Research Article

Comparative study of anti-angiogenic and cytotoxic activity of leaf and leaf callus silver nanoparticles of *Tephrosia villosa* Pers.

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Abstract

Objective: The present study aimed to synthesis silver nanoparticle (SNPs) from leaves and leaf callus of *Tephrosia villosa* and its anti-angiogenesis and cytotoxicity activity. **Materials and methods:** Antiangiogenic activity was conducted on fertilized eggs by modified *in vivo* CAM method and also *in vitro* cytotoxicity activity was studied by MTT assay at different concentration. **Results:** Both samples showed the higher angiogenic activity 94.5±0.5, 92.0±0.1 at the concentration 125 µg/ml. The CAM treated samples displayed distorted vascularation as well as perturbation on existing vasculature. The percentage of vessels inhibition in treated CAM was 13.5±1.5 and 12.5±1.5 at 125 µg/ml concentrations of leaf and leaf callus SNPs respectively. The *in-vitro* anticancer activity of leaf and leaf callus SNPs carried out against MCF-7 cell line by MTT assay showed 74.46 %, 74.35 % cell death at 250 µg/ml followed by 125 µg/ml concentrations with 70.23 and 74.35 µg/ml respectively. The IC_{50} value of leaf and leaf callus SNPs was 63.20 µg/ml, 12.15 µg/ml respectively. When compared the leaf SNPs, the callus SNPs activity was more in the degree of cell mortality. **Conclusion:** This study confirmed that both the leaf and leaf callus SNPs revealed of anti-angioenic and anticancer activity in *in vitro* system. For the commercial and medicinal purpose instead of using wild plant parts, we can use callus. This will reduce the pressure on wild plant collection and also conserve the plant species.

Keywords: Silver nanoparticle, angiogenesis, MTT assay and cytotoxicity

Introduction

Cancer, as an increasingly up-to-date health problem during the last two decades, is now ranked the second cause among the 10 top leading of death around the world. Fortunately, the loss of life has been significantly inhibited by the rapid developments of different diagnostic devices and therapeutic method (Liu et al., 2012). However, among all the current clinical cancer treatments, there is none methodology can only selectively bind and target cancer cells to avoid the toxicity and unwanted side effects of the patients. A new contribution to control this problem is the emergence of nanoparticles synthesis, which opens a new interdisciplinary research area to analyze potential alternative nano-sized materials for cancer diagnosis and treatment (Mollick et al., 2014).

Silver, a noble metal commonly used for preparing jewelry over 200 years ago since discovered, has long been used as a potential anti- bacterial agent with lower toxicity and bacterial resistance properties (Loo et al., 2016; Dipankar and Murugan, 2012). Silver nanoparticles are the greatest importance to expand their biomedical applications and it focuses on describes therapeutic applications of nanoparticles based on their mechanical agents in biocompatible nanocomposites such as micellar nanoparticles; nano capsules (Panyan et al., 2003). Silver nanoparticles have been proved to have great potencial in anti-tumor activity in recent years (Chang et al., 2017; Guo et al., 2015).

The biosynthesis of silver nanoparticles using the plant *Tephrosia villosa* belonging to the family Fabaceae. *Tephrosia villosa* is widely used in traditional Indian medicine as a treatment for dropsy and diabetes (Madhusudhanaa et al., 2010). The taxon is also used as green

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manure in Coffee and Hevea Plantations and as a shade crop in tea plantations (Bosman and De Haas, 1983). Roots, leaves, fruits and twigs of *Tephrosia villosa* showed significant activity against *Culex quinquefasciatus* larvae (Nondo et al., 2011). *Tephrosia villosa* leaves showed reduction in glucose level and pancreatic cells regeneration in alloxan induced diabetes in presence of 20 (29)-lupen-3-ones a compound (Prashant and Krupadanam, 1993; Kim et al., 2001). Four new rotenoid were isolated from seeds and dehydroxyrotenoid and lupenone were isolated from whole plant. The present study aimed to synthesis silver nanoparticle (SNPs) from leaf and leaf callus of *Tephrosia villosa* and its anti-angiogenesis and cytotoxicity activity.

Materials and Methods

Preparation of plant extract

The powdered *Tephrosia villosa* leaf and callus was extracted using 100 ml of ethanol for each sample by using the Soxhlet extractor for 14 hrs (Gafner et al., 1998). The extracts was filtered through Whatmann No.1 filter paper to remove all undissolved matter including cellular materials and other constitutions that are insoluble in the extraction solvent and stored at 4°C used for further experiments.

Preparation of silver nanoparticles

Aqueous solution of 1mM $AgNO_3$ was prepared and used for the synthesis of silver nanoparticles. 10 ml of *T. villosa* ethanol extract of leaf and callus is mixed with 90ml of $AgNO_3$ for the synthesis of silver nanoparticles. The formation of silver nanoparticles of leaf extract was confirmed by UV-visible spectroscopy, Fourier-transform infrared (FT-IR), Energy-dispersive X-ray (EDX), Scanning electron microscopic (SEM), X-ray diffraction (XRD) (Ranjitha et al., 2018).

Anti-angiogenesis assay

Antiangiogenic activity of leaf and leaf callus nanoparticle synthesis sample of *T. villosa* was conducted on fertilized eggs by modified *in vitro* CAM assay method (Parivash Seyfi et al., 2010). Five days incubated fertile white Leghorn chicken eggs were obtained from a local hatchery and they are incubated placed in horizontal position and rotated several times at 37˚C in humified 3 days. The eggs were grouped as per type of SNP_s and sprayed with 70% ethanol and air-dried to reduce contamination from the egg surface. On day 6, 26- gauze needle was used to puncture a small hole in the air sac of the egg, and 2-3 ml of albumen was sucked and sealed. This allows separation of vascularized CAM from the vitelline membrane and the shell. A window was then cut in the shell using a sterile blade and shell was removed with sterile forceps, under Laminar air flow. The window is closed with a cellophane tape after capturing the photographs of the embryo. The eggs were returned to the incubator after the filter paper discs (100 micrograms of SNP_s) of SNP_s are placed on blood vessels of embryo using sterile forceps. After 48 h incubation on 8th day photographs of embryos were taken to obtain the image of CAM after treatment with both SNP_s. At least six eggs were used for each sample dose. The percentage inhibition was calculated using the following equation.

% inhibition = {(vessel number of untreated CAM-vessel number of CAM treated with herbal extract)/vessel number of untreated CAM} x 100.

Cytotoxicity activity

Cell line

Human, Breast Adeno Carcinoma (MCF-7) was obtained from National Centre for Cell Science (NCCS), Pune and grown in Eagles Minimum Essential Medium containing 10% fetal bovine serum (FBS). The cells were maintained at 37˚C, 5% CO_2, 95% air and 100% relative humidity. Maintenance cultures were passaged weekly, and the culture medium was changed twice a week.

Cell treatment procedure

The monolayer cells were detached with trypsin-ethylene di amine tetra acetic acid (EDTA) to make single cell suspensions and viable cells were counted using a hemocytometer and diluted with medium containing 5% FBS to give final density of $1x10^5$ cells/ml. One hundred microliters per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 37˚C, 5% CO_2, 95% air and 100% relative humidity. After 24 h the cells were treated with serial concentrations of the test samples. They were initially dissolved in dimethyl sulfoxide (DMSO) and an aliquot of the sample solution was diluted to twice the desired final maximum test concentration with serum free medium. Additional four serial dilutions were made to provide a total of five sample concentrations. Aliquots of 100 µl of these different sample dilutions were added to the appropriate wells already containing 100 µl of medium, resulting in the required final sample concentrations. Following sample addition, the plates were incubated for an additional 48 h at 37˚C, 5% CO_2, 95% air and 100% relative humidity. The medium containing without samples were served as control and triplicate was maintained for all concentrations.

MTT assay

3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the

MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells.

After 48 h of incubation, 15µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37˚C for 4h. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100µl of DMSO and then measured the absorbance at 570 nm using micro plate reader. The percentage cell viability was then calculated with respect to control as follows

% Cell viability = (A) Test / (A) control x 100

% Cell inhibition = 100 - (A) Test / (A) control x 100

Results

Characterization of silver nanoparticles

In our previous study, we performed Characterization of silver nanoparticles through UV-visible, FTIR, EDX, XRD, SEM and get published (Ranjitha et al., 2018).

Antiangiogenic activity

Antiangiogenic activity of SNPs of leaf, leaf callus samples were tested through *in vivo* CAM model. The 7th day old embryo after treatment for number of blood vessels and their reduction was examined. The analysis of blood vessel was based on evaluation of angiogenesis by measuring the area of inhibition surrounding the applied disc. The inhibition percentage is shown in the table 1. Both samples showed the higher angiogenic activity 94.5±0.5 and 92.0±0.1 at the concentration 125 µg/ml. The figure 1 and 2 respects normal vascularation in the treated CAM which consists of primary secondary and tertiary micro vessels. The CAM treated samples displayed distorted vascularation as well as perturbation on existing vasculature (Figure 1 and 2). The second best angiogenic activity was observed at 250 µg/ml as 86.0 ± 0.1 and 83.5±0.5% leaf and leaf callus against control (92 ± 0.1 and 58 ± 0.1) NaOH and DMSO respectively. The percentage of vessels inhibition in treated CAM was 13.5 ± 1.5 and 12.5 ± 1.5 at 125 µg/ml concentrations of leaf and leaf callus extracts SNPs.

Cytotoxicity activity

The *in-vitro* cytotoxicity activity of leaf and leaf callus SNPs carried out against MCF-7 cell line by MTT assay showed 74.46 %, 74.35 % cell death at 250 µg/ml followed by 125 µg/ml concentration with 70.23 and 74.35 µg/ml

Table 1. Anti-antigenic activity of leaf and leaf callus SNPs of *T. villosa*

S. No	Sample	Concentration (µg/ml)	No. of vessels in treated CAM	% of vessels Inhibition	% Inhibition (Mean±SD)*
1	Leaf SNPs	1000	13±0.57	12.5±0.5	84.5±1.5
2		500	18.1±01	13.0 ± 01	85.5±0.5
3		250	14.5±0.5	12.0±1.5	86.0±01
4		125	14.2±0.5	13.5±1.5	94.5±0.5
5	Leaf callus SNPs	1000	14.1±01	11.5±0.5	77.5±1.5
6		500	16.5±0.5	10±0.5	61.5±1.5
7		250	20±0.01	11±0.12	83.5±0.5
8		125	14.5±0.5	12.5±1.5	92±01
9	NaOH	200	12.5± 0.5	11±0.03	92±01
10	DMSO	200	17±0.03	10±01	58±01

Three eggs used for each samples; Mean ± SD was calculated for % inhibition of each sample

Table 2. Cytotoxicity activity (MCF-7 cell line) of leaf SNPs of *T. villosa*

S. No.	Concentration	Test I	Test II	Test III	Average	Cell Viability
1	250	0.0611	0.0812	0.083	0.0751	25.03333
2	125	0.0891	0.0877	0.0912	0.089333	29.77778
3	62.5	0.096	0.0912	0.1111	0.099433	33.14444
4	31.25	0.2121	0.2222	0.2789	0.237733	79.24444

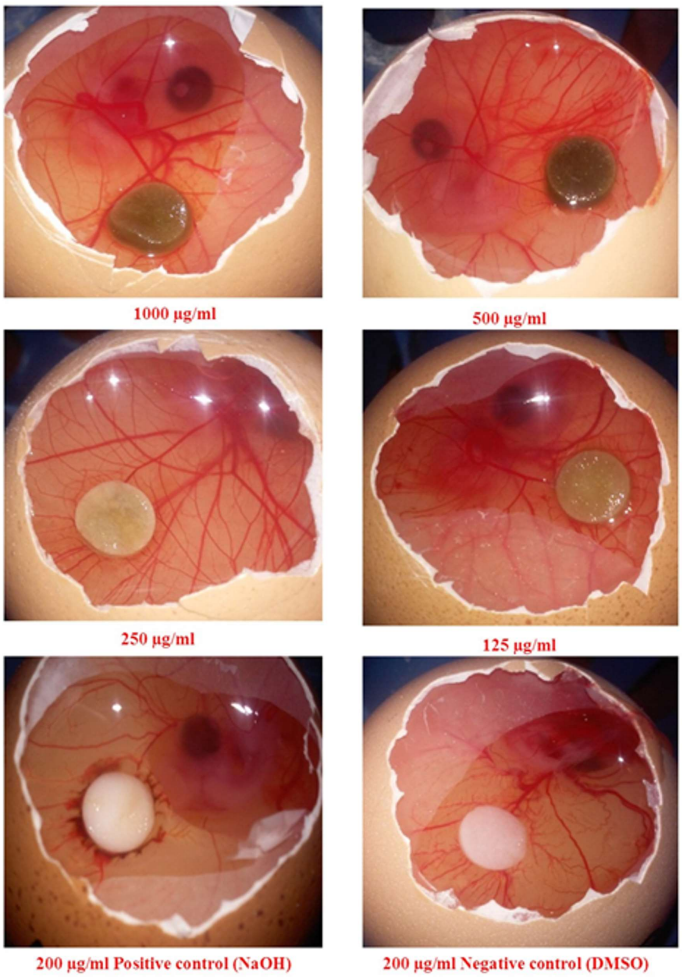

Figure 1. Antiangiogenesis activity of leaf SNPs of *T. villosa*

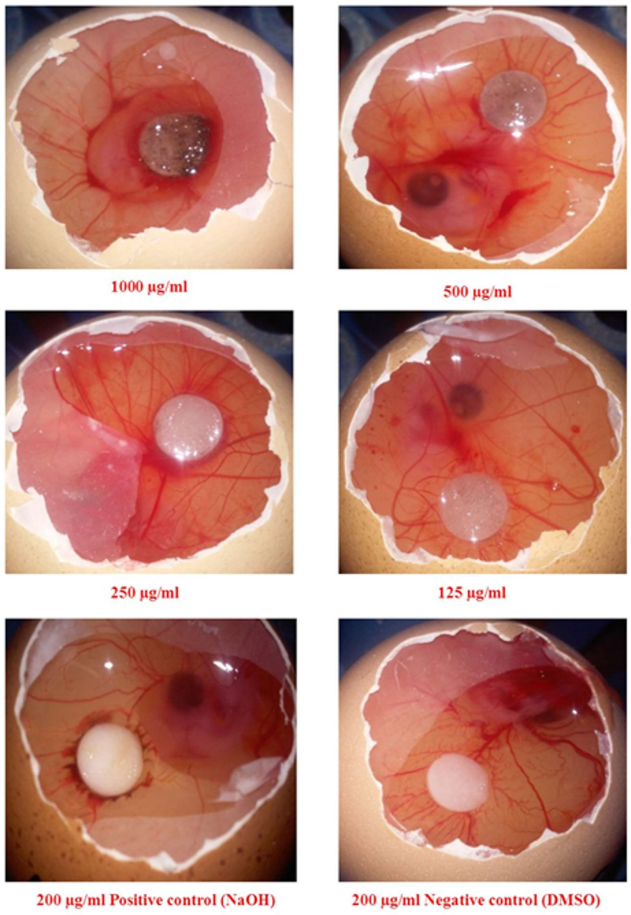

Figure 2. Antiangiogenesis activity of leaf callus SNPs of *T. villosa*

respectively (Table 2 and 3). The IC_{50} value of leaf and leaf callus SNPs was 63.20 µg/ml, 12.15 µg/ml respectively as shown in the figure 3 and 4. From the results of MTT assay, it can be well predicted that the degree of cell mortality rate was directly proportional to the concentration (31.25, 62.5, 125 and 250 µg/ml) of the SNPs.

The normal MCF-7 breast cancer cell lines were spherical in shape which on treatment with SNPs, due to its cytotoxicity activity, cell growth was inhibited and eventually the cell death occurred and aggregated to form round dead cells (Figure 3 and 4). When compared the leaf SNPs the callus SNPs activity was more in the degree of cell mortality.

Discussion

Angiogenesis is essential in tumor growth and metastasis as the process provides necessary oxygen and nutrients for the growing tumor (Folkman, 1971). The present results showed that both leaf and leaf callus SNPs changed the vascularization pattern; both extracts inhibited the new blood vessels formation in the treated CAM as well as distortion of existing vasculature.

The limitations of the available cancer management modalities create an urgent need to screen and generate novel molecules. Despite, well-documented illustrations of phytochemicals being used for prevention and treatment of cancer, their importance in modern medicine remains

Table 3. Cytotoxicity activity (MCF-7 cell line) of leaf callus SNPs of *T. villosa*

S. No.	Concentration	Test 1	Test II	Test III	Average	Cell Viability
1	250	0.081	0.078	0.095	0.084667	25.65657
2	125	0.082	0.084	0.081	0.082333	24.94949
3	62.5	0.093	0.094	0.094	0.093667	28.38384
4	31.25	0.096	0.091	0.094	0.093667	28.38384

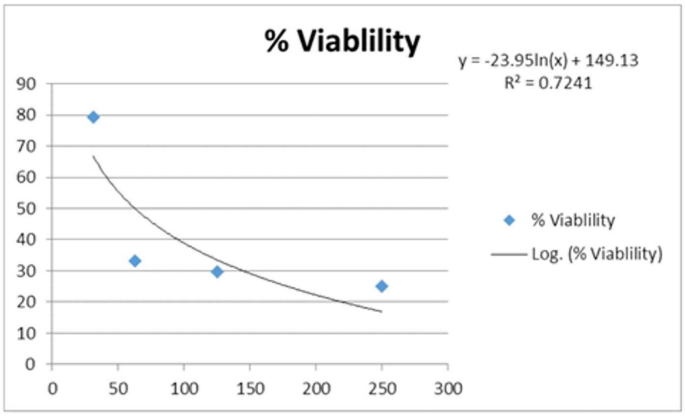

Figure 3. Cytotoxicity activity (MCF-7 cell line) of leaf SNPs of *T. villosa*

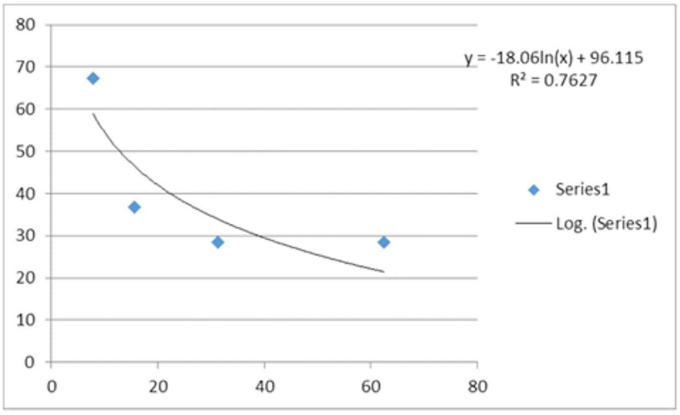

Figure 4. Cytotoxicity activity (MCF-7 cell line) of leaf callus SNPs of *T. villosa*

underestimated. Plants are the storehouse of "pre-synthesized" molecules that act as lead structure, which can be optimized for new drug development. In practice, a large number of cancer chemotherapeutic agents that are currently available in the market can be traced back to their plant resource (Heinrich et al., 2006).

Cancer is one of the most common problems and serious health issue in this world. It has been observed that more than one in three people will develop some form of cancer in their entire lifetime. Based on the origin, there are variety of cancer exist, such as thyroid, prostate, bladder cancer, kidney cancer, pancreatic, breast cancer, melanoma, leukemia with all types, oral cancer, colon-rectal combined cancer, etc. In cancer, cells divide and grow uncontrollably, forming malignant tumors and invading nearby parts of the body. Till date a complete cure for this prevalent disease is yet to be discovered. Chemotherapy could reach all the body part including cancer cells, there may be possibility of occurrence of side effects during treatment. Biological synthesis of silver nanoparticles in nano-biotechnology area has increased its importance to create ecofriendly; cost effective, stable nanoparticles and their applications in medicines, agriculture and electronics are wider.

From variety research on nanotechnology for synthesis of silver nanoparticles it is found that it is safer and better by using natural plants. With the huge plant diversity much more plants are still not explored for the synthesis of nanoparticles and its applications in pharmaceutical and agricultural industries (Rath et al., 2014). In the present study both leaf and leaf callus SNPs showed very good inhibition percentage compare to leaf SNPs the cell inhibition percentage was more in callus SNPs. So it is conformed that both SNPs having very good cytotoxicity activity. In the present study results were compared by the earlier studies on cytotoxicity (Rath et al., 2014; Ramesh and Rajeshwari, 2015).

Conclusion

Based on our results it was clear that the silver nanoparticle of leaf and leaf callus of *T. Villosa* confirms the anti-angiogenesis and cytotoxicity properties. The confirmation of pharmacological properties of both the SNPs, establishing authenticity for the results. In addition, our results revealed that the leaf SNPs was effective in the same way as leaf callus SNPs. Due to the presence of almost similar activities of leaf callus, it can be used for medicinal purpose instead of wild plants. The various activities of SNPs suggest that it can be used as a source for new therapeutic compound development.

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Conflicts of interest: None

References

Bosman MTM, de Haas AJP. 1983. A revision of the genus *Tephrosia* (Leguminosae, Papilionoideae) in Malesia. Blumea, 28(2):421-487.

Chang TY, Chen CC, Cheng KM, Chin CY, Chen YH, Chen XA, Sun JR, Young JJ, Chiueh TS. 2017. Trimn ethyl chitosa-capped silver nanoparticles with positive surface charge:their catalytic activity and antibacterial spectrum including multidrug-resistant strains of *Acinetobacter baumannii*. Colloid Surfaces B, 155:61-70.

Dipankar C, Murugan S. 2012. The green synthesis, characterization and evaluation of the biological activities of silver nanoparticles synthesized from *Iresine herbstii* leaf aqueous extracts. Colloids Surfaces B Biointerfaces, 98:112–119.

Folkman J. 1971. Tumor angiogenesis: therapeutic implications. New England Journal of Medicine, 285(21):1182-6.

Gafner S, Woffender JL, Nianga M, Hostettmann K. 1998. Naphthaquinone from *Newbouldia laevis* roots. Phytochemistry, 48(1):215-216.

Guo D, Zhang J, Huang Z, Jiang S, Gu N. 2015. Colloidal silver nanoparticles improveanti-leukemic drug efficacy via amplification of oxidative stress. Colloid Surfaces B, 126:198-203.

Heinrich M, Bremner P. 2006. Ethnobotany and Ethanopharmacy their role for cytotoxicity drug development. Current Drug Target, 7:239.

Kim EM, Jung HR, Min TJ. 2001. Purification, structure determination and biological activities of 20 (29)-lupen-3-ones from Daedaleopsis *tricolor* (Bull. ex Fr.). Bond. et Sing. Bulletin-Korean Chemical Society, 22:59-62.

Liu W, Li X, Wong YS, Zheng WJ, Zhang YB, Cao WQ, Chen TF. 2012. Selenium nanoparticles as a carrier of 5-fluorouracil to achieve cytotoxicity synergism. ACS Nano, 6:6578–6591.

Loo CY, Rohanizadeh R, Young PM, Traini D, Cavaliere R, Whitchurch CB, Lee WL. 2016. Combination of silver nanoparticles and curcumin nanoparticles for enhanced anti-bio film activities. Journal Agriculture Food Chemistry, 64:2513–2522.

Madhusudhanaa J, Narahari Reddya RV, Reddya BAK, Reddyab V, Gunasekara D, Devillec A, Bodoc B. 2010. Two new geranyl flavanones from *Tephrosia villosa.* Natural Product Research, 24(8):743-749.

Mollick MR, Bhownick B, Mondal D, Maity D, Rana D, Dash SK, Chattopadhyay S, Roy S, Sarkar J, Acharya K, Chakraborty M, Chattopadhyay D. 2014. Cytotoxicity (*in vitro*) and antimicrobial effect of gold nanoparticles synthesized using *Abelmoschus esculentus* (L.) pulb extract via a green route. RSC Advances, 4:37838-37848.

Nondo RSZH, Mbwambo AW, Kidukuli EM, Innocent MJ, Mihale P, Erasto, Moshi MJ. 2011. Larvicidal, antimicrobial and brine shrimp activities of extracts from *Cissampelos mucronata* and *Tephrosia villosa* from coast region, Tanzania. BMC Complementary and Alternative Medicine, 11:33-40.

Panyan J, Labhasetwara V. 2003. Biodegradable nanoparticles foe drug and gene delivery to cells and tissue. Advanced Drug Delivery Reviews, 55:329-347.

ParivashSeyfi, Ali Mostafaie, Kamran Mansouri, DelniaArshadi, Hamid Reza, Mohamadi-Motlagh, Amir Kiani. 2010. *In vitro* and *in vivo* antiangiogenesis effect of Shallot (*Allium ascalonicum*): A heat stable flavonoid-rich fraction of shallot extract potently inhibits angiogenesis. Toxicology In Vitro, 24:1655-1661.

Prashant A, Krupadanam GD. 1993. A new prenylated dehydro rotenoid from *Tephrosia villosa* seeds. Journal of Natural Products, 56:765-766.

Ramesh B, Rajeshwari R. 2015. Cytotoxicity activity of green synthesized silver nanoparticles of *Abutilon indicum l*. leaf extract. Asian Journal of Phytomedicine and Clinical Research, 3(4):124–1.

Ranjitha V, Kalimuthu K, Chinnadurai V, Juliet YS, Saraswathy M. 2018. Green synthesis and antioxidant analysis of *in vivo* leaf and *in vitro* callus of *Tephrosia villosa*. Pharmacognosy Research, 14:S147-53.

Rath M, Panda SS, Dhal NK. 2014. Synthesis of silver nano particles from plant extract and its application in cancer treatment: a review. International Journal Plant, Animal and Environmental Sciences, 4:3.