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Green Carbon Dots Derived From *Acetosa Sagittata* Aerial Parts And *In Vitro* Antidiabetic, Antioxidant And Cytotoxic Properties

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Abstract:
Diabetes mellitus is one of the leading causes of mortality and morbidity worldwide. Several conventional drugs are used to treat hyperglycemic condition in DM but associated with side effects. Carbon dots are alternative small molecules and able to inhibit enzyme activity through their surface functional groups. Green carbon dots are low toxic and biocompatible in nature which are synthesized using renewable precursors. In the present study, the carbon dots were synthesized using hydrothermal assisted method and physiochemical and morphological properties were confirmed using UV-Vis, FTIR, Fluorescence spectroscopy, XRD and HRTEM. Among all three in vitro antioxidant activities DPPH and FRAP assays revealed the efficient free radical neutralizing capability of CDs than ABTS assay with IC_{50} ranges between 74 to 526 µg mL^{-1}. The CDs exhibited effective α-amylase inhibitory activity than α-glucosidase inhibitory activity with lowest IC_{50} recorded for CD_1 sample against α-amylase (61.25 µg mL^{-1}). For cytotoxicity CD_1 shown lowest IC_{50} value against HeLa cell lines (84.44 µg mL^{-1}). Hence the CDs are effective in inhibiting the enzyme activity and neutralizing the free radicals.

Keywords: Carbon dots, *Acetosa sagittata*, Anti-diabetic, Antioxidant, Cytotoxicity

Introduction:
Diabetes mellitus (DM) is a metabolic disorder of protein, fat, and carbohydrate metabolism attributed to low production of insulin or resistance to its action leading to hyperglycemia. All over the world, one of the leading causes of morbidity as well as mortality is DM [1]. The increasing prevalence of DM and adverse effects related to modern medications are also important points of apprehension [2].

Acarbose is a medication clinically used to inhibit *α*-glucosidase and *α*-amylase. Unfortunately, its long-term administration resulted in side effects including abdominal distention and diarrhea. Alternative plant-derived products with better safety potential may also be used for the management of *Diabetes mellitus*. Oxidative stress is a condition arising due to excessive production of free radicals inside the body that leads to oxidation of biologically important molecules [3]. Although many small molecule inhibitors have been investigated from natural and synthetic sources [4]. As an alternative for those organic enzymatic inhibitors, some works have applied carbon-based nanomaterials to modulate the enzymatic activity [5].

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Carbon dots (CDs) represent a novel category of small fluorescent nanomaterials, typically smaller than 10 nm. Their core structure consists predominantly of carbon, enveloped or integrated with heteroatoms and functional groups, varying depending on the synthesis method used [6]. These nanomaterials boast advantageous characteristics such as biocompatibility, low toxicity, stable photoluminescence, water solubility, diverse fluorescence colors, large surface area, and impressive optical properties. Their versatility has led to widespread applications including bioimaging, photocatalysis, drug delivery, chemical and biological sensing, and solar cells [7]. Various synthesis techniques have been devised, including electrochemical and chemical oxidation, laser ablation, pyrolysis, hydrothermal carbonization, and microwave irradiation [8]. Utilizing natural biomaterials offers an eco-friendly and economically viable approach to producing bio carbon dots. Materials such as Psidium guajava leaves, Curcuma longa leaves, Osmanthus leaves, milk vetch, and tea leaves have been investigated for their potential in CD synthesis. Leveraging plant resources not only addresses the need for large-scale CD production but also promotes sustainable practices [9]. Plant materials inherently contain carbohydrates, proteins, amino acids, and other biomolecules essential for surface functionality, eliminating the need for additional reactants for doping, passivation, or post-modification. Despite the promising prospects of plant-derived CDs, there remain

significant challenges hindering their full exploration and utilization in various applications [10,11].

The Nitrogen-doped CDs synthesized using 4-hydroxybenzoic acid and ethylene dimamine shown inner filter effect based fluorescent activated detection of α-glucosidase activity [12]. CDs doped with nitrogen and boron shown fluorescence detection of α-glucosidase enzymatic activity [13]. The Biocompatible CDs prepared using Kappa Carregeenan and phenyl boronic acid successfully detected blood glucose using fluorescence measurements, metformin drug loading and release at different pH conditions [14]. The CDs of *Fructus Crataegi* inhibited sucrase and maltase inhibitory activity in Kunming mice intestine and postprandial hypoglycaemic effect [15]. The *in vivo* study of N-doped CDs loaded with Insulin hormone on Wistar rats shown effective hypoglycemic effect [16]. The CDs synthesized using *Vitis vinifera* seeds shown effective *in vitro* inhibitory α-amylase and α-glucosidase activity and evaluation of glucose uptake capacity by yeast cells [17].

Therefore, only few studies have explored the alpha amylase and alpha glucosidase inhibitory activity of CDs derived from natural resources. The present study aims to synthesize the carbon dots from *Acetosa sagittata* plant aerial parts explore their digestive enzymatic inhibitory activities, antioxidant and cytotoxicity properties.

Methodology:

Green synthesis of CDs from Aerial parts of *Acetosa sagittata* by Hydrothermal assisted method: The aerial parts (leaves, stem and flower) of *Acetosa sagittata* were collected from Adikavi Nannaya University campus. The collected aerial parts were shade dried and pulverized using domestical blender. For the synthesis of CDs, 5 grams of each pulverized powder was dispersed in 100 mL of distilled water and stirred at 100^0C for 1 hour to achieve homogeneous dispersion of particles. Then, this solution was transferred using muslin cloth, and Whatman NO.1 filter paper. Thus, the CDs were purified using 0.22 µm PVDF syringe filters. The purified CDs labelled as CD1, CD2,

Characterization of CDs:

The optical properties like absorbance, fluorescence excitation, emission and functional groups of all CDs samples were evaluated using UV-Visible spectroscopy (Shimadzu UV-2600) Fluorescence spectroscopy (Perkin Elmer) and Fourier transform infrared spectroscopy (Bruker Alpha Ⅱ). The phase analysis of CDs was confirmed using X-ray diffractometer (Powder XRD-Bruker). The morphological features and size were evaluated using High resolution Transmission Electron Microscope (JEOL JEM 2100) at 200 KV.

***Invitro* α-amylase and α-glucosidase inhibitory activity:**

In α-amylase inhibitory activity, the various concentrations of CDs (20-100 µg mL^{-1}) were pre-incubated with α-amylase (1U mL^{-1}) for 15 mins in Phosphate buffer (100mM pH 6.8). Then, the substrate starch (1%) was added to these solutions and incubated at 37^0C for mins. To stop the reaction 3,5 DNS reagent was added and incubated for 5 mins in boiling water bath. Then, the solutions were cooled down to room temperature and diluted with distilled water in 1:5 ration and absorbance was read at 540nm. In α-glucosidase inhibitory activity, the CDs of various concentrations (20-100 µg mL^{-1}) were preincubated with enzyme α-glucosidase (1U mL^{-1}) for 15 mins Phosphate buffer(100 mM pH 6.8). Then the substrate 4- Nitrophenyl β-D-glucopyranoside (PNPG) was added (4.5mM) and again incubated for 10 minutes, the reaction was terminated by adding 0.1 M of Sodium carbonated and absorbance was read at 405nm. Acarbose was used as standard for both experiments.

The % of reaction and % of inhibition were calculated using formula (1) and formula (2) reaction (%)

$$= \frac{The\ amount\ of\ maltose\ absorption\ in\ the\ sample}{The\ amount\ of\ maltose\ absorption\ in\ the\ control} \times 100 \quad \textbf{Formula (1)}$$

$$\text{Inhibition (\%)} = 100 - \text{Reaction (\%)} \quad \textbf{Formula (2)}$$

***Invitro* antioxidant activity:**

In DPPH [2,2-diphenyl-1-picrylhydrazyl] assay, the samples of different concentrations were incubated (20-100 µg mL^{-1}) with 3mM DPPH in dark for 1 hour, and absorbance was read at 517nm. In FRAP assay, the samples of different concentrations added to the FRAP reagent [10mM TPTZ, 40mM HCl and 20mM FeCl3 in 1:1:10 v/v/v proportion in acetate buffer of pH 3.6] and incubated in dark for 1 hour. The absorbance was read at 593 nm. In ABTS assay, 7mM of Azino-bis [3-ethyl benzothiazoline-6-sulfonic acid (ABTS) and 2.45mM of potassium persulphate added in methanol and in 1:1 proportion. The overnight incubated ABTS reagent was diluted with methanol and absorbance set to 0.700 at 734nm. To this reagent different concentrations of CDs were added and incubated for 6 minutes and absorbance was read at 734 nm.

Ascorbic acid was used as standard for all three assays. The percentage of inhibition was calculated according to formula (1) & (2).

Cytotoxicity activity:
A subculture of Hela cells in Dulbecco's modified Eagle's medium (DMEM) with 10% Fetal Calf Serum (FCS) was done. Different concentrations of CD's (20-100 µg mL^{-1}) added to each well along with cells suspension in a 24 well culture plate and incubated at 37^0C in a humidified CO_2. After 48 hr incubation, the Hela cells were observed under and inverted tissue culture microscope with 80% confluence of cells.

MTT assay:
After 48 hr of incubation, the wells were added with [3-(4,5-dimethyl thiazolzyl]-2,5-diphenyl tetrazolium bromide (MTT) and incubated for 3hr in room temperature. The MTT cleaved by mitochondrial succinate dehydrogenase and reductase by viable cells which yields a purple product formazan. After treatment, formazan crystals were dissolved by adding 100 µL SDS in DMSO and absorbance was read at 540nm. using micro plate reader [Lark-LIPR]. The percentage of inhibition was calculated according to formula (1) & (2).

Result and Discussion: The carbon dots derived from the aerial parts of *Acetosa sagittata* synthesized using hydrothermal assisted method. *Acetosa sagittata* is a weedy herbaceous perennial plant rich in primary and secondary metabolites [18], which serves as source for synthesis of carbon dots and do not requires surface functionalization with other atoms such as O, H, N, and S. The mechanism of small metabolites to carbon dots through bottom-up approach. However previous literature supported the synthesis of CD's takes place in three major steps: 1. Hydrolysis and condensation, 2. Self-polymerization / Aggregation, 3. Core transformation and surface passivation [19].

Characterization of CDs:
The CD_1, CD_2, CD_3 samples shown absorbance between 220 to 340nm in UV-Vis spectroscopy analysis Fig 1 which corresponds π-π* of SP^2 carbon core and n-π* transitions. All the CD samples exhibited multiple fluorescent excitations between 510 and 630nm as illustrated in fig.2. The insets of fluorescent excitations represent pale blue to bright blue color fluorescence when exposed to UV light (365nm). The fluorescence properties of CD's is due to C=O by trapping energy of surface functional groups. The functional groups of CD's were identified using FTIR. The absorption bands at 1627 cm^{-1} ,1633 cm^{-1} , 2361 cm^{-1}, 3313 cm^{-1} and 3323 cm^{-1} corresponds to vibrational stretching's of C=O, O=C=O and O-H groups respectively illustrated in fig 3. A strong intense approximately at 2θ=30° represents the amorphous nature of CD's Fig 4., and compared with standard JCPDS card No. 00-050-0926. The Zeta potential of CD's (-1.51 and -3.39mV) considered as neutral and stable in aqueous solutions (fig.5 a-c). The HRTEM analysis shows spherical particles without aggregation and size distribution between 1-8 nm and some of the CD's were also found less than <1nm.

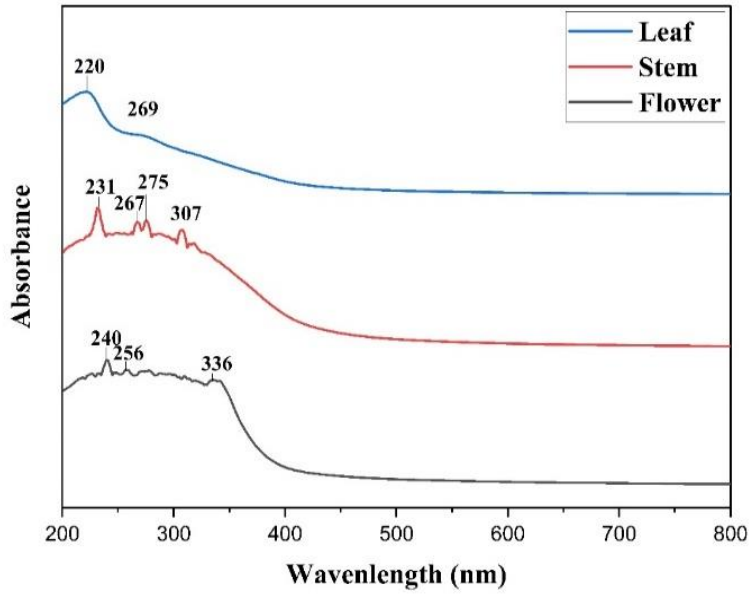

Figure 1: UV-Vis absorbance of CDs.

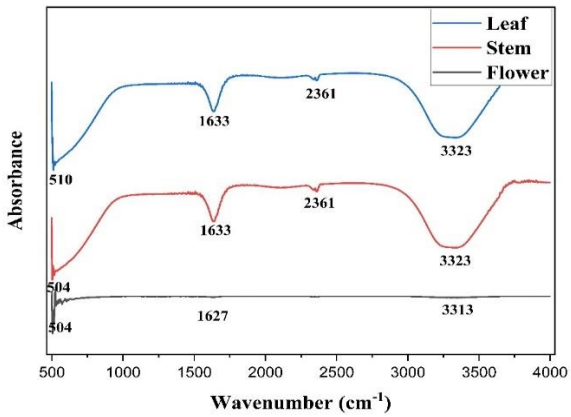

Figure 2: FTIR analysis of CDs

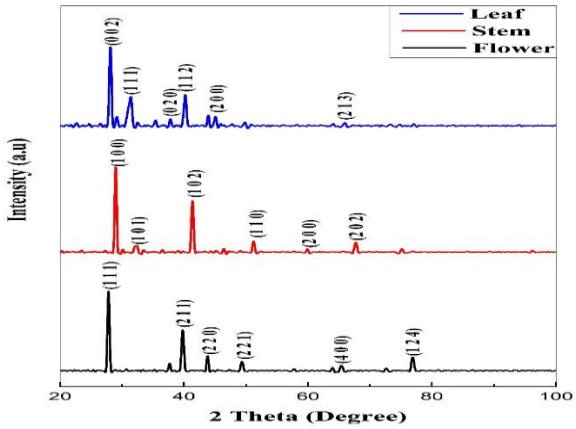

Figure 3:XRD analysis of CDs

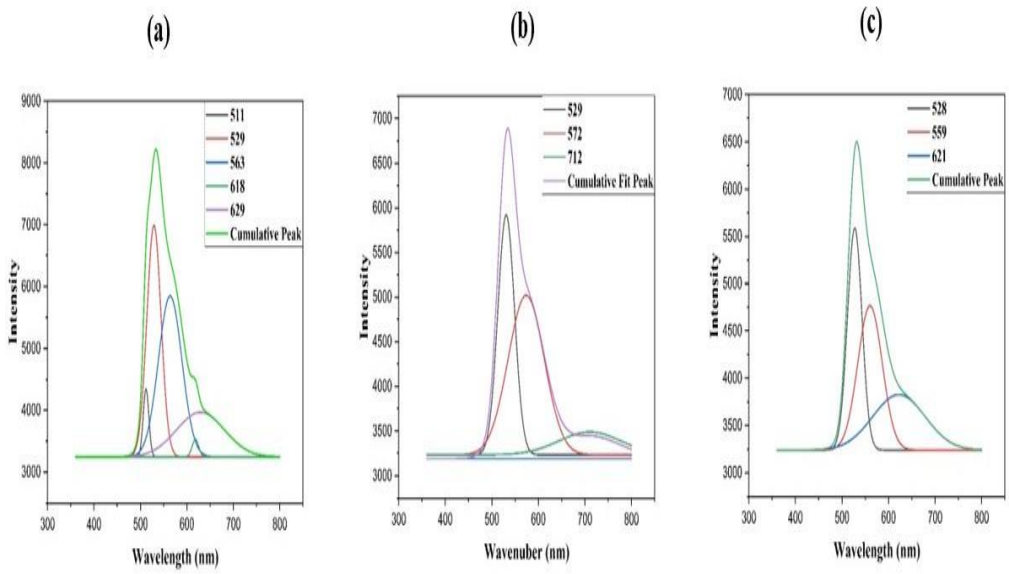

Figure 4: Fluorescence spectroscopy analysis of CDs

***In vitro* antidiabetic activity:** Inhibition of α-amylase and α-glucosidase enzymes are prime important in Type II DM, which delays digestion of carbohydrates and subsequently hyperglycemic condition. Several synthetic drugs are used to reduce the hyperglycemic condition, but associated with side effects. The present study aims to investigate the Enzyme Inhibitory properties of CD's synthesized from renewable resources (plant) which are low toxic and biocompatible [3]. All CD samples were shown similar inhibitory activity on both alpha amylase and alpha glucosidase, For alpha amylase the inhibition ranges between 19-68 %, whereas for alpha glucosidase 7-59% at 20-100 µg mL^{-1} concentrations. The % of inhibitions and the IC_{50} values of each sample were represented in Fig 5& 6 and table 1 respectively. The CD's contains hydroxyl, carboxyl and amino groups as surface functional groups, these functional groups are able to interact with enzyme active site through covalent bonding, hydrogen bonding and electrostatic interactions and inhibits enzymatic activity. Size and surface charges of CD's also influence the activity of enzyme. Enzymes require metal ions as cofactors the CD's are capable in chelating metal ions and subsequently affect the enzyme activity [2].

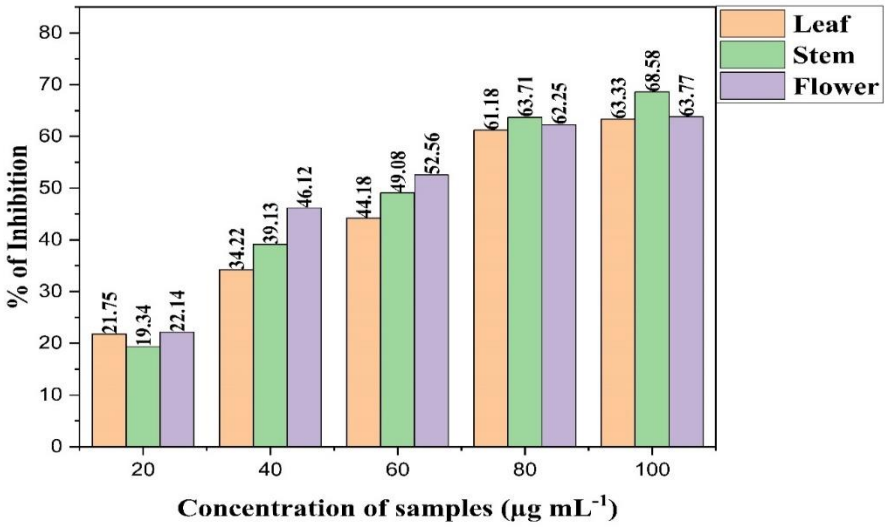

Figure 5: α-amylase inhibitory activity of CDs

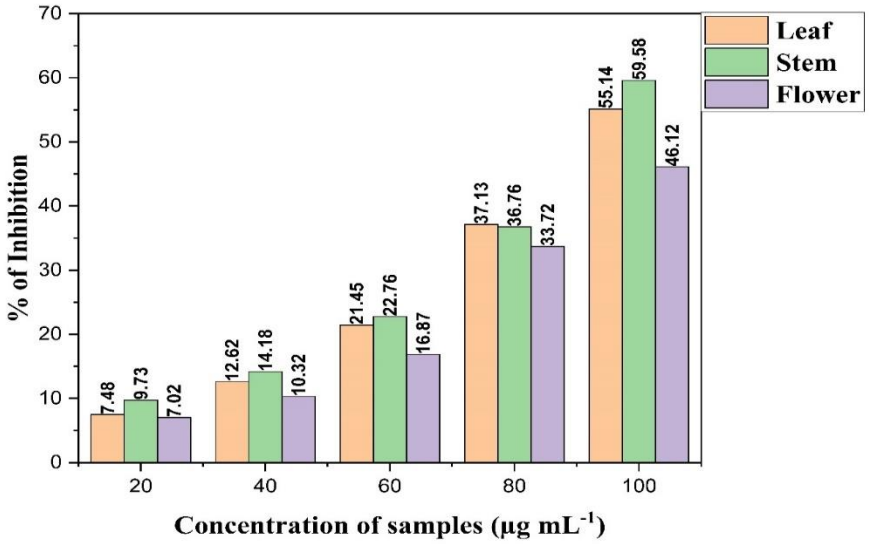

Figure 5: α-glucosidase inhibitory activity of CDs

***In vitro* antioxidant activity:** Carbon dots are well known pro-oxidants as well as antioxidants. CD's are highly effective in neutralizing free radicals [20]. In this study, three invitro assays were used to determine CD's free radical Scavenging activity. DPPH is a nitrogen centered free radical which usually shows absorbance at 517nm. The FRAP assay determines the capacity of CDs to reduce Fe^{3+} TPTZ to Fe^{2+} TPTZ and shows absorbance at 593nm and ABTS assay determines neutralizing the nitrogen cation which exhibit absorbance at 734nm. All CD samples in this study shown excellent antioxidant property with DPPH and FRAP assays than ABTS assay. The DPPH, FRAP and ABTS shown % of inhibitions ranges between 81-87 %, 80-93% and 24-63% respectively. The % of inhibitions and the IC_{50} values of each sample were represented in Fig 7,8 & 9 and table 1 respectively.

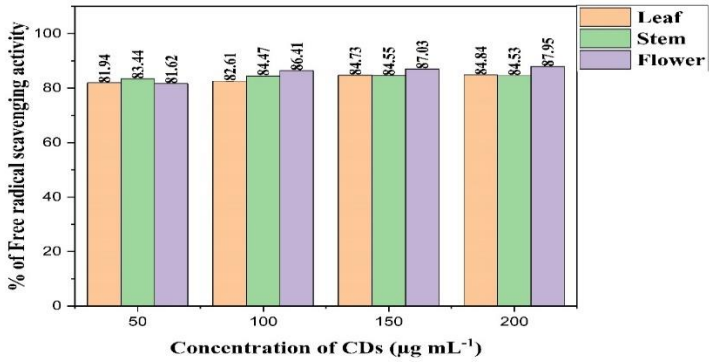

Figure 7: DPPH free radical scavenging activity of CDs

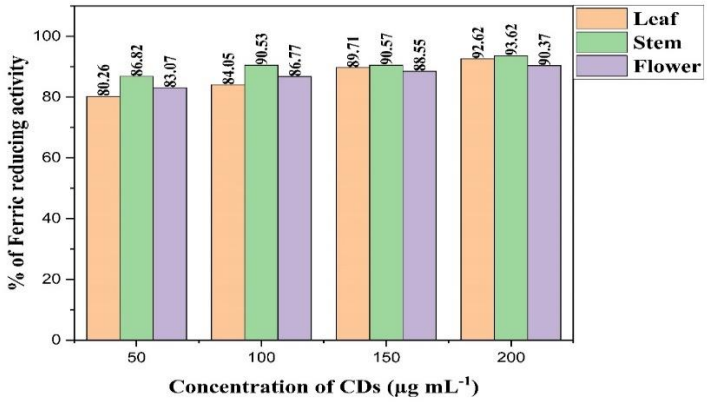

Figure 8: Ferric reducing activity of CDs

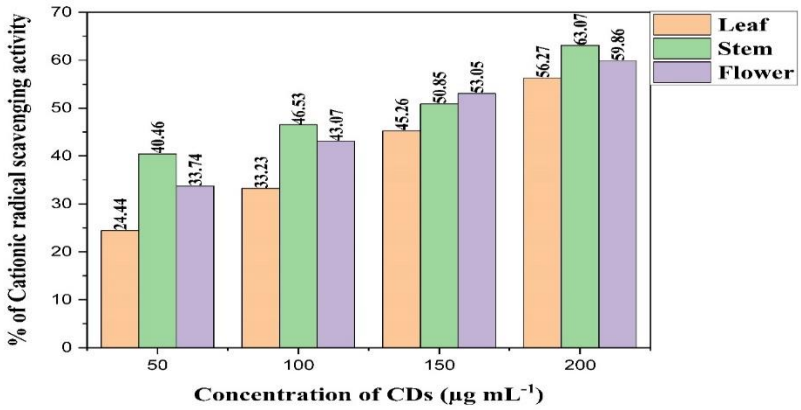

Figure 9: Cationic free radical scavenging activity of CDs

***In vitro* Cytotoxicity properties:** The cytotoxicity properties of CDs were examined on HeLa cell lines. The cytotoxic properties of CDs on cancer cell lines from their ability to induce oxidative stress, apoptosis, disrupt cellular functions [21]. The percentage of cell viability at each concentration and IC_{50} values of each sample represented in Fig 10 and table 1 respectively.

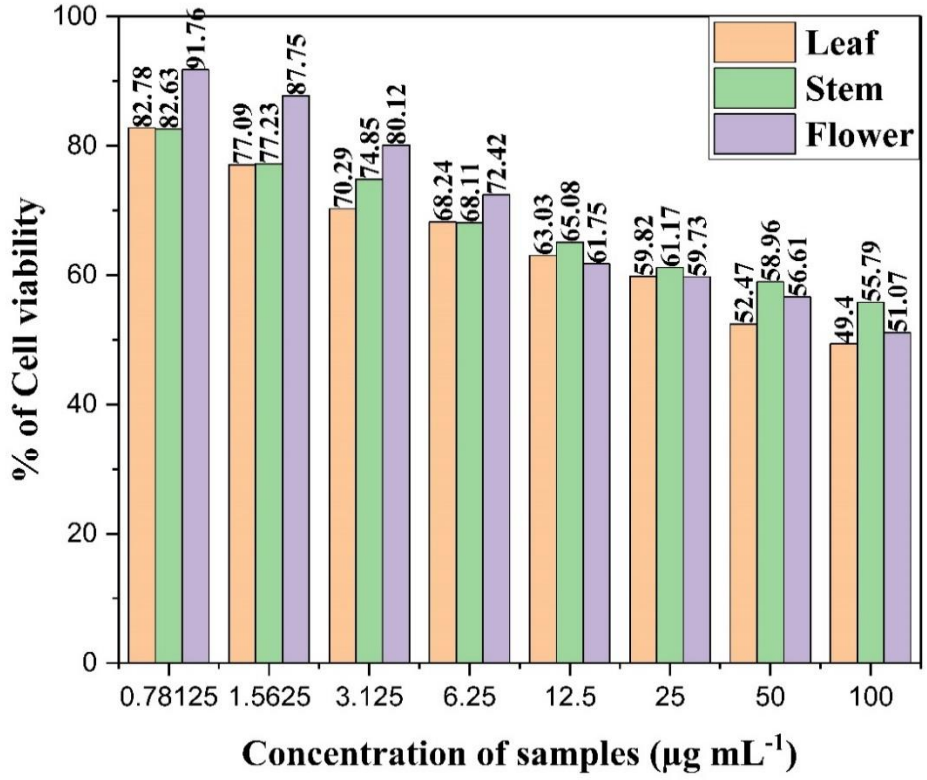

Figure 10: Cytotoxic activity of CDs

Table 1: IC_{50} values of CDs on *in vitro* antidiabetic, antioxidant and cytotoxicity activities

Activity	CD_1 (IC_{50} µg mL^{-1})	CD_2 (IC_{50} µg mL^{-1})	CD_3 (IC_{50} µg mL^{-1})
DPPH free radical scavenging activity	111.91	74.23	175.5
FRAP activity	126.35	155.84	118.14
ABTS cationic radical scavenging activity	189.69	526.39	139.21
α-amylase inhibitory activity	61.25	92.16	109.0
α-glucosidase inhibitory activity	144.08	230.80	79.76
HeLa cell line cytotoxicity	84.44	229.53	498.88

Conclusion:

The study on carbon dots derived from *Acetosa sagittata* aerial parts revealed promising characteristics such as stable photoluminescence, water solubility and diverse fluorescence emissions. The synthesis method utilized a bottom-up approach involving hydrothermal assistance, showcasing the transformation of small metabolites into carbon dots. The CDs exhibited inhibitory activity against α-amylase and α-glucosidase, along with significant antioxidant properties in DPPH, FRAP and ABTS assays. Moreover, the cytotoxicity evaluation on HeLa cell lines demonstrated potential for inducting oxidative stress and apoptosis. These findings underscore the potential of plant-derived carbon dots for various applications in bioimaging, drug delivery and sensing highlighting their biocompatibility and low toxicity for future research and development.

Authors declaration:

The authors declare no conflict of interest to declare.

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