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Research Article

CLINICAL STUDIES OF SYNERGISTIC EFFECT OF MIXTURE OF AEGLE MARMELOS AND HOLARRHENA ANTIDYSENTERICA EXTRACTS AND USE OF *AEGLE MARMELOS IN SILVER NANOPARTICLE SYNTHESIS*

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ABSTRACT

The paper presents evidence-based results of clinical trials on the patients using nanosized particles in homeopathic tinctures of Aegle marmelos (A.M.) and Holarrhena antidysenterica (H.A.). As compared to the commercially available tinctures of A.M. or H.A., the combination of these two drugs prepared in the laboratory showed fast recovery of the patients with doses in liquid form varying according to the age and severity of the diseases. The results were confirmed using bacterial cultures. The in-vitro experiments of these tinctures with Gram positive and gram negative bacterial cultures show considerable improvement over the commercial tincture due to the reduced particle sizes. No toxicity was observed using these drugs in clinical trials. These tinctures thus have better drug resistance against bacteria. A.M fruit extract was used for green synthesis of silver nanoparticles (AgNPs). Plant Metabolites present in fruit extracts of A.M. were used to reduce Ag+ ions to AgNPs in a single-step green eco-friendly synthesis process. FTIR, UV and TEM analysis confirms the above reduction process.

INTRODUCTION

Aegle marmelos is a subtropical plant and grows up to an altitude of 1,200 m altitude from sea level. It grows well in the dry forests on hilly and plain areas. A.M.Is a widely distributed plant and found in sub-Himalayan tracts from Jhelum eastwards to West Bengal, in central and south India. This plant is found almost in all the states of India Among the other parts of the tree, A.M. fruit is reported to be valuable Ayurvedic and Homeopathic medicine for chronic diarrhoea, tonic for heart and brain, anti-viral activity, hypoglycaemic activity, antibacterial activity, anti-proliferative activity and against parasites as reported (Sunita *et al*, 2011). The ripe fruit is aromatic, cooling, alternative, laxative and nutritive. When taken fresh, it is useful in habitual constipation, chronic dysentery and dyspepsia. It also relieves flatulent colic in patients suffering from a condition of chronic gastrointestinal catarrh. Ripe fruit marmalade is used as prevention during cholera epidemics. Powder of the dried fruit pulp is used as febrifuge, antiscorbutic, nauseant, stimulant and antipyretic as reported (Patkar *et al*, 2012). Unripe fruit powder is found to be effective against intestinal parasite *Entamoeba histolytica* and *Ascaris lumberiodes*. Some studies have found the decoction of unripe fruit to be an astringent that is useful in diarrhoea and chronic dysentery (Brijesh *et al*, 2009).

Nanotechnology is a multidisciplinary field, as it combines the knowledge from different disciplines: chemistry, physics, and biology amongst others (Schmid, 2010). Nanotechnology is the art and science of manipulating matter at the atomic or molecular scale and holds the promise of providing significant improvements in technologies for protecting the environment. The technology has excellent prospects for exploitation across the medical, pharmaceutical, biotechnology, engineering, manufacturing, tele-communications and information technology markets (Schmid, 2006).

There are many ways to synthesize nanoparticles such as solid reaction, chemical reaction, co-precipitation and sol gel method, etc. The problem with most of the chemical and physical methods of nanosilver production is that they are very

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expensive and also involve the use of toxic, hazardous chemicals, which may pose potential environmental and biological risks (Saranyaadevi *et al*, 2014). "Green Nanotechnology" is the new term arisen with a lot of consideration that reduces or eradicates toxic substances to be released into environment (Moghaddam *et al*, 2010). For the synthesis of nano-particles, plants are good source for this process. Biologically synthesized nanoparticles are non-toxic, clean, environmentally acceptable, doesn't need high temperature and hazardous chemicals. As we compare to the chemical and physical method their yield is high (Rai *et al*, 2009). Metals were used to synthesize particles of nano size. Biological synthesis can be done by plant mediated synthesis (bark, roots, stem, fruit, leaves and seeds) and microbial synthesis (bacteria, fungi and algae). In this paper, we report a green method for rapid synthesis of AgNPs from A.M. fruit extract and its antimicrobial applications and clinical study of mixture of A.M. and H.A. extract.

MATERIALS AND METHODS

Preparation of Tincture

Aegle marmelos fruits were procured from Vile Parle, Mumbai. Fruit pulp was removed, dried and powdered for preparation of extract since, it is listed in the Homeopathic Pharmacopoeia of India (HPI) guidelines.

Preparation of Extract

10gm Fruit pulp powder was extracted with 100 ml distilled water in Ultra-sonicator bath for 15 min at 37ºC and then filtered with Whatmann paper no.1 to get the fruit pulp extract which was further used for nanoparticle synthesis.

Biosynthesis and Characterization of Silver NPs

For green synthesis of AgNPs, 5ml of fruit extract was mixed with 45ml of 1mM $AgNO_3$ in ultra-sonicator at 37ºC for 10 min and AgNPs of A.M. fruit extract were obtained by observing the colour change from orange to dark brown. The reduction of pure silver ions was observed by measuring the UV-Vis spectrum of the reaction mixture. In this study, a Shimadzu spectrophotometer was used for observing the spectrum at 200 to 800 nm wavelength range. For detection of functional groups responsible for reducing and stabilizing AgNPs, FTIR analysis was carried out. FTIR spectrum in the range 4000–600 cm^{-1} at a resolution of 4 cm^{-1} was used for analysis of the nanoparticles. Morphology of synthesized AgNPs was observed by Transmission Electron Microscopy (TEM) at different magnifications.

Antibacterial activity

Antibacterial activity of bio-synthesized AgNPs, H.A, and A.M. tincture was determined using Disc diffusion method. For this study both gram positive (*Staphyloccus 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 (Table 1).

Clinical Study

This study was done in Dr. Rajesh Barve's Clinic, Mumbai**.** For *in-vivo* study, doses of A.M. and H.A. tincture were given to 17 patients of age groups between 2-90 years with appropriate drops depending on age groups for some weeks (Table 2) (Barve, 2013 and Dabhade *et al*, 2016).

RESULTS AND DISCUSSION

Biosynthesis of AgNPs

Green synthesis of AgNPs was visually observed by colour change. Brown colouration was observed.

Characterization of AgNPs

The shape and size of Green synthesised AgNPs were confirmed by various sophisticated techniques.

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 (Thirumurungan *et al*, 2010). The yellowish brown Colour change was due to reduction of silver ion, which may be the indication of formation of silver nanoparticles (Jain *et al*, 2009).

The characteristic peaks were observed in between 410 nm-440 nm as shown in Figure 1. It is reported that with the size ranging from 2 to 100 nm, this absorption band is assigned to the surface plasmon phenomenon (Safaepour *et al*, 2009). It may be due to the excitation of Surface Plasmon Resonance (SPR) of the synthesised AgNPs (Rashmi, 2009). The SPR band at 430nm confirmed the green synthesis of AgNPs of stem extract (Sathishkumar *et al*, 2009).

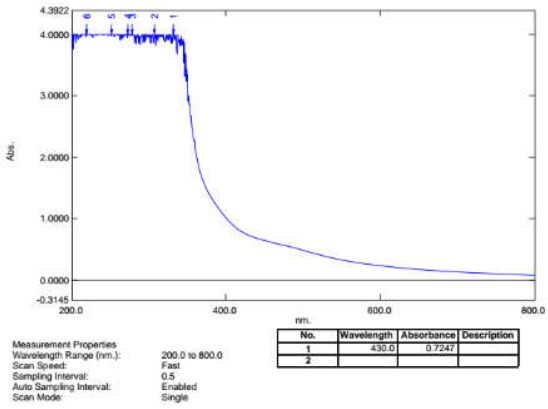

Figure 1 UV-Vis Spectra of AgNPs of *Aegle marmelos fruit*

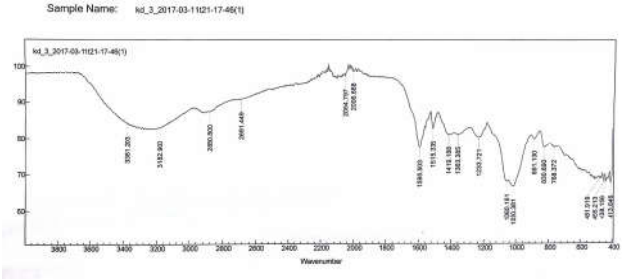

Figure 2 FTIR of *Aegle marmelos* fruit extract

FTIR Spectroscopy

FT-IR analysis revealed the strong bands at 3370, 2234, 1595, and 1419, 1061.25, 760, 519 cm ^{1}. The band at 2234 for O-H stretching corresponds to carboxylic acid, 1595 cm ^{1} for stretching C=C corresponds to aromatic amino groups. The band at 760 cm ^{1} corresponds to C-H stretching of phenyl ring of substitution band, whereas the stretch for Ag-NPs was found around 519 cm^{-1}. (Figures 2 & 3).

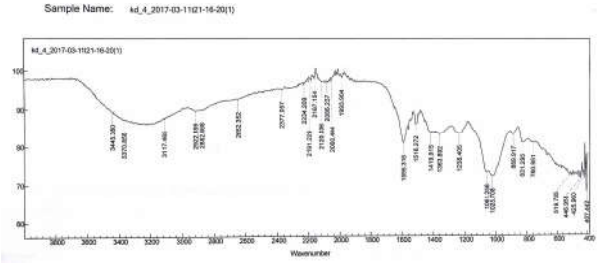

Figure 3 FTIR of *Aegle marmelos* fruit extract AgNPs

TEM

TEM micrograph of AgNPs synthesized by A.M. fruit extract reveals that the particles are spherical in morphology. The nanoparticles were found to be in the size ranges from 20-90 nm which indicates that the particles are polydispersed (Fig. 4).

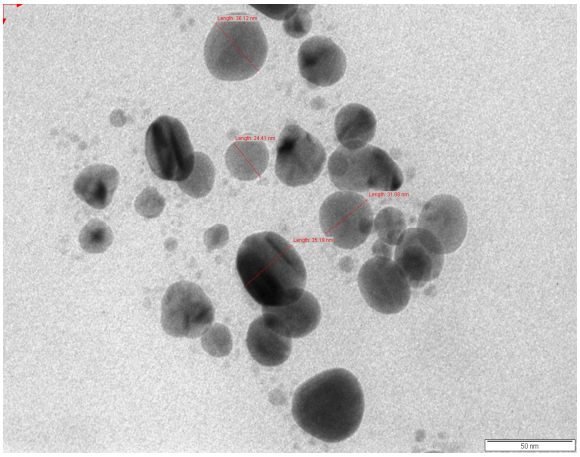

Figure 4 TEM images of AgNPs of *Aegle marmelos* fruit

Antimicrobial study

Table 1 Antibacterial activity

Sample	*E.coli* (Zone of inhibition in mm)	*S.aureus* (Zone of inhibition in mm)
AM	11	07
AM - AgNPs	09	12
HA	13	08
AM+ HA	17	06

Clinical study

0.1% homeopathy tincture of A.M. and H.A fruit was used in clinical studies in 17 patients of age groups between 5-90yrs. The doses used were 8-15 drops in the case of patients suffering from *E.coli* urinary tract infection, IBS (*Irritable Bowel Syndrome*), Acute Gastroenteritis, Dysentery, Ulcerative colitis, Post chemo colic carcinoma, Neuro-endocrine tumor of stomach. The results for the same are reported in Table 2.

CONCLUSION

This paper shows that the prepared doses of combination of H.A. and A.M. extracts and repetition frequency given to the patients of different age groups were less as compared to commercial tinctures being used in clinics. These results were also confirmed *in-vitro* by getting the higher sensitivity of the drug with smaller nanoparticles against bacteria.

The effects of both A.M. and H.A. observed in this study point out the potential of these plants in maintaining the gastrointestinal dysfunctions like IBS (Irritable Bowel Syndrome), Acute Gastroenteritis, Dysentery, Ulcerative colitis, Post chemo colic carcinoma, Neuroendocrine tumor of stomach. Constituents in the tincture may result in better activity due to synergism or lead to decrease in toxicity. The present study validates the use of unripe fruit of A.M. and H.A. stem tincture as an anti-diarrhoeal agent in traditional medicine. The results obtained in the study suggest that the decoction of A.M and H.A. can control several forms of infectious gastrointestinal diseases

The present study completes the objective of 'Green' synthesis of silver nanoparticles by a simple method. We have developed a fast, eco-friendly, simple and economical approach for the preparation of stable silver nanoparticles by reduction of silver nitrate solution with a bio-reduction method using *Aegle marmelos fruit* aqueous extract. The characteristics of the obtained silver nanoparticles were studied using UV-Vis, FTIR, TEM etc. analysis techniques. The experimental results showed that the synthesized silver nanoparticles are stable with an average size of about 30-60 nm. It was confirmed that the synthesized nanoparticles has antimicrobial activity and the AgNPs of A.M. fruit extract proved to be more active against *Staphylococcus aureus*. Green synthesized silver nanoparticles could be very useful in the biomedical field for their potent antibacterial properties.

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Table 2 In Vivo activity

S.No.	Patient details	Clinical diagnosis	Clinical Presentation	Drug and Dose	Result
1	F/51	Neuro endocrine tumour of stomach, recurrent diarrhea, weight loss 6 kg, appetite loss	Gastric polyp, eosinopholic, lymphocytic infiltration colon, with oedema	A.M.+ H.A. tincture 15 drops three times/ day, for 1month	Symptomatic relief of diarrhea + in 10 days, 5kg weight gain
2	F/70	Urinary tract infection with fever 102ºF, chill.Known case of D.M. H,T., Hyperlipidemia	W.B.C 17500 Urine: pus cells250-300/hpf, blood+,proteins+Culture: *E.Coli*+, colony count more than 100000	A.M.+ H.A. tincture 15 drops four times/day for one week	Afebrile in 3days. Urine report after 15 days Pus cells: 3-4 Epith cell: 1-2
3	M/78	Urinary tract infection with fever 101ºF, dysuria,weakness, loss of appetite,(h/o recurrent urinary infection treated previously with antibiotics)	Urine : pus cells: 40-50, RB.C. : occ, albumin: trace *E.coli* infection	A.M.+ H.A. tincture 15 drops four times/day for one week	Afebrile in 2 days .Urine routine after 15 days . Pus cells: 2-4
4	M/7	Acute gastro enteritis after outside food, pain abdomen, nausea, vomiting, stool: 10-12 /day, watery, unformed stool, offensive	-clinical diagnosis	A.M.+ H.A. tincture 10 drops four times/day for three days	A febrile in one day Diarrhea better in 2 days
5	F/66	Dysentery after spoiled milk product, pain abdomen Known diabetic, filariasis	Stool: blood++, pus cells:4-5 Epithelial cells 1-2	A.M.+ H.A. tincture 15 drops three times for three days	Dysentery better in one day
6	F/32	Dysentery, abdomen pain, colic Stools: 7-8 /day	Clinical diagnosis	A.M.+H.A. tincture 15 drops three times for two days	No dysentery next day better in one day
7	F/11	Fever 100ºF, acute gastroenteritis 6-7 stools, pain abdomen, vomiting 2-3 times	Clinical diagnosis	A.M.+H.A. tincture 10 drops four times/day	No pain, no diarrheoa , a febrile in 24 hrs
8	F/72	Irritable bowel syndrome, stool frequency7-8/day diabetis, hypertension	Stool: mucus 1-2, no pus cells	A.M.+H.A. tincture 15 drops three times	Symptomatic relief of I.B.S. After medication Stool freq. 2-3/day
9	M/72	Ulcerative colitis since few years , stool freq 4-5/day Pain abdomen, blood+, mucus+ in stool	Colonoscopic biopsy confirmative Stool; blood+, R.B.C: 8-10 MUCUS ++	A.M. + H.A. tincture 15 drops three times For one month	Stool frq; 2-3 in three days Long term relief after treatment. In last 2 years only had one episode
10	M/68	Urinary tract infection Fever 103ºF, chills, dysuria.	Urine: pus cells: 250-300 RBC: 30-40,blood:+,albumin: +,culture :*E.Coli*++, colony count more than 100000	A.M.+H.A. tincture 15 drops four times/day For one week	Afebrile in 3 days Urine report after one week Pus cells: 3-4/hpf.
11	F/90	Recurrent urinay infection, dysentery unformed stool 4-5/day Diabetes, hypertension	Urine : pus cells: 20-25/hpf Stool: mucus++, rbc 3-4/hpf	A.M.+H.A. tincture 15 drops three times for one week	Stool and urinary symptoms better in 3 days, Urine pus cells 1-2 after one week.
12	F/71	Dysentery, pain colic Stool 7-8/day, mucus +	Clinical diagnosis	A.M.+H.A. tincture 15 drops three times for three days	No symptoms after 3 days
13	F/65	Irritable bowel syndrome, stool freq. increases with anxiety, unformed stool 3-5/day, never at night	Stool report normal	A.M.+H.A. tincture15 drops three times/day and s.o.s	Symptomatic relief after treatment
14	F/79	Adenocarcinoma sigmoid colon, post surgery, post chemo diarrhea recurrent Urgency with unformed stools 5-6/day Weight loss 4-5 kg	Stool: rbc 1-2 Mucus: +	A.M.+H.A. tincture 15 drops three times for one month.	Stool 1-2/ day Relief of symptoms For last 2 years.
15	F/84	Irritable bowel disease, hypothyroidism Stool freq 4-6/ day, urgency+ after eating	T.S.H. 14 Stool : normal	A.M.+H.A. tincture.15 drops three times/ day s.o.s	Symptomatic relief in one day
16	F/74	Recurrent urinary infection,fever101ºF dysuria, weakness, nausea Ischemic heart disease, diabetis	Urine culture: *Ecoli*+, antibiotic resistant strain Pus cells 20-25/hpf s. creat: 1.52	A.M.+H.A. tincture 15 drops four times/day for ten days	Afebrile in 2 days No s/s after 5 days
17	F/75	Ulcerative colitis since many years Recurrent dysentery with mucus and blood Joint pains, hypertension.	Colonoscopy :ulc. Colitis Stool: mucus ++, blood+, rbc 5-6.	A.M.+H.A. tincture 15 drops three times in every episode of dysentery	Symptomatic relief in 1-2 days

Note- F: Female, M: Male

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