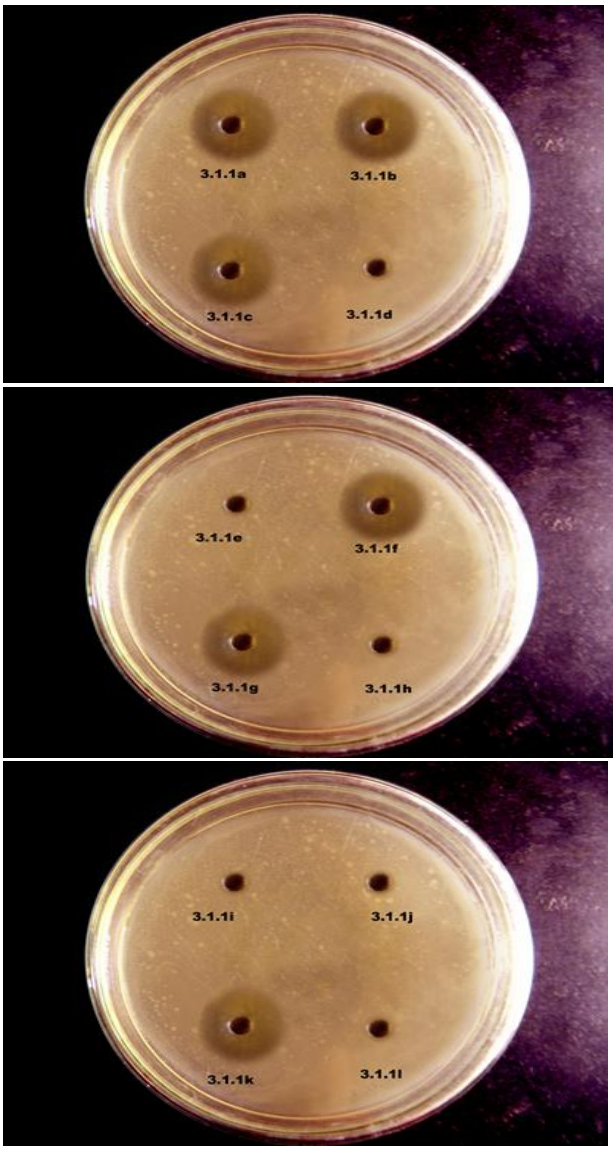


Fig 11-Photographs showing zone of inhibition

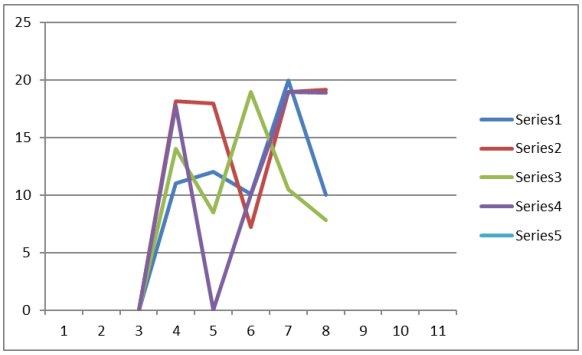


Fig 12-Antibacterial activity of silver nanocrystals synthesized using various concentrations of AgNO3 precursors and aqueous extracts of fresh leaves of Vinca Rosea.

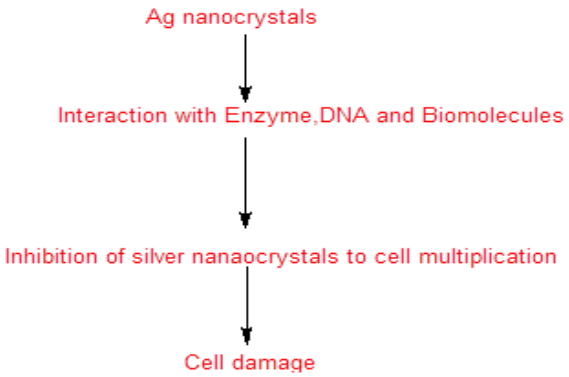


Fig 12- Reaction of silver nanocrystals with bimolecular

However, based on the results of statistical analysis, it was shown that only three treatments were significantly different than the positive controls. The variables were aqueous extracts of Vinca Rosea leaves containing silver nanocrystals against *S. aureus* and *E. coli* bacteria. This information was supported by data that the average size of silver nanocrystals synthesized using extract was relatively smaller than that using the extract of Vinca Rosea .The results of this study were also supported by previous studies that the small size of silver nanocrystals makes these crystals easier to penetrate the outer wall of bacteria, enter the body, destroy the respiratory chain, and thus inhibit cell respiration, causing bacterial death.

CONCLUSIONS

Vinca Rosea healing plant extract of fresh leaves can be used as bioreducing agents to produce silver nanocrystals. The formation of silver nanocrystals in the extract was observed by the color change of Vinca Rosea extract into brownish yellow while of Vinca Rosea extract into grayish brown. Color changes that occur indicate that silver nanocrystals have formed. The silver nanocrystals produced had an increased size due to the increased concentration of AgNO3 solution, but the average size is still in nanometer. Silver nanocrystals contained in the extract were able to inhibit the growth of *S. aureus* and *E. coli* bacteria, and the best antibacterial activity was exhibited by the Vinca Rosea extract containing silver nanocrystals.

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