



Research Article

CLINICAL STUDIES OF GASTRO-INTESTINAL CONDITION USING *HOLARRHENA ANTIDYSENTERICA* STEM TINCTURE AND ITS COMPARISON WITH SILVER NANOPARTICLES

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ABSTRACT

Efficacy of *Holarrhena antidysenterica* (H.A.) stem tincture was studied on the patients with gastro-intestinal conditions. The *in-vitro* experiments of H.A. tincture with Gram positive and Gram negative bacterial cultures show considerable improvement over the commercial tincture due to the reduced particle sizes of the H.A. extract. Plant Secondary Metabolites present in stem extracts of (H.A.) were used to reduce Ag ions to AgNPs in a single-step green synthesis process. FTIR, UV and TEM analysis confirms the above reduction process.

Keywords: *Holarrhena antidysenterica*, Silver NPs, Green synthesis, Cultures, Gastroenteritis, Colitis, DLS, TEM, Zone of inhibition.

INTRODUCTION

Holarrhena antidysenterica, which is also known as Kutaja in India is a plant whose extracts are used in medicines for different ailments like diarrhea, asthma, fevers etc.^{1,2} Different parts of Kutaja have been reported for excellent antibacterial activity.^{3,4} The extract from bark of *Holarrhena antidysenterica* is reported to have anti-diarrheal properties.⁵ The fresh juice of bark is considered good to check the diarrhea. In Bleeding and piles, decoction of Kutaja bark is also very useful. Methanolic extract of H.A stem is also effective in IBS(Inflammatory Bowl Disease).⁶

Nanotechnology is new emerging research field nowadays. Many synthesis methods are available for nanoparticles like chemical synthesis, physical synthesis and lately via green chemistry route or biological synthesis using plant and micro-organisms. Green synthesis provides advancement over chemical and physical method as it is cost effective, environment friendly, easily scaled up for large scale synthesis and with no need to use high pressure, energy, temperature and toxic chemicals etc.⁷ The use of environmentally benign materials like plant leaf extract, bacteria, fungi and enzymes for the synthesis of nanoparticles offers numerous benefits of eco-friendliness and compatibility for pharmaceutical and other biomedical applications as they do not use toxic chemicals for the synthesis protocol.⁸

Silver nanoparticles have proved to be most effective as it has good antimicrobial efficacy against bacteria and viruses. The therapeutic effects of silver particles, when suspended in the solution, depend on their particle size, particle concentration and particle shape. Moreover silver in nature is nontoxic. In such an endeavour, crude extracts of the bark of H.A. were tried without and with the inclusion of silver nanoparticles for antimicrobial activity.

MATERIALS AND METHODS

Preparation of H.A. Tincture and Extract

The H.A. plant has been identified and authenticated by Botanical Survey of India, Pune. The stem of H.A. was collected from Karjat, Maharashtra, washed with sterile distilled water, dried and then made it to powder using grinder.

For tincture preparation stem powder and ethanol were macerated in 1:2 ratio in glass bottle for 15 days in dark and then filtered for further use.

For plant extract- 1 g of stem powder was mixed with 10 cm³ of water and boiled for 10 min. The extracts were filtered through Whatman No. 1 filter paper and stored in refrigerator until further use.

Synthesis of AgNPs

AgNPs were synthesized by mixing 9 ml aqueous AgNO₃ solution (1 mM) with 1 cm³ stem extract and incubated the mixture in an ultrasonic bath for 15 min for reduction of Ag ions. AgNPs synthesis in the reaction mixture was observed by colour change as shown in Figure 1.

Characterization of AgNPs

UV-Vis Spectral Analysis

The bio-reduction of Ag⁺ ions in each sample was observed periodically followed by the dilution with 2 cm³ of double distilled water. This AgNP synthesized mixture was scanned in the range of 200 to 800 nm wavelengths under UV-visible spectrophotometer (Model-Shimadzu UV 1800, Germany). The distilled water was used as a baseline as shown in Figure 2.

FTIR Analysis

For detection of functional groups responsible for reducing and stabilizing AgNPs and H.A. tincture, FTIR analysis was carried out. FTIR spectrum in the range 4000–600 cm⁻¹ at a resolution of 4 cm⁻¹ was used for the analysis of the nanoparticles as shown in Figures 3 and 4.

TEM Analysis

Transmission electron microscopy (TEM) technique was used to visualize the morphology of the AgNPs. A drop of the AgNPs colloidal solution was placed on carbon coated copper grids and later exposed to infrared light (30 min) for solvent evaporation. TEM observations were performed on Transmission electron microscope (PHILIPS model CM 200) operated at an accelerating voltage of 200 kV with the resolution of 0.22 nm as shown in Figure 5.

DLS Analysis

DLS (Dynamic Light Scattering) system model Malvern Nano-ZS was employed to analyse the size distribution as shown in Figure 6.

XRD Analysis

X-ray diffraction (XRD) measurement of AgNPs was carried out using powder X-ray diffractometer instrument in the angle range of 10° - 70° operated at a voltage of 40KV and a current of 30mA with Cu $K\alpha$ radiation in a 2θ configuration. The crystallite domain size was calculated by using Debye-Scherrer formula (Figure 7).

Anti-microbial Activity

Antibacterial activity of bio-synthesized AgNPs, H.A. stem tincture and H.A. commercial tincture was determined using Disc diffusion method. For this study both gram positive (*Staphylococcus aureus*) and Gram negative (*E.coli*) organisms were used. This was performed by measuring the zone of inhibition, which is rapid and inexpensive to determine the susceptibility of a particular test organism to an antimicrobial agent.

In-vivo study

For *in-vivo* study, H.A. stem tincture was given to 10 patients of age groups between 2-75 years with dose 8-15 drops (Table 2.) at Homeopathy Clinic Vile Parle, Mumbai.⁹

RESULTS**Synthesis of Silver Nanoparticles**

The synthesis of AgNPs in the reaction mixture was observed by colour change as shown in Figure 1.

Characterization of AgNPs**UV-Vis Spectroscopy**

It is well known that silver nanoparticles exhibit yellowish brown colour in aqueous solution due to excitation of surface plasmon vibrations in silver nanoparticles.¹⁰ It was observed that as the plant extract was mixed in the colourless aqueous solution of the silver ion complex, it started to change the colour from watery to yellowish brown due to reduction of silver ion, which may be the indication of formation of silver nanoparticles.¹¹ The synthesis of silver nanoparticles was further confirmed by measuring the UV-Vis spectrum of the reaction mixture. The characteristic peaks were observed in between 410 nm-440 nm as shown in the Figure 2. It is reported that with the size ranging from 2 to 100 nm, this absorption band is assigned to the surface plasmon phenomenon.¹² It may be due to the excitation of Surface Plasmon Resonance (SPR) of the synthesised AgNPs.¹³ The SPR band at 430nm confirmed the green synthesis of AgNPs of stem extract.¹⁴

FTIR Analysis

Figure 3 represents FTIR spectra of H.A. tincture. The representative spectra of biosynthesized AgNPs manifests absorption peaks located at about 1003, 1372, 2341, 2930 and 3339 cm^{-1} (Figure 4). The absorption peak around 1033 cm^{-1} is

attributed to the -C-O- stretching.¹⁵ The absorbance peak at 1372 cm^{-1} is assigned to the bending vibration of secondary amines.¹⁶ The peak at 2930 cm^{-1} is attributed to the bending vibration of alkynes. The broad absorbance peak at 3339 cm^{-1} is associated with the presence of characteristic hydroxyl functional groups in alcoholic and phenolic compound.¹⁷

TEM Analysis

TEM provides further insight into the morphology and particle size distribution profile of the AgNPs and revealed pattern similar to the biosynthesized AgNPs characterized using TEM. The data obtained from transmission electron- micrograph showed distinct shape and size of nanoparticles. The particles were spherical in shape in the range of 5-50 nm and uniformly distributed without significant agglomeration as shown in Figure 5.

DLS Analysis

DLS was employed to analyzing quantitative size distributions and a more precise quantity of mono-dispersity in colloidal solutions. The average particle number was found to be 34.6 nm as shown in Figure 6 which is close to that obtained by TEM measurements.

XRD Analysis

The X-ray diffraction (XRD) pattern of silver nanoparticles synthesized from aqueous H.A. stem extract clearly shows the crystalline nature displaying the structural information. The XRD spectrum analysis indicated two different diffraction peaks at 32.12 $^{\circ}$, 46.14 $^{\circ}$, 38.01 $^{\circ}$ and 43.9 $^{\circ}$ (Figure 7). These diffraction lines are obtained at 2θ angles, which have been indexed as (101), (111), (200) and (311) plane of fcc silver by comparing with JCPDS data. Similar observations were reported by Ondari and Nalini for silver nanoparticles synthesized using plant *Tridax procumbens*.¹⁸ The Debye-Scherrer equation was used to determine the average particle size of the nanoparticles.¹⁹

$$D = K \lambda / \beta \cos \theta$$

Where, D is the crystal size of nanoparticles, λ is the wavelength of the X-ray source (1.54 nm) used in XRD, β is the full width at half maximum of the diffraction peak (FWHM), K is the Scherrer constant with a value from 0.9 to 1, and θ is the Bragg angle. According to Debye Scherrer equation the average size was found to be between 10-40nm.

Anti-microbial Activity

From *in-vitro* experiments with bacterial cultures *E.coli* and *S.aureus*, the prepared H.A.tincture showed the inhibition improvement by 7.6%, and 20% respectively as compared to commercial tincture as shown in Table 1. This is represented in Figure 8 and 9 (B and D). The high antimicrobial activity in the ethanolic tincture may be due to the presence of tannins, flavonoids alkaloids and terpenoids in addition to the reduction of nanoparticles in the tincture.

From *in-vitro* experiments of AgNPs against bacterial cultures *E.coli* and *S.aureus*, the AgNPs showed the inhibition improvement by 46.1% against *E. coli* but no activity was seen against *S. aureus* as shown in Table 2. This is represented in Figure 8 and 9 (A and C).

The mechanism of bactericidal effect of the silver nanoparticles is not very well known.^{20, 21} It may be possible that AgNPs adhere to the cell membrane and disturb its permeability. Binding of AgNP to the bacteria depends upon surface area available for interaction.²² AgNPs with small size which have a large surface area available for interaction will give more bactericidal effect than the large particle size.²² It is also possible that AgNPs may penetrate inside the bacteria, causing damage by combining with phosphorus and sulphurous compounds. One

more possible mechanism for the antibacterial activity could be the release of Ag⁺ ions from AgNPs.²²

Clinical Trials

0.1% homeopathy tincture of *Holarrhena antidysenterica* stem was used in clinical studies in 10 patients of age groups between 2-75 yrs. The dosage used was from 8-15 drops in cases of Viral Gastroenteritis, Bacterial gastroenteritis, Protozoal (Giardia & Amoeba) enteritis, Non-specific colitis and Irritable Bowl Disease (IBS). The results are reported in Table 3.

DISCUSSION

This paper reveals the evidence-based results of clinical trials on the use of *H.A.* tincture. From clinical trials, it is confirmed that dose required by the patient is reduced from 20-30 drops of commercial *H.A.* tincture to 8-15 drops of prepared *H.A.* tincture. These results were also confirmed *in-vitro* by getting

the higher sensitivity of the drug with smaller nanoparticles against bacteria.

A simple, stable and eco-friendly method of biosynthesizing AgNPs was successfully developed using *H.A.* stem extract. *H.A.* stem contains more alkaloids that play major roles as reducing as well as capping agents in synthesis of AgNPs. TEM and DLS reports revealed that synthesized AgNPs were crystalline in nature with an average particle size of 30–40 nm. Further, AgNPs revealed to possess an effective antibacterial property against gram negative bacteria and with the reduced particle size, AgNPs improved the culture results at least by 46%. This phenomenon in future can help to reduce drug resistance problem for patients in future.

This biosynthesized method facilitates best alternative for both chemical and other physical methods. Hence, the green chemistry approach can be employed in large-scale production and can be used in many medicinal and technological applications.

Table 1: Anti-microbial activity of *Holarrhena antidysenterica* tincture

Sample	<i>E.coli</i>	<i>S.aureus</i>
Commercial tincture	13mm	10mm
<i>Holarrhena antidysenterica</i> stem tincture	14mm	12mm

Table 2: Anti-microbial activity of AgNPs

Sample	<i>E.coli</i>	<i>S. aureus</i>
AgNPs	19mm	15mm
AgNO ₃ (control)	13mm	14mm

Table 3: In-vivo results of *Holarrhena antidysenterica* tincture on patients

S.No.	Patient details	Clinical Diagnosis	Clinical Presentation	Drug and Dose	Result
1.	M/18 years	Viral gastro enteritis Stool : 3-4 pus cells, watery stool	h/o outside food, vomiting 6-7/day, diarrhoea watery 10-12 /day, crampy pain abdomen, fever	<i>H.A. tincture</i> 15 drops four times a day with water for three days	Pain abdo., fever And vomiting cured in one day, diarrhoea reduced to 2-3/day and settled in 2 days
2.	F / 5 years	Gm-ve Bacterial gastro enteritis Stool: pus cells 15-20, occ.bld ++, RBC +10-12, bacteria +	h/o outside water, vomiting 3-4, pain abdomen with dysentery like stool, fever 102° F	<i>H.A. tincture</i> 8 drops four times a day for three days	Fever, dysentery and pain subsided in 2 days Stool : normal after 2 days
3.	F / 65 years	Giardiasis Stool: giardia cysts++	Pain abdomen, gaseous distension, eructation, unformed stool 3-4/day	<i>H.A. tincture</i> 10 drops twice a day for one week	Gaseous distension , pain abdo., unformed stool was better in two days. Stool report after one week no cysts
4.	M/51 years	Non specific colitis, irritable bowel disease Stool: mucus++ Unformed,	Increased stool freq. 3-4 in morning. Gases, bloating abdomen urgency for stool	<i>H.A. tincture</i> 10 drops twice a day with passiflora tincture 10 drops twice a day	Anxiety and stool frequency was well controlled in two weeks
5.	F /50 years	Amoebiasis, giardiasis Stool: E.Histocysts++ Giardia cysts++ Pus cell- Occ	Pain abdomen, diarrhoea 2-3/day Semisolid stool, gases, flatulence Hiatus hernia, gastritis	<i>H.A. tincture</i> 10 drops twice a day for one week	Stool consistency normal in three days
6.	F / 2 years	Giardiasis Stool: mucus++ Pus cells 15-20 Giardia few Macrophages+ WBC: 11700	Teething child Loose stool 2-3/day greenish yellow Vomiting, fever 100°F	<i>H.A. tincture</i> 8drops four times with sugar water	Fever and diarrhoea settled in 24 hrs
7.	F/ 50 years	<i>E.coli</i> , Gm-ve bacillary dysentery recurrent stool: mucus +, RBC Occ, pus cells 8-10 culture : <i>E.coli</i>	h/o D.M., H.T. rumbling abdomen, stool with mucus pain with urge	<i>H.A. tinct.</i> 15 drops three times/ day for 8 days	Recurrent infection cured in 8 days Stool: no pus cell No bacteria
8.	M/57 years	Colitis since 10 yrs Stool: pus 1-2 Epith: occ	Milk intolerance Wheat intolerance Stool frequent	<i>H.A. tincture</i> 5drops three times with <i>Aegle folia</i> tincture 5 drops	Urge and stool improved in three weeks Weight gain 2 kg

		Mucus+	3-4/day Frequent urge after eating Semiformed stool Weight loss 6-7 kg	three times/day	Still under treatment and improving
9.	M /45 years	Acute gastro enteritis ? bacterial with fever, min dehydration	h/o travel, outside food , water fever 101 f stool 10-12 /day weakness, pain abdo., colic B.P. 100/ 70	H.A. tincture 15 drops 3hrly for two days	Fever controlled in one day, stool freq. 3-4 in one day and 1-2 next day
10.	F / 55 years	Acute gastroenteritis ? viral dehydration k/c/o D.M. v	h/o fried fish diarrhoea 10-12 in 2 hrs vomiting 5-6 Colic + B.P. 90 /70	H.A.tincture 15 drops 2 hourly Followed by Kali carb 1000	Diarrhoea and colic Controlled in one day

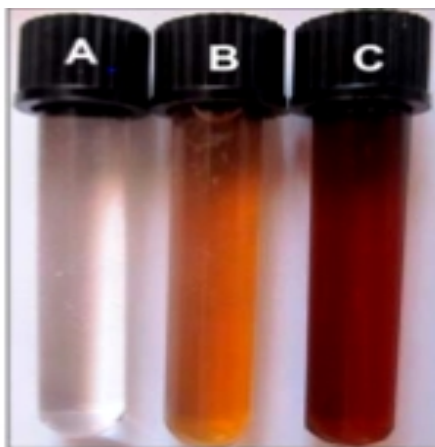


Figure 1: (A)1mM silver nitrate solution, (B) H.A. stem extract and C) Ag NPs of H.A

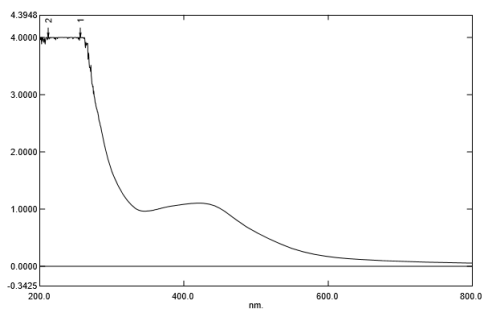


Figure 2: UV-Vis spectra of AgNPs of *Holarrhena antidysenterica* stem extract

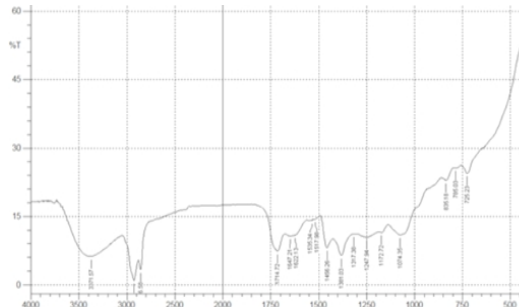


Figure 3: FTIR analysis of *Holarrhena antidysenterica* stem extract

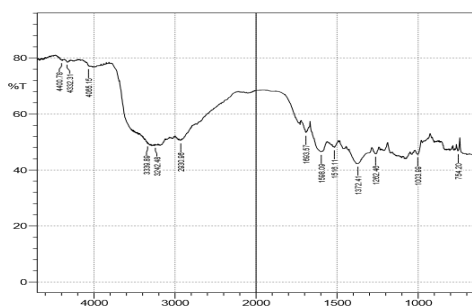


Figure 4: FTIR analysis of AgNPs of *Holarrhena antidysenterica* stem extract

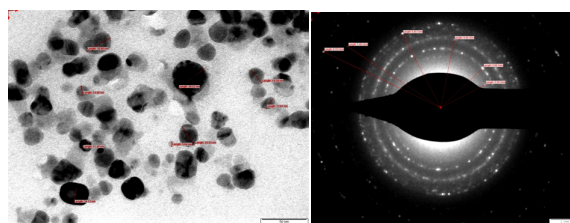


Figure 5: TEM images of AgNPs of *Holarrhena antidysenterica* stem extract

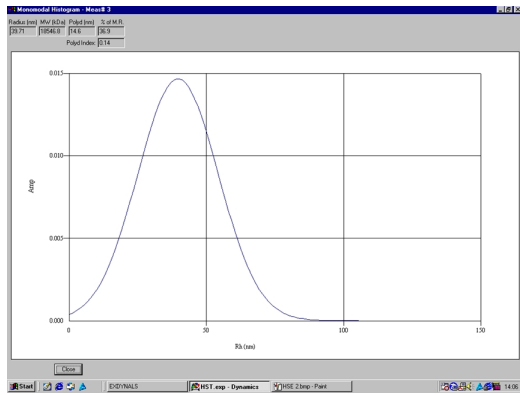


Figure 6: DLS spectrum of AgNPs of *Holarrhena antidysenterica* stem extract

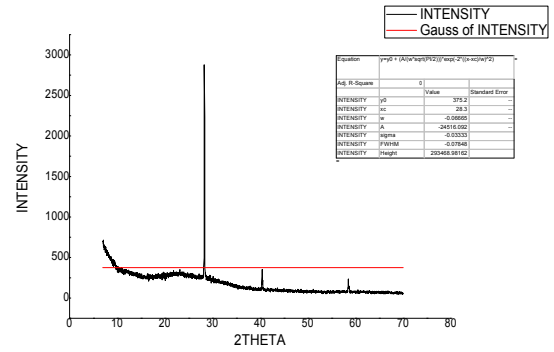


Figure 7: XRD spectrum of AgNPs of *Holarrhena antidysenterica* stem extract

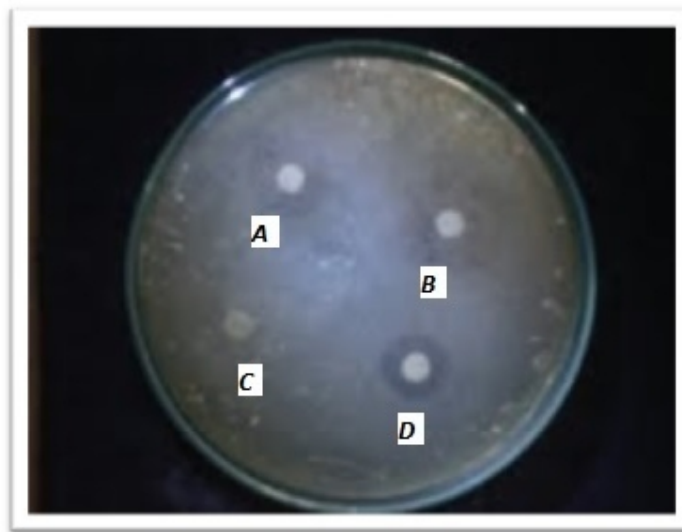


Figure 8: Antimicrobial activity against *S.aureus*. A) AgNPs of *Holarrhena antidysenterica*, B) *Holarrhena antidysenterica* tincture, C) $AgNO_3$ and D) Commercial *Holarrhena antidysenterica* tincture

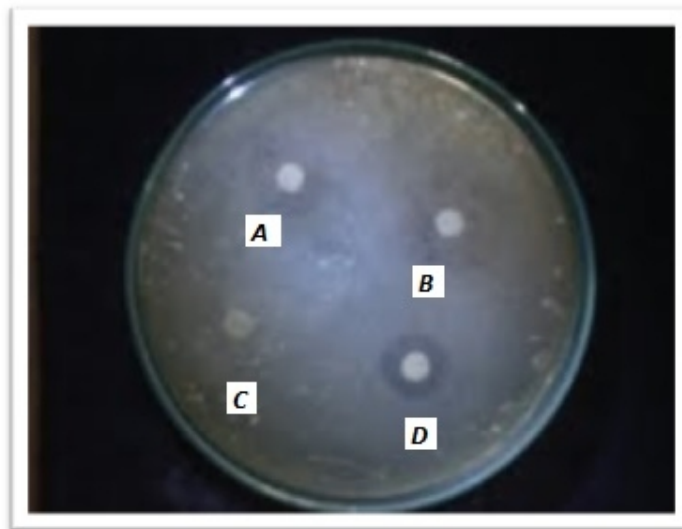


Figure 9: Anti-microbial activity against *E.coli*. A) AgNPs of *Holarrhena antidysenterica*, B) *Holarrhena antidysenterica* tincture, C) $AgNO_3$ and D) Commercial *Holarrhena antidysenterica* tincture

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ABBREVIATIONS

H.A.: *Holarrhena antidysenterica*, AgNPs: Silver Nanoparticles, UV: Ultraviolet-Visible, FTIR: Fourier Transmittion Infra Red, TEM: Transmission Electron Spectroscopy, DLS: Dynamic light Scattering, XRD: X-ray Diffraction.

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