Chapter 3

Nutraceutical evaluation of *Rhynchotechum ellipticum,* a potent wild edible plant consumed by the tribal of North-Eastern region in India and green synthesis of gold nanoparticles using its leaf extract

3.1. Introduction:

With the rapid industrialization and concomitant improvement in quality of life there has been extensive use of various chemicals, heavy metals, electromagnetic waves thereby causing pollution of every kind. The scenario has thrown a major challenge in the form of lifestyle diseases which are caused by unbalanced food habits. The use of junk foods has led to a number of diseases related to nutritional deficiencies. The raised demands for health care have dramatically increased the cost of medical care. Thus, people are trying to achieve a better quality of life by eating more vegetables, fruits, and other plant foods, taking dietary supplements or nutraceuticals, or using nutritional therapy or phytotherapy to replace chemotherapy or radiotherapy[1-3]. Nutraceuticals can play an important role in controlling the lifestyle diseases without any side-effect .No wonder more and more people are turning to nutraceuticals for optimal benefit.

The plants are one of the most important resources of human foods and medicines. With recent advances in medical and nutrition sciences, natural products and health promoting foods have received extensive attention from both health professionals and the conscious public. New concepts have appeared with this trend, such as nutraceuticals, nutritional therapy, phytonutrients, and phytotherapy[4-6]. Nutraceutical rich vegetables have medical health benefits including the prevention and treatment of diseases .The vegetables are rich sources of bioactive compounds such as flavonoids, carotenoids, anthocyanins, vitamins and other polyphenolics[7]. Such compounds play a role in disease prevention/reduce disease risk factors through antioxidant activity. These functional or medicinal foods and phytonutrients or phytomedicines play positive roles in enhancing health, and improving immune function to prevent specific diseases and also hold great promise to reduce side effects and huge health care costs[8]. The future demand of nutraceuticals depend on the consumer perception of the relationship between diet and disease.

Nanoscience and Nanotechnology have added a new dimension to research and development in the field of Biomedical science and Biotechnology and a new promising branch called Nanobiotechnology[9] has emerged. Recently, there has been a surge of research interest in developing eco-friendly synthetic procedure for the nanoparticles without using any toxic materials and without producing any hazardous wastes. Nanoparticles are particles with one of the dimensions less than 100 nm and which have found wide applications in optical, electronics, catalysis, imaging, sensing, drug and gene delivery[10]. Nobel metal nanoparticles are of interest to study and among such particles, the Gold nanoparticles (AuNPs) are of special interest due to their exclusive and tunable Surface Plasmon Resonance (SPR)[11]. The AuNPs have myriad of applications in biomedical field such as drug delivery, tissue tumour imaging, photothermal therapy, immunodiagnostic[12] and determination of heavy metal ions in aqueous solution[13]. The use of AuNPs has the advantage that they are safe, biocompatible and capable of target delivery of therapeutic agents[14].

There are several methods for the synthesis of nanoparticles, such as chemical, physical, mechanical and biological but all the methods excluding the biological ones are costly, using sophisticated apparatus and using toxic reductants, stabilising agents as well[15-17]. Nevertheless these methods produce hazardous wastes and harsh un-reacted toxic reagents thereby making the AuNPs so formed incapable to use in biomedical applications. The Green synthesis of AuNPs using biomass is recommended as an eco-friendly alternative to chemical methods that reduces the maintenance of septic environment and eliminates the generation of toxic by products where the biomass is of plant[18,19], bacterial[20], fungal[21], enzymatic[22] or algal[23] origin. As a result of eco-friendliness and biocompatibility the green synthesis fits for biomedical and pharmaceutical uses.

3.2. Materials and Methods

3.2.1 Plant materials

The leaves of *Rhynchotechum ellipticum* were collected from Meghalaya state, India in December, 2015 and identification was authenticated in our office of Shibpur Botanical garden, Shibpur, Howrah, India. The voucher specimens were preserved in the Plant Chemistry department of our office under registry no BSITS 103. The plant parts were shed-dried, pulverized and stored in an airtight container for further extraction.

3.2.2 Chemicals

The standard phenolic acids (gallic acid, protocatechuic acid, gentisic acid, chlorogenic acid, *p*-hydroxy benzoic acid, vanillic acid, caffeic acid, syringic acid, *p*-coumaric acid, ferulic acid, sinapic acid, salicylic acid and ellagic acid), flavonoids (catechin, rutin, myricetin, quercetin, naringin, apigenin and kaempferol), 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), butylated hydroxytoluene (BHT) were procured from Sigma Chemical Co. (St. Louis , MO , USA). Folin-Ciocalteus’s phenol reagent, potassium ferricyanide, potassium per sulphate, aluminium chloride, ferric chloride , anthrone, sodium carbonate, HPLC-grade solvents (acetonitrile, methanol, water and trifluoroacetic acid), sodium dihydrogen phosphate were purchased from Merck (Germany). Tetrachloroauric acid ($HAuCl_4$) was purchased from SRL. All the chemicals and solvents used were of analytical grade.

3.2.3 HPLC equipment

Dionex Ultimate 3000 liquid chromatograph attached with a diode array detector (DAD) was taken for HPLC analysis. The separation of components was achieved by a reversed-phase Acclaim C18 column (5 micron particle size, 250 x 4.6 mm). 20 μL of sample was injected into the HPLC column. The Chromeleon system manager was used for analyzing the data.

3.2.4 Proximate composition and minerals content in *R. ellipticum*

3.2.4.1 Estimation of ash content

Five gm of powdered leaves of the plant were taken in a silica crucible and heated for about 5-6 h in a muffle furnace controlled at 500 °C. The crucible was cooled, weighed and heated again in the furnace for half an hour. This process was repeated consequently until the weight of the crucible along with sample became constant (ash became white or greyish white). Weight of ash gave the ash content [24]. The ash obtained was preserved for mineral analysis.

$$\text{Ash content (\%)} = \text{Weight of ash} \times 100/\text{Weight of sample}$$

3.2.4.2 Estimation of moisture content

The moisture content of the plant sample was carried out by heating a known amount of fresh leaves in an air oven at 100-110°C and weighed. The loss in weight was considered as a measure of moisture content in the sample [24].

$$\text{Moisture (\%)} = [(\text{Weight of original sample} - \text{Weight of dried sample})] \times 100 / \text{Weight of original sample}$$

3.2.4.3 Estimation of crude fat content

Two gm moisture free leaves were soxhleted with petroleum ether (40-60°C) for about 6-8 h. The petroleum ether extract was filtered and evaporated in a pre-weighed beaker. Increase in weight of a beaker determines crude fat content. Percentage of fat content was calculated using the following formula [24].

Crude fat (%) = Weight of fat in sample × 100/Weight of dry sample

3.2.4.4 Estimation of crude fibre content

The crude fibre content in the plant sample was carried out by warming two gm of moisture and fat-free leaves with 200 ml of 1.25 % sulphuric acid followed by 1.25 % sodium hydroxide solution and with 1 % nitric acid. The solution was filtered and the residue was washed with boiling water and then the residue was dried in an oven at 130 °C to constant weight. The residue was heated in muffle furnace at 550 °C for two hours, cooled in a desiccator and weighed. The crude fibre content was expressed as percentage loss in weight on ignition [24].

Crude fibre (%) = (Weight of residue − Weight of ash) x 100/ Weight of the sample

3.2.4.5 Estimation of crude protein content

The micro Kjeldahl method was adopted for the estimation of crude protein content in the plant was where two gm of samples were digested with concentrated sulphuric acid in a Kjeldahl flask in the presence of 0.5 gm $CuSO_4$ and 5 gm K_2SO_4, until a clear solution was obtained. The digested solution was cooled and diluted with distilled water and an excess of sodium hydroxide solution (40%) was added to the diluted reaction mixture, the liberated ammonia was distilled in steam and absorbed in 25 ml (N/20) sulphuric acid. The excess mineral acid was titrated with known strength of sodium hydroxide and from this, the percentage of nitrogen in the sample was calculated. The amount of protein content determined by multiplying the amount of nitrogen with 6.25 [24].

3.2.4.6 Estimation of carbohydrate content

100 mg of leaves were hydrolysed with 5 ml of hydrochloric acid (2.5 N), cooled to room temperature and neutralised with solid sodium carbonate until the effervescence ceases. The solution filtered in a 100 ml volumetric flask and make up the volume with distilled water. To 1 ml of this solution, 4 ml freshly prepared anthrone reagent (200 mg anthrone dissolved in 100 ml of ice-cold 95% sulphuric acid) were added and heated in a water bath for eight minutes. The mixture was cooled rapidly, a dark green colour appeared and the absorption at 630 nm was measured (UV-visible spectrophotometer Shimadzu UV 1800). The total carbohydrate content was expressed as glucose equivalents using the following equation based on the calibration curve $y = 0.0081x + 0.2475$, $R^2 = 0.9993$ where y was the absorbance and x concentration of glucose in mg/ml [25].

3.2.4.7 Estimation of energy content

The energy (kcal/100gm) content of plant sample was determined by multiplying the values obtained for protein, fat and available carbohydrate by 4.00, 9.00 and 4.00, respectively and adding up the values [26].

3.2.4.8 Estimation of minerals in plant material

One gram of ash of the plant obtained above was dissolved in 30 ml of hydrochloric acid (5 %) solution, filtered and volume make up to 50 ml with double distilled water and minerals were estimated in atomic absorption spectrophotometer (AAS) (AA 800, Perkin-Elmer Germany). The standard solution of each element was prepared and calibration curves were drawn for each element using AAS [27]. All assays were carried out in triplicate and values were obtained by calculating the average of three experiments and data are presented as Mean ± SEM.

3.2.4.9 Antioxidant activities of *R. ellipticum*

3.2.4.9.1 Extraction of plant material (Benzene, chloroform, acetone and methanol)

One gram of each plant materials were extracted with 20 ml each of benzene, chloroform, acetone and methanol with agitation for 18-24 h at ambient temperature. The extracts were filtered and diluted to 50 ml and aliquot were analyzed for their total phenolic, flavonoid and flavonol content, reducing power and their free radical scavenging capacity.

3.2.4.9.2 Estimation of total phenolic content

The amount of total phenolic content of crude extracts was determined according to Folin-Ciocalteu procedure [28]. The tested samples (20 - 100 μl) were taken into test tubes. 1 ml of Folin-Ciocalteu reagent and 0.8 ml of sodium carbonate (7.5%) were added. The tubes were mixed and allowed to stand for 30 min. Absorption at 765 nm was measured (UV-visible spectrophotometer Shimadzu UV 1800). The total phenolic content was expressed as gallic acid equivalents (GAE) in milligram per gram (mg/g) of extract using the following equation based on the calibration curve $y = 0.0013x + 0.0498$, $R^2 = 0.999$ where y was the absorbance and x was the Gallic acid equivalent (mg/g).

3.2.4.9.3 Estimation of total flavonoids

Total flavonoids were estimated using the method mentioned at Seal *et al*., 2015 [28]. To 0.5 ml of sample, 0.5 ml of 2% $AlCl_3$ in ethanol was added. After one hour, at room temperature, the absorbance was measured at 420 nm (UV-visible spectrophotometer Shimadzu UV 1800). A yellow colour indicated the presence of flavonoids. Total flavonoid contents were calculated as rutin equivalent (mg/g) using the following equation based on the calibration curve: $y = 0.0182x - 0.0222$, $R^2 = 0.9962$, where y was the absorbance and x was the Rutin equivalent (mg/g).

3.2.4.9.4 Estimation of total flavonols

Total flavonols in the plant extracts were estimated using the method stated at Seal *et al* 2017 [29]. To 2.0 ml of sample (standard), 2.0 ml of 2% $AlCl_3$ ethanol and 3.0 ml (50 g/L) sodium acetate solutions were added. The absorption at 440 nm (UV-visible spectrophotometer Shimadzu UV 1800) was read after 2.5 h at 20°C. Total flavonol content was calculated as quercetin equivalent (mg/g) using the following equation based on the calibration curve: $y = 0.0049x + 0.0047$, $R^2 = 0.9935$, where y was the absorbance and x was the quercetin equivalent (mg/g).

3.2.4.9.5 Measurement of reducing power

The reducing power of the extracts was determined according to the method described by Seal *et al*, 2017 [29]. Extracts (100 µl) of plant extracts were mixed with phosphate buffer (2.5 ml, 0.2 M, *p*H 6.6) and 1% potassium ferricyanide (2.5 ml). The mixture was incubated at 50°C for 20 min. Aliquots of 10% trichloroacetic acid (2.5 ml) were added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and a freshly prepared ferric chloride solution (0.5 ml, 0.1%). The absorbance was measured at 700 nm. Reducing power is given in ascorbic acid equivalent (AAE) in milligram per gram (mg/g) of dry material using the following equation based on the calibration curve: $y = 0.0023x - 0.0063$, $R^2 = 0.9955$ where y was the absorbance and x was the ascorbic acid equivalent (mg/g).

3.2.4.9.6 Determination of DPPH free radical scavenging activity

The free radical scavenging activity of the plant samples and butylated hydroxyl toluene (BHT) as positive control was determined using the stable radical DPPH (1,1-diphenyl-2-picrylhydrazyl)[29]. Aliquots (20 -100 μl) of the tested sample were placed in test tubes and 3.9 ml of freshly prepared DPPH solution (25 mg L^{-1}) in methanol was added in each test tube and mixed. The absorbance was measured at 517 nm (UV-visible spectrophotometer Shimadzu UV 1800) after 30 min. The capability to scavenge the DPPH radical was calculated, using the following equation:

$$\text{DPPH scavenged (\%)} = \{(Ac - At)/Ac\} \times 100$$

Where Ac is the absorbance of the control reaction and At is the absorbance in presence of the sample of the extracts. The antioxidant activity of the extract was expressed as IC_{50}. The IC_{50} value was defined as the concentration in mg of dry material per ml (mg / ml) that inhibits the formation of DPPH radicals by 50%. Each value was determined from regression equation.

3.2.4.9.7 Scavenging activity of ABTS radical cation

The 2,2´-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation ($ABTS^{\cdot+}$)-scavenging activity was measured according to the method described by Seal *et al*[29]. ABTS was dissolved in water to a 7 mM concentration. The ABTS radicals were produced by adding 2.45 mM potassium per sulphate (final concentration). The completion of radical generation was obtained in the dark at room temperature for 12–16 h. This solution was then diluted with ethanol to adjust its absorbance at 734 nm to 0.70 ± 0.02. To determine the scavenging activity, 1 ml of diluted $ABTS^{\cdot+}$ solution was added to 10 μl of plant extract (or water for the control), and the absorbance at 734 nm was measured 6 min after the initial mixing, using ethanol as the blank. The percentage of inhibition was calculated by the equation:

$$\text{ABTS scavenged (\%)} = (A_{cont} - A_{test}) / A_{cont} \times 100$$

Where, A_c and A_s are the absorbencies of the control and of the test sample, respectively. From a plot of concentration against % inhibition, a linear regression analysis was performed to determine the IC_{50} value of the sample .

3.2.4.10 Quantification of phenolic acids and flavonoids in the methanol extract of *R. ellipticum* by HPLC

3.2.4.10. 1 Preparation of standard solutions

The stock solution of gallic acid of concentration 1mg / ml was prepared by dissolving 10 mg gallic acid in 1 ml HPLC-grade methanol followed by sonication for 10 min and the resulting volume was made up to 10 ml with the solvent for the Mobile phase (methanol and 0.5% aq. acetic acid 1:9). The same method was followed to prepare the standard stock solutions of the phenolic acids and the flavonoids *viz*. protocatechuic acid, gentisic acid, chlorogenic acid, *p*-hydroxy benzoic acid, vanillic acid, caffeic acid, syringic acid, *p*-coumaric acid, ferulic acid, sinapic acid, salicylic acid and ellagic acid, catechin, rutin, myricetin, quercetin, naringin, apigenin and kaempferol. The working standard solutions of concentrations 20, 40, 60, 80 and 100 μg/ml were prepared by further dilution of the standard solution with the mobile phase solvent system. The standard and working solutions were filtered through 0.45 μm PVDF-syringe filter and the mobile phase was degassed before the injection of the solutions.

3.2.4.10. 2 Chromatographic analysis for quantification of phenolic acids and flavonoids

HPLC analyses for the quantification of phenolic acids and flavonoids in the plant extract were performed following the method described by Seal 2016 with minor modification [29]. The analysis were carried out using Dionex Ultimate 3000 liquid chromatograph including a diode array detector (DAD) with 5 cm flow cell and with Chromeleon system manager as data processor. The separation was achieved by a reversed phase Acclaim C18 column (5 micron particle size, 250 x 4.6 mm). 20 μl of sample was introduced into the HPLC column. The method was validated according to the USP and ICH guidelines [30,31]. The mobile phase contains methanol (Solvent A) and 0.5% aq. acetic acid solution (Solvent B) and the column was thermostatically controlled at 25 ^{0}C and the injection volume was kept at 20 μl. A gradient elution was performed by varying the proportion of solvent A to solvent B. The gradient elusion was 10 % A and 90% B with flow rate 1 ml/min to 0.7 ml/min in 27 min, from 10 to 40 % A with flow rate 0.7 ml/min for 23 min, 40% A and 60% B with flow rate 0.7 ml/min initially for 2 min and then flow rate changed from 0.7 to 0.3 ml/min in 65min, from 40 to 44% A with flow rate 0.3 to 0.7ml/min in 70 min, 44% A with flow rate 0.7 to 1ml/min for 10 min duration, solvent A changed from 44% to 58 % with flow rate 1ml/min for 5 min, 58 to 70% A in 98 min at constant flow rate 1 ml/min. The mobile phase composition back to initial condition (solvent A: solvent B: 10: 90) in 101 min and allowed to run for another 4 min, before the injection of another sample. Total analysis time per sample was 105 min.

HPLC chromatograms were detected using a photo diode array UV detector at three different wavelengths (272, 280 and 310 nm) according to absorption maxima of analysed compounds. Each compound was identified by its retention time and by spiking with standards under the same conditions. The quantification of phenolic acids and flavonoids in the leaves of the plant were carried out by the measurement of the integrated peak area and the contents were calculated using the calibration curve by plotting peak area against concentration of the respective standard sample.

3.2.4.11 Biosynthesis of gold nanoparticles using methanol extract of *R. ellipticum*

3.2.4.11.1 Preparation of Au (III) solution

$HAuCl_4$ was purchased from SRL (Sisco Research Laboratory) and used without further purification. $HAuCl_4$ (52.8 mg) was dissolved in de-ionized water (10 ml) to obtain a 13.4 mM Au(III) stock solution.

3.2.4.11.2 Preparation of the leaf extract of *R. ellipticum*

Finely powdered leaves of *R. ellipticum* (3 gm) was suspended in methanol (10 ml) in a test tube, sonicated in an ultrasonicator bath for 45 min and then centrifuged for 10 minutes to obtain a clear supernatant. To know the concentration of the leaf extract, an aliquot of the clear supernatant (2 ml) was taken in a round bottom flask and the volatiles were removed under reduced pressure to afford a sticky solid (1.7 mg). Thus the concentration of the leaf extract was 1700 mg L^{-1} [32].

3.2.4.11.3 Synthesis of Gold Nanoparticles

Aliquots of Au (III) solution (0.2 ml, 13.4 mM each) were added drop-wise to the solution of leaf extract of *R. ellipticum* to prepare a series of stabilized AuNPs where concentration of the extract were 100, 200, 300 and 400 mgL^{-1} and the concentration of Au (III) was fixed at 0.67 mM. UV-visible spectroscopy of the solutions was carried out after 24 h of $HAuCl_4$ and the leaf extract of the plant had been mixed [32].

3.2.4.11.4 Characterization

HRTEM images, SAED and EDX of AuNPs were taken from Technai G2 instrument. UV-visible spectra were recorded in Shimadzu 1601 spectrophotometer. X-ray diffraction (XRD) patterns of the stabilized AuNPs were recorded Bruker-D_8 Advanced with Cu-Kα radiation (λ= 1.54 Å).

3.3 Results and Discussion

3.3.1 Proximate composition and minerals content in *R. ellipticum*

The leaves of *R. ellipticum* were taken for the analysis of proximate composition. The proximate composition of these plants has been presented in Table 1.

The proximate analysis of *R. ellipticum* showed that 100gm of dry plant contain 6.62 ± 0.269 gm ash and 81.67 ± 0.21 gm moisture. The high amount of ash content indicating that this plant was rich in minerals and could provide a substantial amount of mineral elements to our diet [33].

The plant was found to contain protein, fat, fibre and carbohydrate 7.76±0.03 **%,** 11.31±0.18%, 1.52±0.13% and 46.49±0.46% respectively. The energy content of the plant was calculated at 106.78±1.26 kcal/100gm. The fat and fibre content in the plant was particularly high and well compared to that reported for some common vegetables which indicates that the consumption of the plant would be helpful for the absorption of fat soluble vitamins like vitamin A and carotene in the body and might play an important role in decreasing the risks of many disorders such as constipation, diabetes, serum cholesterol, heart diseases, breast and colon cancer, hypertension, etc. [34,35]. The plant are rich sources of protein which can encourage their use in human diets and would be helpful for the proper functioning of antibodies resisting infection [34].

Fruits, and vegetables, are important sources of macro-minerals (Na, K, Ca Mg) and micro-minerals (Fe, Zn, Cu, Mn, Zn, Pb, Cr) which are responsible in maintaining physiological and biological functions of the human body. The leaves of the plant contain a very good amount of sodium (0.48 ± 0.017 mg/gm**)** and potassium (20.66 ± 0.26 mg/gm). The ratio of K/Na was significant in this plant (43.04) which is very much responsible to control the high blood pressure of our body [36]. The leaves of the plant was found to contain 35.10 ±0.27 mg/g calcium which might be beneficial to build strong and healthy bones and also required for the normal functioning of the cardiac muscles [28]. A sufficient amount of Cu, Zn, Mg, Fe and Zn were present in the plant indicating that the consumption of this vegetable might be helpful for preventing iron- deficiency anaemia, nucleic acid metabolism, control the blood- glucose levels and support a healthy immune system[37,38].

Table 1. Proximate composition and minerals content in *R. ellipticum*

Proximate composition	**Amount**	**Minerals**	**Amount (mg/g)**
Ash (%)	6.62 ± 0.269	**Sodium (Na)**	**0.48** ± 0.017
Moisture (%)	81.67 ± 0.21	**Potassium (K)**	20.66 ± 0.26
Protein (%)	7.76±0.03	**Calcium (Ca)**	35.10± 0.27
Fat (%)	1.52±0.13	**Copper (Cu)**	0.0007±0.0005
Carbohydrate (%)	46.49±0.46	**Zinc (Zn)**	0.063 ± 0.0001
Crude fibre (%)	11.31±0.18	**Magnesium (Mg)**	1.32 ± 0.002
Energy (kcal/100gm)	106.78±1.26	Iron (Fe)	0.047 ± 0.001
		Manganese (Mn)	0.057 ± 0.0006

Each value in the table was obtained by calculating the average of three experiments and data are presented as Mean ± SEM

3.3.2 Antioxidant activities of the different solvent extracts of *R. ellipticum*

3.3.2.1 Extractive value

The extractive values of the plant under investigation with four different solvents are depicted in table 2. The result shows that, methanol is the most suitable solvent to obtain the maximum extract from all the plants under investigation in comparison to the other solvents like benzene, chloroform and acetone used for extraction. The leaves of *R. ellipticum* give maximum yield (2.9±0.02 g/100g) when it is extracted with methanol and the least amount is observed with benzene. The differences in the extractive value of the plant materials may be due to the varying nature of the chemical components present and the polarities of the solvent used for extraction.

Table 2. Antioxidant activities of *R. ellipticum* using different solvents

	Benzene	**Chloroform**	**Acetone**	**Methanol**
Extractive value (%)	0.25±0.01	0.275±0.06	0.32±0.04	2.9±0.02
Total phenolic content (Gallic acid equivalent, mg/100gm Plant material)	29.23 ±4.44	43.97 ±6.16	91.41 ±1.97	617.30 ±3.31
Total Flavonoid content (Rutin equivalent mg/100gm Plant material)	15.77±1.38	29.07±0.60	34.00±0.78	197.25±0.82
Total flavonol content (Quercetin equivalent mg/100gm Plant material)	8.99±1.36	20.22±1.23	24.30±1.88	156.37±0.50
Reducing power (Ascorbic acid equivalent mg/100gm Plant material)	21.34±5.22	31.84±4.56	30.03±4.45	209.96±2.49
DPPH radical scavenging activity (IC50 mg dry extract)	5.16±0.43	3.91±0.24	0.17±0.005	0.003 ±0.00003
ABTS radical scavenging activity (IC50 mg dry extract)	0.29±0.008	0.22±0.01	0.08±0.002	0.002 ±0.0008

Each value in the table was obtained by calculating the average of three experiments and data are presented as Mean ± SEM

3.3.2.2 Total phenol, flavonoid and flavonol content in the extract

The screening of the benzene, chloroform, acetone and methanol extracts of the plants revealed that highest amount of phenolic compounds, flavonoid and flavonol were detected in the methanol extract of the plant. The results strongly suggest that phenolics are important components of these plants. The other phenolic compounds such as flavonoids, flavonols, which contain hydroxyls are responsible for the radical scavenging effect in the plants. According to our study, methanol was the most suitable solvent to isolate the phenolic compounds and benzene, chloroform and acetone are the best solvent to isolate the flavonoids and flavonols from the plant materials. The total phenolic component exhibited antioxidant activity through adsorption and neutralization of the free radicals, whereas flavonoid and flavonol showed antioxidant activity through scavenging or chelating process [39,40]. The high content of the phenolic compounds in *R. ellipticum*, can explain their high radical scavenging activity. In this study the methanol extract of *R. ellipticum* showed potent antioxidant activities using DPPH and ABTS assay. The IC_{50} value of DPPH assay of *R. ellipticum* was found to be higher than that of ABTS assay which showed more antioxidant activities. The high radical scavenging property of this plant may be due to the presence of hydroxyl groups that can provide the necessary component as a radical scavenger.

The antioxidant activities of the extractive solution represent an important parameter to evaluate the biological property of the plant. Therefore, it is necessary to characterize and quantify the important compounds like phenolic acids and flavonoids present in the plant and also to validate the method of separation and identification of active constituents.

The HPLC analysis showed (Fig.1, Table 3) the presence of remarkable amount of *p*-coumaric acid (21.72±0.03 mg/gm plant material) in the methanol extract of *R. ellipticum*. Due to the presence of *p*-coumaric acid, the plant is believed to have antioxidant behavior thereby reducing the formation of carcinogenic nitrosamines in the stomach [41].

The plant was found to contain a very good amount of chlorogenic acid which is responsible for reducing hepatic triglycerides levels, thus resulting in weight loss. It also decreases proliferation of new fat cells through its antioxidant effects [42]. One of the important phenolics, ferulic acid which is detected in the methanol extract of the plant in our study and regular intake of the vegetable leads to lower cholesterol level in serum and increases sperm viability [43].

A very significant amount of sinapic acid was detected in the plant under investigation and consumption of this plant would be useful for health promotion because it showed antioxidant, anti-microbial, anti-inflammatory, anticancer, and anti- anxiety activity. [44] An appreciable amount of quercetin, detected in *R. ellipticum* was comparable to the same in apple (0.021 mg/gm), lettuce (0.011 mg/gm) and tomato (0.055 mg/gm) and this is reported to display anti-histamine, anti-cancer as also anti-inflammatory activities [45].

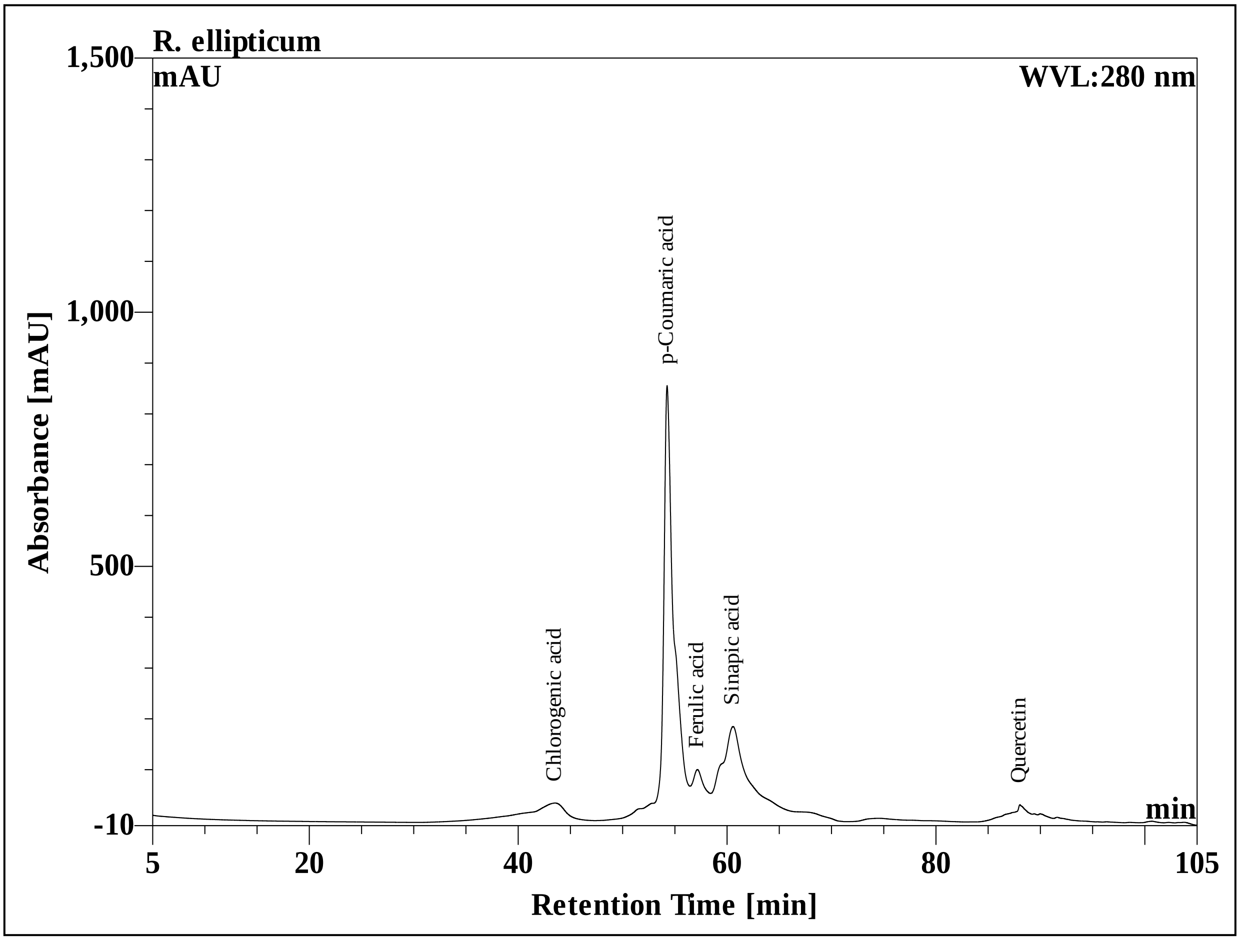

Fig.1. HPLC chromatogram for the quantification of phenolic acids in *R. ellipticum*

Table 3. Phenolic acid and flavonoid content in *R. ellipticum* by HPLC

Phenolic acids/flavonoids	**Amount (mg/gm dry plant material)**	**Phenolic acids/flavonoids**	**Amount (mg/gm dry plant material)**	**Phenolic acids/flavonoids**	**Amount (mg/gm dry plant material)**
Gallic acid	ND	Caffeic acid	ND	Rutin	ND
Protocatechuic acid	ND	Syringic acid	ND	Ellagic acid	ND
Gentisic acid	ND	p-Coumaric acid	21.72±0.03	Myricetin	ND
p-Hydroxy benzoic acid		Ferulic acid	0.018±0.0003	Quercetin	0.149±0.001
Catechin	ND	Sinapic acid	0.073±0.001	Naringenin	ND
Chlorogenic acid	0.524±0.003	Salicylic acid	ND	Apigenin	ND
Vanillic acid	ND	Naringin	ND	Kaempferol	ND

Each value in the table was obtained by calculating the average of three experiments and data are presented as Mean ± SEM

3.3.2.4 Biosynthesis of gold nanoparticles using methanol extract of *R. ellipticum*

The leaf extract of *R. ellipticum* is a rich source of different types of plant secondary metabolites in methanol such as polyphenolic compounds (617.30 ±3.31 mg GAE/100g dry material), flavanoids (265.93±0.82 mg/100g dry extract), flavonols (779.67±6.35mg/100g) etc. HPLC analysis of the leaf extract carried out in our laboratory also supported the presence most of the compounds. The leaf extract of the plant, rich in polyphenolic compounds, can be utilized for the synthesis of AuNPs from $HAuCl_4$.

3.3.2.5 Synthesis of RE-AuNPs and study of its Surface Plasmon Resonance spectroscopy

Antioxidants including polyphenols are well known for their use in the facile synthesis of metal nanoparticles under very mild condition. As the leaf extract of *R. ellipticum* was rich in easily oxidisable plant secondary metabolites including polyphenols, the methanol extract was utilized for the green synthesis of AuNPs at room temperature. To test this, the methanol extract of the plant, contained in vials was treated with $HAuCl_4$ solution (Fig. 2).

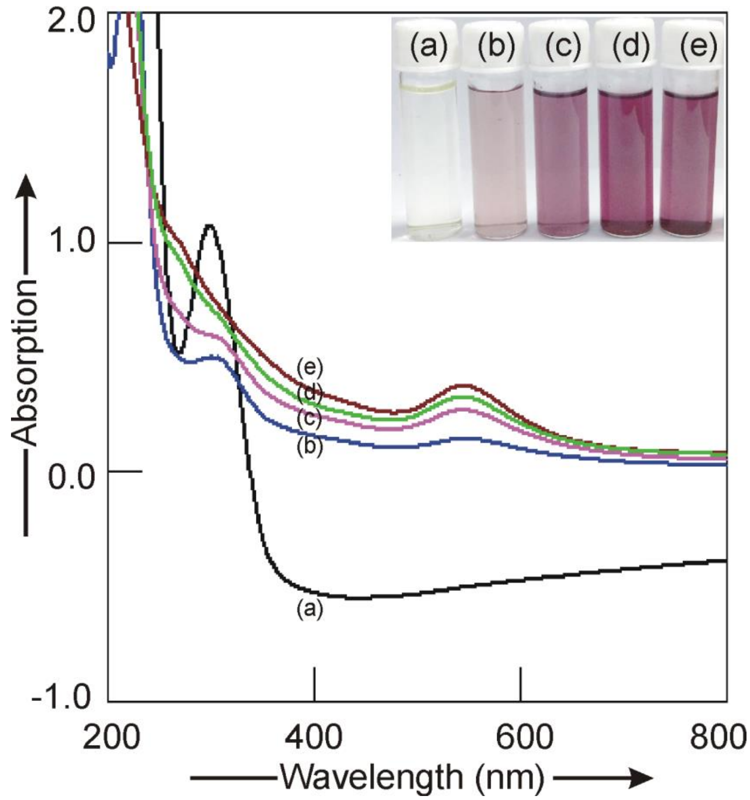

Fig. 2: UV-visible spectra of (a) $HAuCl_4$ (0.67 mM), (b-e) AuNPs at 100, 200, 300, and 400 mgL^{-1} concentrations of leaf extract respectively. Inset: Photograph of the vials containing (a) $HAuCl_4$ (0.67 mM) solution, (b-e) colloidal AuNPs at 100, 200, 300 and 400 mgL^{-1} of leaf extract respectively (after 24 h of mixing)

The appearance of violet to pinkish red coloration after 10 minutes indicating the formation of RE-AuNPs. The intensities of the colors increased on standing the solutions at room temperature for several hours and then remained constant and the RE-AuNPs once formed were stable for several months at room temperature.

The $HAuCl_4$ showed a strong peak at 243 nm and a shoulder peak at 298 nm. This was due to the charge transfer interactions between the metal and the chloro ligands (Fig. 2a). The intensities of these two peaks decreased with increasing concentration of the leaf extract of *R. ellipticum* and new peaks appeared around 530 nm. This is due to surface plasmon resonance (SPR) of the AuNPs, a phenomenon arising due to collective oscillation of the conduction band electrons interacting with the electromagnetic component of the visible light. With increasing the concentration of the leaf extract, a blue shift of the SPR band was observed due to the formation of smaller sized AuNPs. The shoulder peaks observed in the 270-275 nm regions of AuNPs colloids were due to the formation of quinone moiety formed by the oxidation of the phenolic compounds.

3.3.2.6 Mechanism of the formation of Stabilized AuNPs

Leaf extract of *R. ellipticum* is rich source of different types of phytochemicals including polyphenols, flavanoids, flavonols, etc. The o-dihydroxy compounds present in the leaf extract can form a five member chelate ring with the Au(III) ions. Au(III) ions having a very high reduction potential can be reduced to Au(0) with concomitant oxidation of the polyphenols to corresponding quinones. The freshly generated Au(0) atoms in the reaction mixture can collide with each other forming AuNPs which are stabilized by the concomitantly formed quinones, polyphenols and other coordinating phytochemicals. The steric bulk of the backbone of the benzoquinones derivative and other phytochemicals wrapping around the nanoparticles provide robustness against further aggregation of the stabilized AuNPs (Fig. 3).

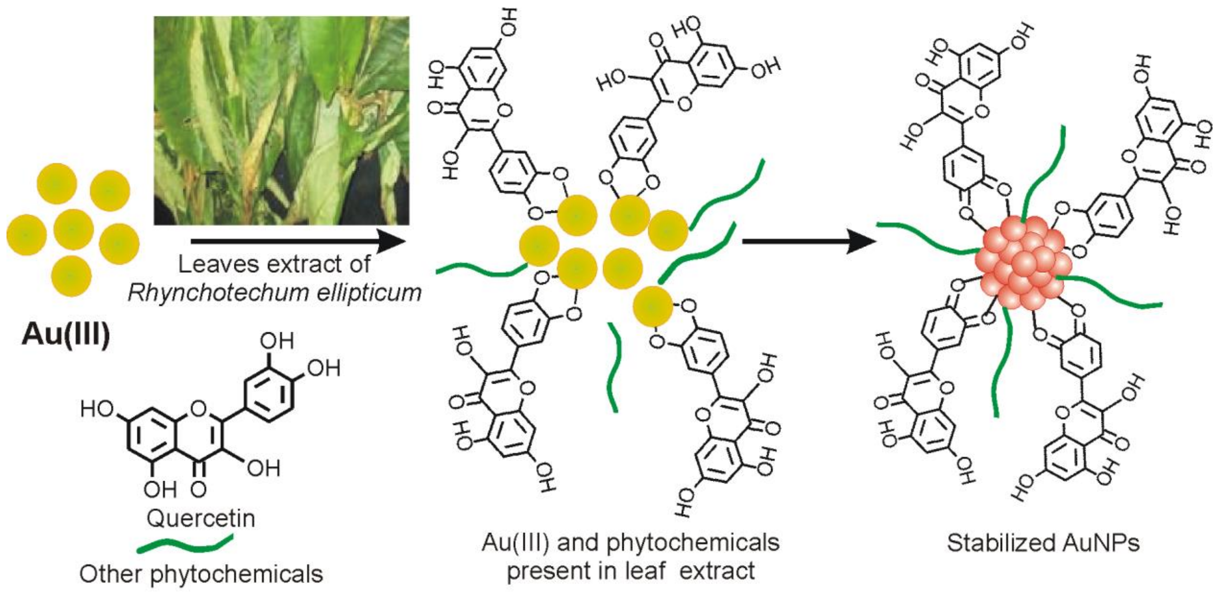

Fig. 3: Mechanism of the formation and stabilization of AuNPs by the phytochemicals present in the leaf extract of *R. ellipticum.*

3.3.2.7 HRTEM, SAED, EDX and XRD studies

High resolution transmission electron microscopy (HRTEM) was carried out to study the size distribution, shape and morphology of the AuNPs formed at particular concentration of the leaf extract of *R. ellipticum*. AuNPs of spherical, triangular, tetragonal, pentagonal and hexagonal shapes were observed. The average size of the AuNPs formed at 400 mgL^{-1} concentration of the leaf extract was 8.3 nm (Fig. 4a-d**)**. The polyphenolic compounds, quinone and other chelating phytochemicals present in the leaf extract could effectively stabilize the smaller sized AuNPs [30]. The formation of the RE-AuNPs was also confirmed from SAED and EDX analysis which showed the presence of Au along with C from the stabilizing organic ligands (Fig. 4 e,f).

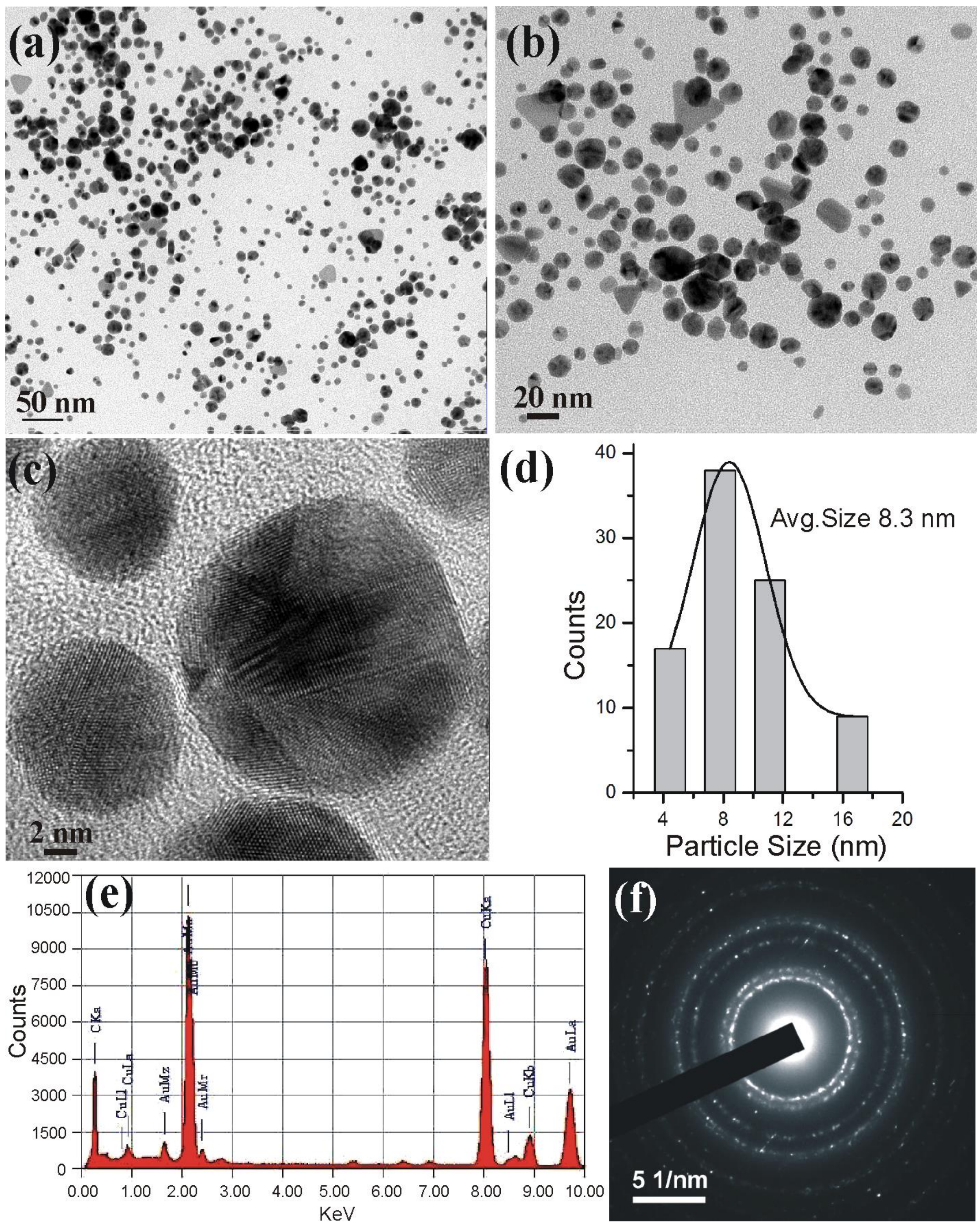

Fig. 4: (a-c) TEM Images of AuNPs obtained from the leaf extract of *R.ellipticum* at 400 mgL^{-1}, (d) Histogram of RE-AuNPs, (e) EDX (f) SAED of stable gold nanoparticles obtained from the leaf extract of *R.ellipticum* at 400 mgL^{-1}.

A colloidal RE-AuNPs sample was coated over a glass plate, the volatiles were removed and X-ray diffraction analysis of the dried RE-AuNPs sample was carried out. The reflections of the planes (111), (200), (220) (311) and (222) at 2θ = 38.1°, 44.4°, 64.7° , 77.6° and 81.7° respectively (Fig. 5) resembled the characteristic reflections of crystalline metallic face centered cubic Au (JCPDS file no. 04-0784). The higher intensity of the 111 plane indicates predominant orientation of this plane compared to the other planes.

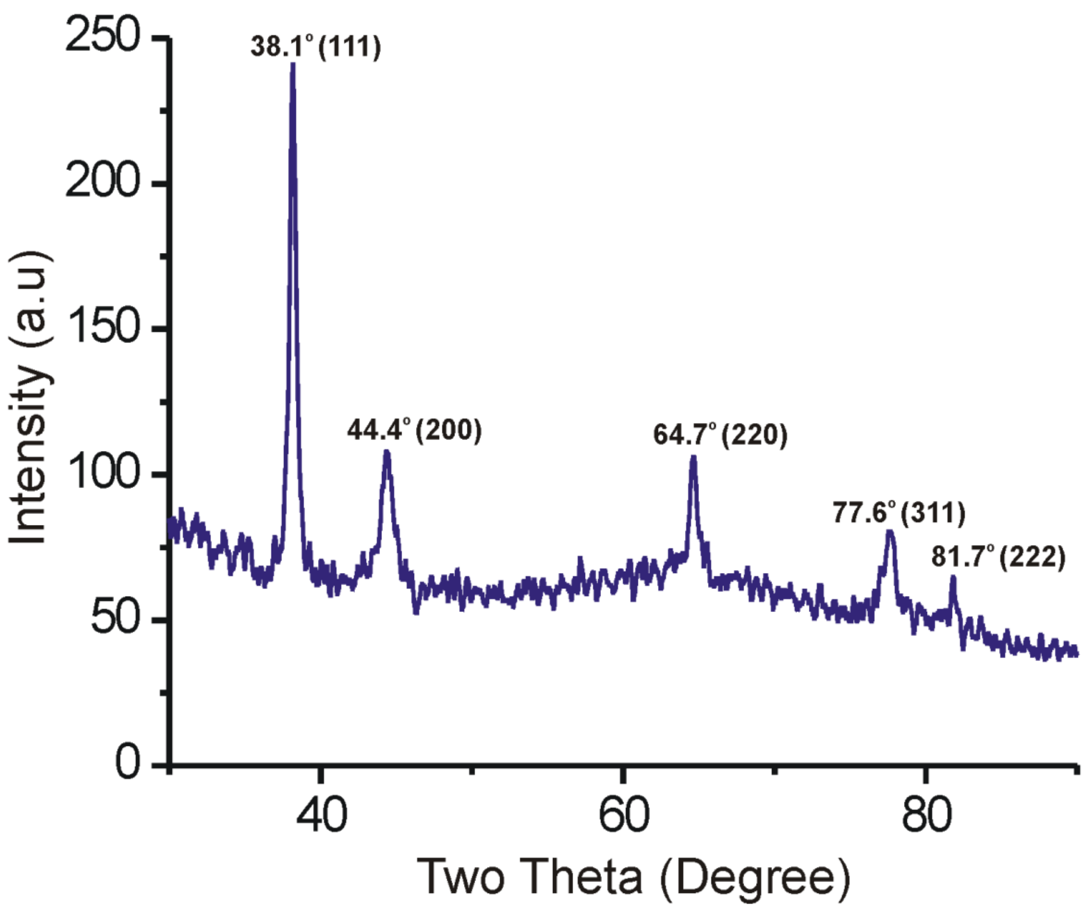

Fig. 5: XRD pattern of stable RE-AuNPs synthesized using the leaf extract of *R.ellipticum* (400 mgL^{-1}).

3.4. Conclusion

The present investigation showed that this wild edible plant are rich in protein, fat, carbohydrate, fiber and vitamins and could provide essential nutrients required for maintaining normal body function. The nutritional property of this plant was similar to and also sometimes better than the common vegetables. The leaf of the plant was also found to be a significantly useful source of various minerals. The minerals, particularly Na, K, Ca, Fe, Cu, Mg and Zn, were present in appreciable quantities. The antioxidant properties and the presence of various phenolic acids and flavonoids inferred that leaves of this plant could be used for the nutritional purpose of human being and adequate protection may be obtained against diseases arising from malnutrition. The presence of significant amount of respective bio-active components in this plant under study and variation of quantity determined ensures its usefulness for the synthesis of gold nanoparticles, without the requirement of any reducing agent. The high amount of phenolic acids present in *R. ellipticum* acted as an electron donor system and ligating agents to form stabilized nanoparticles. Therefore, using this plant extract will be a new and favorable alternative to the current processes to produce metallic nanoparticles in large scale without generating any toxic by-products. HRTEM studies revealed the mostly spherical shape of the AuNPs of average size of 8 nm. As, *R. ellipticum* is non-toxic and edible, *R. ellipticum*-conjugated gold nanoparticles synthesized by the green synthetic method utilizing the active ingredients present in the plant extract will be useful for various biomedical as well as nano-scientific applications.

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