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HYDROCOTYLE SIBTHORPIODES EXTRACT LOADED IN CHITOSAN NANOCAPSULE AS AN EFFECTIVE ANTIBACTERIAL AGENT AGAINST FEW PREVALENT MICROBES IN WOUND

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Hydrocotyle sibthorpiodes, Chitosan nanocapsule with AgNPs, Antibacterial study, Chronic wounds

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ABSTRACT: *Hydrocotyle sibthorpiodes* is a known medicinal plant and is used for treatment against many bacterial and fungal pathogens in a conventional application form. The present study was intended to enhance the activity of *H. sibthorpiodes* plant extract against four bacterial species in combination with clove oil and silver nanoparticles by preparing a chitosan based nanocapsule that cause chronicity in wounds. The size of the synthesized nanocapsules was studied by Dynamic Light Scattering study (DLS) and SEM and was found to be 138.2 d.nm. However, the size of the loaded nanocapsule without AgNPs was obtained with a reduced size of 124.8 d.nm. The analysis of the result for the antibacterial study of the plant extract loaded nanocapsule with AgNPs was also found to be more than the extract alone and nanocapsules without AgNPs with an inhibition zone of 3.9 cm and 3.2 cm against both *S. aureus* as well as *K. pneumonia* respectively which was considered to be more effective than the conventional application of the plant extract for inhibiting the bacterial growth. The chitosan loaded plant extract may not only improve the efficacy of the plant active compounds but will also help in its targeted release thereby providing a good therapeutic benefit to the society with a low cost.

INTRODUCTION: The recent past has shown the increasing research evidences clearly indicating the positive role of traditional plants in the prevention and control of some metabolic disorders [12]. The renaissance happening for the popularity of the herbal medicine can be related to the reasons of growing concern over the safety of synthetic drugs and better results of the natural measures than the drugs without any side effects [6]. The use of biological activity of different medicinal plant species has been studied by popular groups of researchers with a focus on how these plants could benefit the pharmaceutical industry.

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The effectiveness of most of the species of medicinal plants depends on the presence of bioactive constituents such as alkaloids, tannins, flavonoids and phenolic compounds that produces a rapid physiological action in the human body. Due to the lesser side effects and therapeutic benefits, the usage of phytomedicines has been increased thus serving as a crucial source of ancient drugs.

Hydrocotyle a genus of prostrate, perennial or semi-aquatic plants classified in the family Apiaceae is an important medicinal plant species. The herb is widely distributed without any threats to the population. The substantial properties of the species have become a reason for its exploitation for commercial purposes by many drug agencies and have therefore been listed in the red list of IUCN (International Union for Conservation of Nature. Version 2016) as threatened Species. The herb has been used as a remedy in traditional Chinese medicine and ethnic drug for hepatitis in

china. The whole plant is known to possess astringent, anti-inflammatory, anti - helminthic, analgesic and diuretic properties. It is known to have an effective response for rheumatism, sore throat, fungal infection in mouth, thrush, digestive troubles, syphilis, skin diseases and healing wounds. A pounded mixture of *Hydrocotyle sibthorpioides* with flowers of *Musa balbisiana* applied on ulcers leaves a relieving action for skin diseases. In North East, juice of the plant was reported to be used traditionally for typhoid fever by mixing it with honey, rheumatic troubles, skin diseases including syphilis and liver complaints [10]. Also, decoction of the plant is used for treating abscesses, colds, coughs, hepatitis, influenza, sore throat, headaches and urinary problems. Apart from these, the efficacy of different extract of the herb has also been reported in incision and burn wound experimental animal model study [8].

Although, the therapeutic importance of these herbal products has been built for the improvement of human health but the comprehensive application of the products are often restricted due to its low bioavailability and membrane permeability associated with poor lipid soluble compounds. The biologically active constituents of the extracts, such as flavonoids, tannins and terpenoids are highly water soluble but demonstrate a low absorption because of the inability to cross the lipid membrane and higher molecular size which results in poor absorption, less bioavailability and efficacy. Currently, the strategy of applying the plant based phytoconstituents along with biodegradable polymers together form an effective biomaterial as a drug delivery system, developed to enhance the delivery of active compounds against pathogenic microbes mostly prevalent in wound which often results in the delayed healing and chronicity of the wound.

The studies associated with polymeric nanoparticles, liquid crystal (LC) systems, solid lipid nanoparticles (SLNs), liposomes and micro-emulsion are known to effectively allow substances with different properties to be used in the same formulation thereby revolutionizing the drug delivery [3]. Moreover, in addition to the ability to improve the stability of a plant drug, nanotechnology based approach have also targeted the successful combination of active substances with different degrees of hydrophilicity/ lipophilicity and its targeted distribution towards specific tissue and organs [7]. The polymeric nanoparticles has not only been approved to enhance the activity of the encapsulated drugs but also helped to reduce its toxicity. These nanocarriers of submicron size received a remarkable attention as well as advantages for controlled release, good biocompatibility and providing protection to most of the drug from degradation with its unique narrow size distribution and small size.

Biodegradable polymers are self degradable polymers and are either classified as natural or synthetic. Natural polysaccharides based polymers represents major class of biomaterials, which includes agarose, alginate, carageenam and chitosan. Chitosan is a polycationic N-deacetylated product of chitin with significant biological and chemical properties: It is biodegradable, biocompatible and bioactive which is often being blended with other polymer such as alginate, a co-polymer, consisting of linear α-L-glucoronic acid and β-D-mannuronic acid with biocompatible, biodegradable, nontoxic as well as mucoadhesive property [5] to produce a polymeric film. Both the polymers are also known for their effective biomedical applications as well as model extracellular matrices for fundamental biological studies [11].

MATERIALS AND METHOD:

Chemicals: Chitosan (degree of deacylation ≥90%), Sodium Alginate of molecular weight- 216, Pluronic F-68, Glycerol of molecular weight- 92.10 and Acetic acid ultrapure of molecular weight- 60.05 were purchased from HiMedia, India. All the chemicals are of analytical grade. Other chemicals such as Methanol, Ethyl acetate and Acetone were purchased from Merck, India and are of HPLC grade. Milli-Q water was used throughout all the experiments.

Media Used: Microbial culture media were purchased from HiMedia, India. Nutrient Agar (NA), Peptone water, Nutrient Broth.

Collection of Microbial Cultures Used: The bacterial samples were procured from wound patients of Downtown Hospital, Guwahati, India by

using sterile cotton swabs with prior consent. The swabs were immediately immersed into saline and carried to the laboratory. All the cultures were grown on nutrient agar plates and maintained in the nutrient agar (NA) slants at 4 °C. The species of the collected samples were identified in the downtown hospital and few prevalent species were used for the study.

Preservation of Microbial Culture: The bacterial cultures were maintained in nutrient agar (NA) slants. Moreover, for the long term storage all the cultures were maintained in 15 - 20% glycerol stock at -80 °C.

Collection of Plant Sample Used: Fresh whole plant of *Hydrocotyle sibthorpiode* including the aerial parts were collected from different locally grown moist soil areas of Panikhaiti (situated in between 26.22° North latitude and 91.88° East latitude) Guwahati, India in the month of May and June 2015. The sample was collected in a sterile plastic bag and stored at 4 °C to further process. The collected sample was identified to be of correct species with a standard herbarium specimen voucher No. 18135 in the Department of Botany, Gauhati University, Guwahati, India.

Sterilization and Processing of the Plant Sample: The plant samples were washed under running tap water to remove the surface dirt and finally rinsed with distilled water. The collected samples were air dried and powdered. The extraction process was performed by Soxhlet method with methanol as a solvent. 100 gm of powder was weighed and placed in the thimble holder of the apparatus for 5 - 6 hours. The dried extracts were weighted (100 mg stock each) and stored at -20 °C for for use in loading in the nanocapsule.

The preparation of the water extract was done by boiling 100 gram (g) of the powdered plant material with 1000 ml distilled for 1 hour. The extract was then collected and filtered three times and the resulting decoction (about 1000 ml) was evaporated to 20 ml and dried in a vacuum at 50°C. The dried extract was weighted, re-dissolved in distilled water (5 mg/ml stock) and stored at -20 °C for further use in synthesis of the silver nanoparticles.

Biosynthesis of Silver Nanoparticle: Silver nitrate solution of known strength 1 mM was prepared in 100 ml of double distilled water. 1.6 ml of the aqueous plant extract (5 mg/ml) was added to 4 ml of silver salt solution (1 mM) with constant stirring and pH was adjusted to 7.4. The final solution was heated in a domestic microwave for 30 seconds. Further irradiation for 1 minute resulted in an intense yellowish brown coloration which indicated the formation of SNPs.

Preparation of Plant Drug Loaded Chitosan Nanocapsules (CH-NCs) by Solvent Evaporation Method: For the preparation of core-shell nanoparticle (nanocapsule), 8 mg of plant extract and 200 µl of clove oil were prepared in 5 ml of ethanol: ethyl acetate, yielding an organic solution. The final volume of plant-extract solution was added drop wise to the surfactant (Pluronic F68) and the suspension was subjected to ultra sonication for 30 minutes. Finally, the volume of polymer solution (dissolved in 50 ml of 2% acetic acid) was added and stirred overnight at 37 °C. Then after, the solvent was removed under vacuum and the suspension was filtered to remove any stacked mass. The size and shape of the plant drug loaded CH-NCs were studied by Scanning Electron Microscopy (SEM) and Dynamic light scattering histogram (DLS).

Plant Extract Loaded Nanocapsule with Silver Nanoparticles: Polymer nanocapsules with silver nanoparticles were synthesized by solvent evaporation technique. Chitosan, silver nanoparticles and plant extract of definite concentrations were dissolved in ethanol: ethyl acetate and vortexed for 10 minutes to prepare the organic phase. The organic phase was then added into stirred aqueous solution of Pluronic F68. The mixture was allowed to sonicate for 30 minutes and the formed emulsion was stirred overnight at 37 °C to remove the solvent. The synthesized nanocapsules were obtained by centrifugation at 8000 rpm for 10 minutes followed by repeated washing with distilled water.

Antibacterial Evaluation of Loaded Nanocapsule with or without Silver Nanoparticles: To compare the antibacterial activity of the loaded nanocapsules, the test organisms maintained and stored at -80 °C in Nutrient agar (NA) were

initiated by following ATCC guidelines. The aliquots of bacterial cultures incubated with 5 ml of 0.1% peptone water (w/v) were spread evenly onto sterilized Muller Hinton agar plants using sterile cotton swabs. The plated mediums were allowed to dry for few minutes in laminar air flow-hood. 6 mm diameter equidistant wells were made using a cork borer, 2 mm from the edge of the plate. 50 µl (1 mg/ml) of the aqueous dispersion of plant drug loaded nanocapsule, loaded capsules with AgNPs and the plant extract were aseptically poured into the respective agar wells. The plates were incubated at 37 °C or 24 hours. The experiment was performed in triplicates for all the three suspensions.

RESULTS AND DISCUSSION: The formation of Silver nanoparticles showed yellowish brown color in the aqueous solution due to the excitation of surface Plasmon resonance. The complete change in color took around 30 minutes, thereafter no further change in the reaction mixture was observed. This formation was also confirmed by obtaining a respective absorption spectrum that originated through surface plasmon resonance (SPR) with a sharp characteristic peak in the range of 300 - 700 nm **Fig. 1**. The obtained data also affirmed the intake photophysical property of the nanoparticles as well as the size dependent effect of SPR. Scanning Electron microscopy was used to study the surface morphology of the nanoparticles. The image revealed the particles were well dispersed and the predominantly spherical in shape. The average particle size was calculated to be around 13.37 ±10 nm **Fig. 2**.

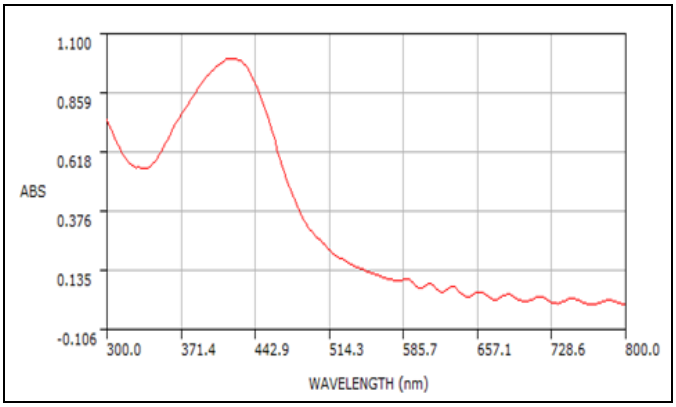

FIG. 1: UV-VISIBLE SPECTRA OF SILVER NANOPARTICLES

Nanocapsule consisting of a liquid core surrounded by a polymeric membrane structure can have several pharmaceutical applications for more rapid absorption of active compounds present in several medicinal plant extracts thereby increasing the bioavailability and efficacy with an increased patient compliance. The double stage process of emulsion-diffusion method has been employed in different research work. In the present study, the similar work has been used as a basis of comparison with few modifications to study the variations in parameters.

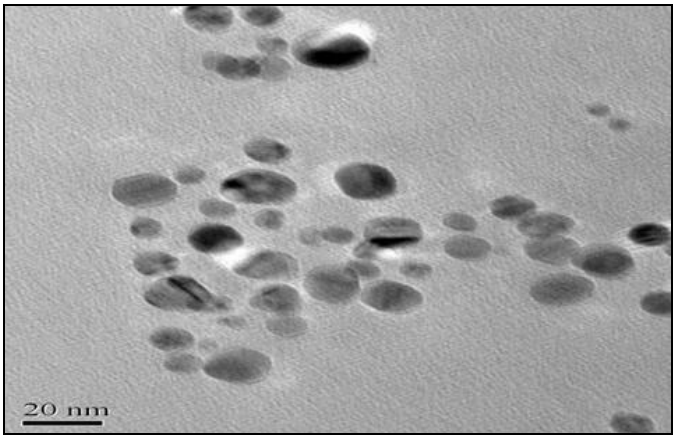

FIG. 2: SEM ANALYSIS OF SILVER NANO-PARTICLES (MAG: 50.00 X) SYNTHESIZED BY *H. SIBTHORPIODES* EXTRACT

In the first step of the process, an emulsion was prepared from oil and plant extract by dissolving in an organic solvent. Following the organic phase, the process enters the aqueous phase which consequently resulted in the precipitation of the polymer around the core containing dissolved plant drug in the oil. For the binding of silver nanoparticles to the plant extract, the suspension was sonicated and the solvent was finally evaporated. The process parameters of the emulsification process and its influence on the size of the nanoparticle were thoroughly investigated by the researchers as depicted in **Table 1** and **2**.

TABLE 1: RESULT OF DLS FOR THE SURFACTANT PLURONIC F-68 WITHOUT AgNPs

Sample	*H. sibthorpiodes* extract (mg)	Solvent: oil ratio:	Average size of the particle (nm)
1	8	2:1	124.8

TABLE 2: RESULT OF DLS FOR THE SURFACTANT PLURONIC F-68 WITH AgNPs

Sample	*H. sibthorpiodes* extract(mg)	Solvent: oil ratio: AgNPs	Average size of the particle (nm)
1	8	2:1:2	138.2

The result of the size obtained in the study has been found to be in agreement with the work done by Akbar *et al.,* 2013 [4]. However, the size of the nanocapsule obtained in the study was found to be approximately similar than the PSAR analysis of the previous data obtained while taking different drug: polymer ratio. The clove oil selected for the study was found to be effective for dissolving the plant extract that may contain both hydrophilic and hydrophobic compounds. The average bigger size of the loaded nanocapsule may be due the adsorption of the silver nanoparticles with the plant extract in the core. The SEM image of the nanocapsule having the core shell structure containing the plant extract and clove oil surrounded by Chitosan polymer is shown in **Fig. 3**.

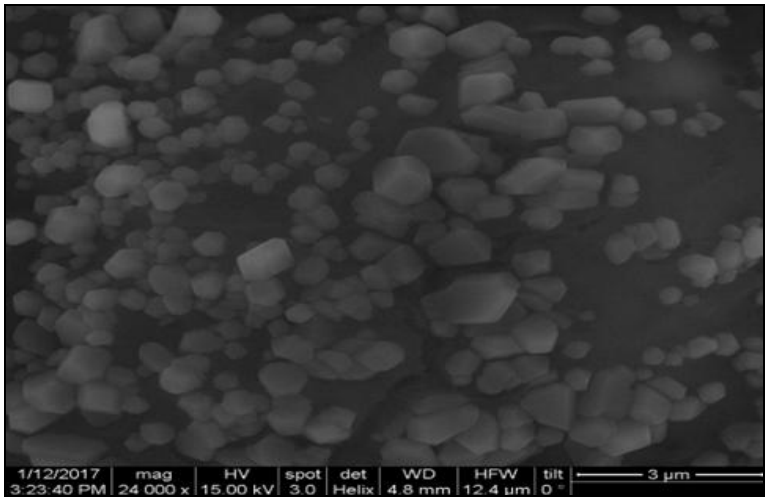

FIG. 3: SCANNING ELECTRON MICROSCOPY OF NANOCAPSULES (MAG: 24000 X) CONTAINING *H. SIBTHORPIODES* EXTRACT WITH PLURONIC, CLOVE OIL AND AgNPs

The evaluation of antibacterial activity of the loaded samples with or without silver nanoparticles was manifested at the sample surface in direct contact with bacterial media. The comparison of the loaded samples with the plant extract was expressed as diameters of inhibition area (cm) as presented in **Table 3**.

The obtained data showed more effective antimicrobial activity by showing greater zone of inhibition against four bacterial strains compared to unloaded extract. It was also observed that the nanocapsules with AgNPs showed the highest activity of inhibition against both *S. aureus* and *K. pneumonia.*

TABLE 3: RESULT OF ANTIBACTERIAL ACTIVITY AGAINST TESTED BACTERIAL SAMPLES

Sample	Bacterial species	ZOI of plant extract	ZOI of plant extract loaded in Chitosan nanocapsule	ZOI of plant extract loaded in Chitosan nanocapsule with AgNPs
1	*S. aureus*	1.2 ± 0.081	2.7 ± 0.127	3.2 ± 0.24
2	*P. aeroginosa*	0.8 ± 0.024	1.3 ± 0.086	2.5 ± 0.44
3	*K. pneumonia*	1.6 ± 0.091	1.9 ± 0.124	3.9 ± 0.69
4	*E. coli*	1.7 ± 0.093	2.0 ± 0.139	2.2 ± 0.57

ZOI: Zone of Inhibition

From the zone of inhibition and increase in fold area data, it is clear that for all the cases, the plant extract loaded into a nanocapsule along with silver nanoparticle is more effective than the extract alone and nanocapsule without AgNPs. *Staphylococcus aureus* has been considered to be MRSA because of its resistant activity against several antibiotics including cefoxicitin and the proportion of the isolate that are resistant to methicillin is rapidly increasing at a greater pace. Moreover, infections caused by methicillin resistant *S. aureus* (MRSA) have been reported as a serious problem not only in health care institutions but also in the community [1]. The result of the study using clove oil as the essential oil showed a significant activity in accordance to a similar study done by Sreenivasan *et al.,* 2006 [9] against both *K. pneumonia* and *P. aeroginosa.* Also, the bactericidal activity of *Hydrocotyle sibthorpiodes* plant extract without loading in a nanocapsule form has been reported to be minimum against the same tested bacterial strains used in the study [2].

CONCLUSION: The purpose of the present study aims at improving the activity of *H. sibthorpiodes* plant extract by encapsulating it in Chitosan nanocapsule with clove oil. The *H. sibthorpiodes* have been reported to contain several classes of bioactive compounds having antibacterial activity against several pathogenic strains of microorganisms but demonstrate a low absorption because of the inability to cross the lipid membrane and higher molecular size which results in poor absorption, less bioavailability and efficacy.

The work, thus, had been established with a novel approach of demonstrating the efficacy of a decelerating drug present in the plant extract for the prevention of bacterial infection as well as the nanoscopic localization of the metallic nanoparticle as often the chronic wound reduces the quality of patient by inflicting significant pain thereby placing a significant burden on the health care system and may serve the society as a good preclinical antimicrobial candidate with low cost.

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CONFLICT OF INTEREST: The authors declared no conflict of interest.

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