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Formulation and evaluation of antibacterial efficiency of nanoparticles of *Tinospora cordifolia*

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Abstract
The green synthesis of nanoparticles by using a plant extract is safe and effective way to increase the efficiency and potency of phytochemicals. Silver has been used since ancient time due to its potent antimicrobial effect and now it is recognized as a nontoxic and safe for human beings. Silver nanoparticles have attracted keen interest due to its specific size ranges 1-100 nm and its unique physical, chemical and biological properties. Out of three methods of preparation biological method are cheap, reliable, safe and nontoxic over physical and chemical methods. Green synthesis technique is a promising approach for synthesis of silver nanoparticles showing antimicrobial effect as its not uses any toxic chemicals and specific higher temperature and pressure. Antimicrobial resistance is, thus, one of the major threats to human health , since it determines an increase of morbidity and mortality as a consequence of the most common bacterial diseases . In present study nanoparticles were prepared from Tinospora cordifolia aqueous and ethanolic extract and tested for antibacterial potency against various multidrug resistant bacterial isolates.

Key Words: *Tinospora cordifolia, antibacterial, nanoparticle, targeting, potential*

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I. Introduction

Color greenish yellow, bloom time December, February hight – 0.5 to 3 ft. difficulty leaves easy to grow, water medium sunlight full sun to partial shade temperature 28 °C, is required for proper growth and propagation(Gitanjali ,2020) . *Tinospora cordifolia* is a glabrous, suckles woody climbing shrub native to India. It thrives well in the tropical region often attain a great height and climbs up the trunk of large trees. The stem is gray and creamy white, deeply cleft spirally and longitudinally, with the space between spotted with large rosette like lenticels. The wood is white, soft and porous and the freshly cut surface assumes a yellow tint when exposed to air leaves are simple, alternate, estipulate long petiolate, chordate in shape showing multicoated reticulate vecnotion.(Joshi et. al, 2016). Kingdom:- plantae, , Order- Rannculales, Family – *menis permaceae*, Genus- *tinospora* , Species – *T. cordifolia* , Clade- Angiosperms, Binomial name- *Tinospora cordifolia*.

II. Material And Method

Maintenance of bacterial culture

Pure culture of *Keliebsell pneumonia, Staphococus aures, Closridium perfiuges* and *Citrobactear freundii* were obtained from Department of Biotechnology of Barkatullah University.

Fig-1: Plant of *Tinospora cordifolia* (original)

Plant collection and extraction

Tinospora cordifolia was collected from campus of Barkatullah University Bhopal in zip lock polythene bags, brought back to the lab. These plant sample were washed in running tap water for 15 minutes and kept in room temperature for dry. After the plant was dries, the leaves and stems of the plant were cut it into small pieces or with the help of, scissors or knives and kept for further drying away from sunlight.

Soxhlet extraction

During operation plant material was gradually filled with condensed fresh solvent (Ethanol and water) from a distillation flask. When the liquid reaches an overflow level, a siphon aspirates the whole contents of the thimble-holder and unloads it back into the distillation flask, carrying the extracted analytes in the bulk liquid. This operation was repeated until complete extraction was achieved after six rounds of soxhlet cycle. After the completion extraction process the collected extract was dried at 50℃ to achieve the desired consistency required for future use, extracted material was then stored in air tight container (Kokatte , 2005).

Yield of extract After extraction, each cycle was analysed for % yield of the extract is calculated in percentage by the formula:

% Yield =wt. of the extract / wt. of the plant material× 100

Phytochemical analysis The commonly known phytochemicals from *Tinospora cordifolia* are cardiac terpenoids, steroids ,saponin, tannin, flavonoids and alkaloids. Extracts were subjected to various chemical tests in order to determine the secondary plant constitutuents present by employing standard procedures as follows. (all the tests were done in triplicates) (Hardone 1973, Evans and trease 1989, Kokate2005).

Total ash Presence of ask in any drug (natural) is a limited factor which can interfere with the pharmacological properties of extract. During validation and formulation ask value must be in lower rang, lower the value higher the potency of the extract. To determine total ash about 2 of the air-dried extract was placed in a crucible, extract was then spread in an even layer and ignited and gradually the temperature was increased to 450℃ until it is white, indicating the absence of carbon. The content was cooled in a desiccators and weighed. Ash value can be calculated by using formula:- Ash value = Initial Weight – final Weight × 100/ Initial weight Uprieti et. al., (2013).

Water soluble ash The total ash obtained from 2g extract was boiled with 25 ml of distilled water for 5 minutes. The insoluble matter was collected on an ash less filter-paper, washed with hot water and ignited to constant at low temperature. The weight of the insoluble matter was subtracted from the weight at total ash, represent the water soluble ash. The percentage of water soluble ash was calculated with reference to the air dried drug Uprieti et. al.,(2013).

Test for cardiac glycosides Test for Cardiac Glycosides 0.5 ml of each extract was treated with 0.2 ml glacial acetic acid then 1 drop of 3.5%ferric chloride ($FeCl_3$) was added to the solution. This was layered with 1 ml of concentrated HSO_4. A reddish brown ring was occurred at the interface indicates the presence of cardiac glycosides.

Test for terpenoids 0.5 ml of plant extract was added to the test tube then 2ml of chloroform was mixed to the solution. 3 ml of concentrated HCl was added carefully from the wall of the test tube, to form a lower layer. Occurrence of reddish-brown colour at the interface indicated the presence of terpenoids.

Test for steroids Test for Steroid 0.5ml of extract was dissolved in 3ml of chloroform. The solution was filtered, 2ml of concentrated H_2SO_4wasadded to the filtrate to form a lower layer. A reddish-brown colour ring at the interface was indicated the presence of steroid.

Test for saponin Test for Saponin 0.5ml of extract was taken in the test tube, and then 5ml of distilled water was added to it. The solution was vigorously shaken and stable persistent froth was observed for the presence of saponin.

Test for tannin Test for Tannin 0.5ml of extract and 5ml of distilled water was taken in test tube then it was boiled then filtered. Few drops of concentrated H_2SO_4 and 1% $FeCl_3$ were added to the filtrate. Deep green, brownish green or blue black coloration was indicated the presence of tannin.

Test for flavonoid Test for Flavonoid 0.5ml of extract and 5ml distilled water was added to test tube then it was filtered. 5ml of diluted ammonia solution was added to the filtrate then concentrated $H_2SO_4$was added. A yellow coloration indicated the presence of flavonoid. The yellow color disappeared on standing.

Test for alkaloids Test for Alkaloid 0.5 ml dried extract was taken and 3ml of methanol was added to it. Then 300µl of acetic acid (10% of methanol) was added to the solution ammonium hydroxide was added drop wise. Appearance of precipitate indicated the presence of alkaloid.

Biosynthesis of nanoparticles and antibacterial activity

Aqueous solution of silver nitrate (1mM) was prepared and mixed with fresh plant aqueous and alcoholic extract at a ratio of 8:2 This solution was placed on a shaker with magnetic stirrer in the room temperature at 27 ± 2°C for 24 h. All stages of the experiment were implemented in triplicates . Silver nanoparticles synthesized by plant extract were tested for antibacterial activity agar well diffusion method against pathogenic bacteria.

Well diffusion assay well diffusion method

For *In vitro* antibacterial screening agar plates are made, then the bacteria culture are poured and with the help of a spreader inoculums were spread on petriplates. After that small holes were punctured on the agar plates. In those wells extracts (both ethanol and aqueous) of different dilution series ranging from 2µl- 10µl were poured with the help of a micropipette and these plates were kept in the incubator for 24 hours at 37°C . After incubation the zone of inhibition bacteria is noted presence of clear zone indicates the potency against the bacterial strains. The zone of inhibition is calculated with the formula πr^2(these experiments were also done in triplicate)Cappuccino and Sharma (2006).

In this method 20 µl bacterial cultures were seeded on the surface of Nutrient agar media plates by spread plate technique. Media was punched with a sterile cork borer to make open wells (4 mm) in all plates. Different colloidal solutions of silver nanoparticles (2µl, 4 µl, 6 µl and 8 µl) synthesized above were poured in separate wells. Then these plates were incubated at 37°C for 24 hrs. After 24 hours incubation antibacterial activities of different nanoparticles sample were recorded in terms of diameter of inhibition zone measured in mm by using scale.

Characterization of silver nanoparticles

The brown colloidal solution containing silver nanoparticles obtained in above experiments is further subjected to following characterization techniques for determination of their physical, chemical properties viz., size, stability etc.

UV-Visible spectroscopic analysis

Change in colour of the water extract incubated with 1 mm silver nitrate solution visually observed over a period of time indicates the bio reduction of silver ions to silver nanoparticles. The colloidal brown solution was monitored by absorption measurements carried out on UV–Visible Spectrophotometer (UV 1800 spectrophotometer Schimadzu) at a resolution of 1 nm between 300 to 600 nm (which is a characteristic wavelength absorption range for silver nanoparticles) wavelength range for confirming the synthesis of silver nanoparticles in the solution (Devi and Joshi., 2015). For absorption measurements, different brown colloidal solutions were poured in cuvette and placed in sample holder where wavelength of specific range is passed through it and absorption values are displayed in the form a spectra. Maximum absorption at a particular wavelength was depicted as a peak .

III. Results And Discussion

YIELD OF EXTRACT

%yield of ethanolic extract is 25.3±5.0 and for aqueous extract is 33.3±3.4

Total ash value of extracts

Ash value of both extract was calculated and results of ash value in ethanolic extract is 1.5±0.26 and water soluble ash is 1.08±0.3,for aqueous extract water soluble ash ia 0.65±0.08 and total ash value is 0.73±0.1

Phytochemical analysis of extract

Table: Phytochemical analysis of ethanolic and aqueous extract of *Tinospora cordifolia*.

S NO.	PHYTOCHEMICAL COMPOUND	ETHANOLIC EXTRACT	AQUEOUS EXTRACT
1	TANNINS(cathecolic)	-	+
2	VOLATILE OIL	+	-
3	REDUCING SUGAR	+	+
4	SAPONINS	+	+
5	GLYCOSIDE	-	-
6	ALKALOID	+	-

+=present,-=absent

Biosynthesis of silver nanoparticles

Aqueous solution of silver nitrate (1mM) was prepared and mixed with fresh plant extract of *Tinospora cordifolia* at a ratio of 8:2 .This solution was placed on a shaker with magnetic stirrer in the room temperature at 27 ± 2°C for 24 h. All experiment were conducted in triplicate .

Screening for antimicrobial activity

In one of the previous study free plant extract fails to inhibit the growth with of *Kliebsella pneumonia* but, nanoparticles prepared with plant extract are effective against *Kliebsella pneumonia* population. It is evident from the results that the nanoparticles inhibit the population of *Clostridium perfringes* and *Staphlococcus aureus* more effectively compared to free extract. Nanoparticles inhibit population of *Citrobacter freundii* while free plant extract fails to inhibit the population .

Nanoparticle preparation:

1N $AgNO_3$ solution was prepared by mixing 10ml D/W in 0.1g $AgNO_3$, the solution for nanoparticles prepared by mixing 16 ml of D/W with 3ml of $AgNO_3$ and 1ml plant extract was added. With continuous stirring for 24 hours at room temperature, gradually the colour of the solution changes to red. The intensity of the colour increased, which confirmed Ag ion reduction and the formation of Ag-NPs. Silver nanoparticle surface plasmon excitation causes colour change in the solution, which is the primary and notable evidence for the formation of Ag-NPs.

UV–Vis analysis

UV–Vis absorption spectrum of Ag-NPs was recorded in broad bell-shaped spectrum curve was obtained from UV–Vis analysis. Various metabolites from plant extract introduced to solution make the Plasmon band broad because they may be read in this spectrophotometric range, too. Surface plasmon resonance (SPR) of silver occurs at 300nm. to 600 nm. This peak increased with time up to 360 min. According to Mie theory, spherical nanoparticles show only a single SPR band. The number of peaks increases by increasing diversity of particles shapes . Then, it can be concluded that biosynthesized Ag NPs are unanimously spherical in nature. In present study nanoparticles of all the extract are giving λ mean of 440- 500 nm. Nanoparticles gives λ mean at around 460-500 nm. Result confirms that at nanoparticles formulated in present research are stable and intact .

Stability of nanoparticles

The activity was determined by spectrophotometer analysis. All the nanoparticles formed give lambda mean at 380-400 nm up to 15 days of preparation which confirm the integrity and stability of nanoparticles formed. The total ash value was calculated 0.60gmfor ethanolic and aqueous leaf extract respectively. For water soluble ash the values are1.5gm, 0.65gm for ethanolic and aqueous extract.

***TABLE ZONE OF INHIBITION OF NANOPARTICLES OF ETHANOLIC EXTRACT OF Tinospora cordifolia* leafs.**

S NO	Dose of plant extract and nanoparticles in µl	Area of zone of inhibition in cm^2			
		Kliebsella pneumonia	*Staphylococcus aureus*	*Citrobacter freundii*	*Clostridium perfringes*
1	2	0.1	0.5	0.3	0.2
2	4	0.2	0.1	0.4	0.2
3	6	0.2	0.2	0.5	0.3
4	8	0.7	0.2	0.6	0.3

** No zone of inhibition was observed.

##results are the mean value of triplicate.

In the previous study of nanoparticles preparation the growth were inhibit in *Kliebsella pneumonia* in the area*0.*3cm^2,0.6cm^2,0.6cm^2,1.54cm^2, *Staphylococcus aureus* in the area0.78cm^2,0.3cm^2,0.6cm^2,0.6cm^2,*Citrobacter freundii* in the area 0.8cm^2 ,0.12cm^2 ,0.28cm^2 ,0.28cm^2 area *Clostridium perfringes* in the area 0.6cm^2,0.6cm^2,0.8cm^2,0.8cm^2.

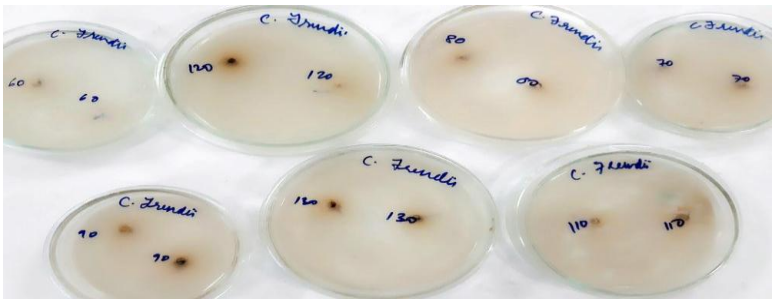

Fig Agar plates showing susceptibility of *Clostridium perfringes, Citrobacter freungii, Kliebsella pneumonia* and *Staphylococcus aureus* through zone of inhibition against ethanolic extract of *Tinospora cordifolia*

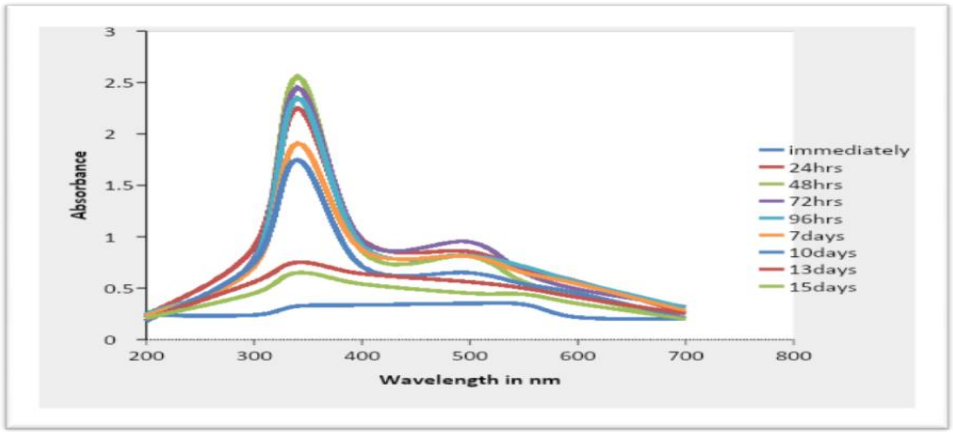

Fig. - graph showing the activity of nanoparticles (15 days)

STABILITY OF NANOPARTICLES

The activity was determined by spectrophotometer analysis. All the nanoparticles formulated have lambda max at 380-400 nm. Stability of nanoparticles remains intact up to 15 days from preparation which confirm the integrity and stablility of nanoparticles .

From both the result we can conclude that method which was adopted by to form nanoparticles is best suited for plant extract and stability up to 15 days is a good sign and hold a future properties for plant extract nanoparticles for application use.

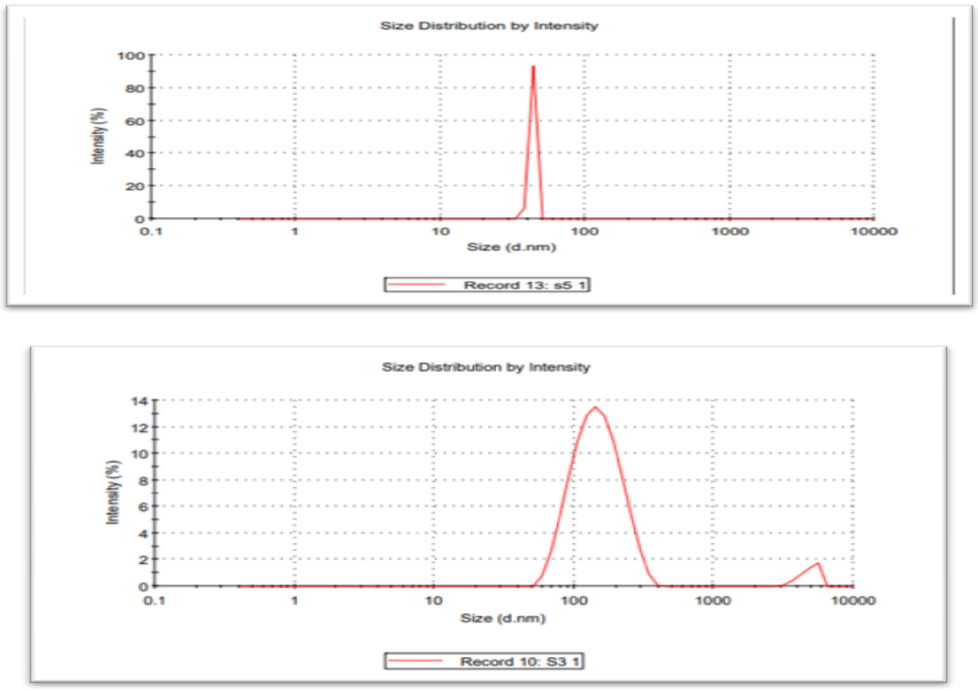

Fig Zeta potential analysis (A) Fig Zeta potential analysis (B)

When nanoparticles of *Tinospora cordifolia* was given to all the four bacterial culture zone of inhibition at 8µl concentration was 0.7cm^2, 0.2cm^2, 0.6cm^2, 0.3cm^2 for *keliebsella pneumonia, Staphylococcus aureus, Citrobacter freundii and, Clostridium perfringes.*

Future recommendation

In place of crude extract purified extract like (tannins, saponins, flavonoid, glycosides etc.) can lead to a potent drug discovery. These plants can be further exploited as potent alternative to current drug therapy against bacterial infection. Nanoparticle of pure phytochemical can be exploited in future which will definitely leads to positive and effective outcomes .

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