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Evaluation of cell viability of gold nanoparticle-reduced graphene oxide composite on MCF-7 Cell lines

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Plant mediated synthesis of nanoparticles is a better substitute for chemically synthesized nanoparticles. These nanoparticles facilitate more advantages in cancer treatment. In this study, biosynthesis of gold nanoparticles has been carried out using aqueous extract of *Eichhornia crassipes* (Mart.) Solms by the hot air-oven method. The effect of variation of plant extract concentration was carried out to optimize the formation of gold nanoparticles by this method. Reduced graphene oxide was prepared by treating graphene oxide with aqueous extract of *E. crassipes* as a capping agent. Nano composite was prepared using an equal ratio of reduced graphene oxide and nanogold by sonication method. Prepared metal and non-metallic nanoparticles were characterized by analytical spectroscopic tools *viz*. UV-Visible spectrometer, FTIR spectrophotometer, and Field Emission Scanning Electron Microscope. Cell viability of aforesaid nanoparticles was examined by MTT assay against MCF-7 cell lines. Among all the nanoparticles, reduced graphene oxide exhibited 23% cell viability at 250 µg /mL and IC_{50} at 140 µg /mL against MCF-cell lines. Doxorubicin was used as a standard. The result of this study reveals the cytotoxicity of the prepared nanoparticles and nanocomposite against MCF-cell lines.

Keywords: Cell viability, *Eichhornia crassipes*, MCF-7 cell line, Nanoparticles

Research in drug delivery is one of the promising applications in the field of pharmaceutics to achieve a disease- free human life. A new drug should be developed in such a way that it should not be dangerous to human welfare during its action on targeted organisms. Among the non-communicable diseases threatening public health, cancer is the second leading cause of death all over the world (WHO, 2018)[1]. The cancer rate is expected to grow from around 12 million in 2007 to 18 million by 2020 (Centers for Disease Control and Prevention, 2016)[2]. Specific cancer- targeting property adds to the advantages of nanoparticles in cancer research[3]. Uncontrolled cell growth in cancer can be combated through the use of nanoparticles which in micrograms, offers effective cell inhibition. Hence this research paper focuses on anticancer activity using gold nanoparticles and its composites.

E. crassipes is a tropical species under the Pontederiaceae family. A Literature review has revealed antimicrobial activity[4], anti-inflammatory activity[5], and many pharmaceutical properties. Methanol extract shows mild anticancer activity against HeLa cell lines at 32.33 µg /mL[6]. Crude extract shows high anticancer activity against hormone- dependent tumor types like cervix and breast cancer[7]. The selection of methods for drug production should minimize the environmental impact. It can be attained by the environmental friendly approaches[8]. The use of nanotechnology research in drug delivery was tremendously increased with the development of new nanoparticles. Bio-mediated syntheses are steered to provide metal nanoparticles. The colour of the gold nanoparticles is correlated to the size and shape of the nanoparticles. This in turn also depends upon the method of synthesis. Purple coloured gold nanoparticles have been prepared by various methods such as microwave method, high- temperature method, ultrasonic homogenizer method and sonication[9]. In the present study the hot air oven method was used to prepare gold nanoparticles using water hyacinth extracts.

Graphene and graphene oxide as the foundation of nano composites, have garnered a lot of interest among researchers of several fields[10]. Graphene- based nanoparticles are used in cancer treatment[11]. Gold nanoparticle decorated reduced graphene, synthesized using antimicrobial nisin peptides, show ~80% cell inhibition against MCF-7 cell lines at 10 µg /mL[12].

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Toxicity of graphene oxide on human fibroblast cells and mice depends upon the time and dosage. At low concentration (0.1- 0.25 mg) graphene oxide is not toxic to the human fibroblast cells but it exhibits chronic toxicity at higher concentrations (> 0.4 mg)[13,14].

The aforesaid significance of gold nanoparticles, reduced graphene, and nanocomposite has been the impetus behind this present work for developing new anticancer agents against MCF-7 cell lines.

Materials and Methods

Materials

All the chemicals employed in the study were of AR (Analytical Reagent) grade. Gold chloride was purchased from HIMEDIA. Doubly distilled water was used throughout the study. The chemicals and samples were weighed by using Electronic balance (Shimadzu AUW220D, model number D445910303). Magnetic stirrer (REMI 1MLH) used for stirring. Hot air Oven (Sigma Scientific instruments, 181818 size, 1750W, SL.NO:21470) was used to synthesize gold nanoparticles.

Preparation of reagents for the study

Hydrogen peroxide (30%) was prepared and stored in the brown bottle for further use. This solution was used for the preparation of graphene oxide. Hydrochloric acid (10%) solution and 3 mM gold chloride solutions were prepared and refrigerated for further use.

Preparation of plant extract

Eichhornia crassipes (Mart.) Solms was collected from a local water body in Coimbatore. The plant sample was identified by the Botanical Survey of India, Southern Regional Center, Coimbatore -02. The identification number is BSI/SRC/5/23/2011-12/Tech[5]. *E. crassipes* (1.5 kg) was extracted with doubly distilled water (1000 mL) for 6 h at 100°C. Then the hot solution was filtered using Whatmann no.42 filter paper and the filtrate is allowed to dry.

Phytochemical screening of the extract

Preliminary phytochemical tests of the extracts of *E. crassipes* were carried out as per standard procedure reported by the previous workers[15].

Synthesis of gold nanoparticles (GS) by oven heating method

Aqueous extract of *E. crassipes* (1 g) was sonicated for 30 min with doubly distilled water (100 mL) at 30°C. Then the extract was filtered using Whatmann filter paper. The filtrate was used in the preparation of GS. The gold nanoparticles were synthesized by treating different ratio (1:1, 2:1, 3:1, 4:1, 5:1) of *E. crassipes* aqueous extract and gold chloride, respectively. The formation of GS was confirmed by the colour change from yellow to wine red colour and the corresponding UV absorbance was recorded.

Preparation of Graphene oxide (Modified Hummer's method)

Graphite powders (10g) were treated with conc.H_2SO_4 (120 mL).The reaction was stirred in an ice bath constantly for 1 h. $KMnO_4$ (20g) was added gradually over a period of 1 min at temperature 20°C for 3 h. Doubly distilled water was added to the mixture and the temperature maintained below 100°C. Finally, 30% H_2O_2 was added until the mixture turned yellow colour. The obtained mixture was filtered using 10 % HCl to remove the metal ions and repeated washing with double distilled water to obtain a powder of pure graphene oxide.

Preparation of plant extract

E. crassipes aqueous extract (100 mg) was sonicated with doubly distilled water (100 mL) at 30°C for half an hour. Then it was filtered using Whatmann –42 filter paper and kept for further study.

Reduction of Graphene oxide (RS) using plant extracts

The prepared Graphene oxide (200 mg) was sonicated with doubly distilled water (400 mL) for 5 min. The GO solution treated with *E. crassipes* aqueous extract (30 mL) was then taken in a 500 mL RB flask and refluxed at 90°C- 100°C for 6 h. The brown colour solution changed to a black colour indicating the complete reduction reaction. Further, it was sonicated for 1 h and filtered using Whatmann -42 filter paper. The rGO obtained was then dried and stored in screw cap glass bottles[16].

Preparation of nanocomposite (CS)

GS and RS were taken in 1:1 ratio and sonicated for 1 h. The composite was centrifuged at 100 rpm for 15 min. This filtrate was taken for further analysis.

Characterization of GS, RS, and CS

The synthesized GS, RS and CS were separated by centrifuging the solution using a centrifuge machine (REMI RM12C) at 100 rpm for 10 min. Synthesized nanoparticles were characterized by using UV Spectrometer (Biospec-nano (230 V). FTIR spectral measurements were recorded in Perkin Elmer FTIR-00585 spectrophotometer. The surface morphology of GS, RS, and CS were analyzed in TESCAN MIRA3

FESEM with EDX using SUTW- SAPPHIRE model detector. The nanoparticles were coated onto 8mm glass plates and analyzed in FESEM. The sputtering of the sample was done using SC7620 Sputter Coater under the nitrogen atmosphere. The samples coated on glass plates were placed on a small strip of carbon tape. In order to make the glass plate conductive, a small piece of carbon tape was placed between a glass plate and aluminium stub. The stubs were placed in the Sputter coater and sputtered at 10 mV for 60 seconds before FESEM analysis. Distribution of the particles and elemental analysis was carried out using EDAX.

MTT assay for determination of cell viability of the synthesized GS, RS, and CS

The synthesized GS, RS and CS were tested for cell viability against MCF -7 cell line by MTT assay.

The human breast cancer cells (MCF-7) cells from the National Center for Cell Science -Pune, India was used in the study. The MCF- cells were kept in DME medium with L- glutamine (2 mM) and balanced salt solution (BSS). The medium was adjusted to contain Na_2CO_3 (1.5 g/L), nonessential amino acids (0.1 mM), sodium pyruvate (1 mM), l-glutamine (2 mM), glucose (1.5 g/L), 10 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid and 10% fetal bovine serum (GIBCO, USA). Penicillin and streptomycin (100 IU/100 µg) were adjusted to 1 mL /L. The cells were maintained at 37°C with 5% CO_2 in a humidified CO_2 incubator.

Evaluation of cell viability

The inhibitory concentration (IC_{50}) value was evaluated using an MTT [3-(4, 5- dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide] assay. Both cells were grown (1×10^4 cells/ well) in a 96-well plate for 48 h in to 80% confluence. The medium was freshly replaced with diluted synthesized compounds and the cells were incubated for 48 h. The culture medium was removed; 100 µL MTT solution was added to each well and incubated at 37°C for 4h. After removing the supernatant, 50 µL DMSO was added to each of the wells and incubated for 10 min to solubilize the formazan crystals. The optical density was measured at 620 nm in an ELISA multiwell plate reader (Thermo Multiskan EX, USA). The Optical density value was used to calculate the percentage of viability using the following formula.

$$\%\,\text{cell viability} = \frac{\text{OD value of sample}}{\text{OD value of control}} \times 100$$

A graph was plotted between percentage cell viability and sample concentration in µg/mL. The SPSS statistical software (17.0 version) and Microsoft Excel (2007) were used for the analysis.

Results and Discussion

The consolidated results of cell viability of GS, RS and CS are discussed below.

Biogenic synthesis of gold nanoparticles

Phytochemical screening of aqueous extract of *E. crassipes* revealed secondary metabolites such as alkaloids, flavonoids, sterols, and carbohydrates to be present in the extract. Terpenoids, sugars, proteins, phenolic acids and alkaloids, play an important role in the bio-reduction of metal ions, yielding nanoparticles[17]. A main terpenoid of Eugenol extracted from *Cinnamomum zeylanisum* plays an important role in the reduction of $HAuCl_4$[18]. Protein molecules showed high reducing activity and potential for attracting metal ions to the molecule that are responsible for reduction resulting in the formation of nanoparticles[19]. Proteins present in the extract of mulberry leaves are involved in the formation of gold nanoparticles[20]. Aspartic acid is involved in the reduction of chloroaurate ion to form gold nanoparticles[21].

In the present study, an alternate method has been employed to produce gold nanoparticles using aqueous extract of *E. crassipes* as a capping agent. The aqueous extract and gold chloride (1:1, 2:1, 3:1, 4:1, 5:1) were kept in a hot air oven and monitored periodically for the formation of gold nanoparticles (Table 1). Visible colour change from yellow to wine red indicates the formation of gold nanoparticles.

The time required for the formation of AuNPs increases with increase in extract concentration. The reaction between functional groups in the plant metabolites and gold chloride are responsible for

Table 1 — Effect of varying concentrations of *E. crassipes* on the formation of gold nanoparticles

Aqueous extract: $AuCl_3$	Time (min)	Color of the nanoparticles
1:1	5	Wine red
2:1	10	Wine red
3:1	15	Wine red
4:1	26	Wine red
5:1	35	Wine red
5:1	40	Blue

increased time of formation of gold nanoparticles with increase in time[22]. The chemical composition of the extract varies with the type of the parts used and extraction procedure. Time factor also influenced the delay of nano formation due to the aggregation[23]. The bio-active reducing agents present in the plant extract form zero valent gold and it also causes agglomeration of gold ions[24]. The agglomeration of the particles leads to bathochromic shift in Surface Plasmon Resonance (SPR) band due to the inter particle plasmon coupling, resulting in the increase in size of the nanoparticles with increase in time and evidenced by the colour change from wine red to blue[25]. Considering the rapid time of formation, a 1:1 ratio is suitable for synthesizing gold nanoparticles. The gold nanoparticles were formed within 5 min at 45°C. This method is obviously simple as it provides uniform heating by forced hot air circulation[26].

Characterization of synthesized GS, RS and CS

The UV absorbance for the GS gives absorption bands in the region 510-535 nm range (Fig. 1A). RS gave UV absorbance band at 262 nm (Fig. 1B). The UV–Visible spectrum of graphene oxide shows an absorption peak at 234 nm. The absorption peak at 274 nm for graphene confirms the reduction reaction[16]. A redshift UV for reduced graphene oxide which is due to the electronic configuration in graphene in the reduction of graphene oxide. This absorption peak is attributed to n– π* transition of C–O bonds embedded by exfoliation and intercalation on the graphene[27]. UV-Visible spectrum of CS showed the absorption peak of GS and RS (Fig. 1C).

FTIR is a rapid, non- destructive, time- saving method that can detect a range of functional groups and is sensitive to changes in molecular structure. The FTIR of RS shows the absence of carbonyl peak indicating the formation of reduced graphene oxide (Fig. 2A). The FTIR spectra of GS (Fig. 2B) shows absorption peaks at 3352.28, 1639.49, and 601.79 cm^{-1} due to the presence of –OH, -C=C and C=O, respectively. All the above peaks indicate the functional groups of the metabolites in the plant extract embedded gold nanoparticles.

The surface morphology of GS, RS, and CS were analyzed using Field Emission Scanning Electron Microscopy (FESEM) with EDAX. It shows highly-wrinkled layers of RS with 510 nm (Fig. 3A & B). A spherical shaped gold nanoparticle with 50-200 nm in size was obtained using *E. crassipes*[9]. In the present study formation of gold nanoparticles using water hyacinth aqueous extract by hot air oven was confirmed by FESEM and EDAX analysis. The average size of the synthesized gold nanoparticles was found to be less than 50 nm (Fig. 4A & B). The distributions of the GS were studied using elemental mapping (Fig. 4C). The

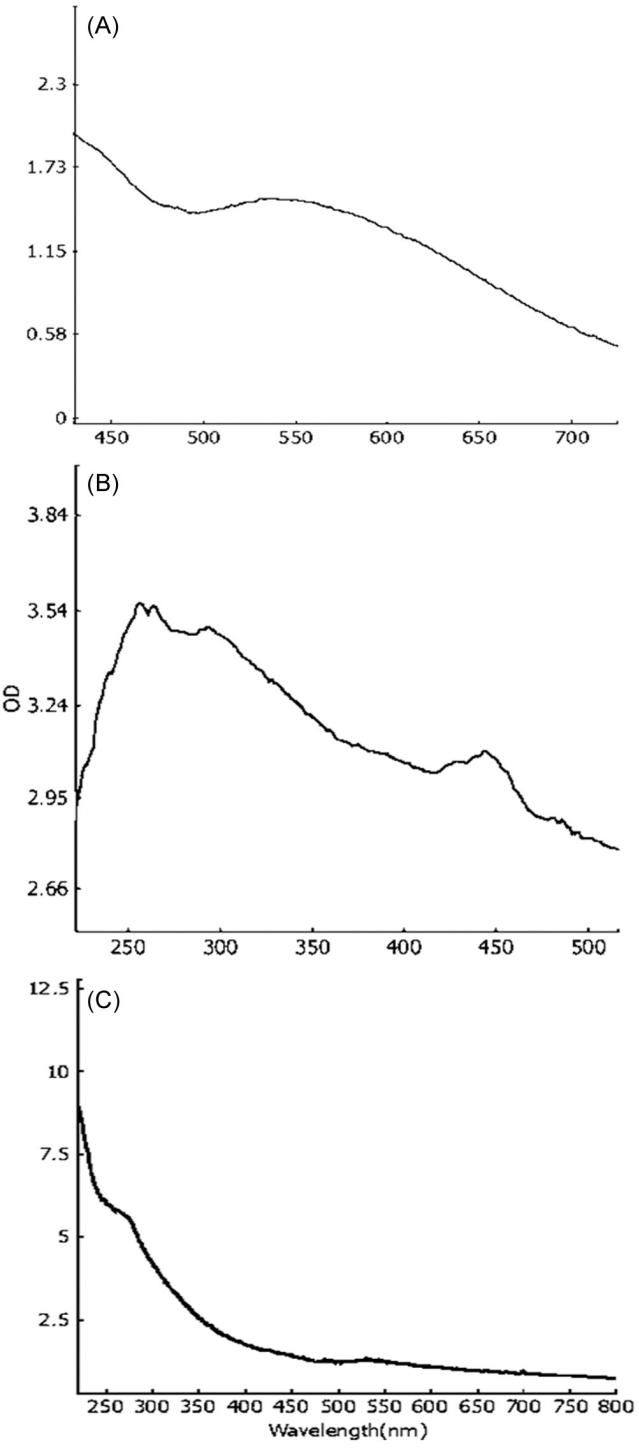


Fig. 1 — UV-Visible spectrum of (A) GS; (B) RS; and (C) CS

surface morphology of the CS revealed gold nanoparticles to be strongly bound to the surface of the graphene layer (Fig. 5A). EDAX results show the reduced graphene oxide and gold nanoparticles uniformly distributed in CS (Fig. 5B & C).

Results of Cell viability of the synthesized GS, RS and CS using MTT assay

The results of the cell growth inhibition of MCF-7 breast cancer cell lines through *in vitro* MTT assay using samples GS, RS, and CS at concentrations 50, 100, 150, 200, 250 µg/mL are given below (Table 2). The experiments were done in triplicate (mean ± SD). Doxorubicin was used as a standard. IC_{50} values were calculated at 48 h. Statistical significance noted using ANOVA and *P*- value <0.01 was considered significant.

Initially, the percentage of cell viability of doxorubicin was calculated. At lower concentration, the doxorubicin (standard) shows less toxicity against MCF-7 cell lines. An Increase in the concentration of doxorubicin from 50-250 µg/mL, decreases the viability of MCF-7 cell lines from 96-17% (Fig. 6D). Gold nanoparticles from *Gymnema sylvestre* showed 38 % of inhibition at the maximum concentration of 250 $\mu g/mL^{-1}$ against Hep2 cells[28]. Flower-shaped gold nanoparticles using the aqueous extracts of *Kedrostis foetidissima* showed 88 % cell viability against MG-63 cell lines at 200 µg/mL[22]. Spherical shaped silver and gold nanoparticles synthesized using *Acalypha indica* Linn show 40% inhibition against MDA-MB-231 cells at 100 µg/mL[29]. Nanoparticles from *Euphorbia hirta* L. (EH-ET) (aerial part) showed IC_{50} value 335 µg/mL against MCF-7 cell lines[30]. The present study revealed that as the concentration of the gold nanoparticles increases,

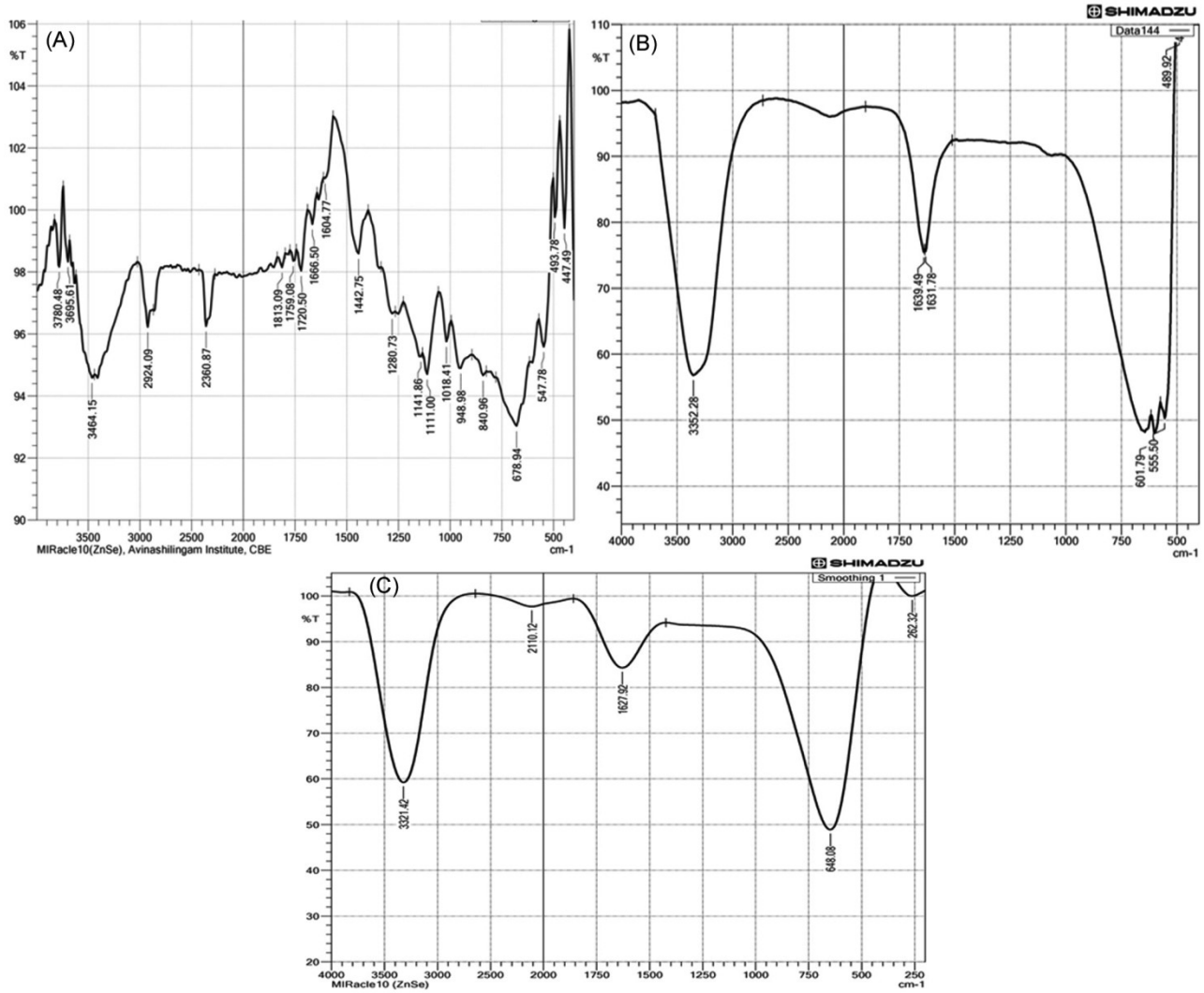
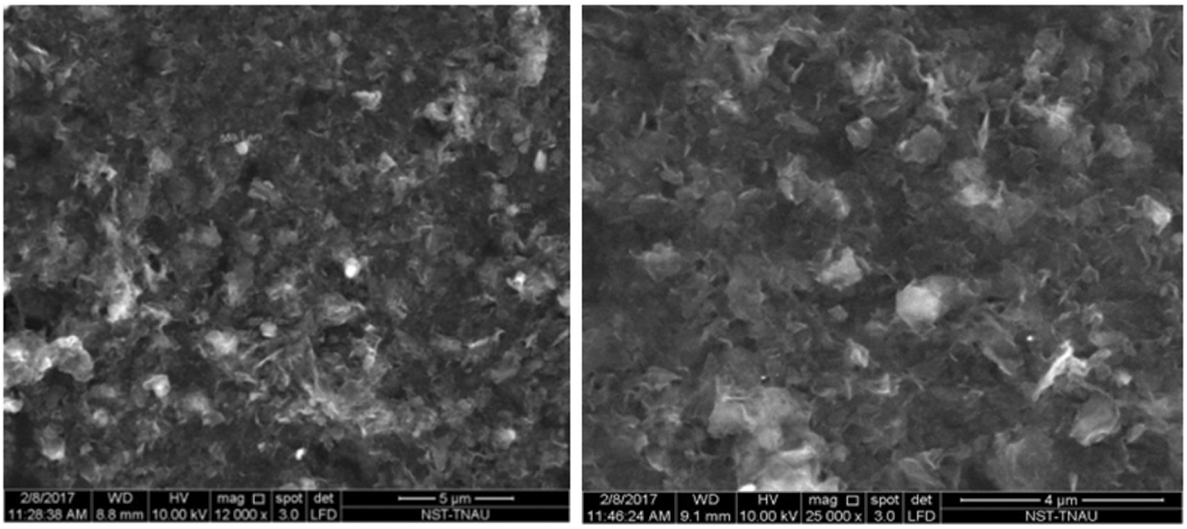
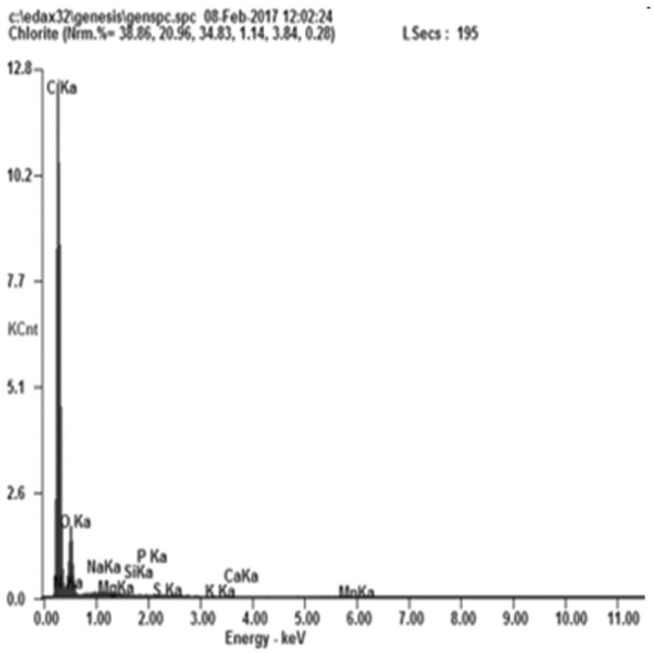


Fig. 2 — FTIR spectra of (A) GS; (B) RS; and (C) CS

(A)



(B)

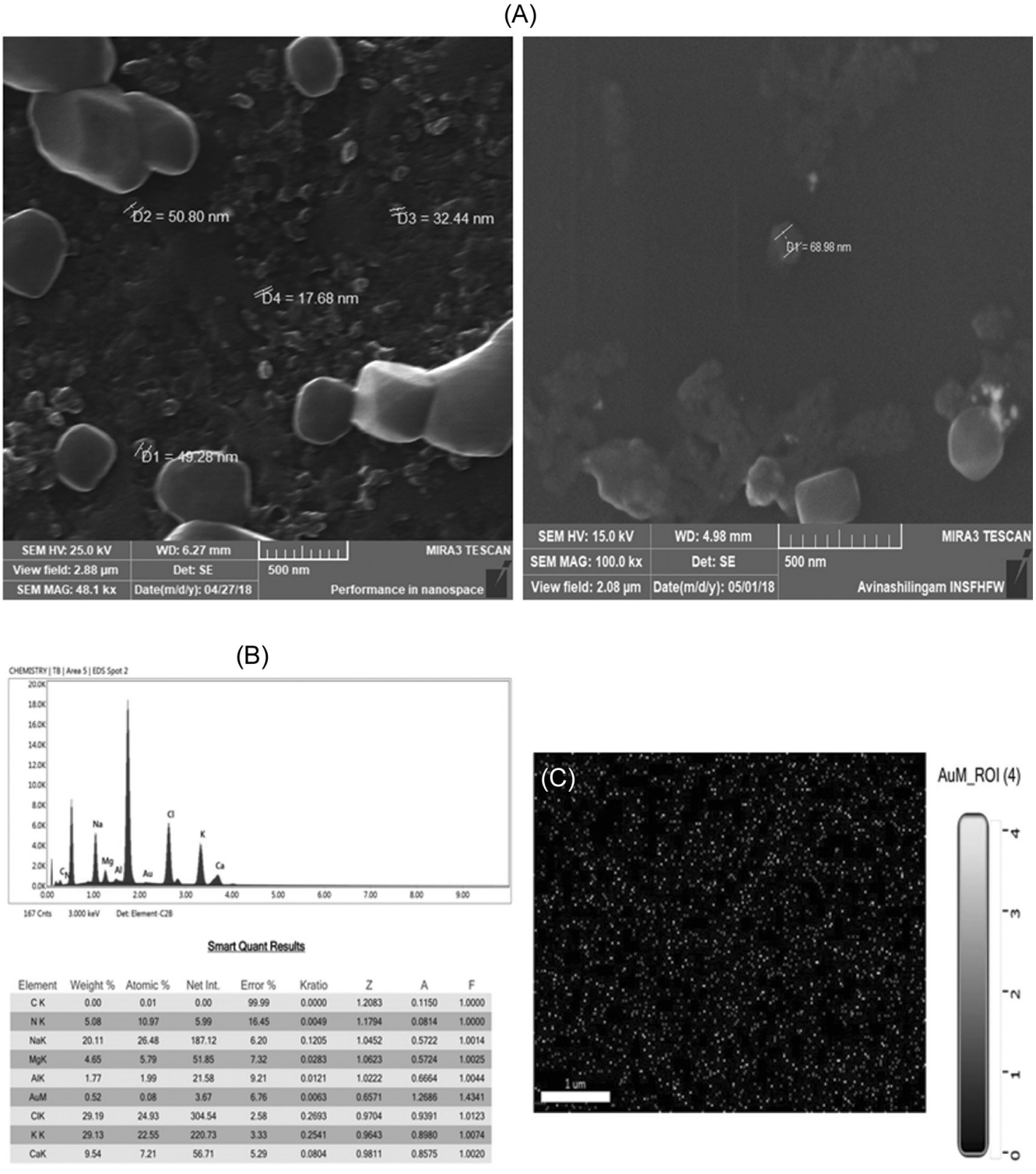


Element	Wt %	At %
CK	59.06	63.99
NK	24.39	22.66
OK	16.23	13.2
NaK	0.07	0.04
MgK	0.08	0.04
SiK	0.02	0.01
PK	0.04	0.02
SK	0.05	0.02
KK	0	0
CaK	0.05	0.02
	0	0

Fig. 3 — Surface morphology of reduced graphene oxide (A) FESEM image of RS; and (B) EDAX spectrum of RS

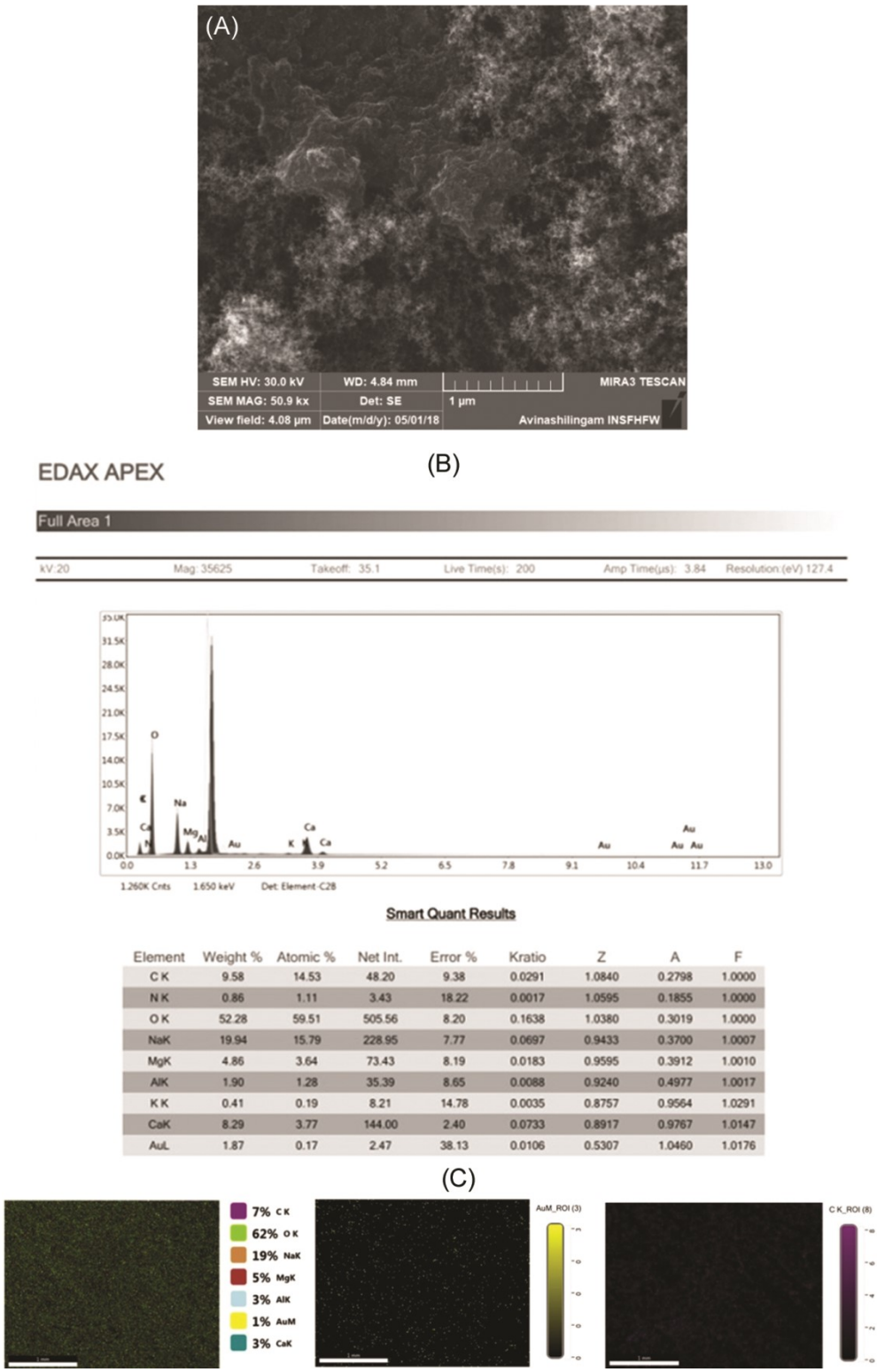
cell growth decreases (Fig. 6A). At 50 µg/mL GS showed only 4% cell inhibition efficiency. As the concentration of GS was increased from 50 to 250 µg/mL, cell inhibition also increased from 4 to 65% and it's showed significance $P_{0.033} < 0.05$. RS had less cytotoxicity activity at 50 µg /mL. An Increase in the concentration of the RS decreases the percentage of cell viability (77% cell inhibition at 250 µg/mL) (Fig. 6B). The significance result of RS is $P_{0.037} < 0.05$. The cytotoxicity of reduced graphene oxide higher than GO due to the generation of oxygen molecules with more reactive tendency and the sharp edges in their graphene sheets contain very high aspect ratio. The excellent and ideal behaviour of rGO have capable to directly interact with microorganisms[31]. The cell viability of MCF -7 cell lines decreases with the increase in the concentration of nanocomposite (CS) (Fig. 6C). CS showed less cytotoxicity at 50 µg/mL and significance $P_{0.040} < 0.05$. At minimum concentration, the intake of nanoparticles

by cancer cell lines is very less so that the (Table 2). cytotoxicity activity is more at higher concentration



Element	Weight %	Atomic %	Net Int.	Error %	Kratio	Z	A	F
C K	0.00	0.01	0.00	99.99	0.0000	1.2083	0.1150	1.0000
N K	5.08	10.97	5.99	16.45	0.0049	1.1794	0.0814	1.0000
NaK	20.11	26.48	187.12	6.20	0.1205	1.0452	0.5722	1.0014
MgK	4.65	5.79	51.85	7.32	0.0283	1.0623	0.5724	1.0025
AlK	1.77	1.99	21.58	9.21	0.0121	1.0222	0.6664	1.0044
AuM	0.52	0.08	3.67	6.76	0.0063	0.6571	1.2686	1.4341
ClK	29.19	24.93	304.54	2.58	0.2693	0.9704	0.9391	1.0123
K K	29.13	22.55	220.73	3.33	0.2541	0.9643	0.8980	1.0074
CaK	9.54	7.21	56.71	5.29	0.0804	0.9811	0.8575	1.0020

Fig. 4 — FESEM image of gold nanoparticles using *E. crassipes* by hot air oven method (A) Surface of the GS; (B) EDAX spectrum of GS; and (C) Uniform distribution of GS



Element	Weight %	Atomic %	Net Int.	Error %	Kratio	Z	A	F
C K	9.58	14.53	48.20	9.38	0.0291	1.0840	0.2798	1.0000
N K	0.86	1.11	3.43	18.22	0.0017	1.0595	0.1855	1.0000
O K	52.28	59.51	505.56	8.20	0.1638	1.0380	0.3019	1.0000
NaK	19.94	15.79	228.95	7.77	0.0697	0.9433	0.3700	1.0007
MgK	4.86	3.64	73.43	8.19	0.0183	0.9595	0.3912	1.0010
AlK	1.90	1.28	35.39	8.65	0.0088	0.9240	0.4977	1.0017
K K	0.41	0.19	8.21	14.78	0.0035	0.8757	0.9564	1.0291
CaK	8.29	3.77	144.00	2.40	0.0733	0.8917	0.9767	1.0147
AuL	1.87	0.17	2.47	38.13	0.0106	0.5307	1.0460	1.0176

Fig. 5 — FESEM and EDAX spectrum of nano composite (A) Surface morphology of CS; (B) Elemental analysis of CS; and (C) Uniform distribution of carbon and gold nanoparticles

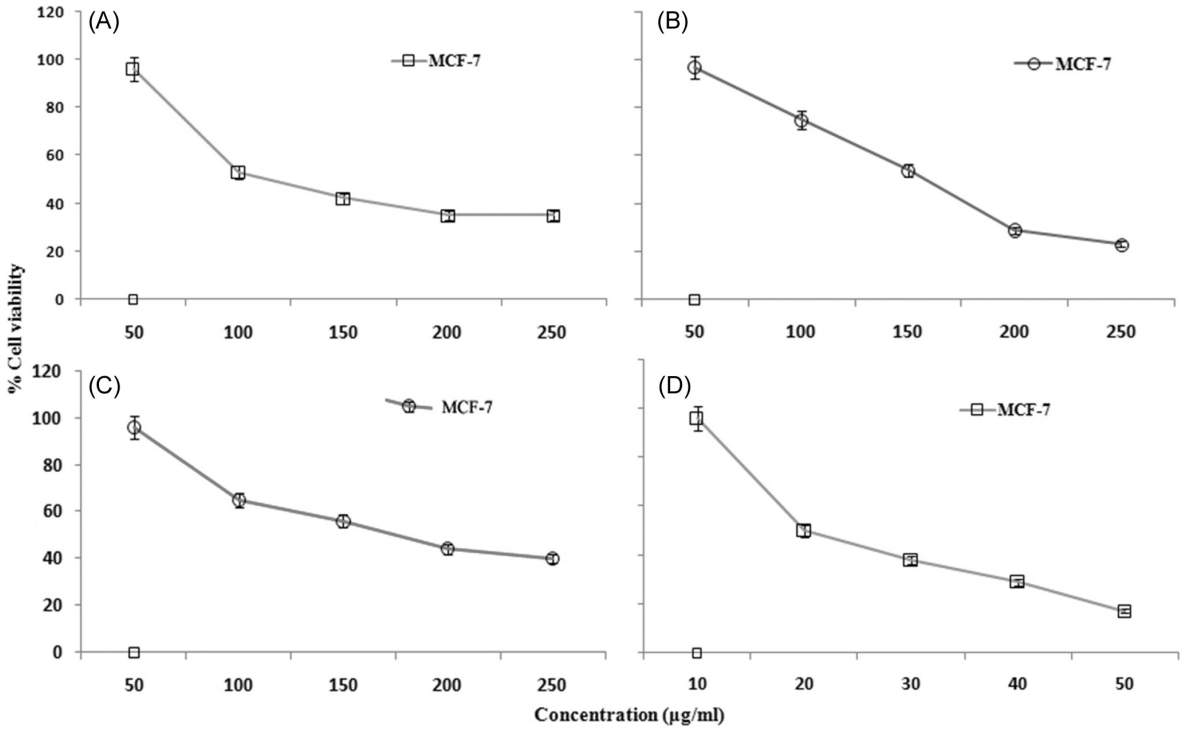


Fig. 6 — Percentage Cell viability of synthesized nanoparticles (A) GS; (B) RS; (C) CS; and (D) Doxorubicin against MCF-7 cell lines

Table 2 — Percentage of Cell viability of GS, RS, and CS against MCF-7 cell lines

Concentration of (µg/mL)	% Cell viability			
	GS	RS	CS	DOX
50	96	97	96	96
100	53	75	65	50
150	42	54	56	38
200	35	29	44	29
250	35	23	40	17

The IC_{50} of GS, RS, and CS against MCF-7 cell line were calculated. IC_{50} value found to be 16 ± 1.5 µg/mL concentration for Doxorubicin. GS and CS showed IC_{50} value at 120 ± 1.0 µg/mL due to the binding property of gold nanoparticles which is strongly bound to the surface of carbon sheets of RS. Water hyacinth rGO (RS) shows IC_{50} value at a very high concentration of 140 ± 0 µg/mL when compared to gold nanoparticles and composite. These results portray that the samples which are used in the study compared with the standard are appreciable. The present research work substantiates the use of nanocomposite and also water hyacinth in anti-cancer studies.

Conclusion

The gold nanoparticles were successively synthesized using aqueous extract of *E. crassipes* under hot air oven within 5 min. By this method, stable gold nanoparticles of size <50 nm were produced. A simple refluxing method was used for the synthesis of reduced graphene oxide using aqueous extract of *E. crassipes*. Phytochemical analysis reveals the presence of alkaloids, flavonoids, sterols, and carbohydrate which may be responsible for the reduction of metal and GO. Nano composite was prepared under sonication method for 1 h. The formation of nanocomposite, metal, and non-metallic nanoparticles were monitored by UV–Visible spectrometer and FTIR. FESEM analysis clearly portrays flake- like the structure of rGO and binding properties of gold nanoparticles with reduced graphene oxide. The cell viability studies of reduced graphene oxide show a high percentage of cell inhibition (77%) at 250 µg/mL against MCF-7 Cell lines. Synthesized gold nanoparticles and nano composites also showed higher cell inhibition of 45 and 60 %, respectively. The results of the cell viability of synthesized nanoparticles showed significant ($P_{0.033}$< 0.05) results. The present study suggests the use of metal and non-metallic nanoparticles synthesized using water hyacinth extract for cancer treatment.

Conflict of interest

All authors declare no conflict of interest.

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