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Green Synthesis of Tecoma Stans Flower and Leaf Extracts: Their Characterization and Anti-OProliferative Activity in Colorectal Cancer Cell Lines.

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ABSTRACT

Since times immemorial, many plant species were utilized to cure severe ailments. A wide range of diversification has been observed in various medicinal plants which indeed able to cure several deadly diseases. Presence of secondary metabolites is a high priority for their medicinal characteristics. In this study, we focused on T.stans (Yellow bells), a shrub that grows profoundly in tropical and sub-tropical regions. Despite many studies the medicinal values of this plant species, yet not much research has been done in the cancer treatment and nanomedicine. Green synthesized silver Nano-particles is an eco-friendly approach to deliver the drug to the target size. Nano appearance is an add-on advantage of these compounds- hence emerging in the field of medicine. Our current study, focused on Colorectal cancer, is the 4th deadliest one globally. Hence the synthesized AgNPs of T.stans flower and leaf extracts showed cytotoxic and wound healing properties on colorectal cancer cell lines (HCT 116 and SW 480). Synthesis of AgNPs confirmation is done by UV-Vis spectrophotometry and Particle size analyzer. All the results showcase the beneficial effects of AgNP synthesized plant extracts and may be used as a novel medicine in the field of chemotherapy.

INTRODUCTION

In nature, plant species play a magnificent role to treat several alignments caused by bacteria, virus, nematode and fungal species. Various cultures and several demographics use their naive plant species which possess several bio active compounds to cure deadly diseases. Secondary metabolites are the primary sources to exhibit the bio activity in most of the plant species. Tecoma stans, called as yellow bells, is a popular ornamental plant belongs to the family Bignoniaceae and is popularly distributed in both tropical and sub-tropical regions.

Tecoma stans is a unisexual, deciduous shrub with 5.6-7 m in height. In the study of Costantino et al. (2003), the flower and leaf infusions can be taken orally for diabetes and stomach pains (1). A strong leaf and root decoction can be taken orally as a diuretic, to treat syphilis or for intestinal worms (2). T. stans is known to have various medicinal and therapeutic properties. T. stans leaves contain potent anti-inflammatory and analgesic activities that have been evaluated as antibacterial agents against the growth of some human bacterial strains (3). In addition, the flowers and barks are used traditionally for the treatment of various cancers (4). Amongst nine plants studied, T. stans was found to give the best of inhibition zones against the fungal activity. T. stans possesses various bioactive compounds such as saponins, flavonoids, alkaloids, phenols, steroids, anthraquinones, tannins, terpenes, phytosterols, triterpenes, hydrocarbons, resins, volatile oil and glycosides (5). The methanol extract of T. stans leaf was reported to possess significant wound healing property (6). Additionally, the ethanol, methanol and water extracts of T. stans had been reported for good antimicrobial effects on some human pathogenic bacteria and antioxidant activities.

Taxonomic classification:

Kingdom: Planta

Clade: Angiosperms

Order: Lamiales

Family: Bignoniaceae

Genus: Tecoma

Species: stans

Nano technology is currently an emerging field with dynamic extensions in medical, electronic and structural designs. The tiny size: which reflects their name gets a maximum benefit for their existence. The flexibility in their size help these particles to enter minute blood vessels to deliver the drug and hit the target at point. This feature made Nano particles to form a major platform in Nano medicine and drug delivery.

Colorectal cancer is listed as the third emerging cancer in fatality and fourth most dominating cancer globally in mortality rate. Around 25% of cases are exhibiting the recurrence annually (7). In recent years, recurrence of the disease has become a major concern. This led to the development of tumour in most aggressive way. Standard drugs such as oxaliplatin controls the growth of tumour at the initial stages, but tumour becomes resistance after a period of time. Hence, using bioactive compounds with the combination of nanoparticles increases the treatment efficiency.

Although Tecoma stans plant parts exhibit diverse medicinal properties in the treatment of various diseases, Silver nano

particle synthesis using methanolic extracts in cancer treatment were not evaluated scientifically. Primary studies in this aspect could create an open platform in the field of clinical research and in combinational therapy.

Considering this, in our current study, we have used Tecoma stans flower and leaf extracts to evaluate their role in anti-proliferation and wound healing properties. AgNPs were synthesized using methanolic extracts and characterization studies were performed using UV-Vis spectrophotometry. The synthesized AgNPs were further studied to determine their size and charge using particle size analyser and zeta potential. Functional group characterization was performed using FT-IR study. Anti-oxidant properties were examined in comparison between both crude extracts and AgNPs. Anti-microbial activity was studied against both gram positive and gram-negative bacteria. Anti-proliferative activity was studied using HCT-116 and SW 620 cell lines. HEK 293 was used as a control. Wound healing properties were studied using different concentrations of AgNPs.

MATERIALS AND METHODS

Collection of leaf and flower extracts

The leaves and flowers of Tecoma stans (commonly called as yellow bells) were collected from the trees growing around the Sri Padmavati Mahila University (SPMVV), Tirupati, Chittoor district, Andhra Pradesh, India. The collected plant parts were rinsed with double distilled water to remove the dirt present on the surface and then chopped into small pieces. The leaves and flowers were allowed to shade dry for about 7-10 days.

Preparation of plant extracts

The nanoparticles were synthesized by using plant extract, which reduce the generation of hazardous substances. Tecoma stans flower and leaf extracts were taken and shade dried for about one week. The shade dried flowers and leaves were grinded separately with the help of blender and the coarse powder was collected in Zip lock covers for future use. 7.5gms of powder was weighed and Soxhlet with 250ml of 80% methanol which was used as a solvent system for extraction by using Soxhlet extractor for 7 cycles up to 8 hours at 64°C until the solvent in siphon tube become colourless. Rotary Flash evaporator was used to the remove the solvents from the plant sample by evaporation at 64ºc for 1 hr. The solvent (methanol) was separated and collected in the tube and can be reused. The crude extract was collected in a Petri dish and allowed to air dry. The collected extract was stored in scintillation flask and can be used for analysis.

Preparation of AgNPs

The crude sample was prepared with a concentration of 10mg/ml by adding distilled water. 2ml of crude sample was taken and the volume was made up to 5ml by adding distilled water.10ml of 1mM Silver Nitrate (AgNO3) solution was added to the extract and kept in water bath for reduction reaction at 70 - 80ºc for about 30 mins. The colour change observed indicates the AgNP´s were synthesized and peak was observed.

Phytochemical analysis for leaf extracts

1. Test for alkaloids

(a)Mayer's test (potassium mercuric iodide)

To few ml of filtrate, a few drops of Mayers reagent was added along the sides of the test tube.

(b)Wagner's test

To 1ml of plant extract, few drops of Wagner's reagent was added along the sides of the test tube.

2. Test for Tannins

(a) Ferric Chloride Test

0.5g of the extract was boiled in 10ml of distilled water and filtered. 5ml of filtrate taken in test tube and few drops of 0.1% FeCl3 was added.

3. Test for Saponins

(a) Froth Test

About 0.2 g of the extract was shaken with 5 ml of distilled water and then heated to boil. Frothing shows the presence of saponins.

4. Test for Phenols

(a) FeCl3 test

50ml of the extract is dissolved in 5ml of distilled water. To this few drops of neutral FeCl3 was added.

5. Test for steroids

(a) Lieberman Burchard´s test

20mg of the extract was treated with 2.5ml of acetic anhydrite and 2.5ml of chloroform. Then H2SO4 was added slowly.

6. Test for terpenoids

(a)Salkowski´s test

Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of Conc. solution of H2SO4.

7. Test for Flavonoids

(a) Lead acetate test

To 1ml of extract 1ml of 10% lead acetate was added.

(b)Alkaline reagent test

Extracts were treated with few drops of NaOH solution. Formation of intense yellow colour, which become colourless on addition of dilute acid.

8. Test for glycosides

(a) Cardiac glycosides

To 5 ml of extract 2ml of glacial acetic acid, a few drops of FeCl3 and 1ml of H2SO4 was added.

Anti-microbial activity:

Bacterial strains used for antimicrobial activity are Escherichia coli, Bacillus subtilis, Klebsiella Pneumonia, and Staphylococcus-obtained from microbiology lab in our department. These strains are activated at 37ºc for 24hr. Antibacterial activity was assayed by agar well plate method and used to detect anti-bacterial activities of synthesized nanoparticles. After solidifying the media with bacterial culture, the wells were made by using sterile cork borer. Wells of 6mm size were made into the agar set plate containing the bacterial culture and lower portion was sealed with a little molten agar media. Different concentrations of (10, 20, 30, 40µg/ml) were placed into each well. The crude extract was placed in the middle of the Petri plate. The culture plates were incubated at 37ºc for 18-24hrs and antibacterial activity was evaluated by measuring the radius of the zone of inhibition. The zone of inhibition was compared with that of standard antibiotic (levoflaxin) plates.

ANTI-OXIDANT ASSAY

Different concentrations of synthesized nanoparticles (100,200,300,400,500µl) were taken in different vials and then make up to 1ml with methanol. Ascorbic acid was taken as standard and DPPH was used as control. Add 1ml of 0.1mM DPPH solution to the test tubes. The test tubes were shaken and incubate for 30min in darkness. The absorbance of the samples was measured against blank (methanol) at 517nm by using UV-Spectrophotometer. DPPH scavenging activity was expressed as the % of inhibition of the free radical DPPH.

% of inhibition = (A control –A sample / A control) X 100

A sample =absorbance of the sample

A control = absorbance of DPPH

MTT ASSAY

When the cultured cells reached 80% confluency, cells were trypsinized using 0.25% trypsin followed by counting and respective seeding(1x105) in a 96 well plate and kept in incubator which contains 5% of CO2, at 37ºC for 24hrs. C1 - Medium control, C2 – Cell control, C3 – Drug control were used as controls. S1, S2, S3, S4, S5, S6 were taken as standards. After 24 hrs, the cells were treated at various concentrations of flower and leaf AgNP extracts respectively (25,50,100,200,300 and 400µg/ml). After 24 hrs 10µl of MTT solution was added to each well including control and incubated for 2-4 hrs in incubator. After incubation period 100µl of solubilisation solution was added and stirred gently on gyratory stirrer to ensure the crystal dissolution. Absorbance was taken on ELISA reader at 570nm with a reference wavelength higher than 650nm. The graph was plotted by taking concentration on X-axis and OD values on Y-axis.

Wound healing assay:

1*106 cells were seeded in a 6 well plate and the cells were incubated in a CO2 incubator for 24hrs. Once the cells reach 90% confluency, a scratch was given by using a 10µl sterile tip in a straight line. By taking the IC50 value as a reference, the cells were treated with two different concentrations. At 0 hour an image was captured using inverted microscope and ZEN software was used for analysis. To check the efficiency of migration, another image was captured after 24 treatment. The migration distance was calculated using image J.

RESULTS AND DISCUSSION

Green synthesis of silver Nano particles

Colourless plant extracts which were prepared form the powdered leaf and flowers of T.stans contain several molecules which have the potential to reduce silver ions to AgNPs by changing the colour to dark green after heating at 80ºC for 20 minutes.

a- Represents synthesis of AgNPs using leaf extracts 1b-Represents synthesis of AgNPs using flower extracts of T. stans.

CHARACTERIZATION STUDIES

UV-Visible spectrophotometry

The optical property of AgNPs can be determined by using UV-Vis spectrophotometry. A surface plasmon resonance spectrum shows a characteristic peak at a range of 300nm-700 nm confirms the synthesis of AgNPS. T. stans flower shows peak absorbance at 436 nm and T. stans leaf shows maximum absorbance at 446 nm.

– Represents the UV absorption peak of T. stans flower and leaf extracts.

Particle size determination

The particle size of the AgNPs obtained is detected by intensity and laser diffraction which are poly dispersed mixture solution. The size of synthesized T. stans flower AgNPs ranging from 30-50nm and the size of leaf extracts were ranging from 60-111.7 nm . Particle size analyser was used to study the method. Denotes the Particle size of both flower and leaf extracts of T. stans.

Zeta potential analysis

The electrostatic repulsive force between the nanoparticles depends on the charge which is present on the surface of the particle. The negative value of zeta potential confirms the repulsion among the particles and thereby increases the stability of the formulation and prevents the nanoparticles from agglomeration in the medium, leading to long term stability. The zeta potential of the AgNPs of Tecoma stans leaf extract was found to be -17.1mV and flower extract is found to be – 31.5mV. It was concluded that the AgNPs synthesized with Tecoma stans leaf extract was moderately stable and AgNPs of flower extracts were highly stable.

Fig 4a shows the zeta potential of the AgNPs of Tecoma stans leaf extracts (-17.1mV) and 4b represents the Zeta potential of flower extracts (-31.5mV).

Phyto chemical screening of leaf extracts

Phyto chemical analysis was performed using standard protocols. These tests confirmed the presence of secondary metabolites such as Alkaloids, Flavonoids, Glycosides, Phenols, Tannins, Steroids, Terpenoids and Saponins in leaf extracts of T. stans. The confirmation of secondary metabolites was observed by respective colour change (fig 5).

Figure 5 and Table 1 denotes the presence of secondary metabolites in leaf extracts of T.stans.

FTIR analysis

FTIR Pattern of the synthesized nanoparticles was studied. It ranges from 500-3500cm-1. (fig 6a and 6b) shows functional groups of crude leaf extract and synthesized AgNPs of Tecoma stans. FTIR spectrum of crude leaf extract showed significant peaks at 2852.16, 1383.45, 667.87,1019.50, 3399.88, 1743.38 which corresponds to the presence of alkanes, alkenes, alkyl halides, alcohols, ketones, esters and amides. The Tecoma stans leaf AgNPs showed significant peaks at 2854.58, 2923.81, 668.20, 1089.31, 1270.70, 1319.29, 1626.01, 1741.96, 2854.58, 2923.91,3420.81, 1270.70,1626.02, 779.91 which corresponds to the presence of alkanes, alkenes, alkynes, alkyl halides, alcohols, ethers, aldehydes, ketones, carboxylic acids, esters, amides and aromatic compounds (table 2). Figures 6c and 6d represents the FT-IR peaks of T.stans crude and AgNPs of flower extracts. T.stans crude flower and AgNPs posses functional compounds such as alkyl halides, alkanes, alkyls, amides, carboxylic acids and ketones (Table 3). Figure 6a,6b,6c and 6d shows FTIR spectra of both crude and AgNP synthesized particles of both T.stans leaf and flower. Table 2 and table 3 shows the Functional compounds present in both crude and AgNP extracts of leaf and flower.

Anti-microbial activity

The anti-bacterial activity of the sample was identified by measuring the zone of inhibition. The size of zone of inhibition is a measure of the compound's effectiveness. The anti-microbial activity of Tecoma stans leaf extract was studied at different concentrations (10, 20, 30, 40µg/ml) against Escherichia coli, Bacillus subtilis, Klebsiella pneumonia, Staphylococcus aureus. It was confirmed that the antibiotic (levofloxacin) and plant extracts has shown the zones of inhibition.The plant extract showed antibacterial activity at different ranges. The maximum zone of inhibition was observed in E. coli followed by K. pneumonia, S. aureus. Zone of inhibition was measured and calculated. (Table 4 a and b)

The leaf AgNP extracts has showns a better zone of inhibition compared to the flower AgNP extracts of T.stans. (figure 7 a-d).

FREE RADICAL SCAVENGING ACTIVITY

Antioxidant activity of Silver nanoparticles synthesized from Tecoma stans leaf extract was studied by DPPH free radical scavenging assay. This method is dependent on the reduction of DPPH radical to the ion-radical form DPPH-H in the presence of a hydrogen donating antioxidant. The radical scavenging activity (RSA) values of AgNPs and crude extract values were represented in the table 5. The RSA of AgNPs was increased with increasing in the concentration and the highest RSA was observed at highest test concentration of 500µg/ml used in this assay was found to be 70.694% (fig 8a) (Table 5).

MTT ASSAY

Methanolic extract of Tecoma stans was applied to the HCT116 cell line. The IC50 doses were measured and incubated at 37ºC in darkness. HCT 116 cells with DMEM was replaced every 3 days and incubated for 24hr. The number and diameter of colonies within each cell was counted each day under the microscope and the images were captured for the representative fields.

Figure – 9-a,b,c,d are the microscopic observations of HCT 116 cell line at different test sample concentrations.

WOUND HEALING ASSAY

Figure 12 a and b represents the wound healing activity of the Tecoma stans flower AgNPs extract against SW480 cell line as well as T.stans leaf in HCT 116 cell lines. and c) represents the pictoral representation of area covered after 24 hours.

DISCUSSION

The biological synthesis of silver nanoparticles using Tecoma stans leaf and flower extracts provides environmentally friendly, simple and efficient route for synthesis of benign nanoparticles. Phytochemical screening studies of leaf extracts shows the high concentration of bioactive compounds such as terpenoids, flavanoids, tannins, alkaloids, saponins.

The leaf nanoparticles contain functional groups as amines, alcohols, ketones, aldehydes etc., which were found from the characterization using UV-visible spectrophotometer, zeta potential, particle size analyzer. FTIR analysis for flower exracts was performed and confirmed the presence of different functional groups. The data concludes that a greater number of functional compounds are present in Tecoma stans flower AgNPs extract than in crude sample.

DPPH radical scavenging activity of the synthesized AgNPs was increased with increasing the concentration in T.stans leaf extracts. The free radical scavenging activity is more in AgNPs extract when compared with the crude extract of Tecoma stans flower.

The synthesized leaf AgNPs possess antibacterial activity against different pathogenic species such as E.coli, B.subtils,S.aureus, K.pnuemonia. The antimicrobial activity of Silver nanoparticles of Tecoma stans flower when compared with the standard drug shows less antimicrobial activity.

Cytotoxicity assay reveal that the leaf extracts possess antiproliferative activity and IC50 value was determined in both leaf and flower extracts.

The wound healing assay was performed in order to check the healing activity of the AgNPs extract of Tecoma stans flower and

leaf. With an increase in its concentration we can see the number of cells decreasing with in the scratch that was made. Hence it was proven that it has wound healing property.

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