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Effect on Healing Potential of Biosynthesized Silver Nanoparticles in Streptozotocin induce Diabetic Rat Wound Model

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ABSTRACT

Background: Diabetes mellitus is a complex chronic condition that results in hyperglycemic environment affecting the metabolism of proteins and lipids, promotes inflammation and delays wound healing.

Methods: A total of n=30 Wistar rats of either sex were divided into five groups with 6 animals in each. Diabetes mellitus was induced using streptozotocin @ 35 mg/kg body weight and excision wound model was developed in all the animals. Group I and Group II were considered as healthy and diabetic control group respectively. The wounds of Group III, IV and V were treated with topical application of *Carica papaya* synthesized silver nanoparticles @ 62.5 µg/ml, *Carica papaya* leafextract @ 31.25 mg/ml and 10% povidone iodine respectively.

Result: Present study concluded that the characteristics of silver nanaoparticles synthesized form *C. papaya* leaf extract make them suitable for the treatment of wound healing in diabetic subjects.

Key words: Diabetes, Extract, Nanoparticles, Plant, Silver, Wound.

INTRODUCTION

Diabetes Mellitus is a metabolic disease characterized by the presence of chronic hyperglycaemia resulting from defects in insulin secretion, insulin action or both (Fagninou *et al.*, 2019). Hyperglycemia results in glycation of biological macromolecules such as proteins, nucleic acids and lipids that block the insulin signaling pathway and promote inflammation and over time this leads to damage, dysfunction and failure of many organ systems (Singh *et al.*, 2015). Diabetes is the leading cause of delayed wound healing (Iglay *et al.*, 2016). The diabetic wound arrest in chronic inflammatory phase that hinders the formation of mature granulation tissue and results in reduced tensile strength of wound (Landen *et al.,* 2016).

Antibiotics drug resistance has become a major problem worldwide because of the abusive use of antibiotics. Topical antibiotics for wound healing generally create a development of antibiotic resistance because of poor perforation into deep skin infections (LeBlanc *et al.*, 2006). In the present situation, nanoscale materials have came up as novel antimicrobial agents because the mechanisms by which nanoparticles inhibit microbial growth are different from the mechanism of prevention of bacterial growth by antibiotics (Khandel *et al.*, 2018, Melinamani *et al.*, 2021). Medicinal Plant extracts having phytoconstituents and enzymes have important role in phytofabrication of metal nanaoparticles (Jacob *et al.*, 2011). Expoliting the potential of medicinal plants, in the green synthesis routes is significant because the current therapeutic approaches have toxicity and resistance issues. The pharmaceutical companies and the researchers are in search of novel antibacterial agents to solve the problem of development of antibiotic resistance against pathogenic bacteria and to induce the diabetic wound healing process.

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Therefore the present work was conducted to evaluate the efficacy of green synthesized AgNPs to tackle the complexity of wound healing in diabetic subjects.

MATERIALS AND METHODS

Preparation of plant extract

Aqueous extract of *C. papaya* was prepared using freshly collected leaves (25 gm). Leaves were washed thoroughly, diced into fine pieces and transferred to sterile 250 ml conical flask. Milli-Q water 200 ml was added in flask and heated at 60°C for 5-10 min and incubated on sand bath for 30 min. This extract was filtered through normal filter paper and stored at 4°C.

Synthesis of silver nanoparticles

Aqueous leaf extract and freshly prepared 1 mM aqueous

solution of silver nitrate solution were mixed in the ratio 1:4 in a conical flask and heated in sand bath at 60°C for 30 minutes. AgNPs precipitate was centrifuged and dried in oven to obtain powder. Characterization of *C. papaya* leaf synthesized silver nanoparticles (CPAgNP) was done by UV-VIS spectra analysis (T60 UV/VIS Spetrophotometer, LabIndia), Scanning Electron Microscope-Leo 435 VP, Fourier transform infrared (FTIR) (Bruker-FTIR Spectrophotometer) analysis and Zeta Analysis (Zetasizer Ver.7.12, Malvern Instruments Ltd., UK).

Experimental animals

Healthy Wistar rats (160-180 g) (n=30) of either sex were selected from Small Animal Laboratory, College of Veterinary Science and A.H., Anjora, Durg, Chhattisgarh, India, housed in polypropylene cages and maintained at 26±2°C, relative humidity 44-56%, light and dark cycles of 12:12 hr, respectively, for one week before and during the experiment. Animals were given standard rodent pellet diet and ad libitum water. The study protocol was approved by the Institutional Animal Ethics Committee (VCA/VPB/1/1/2021 dated 17/8/2021) and conducted experiments on the basis of CPCSEA guidelines.

Induction of diabetes in rat model

The rats were fasted for one night prior to administration of streptozotocin (STZ). Type 1 diabetes model was induced by a single dose STZ@ 35 mg/kg intraperitoneally dissolved in 0.1 M citrate buffer, pH 4.5. Animals with blood glucose level >180 mg/dl confirmed the establishment of diabetic model.

Excision wound model in diabetic rats

Excision wound model was developed in diabetic rats (n=30) after anesthesia and proper sterilization a circular piece of full thickness (approximate 100 mm^2 radius) of skin was cut off with curved scissor from both sides of vertebral column in pre-determined dorsal thoracic area of the rats. Wound area was measured by using a measuring scale.

Experimental animals were divided into five groups of 6 animals each. The wounds of group I rats were given normal saline intraperitoneally and kept as healthy control to observe placebo effect. Diabetes was induced in rats of group II, III, IV and V. The wounds of group II rats was not treated with any compound and kept as diabetic control. The wound of group III, IV and V rats were treated with topical application (twice daily) of CPAgNP @ 62.5 µg/ml, Aqueous Extract pf *Carica papaya leaves* (AECPL) @ 31.25 mg/ml and standard 10% povidone iodine respectively.

Wound healing parameters

Wounds were measured with scale on the day of wounding and then subsequently on the alternate days until complete healing. Wound kinetics was calculated as given in the formula below:

$$\text{Wound area\%} = \frac{\text{Wound area on day (n)}}{\text{Wound area on day (0)}} \times 100$$

Collection of samples

Granulation tissue was also collected on 7th day for the assessment of biochemical parameters of wound healing.

Estimation of connective tissue parameter

Wet tissue 250 mg approximately was dried and hydrolysate was prepared for the estimation of hydroxyproline (Newman and Logan, 1950), hexosamine (Dische and Borenfreund, 1950) and hexuronic acid (Bitter and Muir, 1962) and represented as µg/mg protein).

RESULTS AND DISCUSSION

The formation of silver nanoparticles was primarily monitored by using the UV-Vis Spectrophotometer at a wavelength range of 300-800 nm. A single, strong and broad SPR peak in UV-visible spectrum of the green synthesized silver nanoparticles using the *Carica papaya* extract was observed at 400 nm (Fig 1). The event clearly indicated that the reduction of the ions occurs extracellularly through reducing agents released into the solution by *C. papaya* leaf extract. No colour change was observed in control treatment (without silver ions) when kept in the similar conditions. Megiel, (2017) reported surface plasmon resonance peak range of silver nanaoparticle within 410-460 nm region and Elamawi *et al.*, (2018) reported single strong peak at 383 nm wave length.

FTIR spectroscopy is used to the biomolecules involved in the synthesis of nanoparticles. The FTIR spectrum of the CPAgNP is shown in (Fig 2). The observed intense bands were compared with standard values to identify the functional groups and it is possible to quantify secondary structure in CPAgNPs.

Present study reported the broad spectrum ranges from band at 3465 cm^{-1} to 415 cm^{-1}. Li *et al.*, (2007) also reported that the broad spectrum in FTIR demonstrates the existence of AgNP. FTIR spectrum revealed band set at 3465.80 cm^{-1}, 3136.95 cm^{-1}, 2917.61 cm^{-1}, 1625.08 cm^{-1}, 1511.51 cm^{-1} and 1384.81 cm^{-1}. The FTIR spectrum explains the interaction of AgNPs with leaf biomolecules of *C. papaya*. It showed the broad band at 3465.80 cm^{-1} and 3136.95 cm^{-1} are due to the stretching vibrations of -O-H group and -N-H. Shanmugaiah *et al.*, (2015) also reported that the absorbance peaks at 3432 cm^{-1} can be assigned to N-H stretching vibration. Bands observed at 2917.61 cm^{-1} and 2849.77 cm^{-1} region arising from C-H stretching of aromatic compound. According to Karthik *et al.*, (2013) 2925 cm^{-1} can be associated to C-H stretching vibration. Band observed at 1625.08 cm^{-1} region arising from C=C stretching of aromatic compound. Our findings corroborated with the reports of Annamalai and Nallamuthu (2016) that the band at 1631 cm^{-1} can be attributed to the C=C stretching vibration. The peak at 1384.81 cm^{-1} corresponding to C-H vibration (Deepa *et al.*, 2013) and at 1033 cm^{-1} can be allocated to C-O stretching vibration (Rai *et al.*, 2015).

Thus, the FTIR study reveals the multifunctionality of the aqueous extract of *C. papaya* leaves where reduction

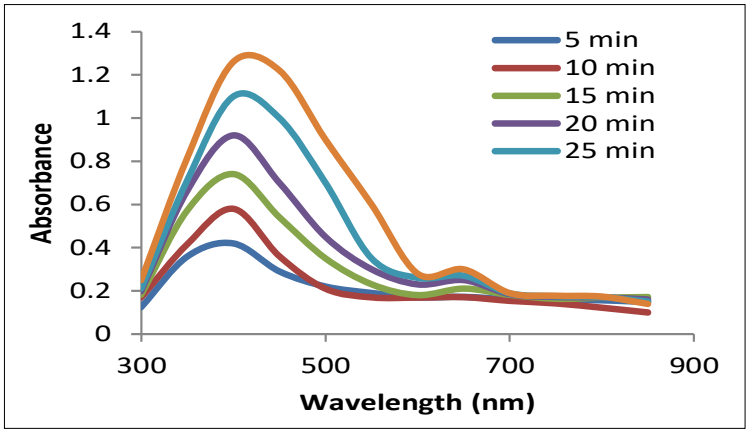

Fig 1: UV-Vis spectra showing absorbance of silver nanoparticles synthesized from *Carica papaya* leaf extract with different time intervals.

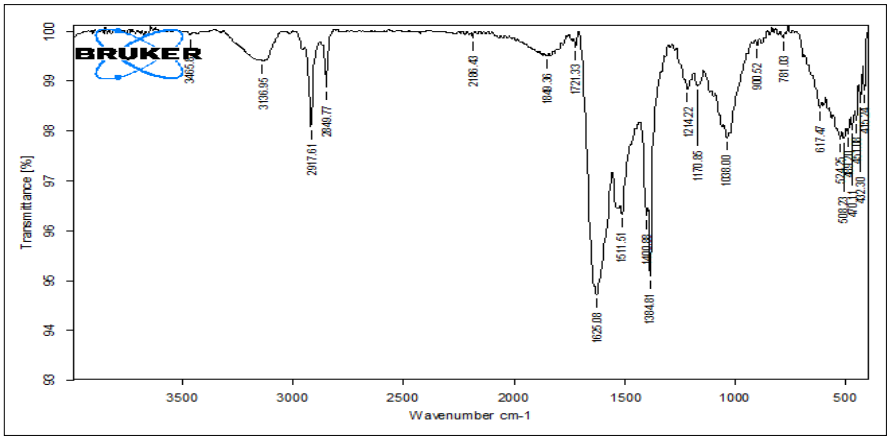

Fig 2: FTIR spectrum of the biosynthesized AgNPs using *C. papaya* leaf extract.

and stabilization occur simultaneously results in the fabrication of silver nanaoparticles and may also lead to their possible stabilization and prevention of agglomeration.

Measurement of zeta (ξ) potential

Fig 3 presented the zeta potential distribution for the aqueous colloid nano silver solution, with a zeta potential -37±2.33 mV. The metallic nanoparticles having large positive and negative charge repels each other and the nanoparticles having lower zeta potential values agglomerate due to absence of repulsive force that prevents such aggregation (Zhang *et al*., 2008). Present study recorded the average value towards the negative side which showed the efficiency of AECPL as capping materials in stabilizing the nanoparticles.

In terms of particle size, it was observed that the colloidal solution of silver nanoparticles contains particles of different sizes and shows a peak of mean particle size 130.2±36.46 nm.

Our finding were in agreement with Ashour *et al*., (2015) who reported that the negative surface charge could be assigned to the adsorption of phytochemicals onto the surface of the NPs present in the aqueous plant extract. Our result shows larger size AgNP but this represented that zeta sizer gives the hydrodynamic size (the size of the nanoparticle plus the liquid layer around the particle).

Scanning electron microscopy imaging

Scanning electron microscopy revealed the average size of 12-83nm (Fig 4a and 4b). However, in a few images the particle sizes as big as 98 nm particles and as small as 5nm were observed (Fig 4a). This indicated that there might be aggregation of the smaller particles is to form comparatively bigger particles surrounded by smaller particles. The spot type pattern suggested the presence of single crystalline particles.

Wound healing measurements

Invasion of opportunistic microbeshampers the healing of diabetic wound, leading to chronic nonhealing wounds. Oxidative stress at the wound site inhibit the healing process; hence, an antimicrobial with antioxidant properties may prove to be beneficial. The STZ induced diabetic rat model has also been used to evaluate the wound healing potential of CPAgNPs and AECPL as compared to povidone iodine (10%). Fig 5 illustrates the percentage of wound contraction. Total phenolic content preset in *Carica papaya* leaf extract is responsible for the antioxidant effect of CPAgNP (Fig 2).

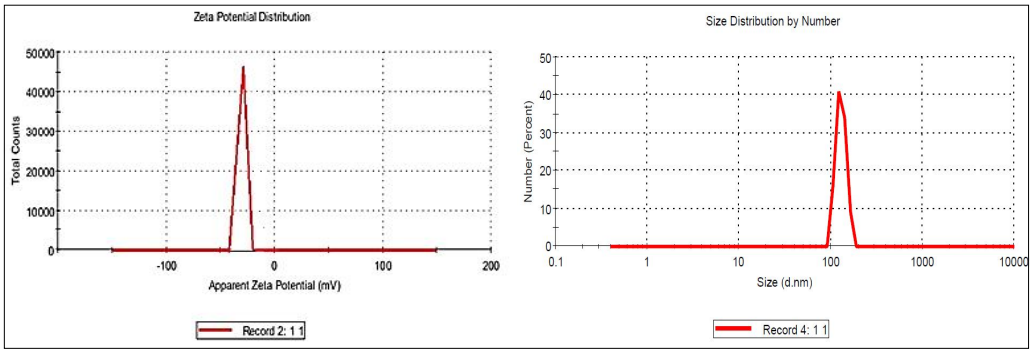

Fig 3: Zeta potential value and size of synthesized CPAgNPs.

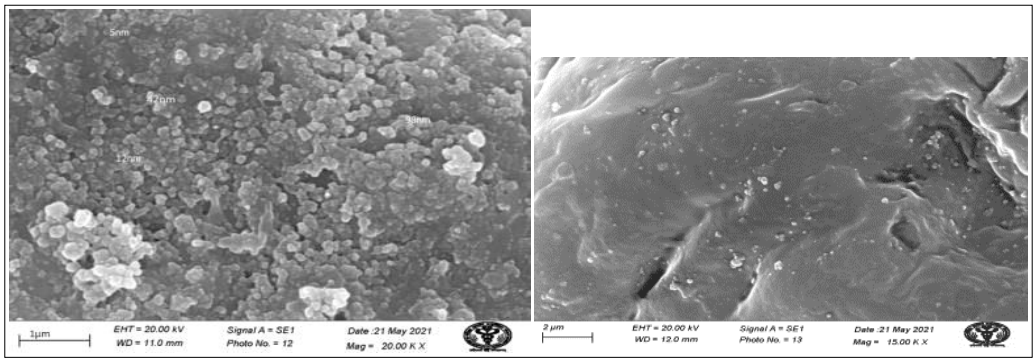

Fig 4: SEM image of the biosynthesized silver nanoparticles (a) size 12-98 nm (b) 5 nm.

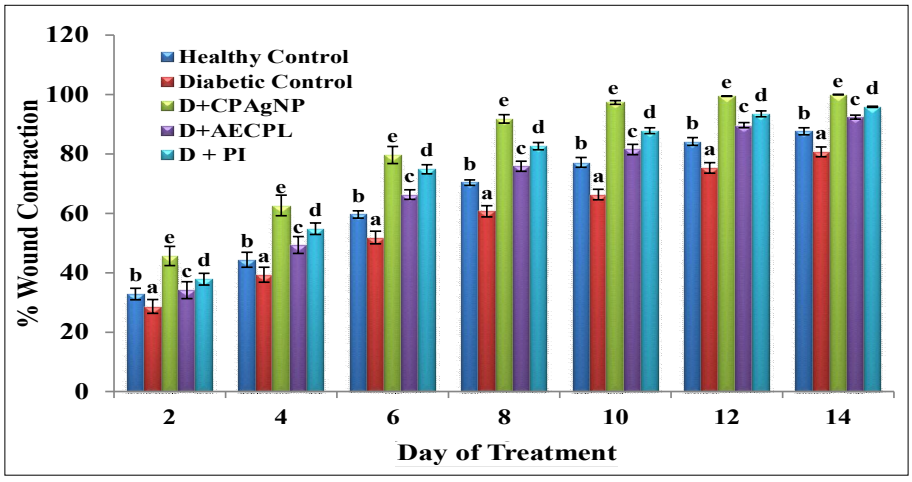

Fig 5: Effect of CPAgNp, AECPL and PI on wound contraction percentage in streptozotocin induced diabetic rats. Values are mean±SEM; n=6 in each group.

The quantitative analysis of wound healing involved measuring the initial wound size (0 day) along with healing towards wound closure (14th day) as illustrated in (Fig 6, 7). The results show cased complete wound closure with CPAgNPs on 14th day, thus confirming had significantly ($P<0.001$) faster healing effect as compared to other treatments and diabetic control group. The impaired healing in diabetic rats may be due to dysfunction of fibroblast, epidermal cells and high level of metalloproteases (Lodhi and Singhai, 2013).

Administration of CPAgNPs and AECPL in group III and IV respectively led to the stimulation of reepithelization at wound site. Group V rats received povidone iodine solution also has positive effect on wound healing. Wounds in all the treatment groups demonstrated epidermal reorganization with complete restoration of normal wound architecture. As per Dunnett's test the per cent healing was significantly increased in group III, IV and V as compared to diabetic control group.

The results obtained showed efficient wound healing potential of silver nanoparticles as compared to pre-existing drug povidone-iodine, *i.e.*, the percentage reduction in wound area after therapy was 100% on day 14th day in case of biosynthesized silver nanoparticle(CPAgNP) treated

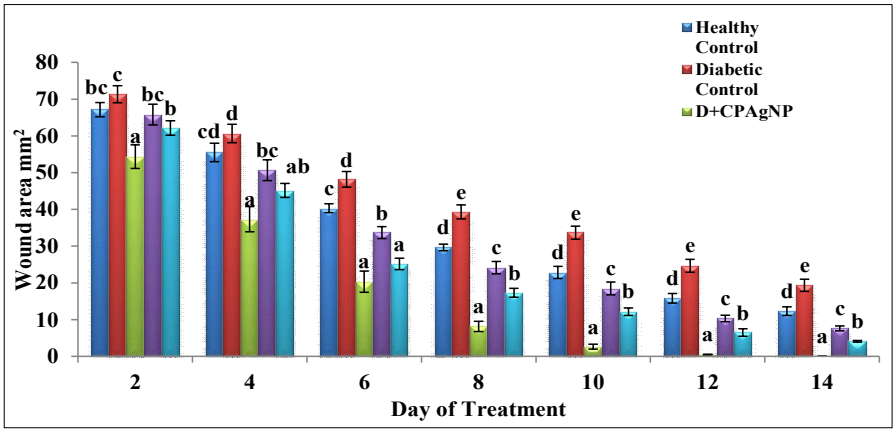

Fig 6: Effect of CPAgNp, AECPL and PI on wound kinetics in streptozotocin induced diabetic rats. Values are mean±SEM; n=6 in each group.

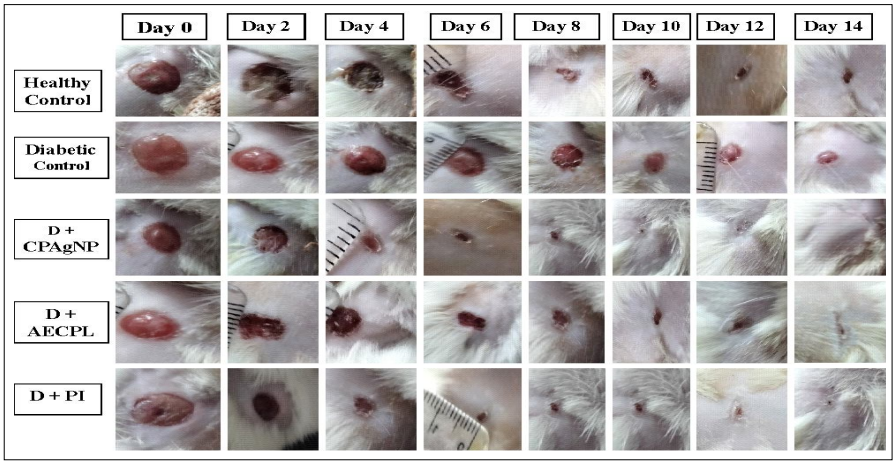

Fig 7: Effect of CPAgNp, AECPL and PI on wound kinetic.

group, 92.39% reduction in AECPL treatment group, 95.89% reduction in case of povidone-iodine-treated animals, 80.67% reduction in case of diabetic control group (diabetic) and 87.67% reduction in healthy control group (non-diabetic). Chauhan *et al*., (2018) also reported efficient wound healing (96.9%) potential of biosynthesized silver nanoparticles as compared to drug povidone-iodine (64.28%).

Compared with conventional topical agents povidone Iodine, CPAgNPs possess considerable antibacterial activity and repair tissues faster and more efficiently and can be recommended for treatment of acute and chronic wound in diabetic animals.

Biochemical parameters of wound healing

Biochemical analysis showed increased hydroxyproline content (132.5±4.60 µg/mg of protein), hexosamine (146.66 ±2.10 µg/mg of protein) and hexuronic acid (17.5±0.92 µg/mg of protein) in group III animals treated with CPAgNPs as compared to 85.00±3.16 µg/mg of protein, 120.00±2.58 µg/mg of protein and 7.5±1.02 µg/mg of protein respectively in diabetic control group (Fig 8). As per our record a significant increase ($P<0.05$) of hydroxyproline, hexosamine and hexuronic acid values were observed in all the treatment groups as compared to diabetic control group. Increased hydroxyproline is a reflection of increased cellular proliferation and there by increased collagen synthesis. Increased hexosamine and hexuronic acid content reflects the stabilization of collagen molecules by enhancing electrostatic and ionic interactions (Nayak *et al*., 2009). Silver nanoparticle mediate secretion of extracellular matrix facilitating collagen production which corresponds to the re-epithelialization phase of wound healing (Tavakoli and Klar 2020). Collagen not only confers strength and integrity to the tissue matrix but also plays an important role in homeostasis and in epithelialization at the latter phase of healing (Dwivedi *et al*., 2017). Group IV animals which received treatment of AECPL also showed significant increase of collagen synthesis and stabilization at the site of wound as compared to normal and diabetic control group animals. The enhanced synthesis of hydroxyproline and hexosamine and hexuronic acid in all the treated rats provide strength to repaired tissue and also healing pattern.

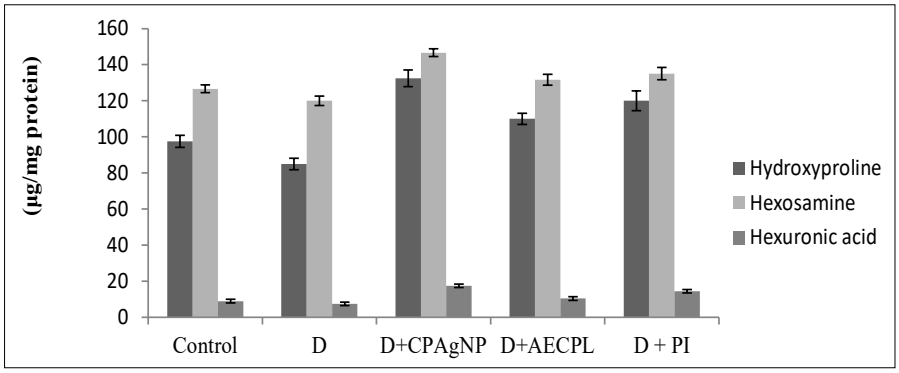

Fig 8: Effect of CPAgNp, AECPL and PI on hexosamine content in granulation tissue (on 7th day) in streptozotocin induced diabetic rats. Values are mean±SEM; n=6 in each group.

CONCLUSION

The result showed potent wound healing capacity as evident from the wound contraction; increased antioxidants and increased biochemical parameters in healing tissue have thus validated the claim of wound healing potential of CPAgNPs in diabetic rat wound model.

Conflict of interest: None.

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