Indian Journal of Chemical Technology
Vol. 28, September 2021, pp. 580-586

In vitro anti-oxidant and In-vitro anti-diabetic studies of silver, gold and copper nanoparticles synthesized using the flowers of *Azhadirachta indica.*

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Received 06 June 2020; accepted 1 September 2021

Nanotechnology industry focuses primarily on promoting nanoscience as a "green" technology to provide a way for less-toxic environmentally benign economic expansion and to synthesize eco- friendly and innocuous drugs to cater to the needs of the contemporary world. An attempt has been made to prepare silver, gold and copper nanoparticles using the aqueous flower extract of *Azhadiracta indica* (neem). In-vitro anti-oxidant efficacy of these metal nanoparticles (NPs) were investigated through i) Hydrogen atom transfer (HAT) reaction based assays- (ABTS scavenging effect and Hydroxyl radical scavenging effect) and ii) Electron transfer (ET) reaction based assays(DPPH radical scavenging activity and NO free radical scavenging activity). CuNPs exhibits better activity compared to AgNPs and AuNPs. In vitro anti-diabetic test was conducted for all the three NPs through three methods namely inhibition of α- glucosidase enzyme assay, inhibition of alpha amylase enzyme assay and Non-enzymatic glycosylation of hemoglobin assay. CuNPs had a remarkable anti-diabetic activity which was close to the standard acarbose.

Keywords: Anti-diabetic, Anti-oxidant, Metal nanoparticles, Neem flower, *A Indica*

Nanomaterials have evolved as one of the most promising field in the research for improved antioxidants. Diverse nanomaterials, including possessing inherent anti-oxidant, anti-microbial and cytotoxic properties, which depend on their surface properties, and not on their functionalization, have been reported[1-7]. Reactive oxygen species (ROS) viz., single oxygen, peroxynitrite, hydrogen peroxide, superoxide and hydroxyl radical are the normal byproducts of cellular breath that can cause cellular disintegration by oxidation process[8]. A better understanding of new nanoparticles with enhanced properties, as well as their combination into a unique form with enhanced antioxidant properties, will shape up the future of nano antioxidant-mediated treatments[9]. Although a wide spectrum of synthetic drugs are being used recently. Many drugs had disturbing side effects such as colic, borborygmus and diarrhoea[10-12]. Several researchers have attempted to identify alternative anti-diabetic compounds involving metal ions such as V^{3+}, Zn^{+2}, Mn^{+2}, Cu^{+2}, Cr^{+3}, and W^{+6} [13-20]. Synthesis of metal nanoparticles through ecofriendly green route are more effective and efficient[21]. An elaborate description of the synthesis of different metal nanoparticles through environmentally benign green route is reported[22]. Early reports have highlighted the importance of metal nanoparticles synthesized through non-toxic environmentally benign green conditions, which is of crucial importance to address the growing needs on the overall toxicity of metallic nanoparticles (MNPs) for medical and technological applications[23]. Among these silver (AgNPs), have been studied extensively[24-26]. Fabrication of CuNPs using plant metabolites were investigated at length[27]. *Azhadirachta indica* (Neem) plant is one of the most studied medicinal plants, the different parts of which is explored by researchers and whose anti-diabetic activity and anti-oxidant activity is well established[28]. The flowers of this plant possess wide variety of phytochemicals. The GC analysis of methanolic and n-hexane extracts of neem flower, reveals the presence of many important phytochemicals[29]. The green aqueous extraction of the phytochemicals present in neem flower, (called Neem flower Aqueous Extract, NFAE) and the syntheses of silver, gold , copper nanoparticles using this extract, (AgNPs, AuNPs and CuNPs, collectively referred as NFAE-MNPs), which was the authors' novel attempt, has been reported by the authors earlier [30-32]. The objective of the current study is to investigate he anti–oxidant and anti-diabetic efficacy of NFAE-MNPs.

Experimental Section

Chemicals and reagents

Chemicals utilized were procured from Sigma-Aldrich and used as received.

Preparation of neem flower aqueous extract (NFAE)

Azhadirachta indica flowers were collected, between the months of March and July, dried under sunshade for seven days and made into fine powder. About 500 g of this powder was macerated, boiled in 500 mL deionized water, maintaining a basic *p*H, for two hours. The solution was allowed to stand overnight, digested twice and filtered using a Whatmann No1 filter paper. The filtrate was immediately used for the synthesis of AgNPs, AuNPs and CuNPs.

Preparation of electrolyte stock solutions and biosynthesis of metal nanoparticles (NFAE-MNPs)

Stock solutions of $AgNO_3$, $HAuCl_4$ and $CuSO_4$ were prepared in the range of 0.1mM to 2mM. Various specifications such as substrate concentration, measure of plant extract, *p*H and the variations in temperature were optimized using UV-Vis spectroscopic method. The optimized conditions were used to synthesis all the three NFAE-MNPs [30-32].

Characterization techniques

The green synthesized MNPs were characterized utilizing the analytical instrument namely UV-Visible spectroscopy, FTIR spectroscopy, XRD measurements and electron microscopic measurements (130Kv Hitachi-S 3400N).

Anti-oxidant assay

The *in-vitro* anti-oxidant activity of the green synthesized nanoparticles was studied through four methods which are classified under two headings as follows: 1. Electron transfer (ET) reaction based assays: DPPH Radical Scavenging Activity, NO free radical scavenging activity; 2.Hydrogen atom transfer (HAT) reaction based assays: ABTS scavenging effect, hydroxyl radical scavenging effect.

Scavenging activity of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical

This important radical scavenging activity was executed in pursuant to the methodology reported amidst certain modifications[33].s All the three NFAE-MNPs were mixed with methanol and sonicated for two minutes. Thus the stock solution was prepared (10 mg/100mL) individually. From this stock solution different concentrations of 20µg/mL to 100µg/mL were taken in separate test tubes. A 0.004% (w/v) solution of DPPH in methanol, prepared afresh was added to each of these test tubes and vortexed thoroughly. After an elapse of 10 minute interval the decline in the absorbance was noted at 517 nm with the repeated procedure to get duplicate values. Ascorbic acid at the same concentration was used as the control. For the use of blank reagent methanol (95%) was used. Percentage of the DPPH free radical scavenged was tallied by adopting the following relation

$$\% \text{ scavenging} = [(Abs_{control} - Abs_{test\ sample}) \times 100] / Abs_{control}$$

Where, $Abs_{control}$ refers to the absorbance of DPPH + methanol and $Abs_{testsample}$ denotes the absorbance of DPPH + sample (NFAE-MNPs / standard).

NO free radical scavenging assay

This experiment was conducted for all the three NFAE-MNPs estimated according to the earlier described method with certain modifications[34]. A 5 mM saline solution of Sodium nitroprusside solution buffered using phosphate was mixed with diverse concentrations of NFAE-MNPs and nurtured at 25° C for 150 min. The samples were then vortexed with Griess reagent and the absorbance was observed at 546 nm. The positive control was ascorbic acid. The IC_{50} values for the standard besides each of the test sample were determined as follows,

$$\% \text{ scavenging} = [(Abs\ control - Abs\ test\ sample)/Abs\ control] \times 100$$

$Abs_{Control}$ refers to the absorbance of the ABTS radical which together with methanol and Abs sample indicates the absorbance of the ABTS radical in conjunction with the sample (NFAE-MNPs or the standard).

2, 2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical scavenging assay

This assessment was conducted for all the three NFAE-MNPs as described earlier with minor modifications[34]. 45µL of 0.138 M solution of potassium persulphate mixed with 3.88M ABTS solution and left in dark condition for 15 h at ambient temperature. A mixture of two solutions namely 1 mL of ABTS solution and 88 ml of 50% ethanol was considered as the working solution. 25 µL of various concentrations of NFAE-MNPs (20µg/mL to

100µg/mL) were blended with 250 µL of ABTS working solution and the maximum absorbance was determined at 734 nm, with ascorbic acid as the standard. The study was done in duplicate and the percentage inhibition was calculated as follows.

$$\text{Inhibition (\%)} = [(\text{Abs}_{\text{Control}} - \text{Abs}_{\text{test sample}}) \times 100] / \text{Abs}_{\text{Control}}$$

$\text{Abs}_{\text{Control}}$ is the absorbance of the ABTS radical including methanol and $\text{Abs}_{\text{test sample}}$ refers to the absorbance of the ABTS radical including the sample (NFAE-MNPs/standard).

Hydroxyl radical scavenging assay

The significant and most important assay, namely the OH radical scavenging activity, was ascertained according to the procedure described in earlier study[34]. 75 µL of each of the three NFAE-MNPs solution (20-100 µg/mL in methanol) was mixed with 90 millimoles of sodium phosphate buffer solution, 1.5 micromoles of deoxyribose, 1.5 micromoles of $FeSO_4$ -EDTA , 1.5 millimoles of H_2O_2 and 525 µL deionized water. After an incubation time of one hour, at 37 °C, process the reaction was interrupted through the inclusion of a mixture of 750µLof CCl_3COOH (2.8%) and 750 µL of sodium thiobarbiturate(1% solution dissolved in 0.05M sodium hydroxide), boiled, cooled and was measured at 520 nm. The analysis was performed in duplicate with ascorbic acid as standard and methanol as blank. The extent of hydroxyl radical scavenging activity as the percentage, was evaluated using the relation

$$\text{\% scavenged} = (\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})/\text{Abs}_{\text{control}},$$

In the above relation, $\text{Abs}_{\text{control}}$ indicates the absorbance of the control (all the reagent except the test sample) and $\text{Abs}_{\text{sample}}$ refers to the absorbance of NFAE-MNPs/ ascorbic acid.

Anti-diabetic assay

All the three biosynthesized NFAE-MNPs were evaluated for their in-vitro anti-diabetic activity by the following three procedures.

Non Enzymatic Glycosylation of Hemoglobin Assay

A modified procedure is adopted to estimate the extent of non-enzymatic glycosylation of hemoglobin[35,36]. 1g/L solutions of all the three NFAE-MNPs and α-Tocopherol using DMSO were prepared. To the disparate concentrations obtained from stock solution (20-100µg/mL), Gentamycin (0.02% in 0.01 M phosphate buffer) (1 mL), glucose solution (1 mL) and hemoglobin solution (1 mL) were added. After an incubation period of 72 h, in dark, the absorbance was read at 520 nm. The method to prepare the control was akin to the one stated earlier, omitting the addition of extract. The well-known pharmaceutical standard, α-Tocopherol was utilized in this measurement and the inhibition percentage was computed as follows

$$\text{Percentage of Inhibition} = [(\text{Abs}_{\text{sample}} - \text{Abs}_{\text{control}}) \times 100] / \text{Abs}_{\text{sample}}$$

Inhibition of α- glucosidase enzyme assay

The scope of this measurement was observed by following the procedure already stated with minor modifications[37]. The assay mixture consist of 15micromoles of sodium phosphate buffer (*p*H 7), 100 µL of α -glucosidase, and test samples (standard/NFAE-MNPs) were considered at five different concentrations of 20, 40, 60, 80 and 100 µg/mL in dimethyl sulphoxide. After the denouement of maintenance of 10 min, at 37°C, 0.1 micromoles of paranitrophenyl α-D-glucopyranoside dissolved in 100mM Na_3PO_4 buffer was combined with the mixture. This was followed by incubation for 20 min at 37°C and terminating the process by the addition of 50 micromoles of sodium carbonate solution followed by the measurement of absorbance at 405 nm. The outcome of the measurement was communicated as percentage of the blank control. The sample with α-glucosidase, and without NFAE-MNPs, was considered as the control considering 100% enzyme activity. Acarbose performs the role of standard of reference.

$$\text{\% Inhibition} = (A_{\text{control}} - A_{\text{sample}})/A_{\text{control}} \times 100$$

Where A_{control} = absorbance without standard/NFAE-MNPs; A_{sample} = absorbance with standard/ NFAE-MNPs.

Inhibition of alpha amylase enzyme assay

This measure was performed using dinitrosalicylic acid reagent (DNSA) as stated in literature[38]. The mixture which is to be investigated consists of 10 micromoles of a buffer solution (prepared using Na_2HPO_4-$7H_2O$ and $NaH_2PO_4H_2O$ at *p*H 7), 100 µL of α-amylase solution and test samples (standard/NFAE-MNPs) at five different concentrations namely 20, 40, 60, 80 and 100 µg/mL in DMSO. After the culmination of an incubation time

of 20 min at 37°C, starch solution (containing 2.5mg starch prepared using the buffer), was added to the tubes and incubated for 15 min at 37°C. Termination of the process was done by including DNSA (1 mL) followed by incubation for 10 min. The tubes were air-cooled to ambient temperature and were assessed at 540 nm. The sample which was considered as the reference constituted all other reagents and the enzyme barring the sample to be inspected. Acarbose was taken as the standard. The extent of inhibition was computed according to the ensuing relation,

$$\text{Percentage of inhibition} = [(A_{I\text{-}540} - A_{E\text{-}540})/A_{I\text{-}540}] \times 100$$

In the above relation, $A_{I\text{-}540}$refers to absorbance determined without adding standard/NFAE-MNPs; $A_{E\text{-}540}$indicates the absorbance achieved with standard/NFAE-MNPs

Results and Discussion

Various parameters such as the electrolyte concentration, the aqueous plant extract volume, pH of the medium and the reaction temperature play a significant role in the morphology and size in the NPs. All the three MNPs were synthesized after optimizing these conditions. The UV-Vis, FTIR, XRD and TEM results of all the three MNPs have been published by the authors' earlier[33-35]. Each of the three MNPs synthesized shows the characteristic of SPR bands. The presence of organic moieties capped on the synthesized MNPs could be well understood from the FTIR spectrum of these NPs. While the XRD pattern of NFAE-AgNPs shows peaks at 2θ values 38.1°, 44.3°, 64.5° and 77.4°, that of NFAE-AuNPs had 2θ values indexed at 37.9°, 44.2°, 64.3° 77.4° and that of the NFAE-CuNPs had peaks at 2θ values at 43.5°, 50.6°and 74.3° revealing the FCC crystal system all these distinct diffraction peaks. The mean crystallite size was calculated using the Scherer's formula, with 16.11nm, 38.48nm and 36.59nm for NFAE-AgNPs, NFAE-AuNPs and NFAE-CuNPs respectively. TEM results were in close correlation with these values. All the three green synthesized NFAE-MNPs were appraised for their free radical scavenging capacity and the evaluation was done in comparison to ascorbic acid as the standard. The results are shown in Fig. (1a, 1b, 2a, 2b) and the concentration required for 50% inhibition, (IC_{50}), is shown in Table 1. The values of correlation coefficient, indicated as R, and the determination coefficient, indicated as R^2, are also tabulated. Elevated values of R and R^2 indicate the goodness of fit in these observations.

DPPH radical is nitrogen centered, room temperature stable free radical, which is regularly used to assess the antioxidant activity of nanoparticles. The antioxidant activity of NFAE-MNPs can be determined spectrophotometrically by monitoring the change in color of DPPH from violet to yellow. Towards DPPH radical, all the three NFAE-MNPs showed increasing inhibitory activity as the concentration of the NFAE-MNPs were increased. Among the three nanoparticles, NFAE-CuNPs shows best results (Fig. 1a) as against the standard. A maximum inhibition of 78.72% is seen at a concentration of 100µg/mL. Though the IC_{50} value was nearly double as that of the standard, it was better than that reported[40]. NFAE-AuNPs exhibited a % activity intermediate between NFAE-AgNPs and

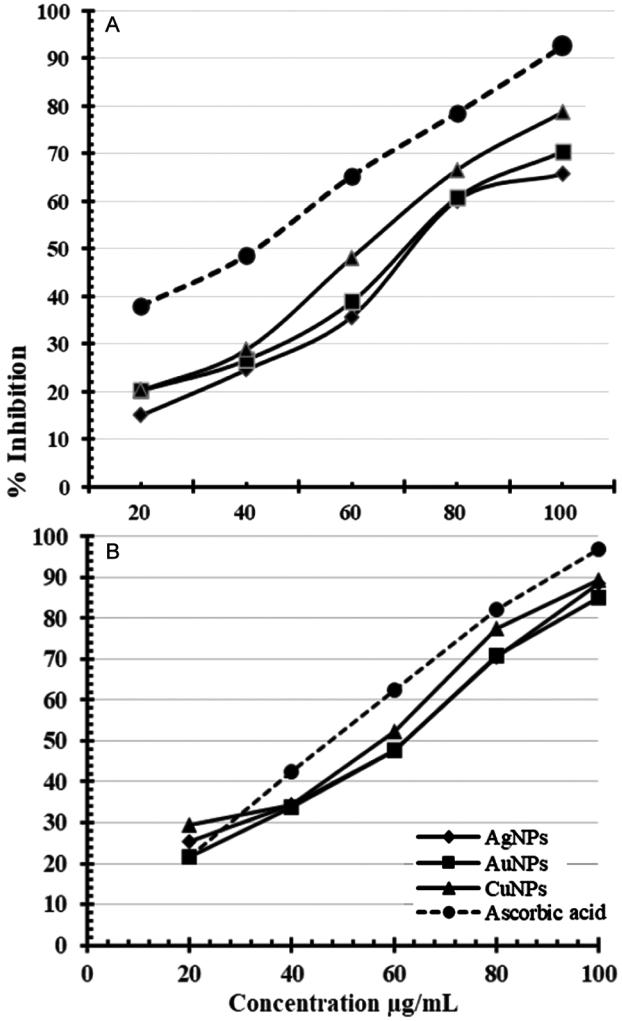

Fig. 1 — Results of DPPH Radical Inhibition assay

NFAE-CuNPs. It is suggested that the organic moieties present as the capping agent interacts differently on the basis of their structures, which results in different % of inhibition.NO radical scavenging ability of the nanoparticles are assessed using Griess reagent, which forms a coloured complex called form a zone. The % inhibition determined for NO radical scavenging assay is presented in Fig. 1b. As the concentration was increased beyond 40µg/mL, an abrupt increase in the% inhibition was observed, similar to that observed in DPPH assay, for all the NFAE-MNPs. the IC_{50} values (Table 1) is indicative of a better inhibitory ability towards NO than DPPH. Once again NFAE-CuNPs exhibited better NO radical inhibition, with a value of 84.72% at 100µg/mL concentration. NFAE-AgNPs and NFAE-AuNPs reached a maximum inhibition of 79.72% and 79.45% respectively. This sort of an increased inhibitory activity might be due to the presence of thiol groups in NFAE[43].As the concentration of all the three test samples have increased, the ABTS cation radical scavenging activity also increased markedly. NFAE-CuNPs exhibits maximum inhibition of 88.72% as against the standard which shows 90.6%. The hydroxyl radical scavenging assay of all the three NFAE-MNPs showed very good activity, with NFAE-CuNPs showing the maximum inhibitory percentage of 89.445. The IC_{50} values (Table 1) were also near the standard indicating the ability of the three test samples to behave as efficient anti-oxidants. The presence of a plethora of able binding and strongly interacting organic moieties and components on the expanse of the biosynthesized NFAE-MNPs, confirmed from their FTIR spectra[31-33], in combination with particle size and other inherent properties are suggested to be the reasons for the anti-oxidant behavior. In addition, the mechanism for the

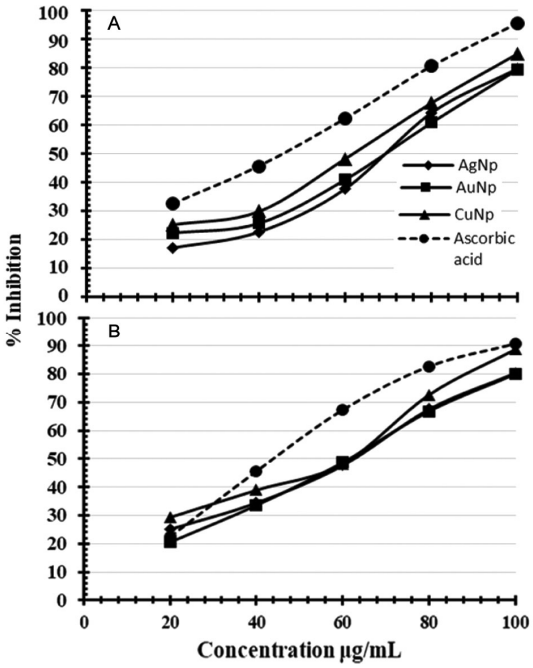

Fig. 2 — Results of NO Free Radical Inhibition assay

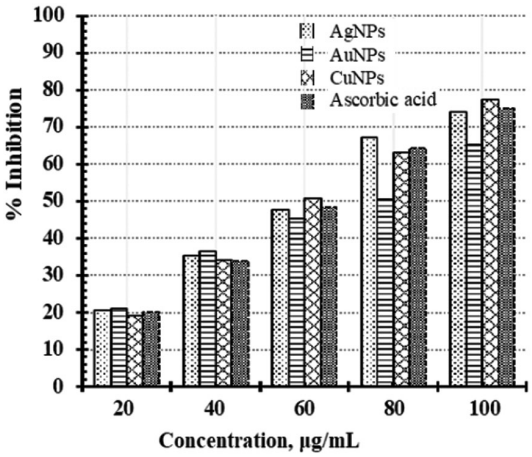

Fig. 3 — Results of ABTS Scavenging activity assay

Table 2 — Results obtained for all the three anti – diabetic assays performed for all the three NFAE-MNPs . IC_{50} , R and R^2 values are compared with the control .

Method		NFAE-AgNPs	NFAE-AuNPs	NFAE-CuNPs	Control
Non enzymatic glycosylation	IC_{50} (µg/mL)	61.42	72.17	61.43	62.26
of Hemoglobin assay	R	0.989	0.982	0.993	0.997
(control:	R^2	0.980	0.965	0.987	0.996
α –Tocopherol)					
Inhibition of α- glucosidase	IC_{50}(µg/mL)	48.01	58.66	44.85	43.8
enzyme assay	R	0.974	0.996	0.987	0.975
(control : acarbose)	R^2	0.9501	0.993	0.975	0.952
Inhibition of alpha amylase	IC_{50}(µg/mL)	44.07	45.72	37.41	40.879
enzyme assay	R	0.961	0.995	0.993	0.987
(control : acarbose)	R^2	0.925	0.992	0.986	0.974

anti-oxidant efficacy involves an electron shuttle enzymatic metal reduction process[44]. This may be influenced by the size and charge on the metal ion.

The results of the anti-diabetic assays performed with the three NFAE-MNPs are given in Figs. 3 and 4.

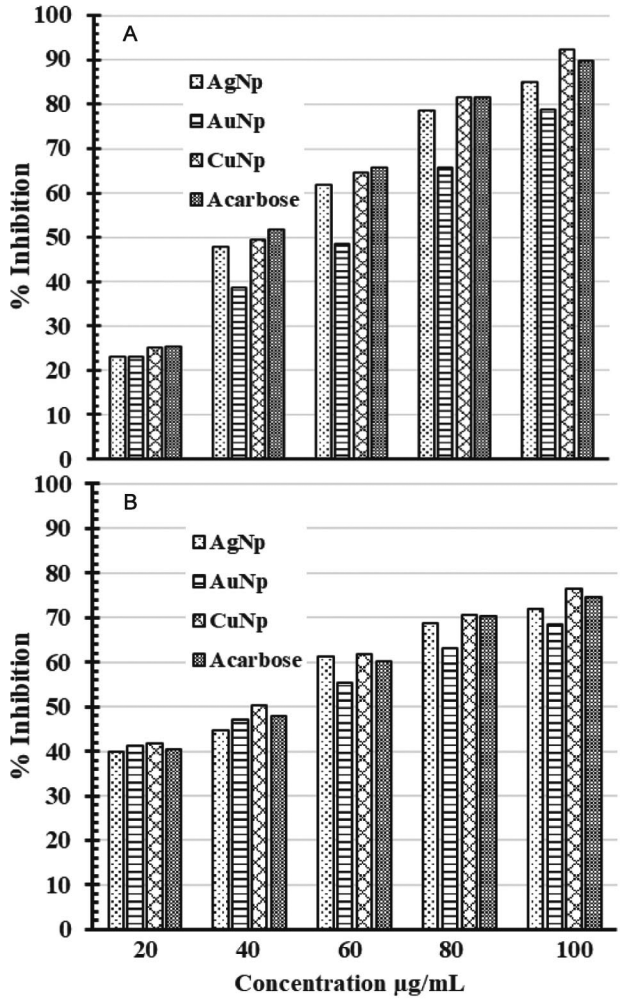

Fig. 4 — Results of hydroxyl radical scavenging activity assay

The Table 2 lists the IC_{50}values, R and R^2 values for the three assays. It can be well inferred from the results that all the three test samples possess the ability to inhibit the enzymes responsible for increase of glucose concentration in blood plasma. These enzymes favour glycogenolysis and gluconeogenesis pathways thereby causing an increase in blood glucose levels. Inhibition of these enzymes can be considered as one of the treatments for diabetes. In the present study it is understood that NFAE-CuNPs and NFAE-AgNPs inhibit α-glycosidase and α-amylase enzymes effectively.

Conclusion

The excellent anti-oxidant efficacy of the NFAE-MNPs in scavenging DPPH, NO, ABTS and OH radicals along-with the anti –diabetic potential of them in inhibitingα-amylase , α-glucosidase and controlling the glycosylation of hemoglobin has been reported. These nanoparticles can potentially overcome many constraints of small-molecule antioxidants, by displaying superior bio-availability, higher stability and the possibility to reach precise targets.

In conclusion, these biogenic MNPs are dependable, efficacious, foreseeable, scalable, and reproducible, do not involve hazardous chemicals, contains the organic compounds in the plant engaged as capping, reducing as well as stabilizing agents. Therefore these are secure, convenient to be used for radical-scavenging, cancer treatment and glycosidase suppression remedies. In the present study promising results were obtained for NFAE-CuNPs. CuNPs, being more bio-compatible and not detrimental, has a preference over other metal nanoparticles with medicinal relevance[45]. The exact mechanism of anti-oxidant and anti-diabetic properties exhibited by these metal nanoparticles need further research.

Table 1 — Results obtained for all the four anti –oxidant assays performed for all the three NFAE-MNPs . IC_{50} , R and R^2 values are compared with the standard ascorbic acid

Method		NFAE-AgNPs	NFAE-AuNPs	NFAE-CuNPs	Ascorbic acid
DPPH radical scavenging assay	IC_{50} (µg/mL)	74.19	69.77	61.9	38.99
	R	0.981	0.983	0.993	0.998
	R^2	0.963	0.967	0.986	0.996
NO radical scavenging assay	IC_{50} (µg/mL)	66.87	65.57	58.52	43.50
	R	0.979	0.977	0.984	0.998
	R^2	0.958	0.955	0.970	0.997
ABTS radical scavenging assay	IC_{50} (µg/mL)	58.54	60.04	52.7	46.48
	R	0.993	0.999	0.982	0.984
	R^2	0.986	0.997	0.965	0.968
OH radical scavenging assay	IC_{50} (µg/mL)	55.96	57.86	51.92	48.30
	R	0.988	0.994	0.981	0.998
	R^2	0.976	0.987	0.963	0.996

Acknowledgement

The authors thank the Convener, ICACSEM-2020 for selecting this manuscript for publication. Authors also sincerely thank the Management and Principal, Ramakrishna Mission Vivekananda College, Chennai 600004, for their continuous support and motivation.

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