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Appendices

Appendix 1

Qualitative phytochemical analysis

of alkaloids Kumar *et al.*, 2009

Mayer's test (Evans 1997)

A fraction of the extract was treated with Mayer's reagent (1.36 g of mercuric chloride and 5 g of potassium iodide in 100 ml of distilled water) and observed for the formation of cream coloured precipitate.

Dragenodorff's test

An aliquot of the extract was tested with Dragendorff's reagent [Solution A: Bismuth nitrate (0.7 g) in glacial acetic acid (2 ml) and distilled water (8 ml)], [Solution B: Potassium iodide (4 gm) in glacial acetic acid (10 ml) in water (20 ml)], Mixed solutions A and B and diluted to 100 ml with distilled water and observed for formation of reddish orange precipitate.

Wagner's test (Wagnner 1993)

A fraction of the extract was treated with Wagner's reagent (1.2 g of iodine and 2 g of potassium iodide in 100 ml of distilled water) and observed for the formation of reddish brown coloured precipitate.

Hager's test

To a few ml of filtrate, 1 or 2 ml of Hager's reagent (saturated aqueous solution of picric acid) was added. A prominutesent yellow precipitate indicated the test as positive.

Detection of phenols (Raaman, 2006)

Ferric chloride test

To a fraction of the extract, 5% Ferric chloride ($FeCl_3$) solution was added and observed for the formation of deep blue colour.

Lead acetate test

A fraction of the extract was treated with 10 % lead acetate solution and observed for the formation of white precipitate.

Detection of Flavonoids (Ayoola *et al.*, 2008)

Aqueous sodium hydroxide test

To a fraction of the extract, a drop of 1N aqueous sodium hydroxide solution was added and observed for the formation of yellow orange colouration.

Schinodo's test

A fraction of the extract was treated with a piece of magnesium turnings followed by a few drops of concentrated hydrochloride acid, heated slightly and observed for the formation of dark pink colour.

Sulphuric acid test

To a small amount of the extract, added a few drops of conc. sulphuric acid. Orange colour indicated the presence of flavonoids.

Alkaline reagent test

An aqueous solution of the extract was treated with 10% ammonium hydroxide solution. Yellow fluorescence indicated the presence of flavonoids.

Detection of tannins (Edeoga *et al.*, 2005)

5 ml of the extract was treated with a few drops of lead acetate. A yellow precipitate indicated the presence of tannin.

Identification of glycosides (Raaman, 2006)

For detection of glycosides, 50 mg of extract was hydrolysed with concentrated hydrochloric acid for 2 hours in a water bath, then filtered and the hydrolysate was subjected to the following tests.

Borntrager's test

To 2 ml of filtered hydrolysate, 3 ml of chloroform was added and shaken. The chloroform layer was separated and equal volume of 10 % ammonia solution was added to it Pink violet or red colour indicated the presence of glycosides.

Legal's test

50 mg of the extract was dissolved in pyridine and Sodium nitroprusside solution was added and made alkaline using 10 % sodium hydroxide. Presence of glycosides was indicated by change in colour from pink to blood red (Zohra *et al.*, 2012)

Kellar Killani test

The ethanolic extract was dissolved in 2 ml of glacial acetic acid containing a trace of ferric chloride. Same amount of ferric chloride dissolved in 1 ml of conc. sulfuric acid along

the sides of the test tubes was added to settle at the bottom. Appearance of a reddish green colour at the junction of two reagents within 2-5 minutes spreading slowly into the acetic acid layer confirmed the presence of cardiac glycosides.

Detection of saponin (Kumar *et al.*, 2009)

Frothing test

A portion of the extract was added alongwith 5 ml of distilled water in a test tube. The solution was shaken vigorously and observed for stable persistant froth.

Sodium bicarbonate test

In a test tube, about 5 ml of extract was added followed by a drop of sodium bicarbonate. The mixture was shaken vigorously and kept for 3 minutes. The formation of a honey-comb like froth showed the presence of saponins.

Detection of terpenoids (Edeoga *et al.*, 2005)

5 ml of extract was mixed with 2 ml of chloroform and 3 ml of conc. sulphuric acid was carefully added to form a layer. A reddish brown colouration at the interface formed to idicate the presence of terpenoids.

Detection of steroids (Edeoga *et al.*, 2005)

Liebermann Burchard reaction

A portion of the extract was treated with 10 ml chloroform and filtered. 2 ml of filtrate was treated with 2 ml of acetic anhydride and 2 ml ōf conc. sulphuric acid. Blue, green ring indicated the presence of steroids.

Test for phytosterols

To 1 ml of plant extract, equal volumes of chloroform and 3 drops of concentrated sulphuric acid were added. Formation of brown ring indicated the presence of steroids and formation of bluish green colour indicates the presence of phyosterols.

Test for Amino acids (Kumar *et al.*, 2009)

Ninhydrin test

To 2 ml of sample few drops of Ninhydrin reagent was added and kept in a hot water bath for 20 minutes. Appearance of purple colour indicated the presence of amino acids in the sample.

Test for Protein (Gahan, 1984)

Biuret test

To 2 ml of extract 1 ml of 40 % Sodium hydroxide solution and 1 to 2 drops of 1% copper sulphate solution were added. A violet colour indicated the presence of peptide linkage in the molecule.

Appendix 2

Determination of Total Phenols

(Aiyegoro *et al.*, 2010)

Principle

Phenols react with phosphomolybdic acid in Folin-Ciocalteau reagent to produce a bluecoloured complex in alkaline medium, which can be estimated spectrophotometrically at 650nm.

Reagents

1. Stock I: 10 mg of gallic acid was dissolved in 10 ml of distilled water

Stock II: From stock I took 1 ml and diluted with 9 ml of distilled water (100 mg/ ml)

Standard: 2 ml of stock II was diluted with 8 ml of distilled water. 1 ml of working standard contains 20 mg of gallic acid.

2. Folin- Ciocalteau reagent (1: 10)

3. 20% Sodium carbonate

Procedure

The amount of phenol in the hydroethanolic extract was determined by using the previously described Folin-Ciocalteu's method**.** To 0.5 ml of the extract (1 mg/ml), 2.5 ml of 10% Folin-Ciocalteu reagent and 2 ml of Sodium carbonate (2 % w/v) were added. The resulting mixture was incubated at 45˚C for 15 minutes with intermittent shaking. The absorbance was measured at 765 nm. Results were expressed as milligrams of gallic acid (0.1-0.5 mg/ml) dissolved in distilled water.

Appendix 3

Determination of Total flavonoids

(Chang *et al.*, 2002)

Principle:

Flavanoids present in the sample react with Sodium nitrite & Aluminum chloride to give a red color product which is measured at 510 nm. The intensity of colour developed is directly proportional to the amount of flavanoid in the sample.

Reagents

1. Preparation of standard – 1 mg /1 ml.

   10 mg of quercetin dissolved in 10 ml methanol. 1 ml contains 1mg of flavanoid.

2. 5 % Sodium nitrite

3. 10 % Aluminum chloride

4. 1 mM sodium hydroxide

Procedure

To 1ml of the hydroethanolic extract (1 mg/ml), 3 ml methanol, 0.2 ml of aluminum chloride, 0.2 ml of potassium acetate and 5.6 ml of distilled water were added. The mixture was incubated at room temperature for 30 minutes and the absorbance measured at 420 nm. Results were expressed as milligram of gallic acid (0.1 – 0.8 mg/ml) dissolved in distilled water.

Appendix 4

Estimation of DPPH radical scavenging activity

(Mensor *et al.*, 2001)

Principle

DPPH (2,2- diphenyl- 2-picryl hydrazyl), a stable free radical, when acted upon by an antioxidant is converted into diphenyl-2-picryl hydrazine with a colour change from deep violet to light yellow colour. This can be quantified spectrophotometrically at 518 nm to indicate the extent of DPPH scavenging activity by the plant extracts.

Reagents

1. DPPH (0.3mM in methanol)
2. Methanol

Procedure

The extracts of sample (25 µl) and 0.48 ml of methanol were added to 0.5 ml of methanolic solution of DPPH. The mixture was allowed to react at room temperature for 30 minutes. Methonal alone served as blank and DPPH in methanol, without the plant extracts, served as positive control. After 30 minutes of incubation, discolouration of the purple colour was measured at 518 nm. The radical scavenging activity was calculated as follows

$$\text{Scavenging activity (\%)} = \frac{\text{A (control)} - \text{A (sample)}}{\text{A (control)}} \text{ X } 100$$

Appendix 5

Determination of Ferric reducing antioxidant power radical scavenging activity

(Benzie and Strain, 1996)

Principle

The method is based on the reduction of $Fe3^{+}$ 2,4,6- tripyridyl-s-triazine (TPTZ) complex (colorless complex) to $Fe2^{+}$- tripyridyltriazine (blue colored complex) formed by the action of electron donating antioxidants at low pH. This reaction is monitored by measuring the change in absorbance at 593 nm.

Procedure

The Ferric reducing antioxidant power (FRAP) reagent was prepared by mixing 300 mM acetate buffer, 10 mmol/liter TPTZ in 40 mM Hydrochloric acid and 20 mM Ferric Chloride Hexahydrate ($FeCl_3.6H_2O$) in the proportion of 10:1:1 at 37°C. Freshly prepared working Ferric reducing antioxidant power reagent was pipetted using 1-5ml variable micropipette (3.995 ml) and mixed with 5µl of the appropriately diluted plant sample and mixed thoroughly. An intense blue color complex was formed when ferric tripyridyltriazine (Fe3+ TPTZ) complex was reduced to ferrous ($Fe2^{+}$) form and the absorbance at 593 nm was recorded against a reagent blank (3.995 ml Ferric reducing antioxidant power reagent + 5 µl distilled water) after 30 minutes incubation at 37°C. All the estimations were performed in triplicates. The calibration curve was prepared by plotting the absorbance at 593 nm versus different concentrations of Ferrous Sulfate. The concentrations of Ferrous sulfate were in turn plotted against concentration of standard antioxidant trolox. The Ferric reducing antioxidant power values were obtained by comparing the absorbance change in the test

mixture with those obtained from increasing concentrations of $Fe3^{+}$ and expressed as mg of Trolox equivalent per gram of sample.

The FRAP value was calculated using the following equation:

$$\text{FRAP value} = [(A1 - A0)/(Ac - A0)] \times 2$$

where A0 is the absorbance of the blank, A1 is the absorbance of the sample and Ac is the absorbance of the positive control

Appendix 6

Total antioxidant capacity (TAC) assay

Phosphomolybdate method (Lallianrawna *et al.*,2013)

Principle

The total antioxidant capacity assay was based on reduction of phosphate-molybdenum (VI) to phosphate-molybdenum (V) which is green in colour recorded by the absorbance at 695 nm. The reagent solution containing 600 mM sulfuric acid; 28 mM sodium phosphate: 4 mM ammonium molybdate in the ratio 1:1:1 by volume was prepared in which ammonium molybdate served as the source of molybdenum.

Reagents

1. Sulfuric acid (600 mM)
2. Sodium phosphate (28 mM)
3. Ammonium molybdate (4 mM)

Procedure

Samples of 0.2 mg concentration were allowed to react with 1 ml of reagent solution and incubated at 95˚C for 1 h 30 minutes, after which cooled and performed the measurement at 765 nm. It was compared with standard gallic acid (0.2 mg) and all the tubes were made upto 1.1 ml. The total antioxidant capacity can be calculated using the formula,

$$\text{TAC} = \frac{\text{OD test} \times \text{concentration of standard (mg)} \times \text{made up volume}}{\text{OD of the standard}}$$

Appendix 7

Estimation of Catalase Activity

(Luck, 1965)

Principle

The UV light absorption of hydrogen peroxide (H_2O_2) can be easily measured between 230-250 nm. On decomposition of hydrogen peroxide by catalase, the absorption decreases with time. The enzyme activity can be estimated by this decrease in absorption.

Reagents

1. Phosphate buffer: 0.067 M (pH 7.0)

Dissolved 3.522 g of Potassium dihydrogen phosphate (KH_2PO_4) and 7.268 g of Dipotassium hydrogen phosphate ($KHPO_4.2H_2O$) in distilled water and made up the volume to one litre.

2. Hydrogen peroxide in phosphate buffer (2 mM)

Dissolved 0.16 ml of hydrogen peroxide (10% w/v) to 100 ml phosphate buffer, prepared fresh. The absorbance of the solution was 0.5 at 240 nm with 1 cm light path.

Procedure

20% homogenate of the plant sample was prepared in phosphate buffer (0.006 M, pH 7.0) and the homogenate was employed for the assay. The sample was read against a control without homogenate, but containing the hydrogen peroxide - phosphate buffer. To the experimental cuvette, 3 ml of hydrogen peroxide -phosphate buffer was added, followed by the rapid addition of 40 µl enzyme extract and mixed thoroughly. The time interval required for a decrease in absorbance by 0.05 units was recorded at 240 nm. The enzyme solution containing hydrogen peroxide free phosphate buffer served as the control. One enzyme unit was calculated as the amount of enzyme required to decrease the absorbance at 240 nm by 0.05 units.

Calculation

The catalase value was calculated using the following equation

$$\text{Units/ml enzyme} = \frac{(3.45)\ (\text{df})}{(\text{time})\ (0.1)}$$

where:

3.45 = corresponds to the decomposition of 3.45 µmoles of hydrogen peroxide in a 3.0 ml reaction mixture producing a decrease in the A240 from 0.45 to 0.40

df = dilution factor

time = minutes required for the A240 to decrease from 0.45 to 0.40 absorbance units

0.1 = milliliter of enzyme added to the cuvette

Appendix 8

Estimation of Peroxidase Activity

(Reddy *et al.*, 1985)

Principle

Peroxidase catalyses the conversion of hydrogen peroxide (H_2O_2) to water (H_2O) and oxygen (O_2) in the presence of the hydrogen donor pyrogallol. The oxidation of coloured product called purpurogalli can be measured spectrophotometrically at 430 nm with the specified time interval. The intensity of the product is proportional to the activity of the enzyme.

Reagents

1. Pyrogallol (0.05 M in 0.1 M phosphate buffer, pH 6.5)

   630 mg of pyrogallol in 100 ml of 0.1 M phosphate buffer.

2. Hydrogen peroxide (H_2O_2) (1 % in 0.1 M phosphate buffer)

Procedure

The plant samples were prepared as 20% homogenate in 0.1M phosphate buffer (pH 6.5) and used for the assay. Pyrogallol solution (3.0 ml) and enzyme extract (0.1 ml) were pipetted out into a cuvette. The spectophotometer was adjusted to read zero at 430 nm followed by the addition of 0.5 ml of 1% hydrogen peroxide and mixed. The change in absorbance was recorded every 30 seconds upto 3 minutes.

Calculation

Change in absorbance / minutes = X

Weight of the plant material taken = 300 mg

Volume of the extract taken for the assay = 0.02 ml

Change in absorbance for 1.5 ml extract = (X / 0.02 ml) x 1.5 - Y

(i.e) peroxidase activity in 300 mg plant tissue = Y

Peroxidase activity / g plant tissue = Y x (1000 / 300) units

Appendix 9

Estimation of Polyphenol Oxidase (PPO)

(Esterbauer *et al.*, 1977)

Principle

Polyphenol oxidases are copper containing proteins of wide occurrence in nature, which catalyes the aerobic oxidation of phenolic substrates to quinines, which are auto oxidized to dark brown pigments generally known as melanins, which can be estimated spectro-photometrically at 495nm.

Reagents

1. Tris hydrochloride (Tris HCl) (50 mM, pH 7.2)
2. Sorbitol (0.4 M)
3. Sodium chloride (10 mM)
4. Catechol (0.01 M) in phosphate buffer (0.1 M. pH 6.5).

Procedure

The leaves were homogenized in about 20 ml medium containing 50 mM Tris HCl, pH 7.2, 0.4 M sorbitol and 10 mM Sodium chloride. The homogenate was centrifuged at 2000 rpm for 10 minutes and the supernatant was used for the assay. The assay mixture contained 2.5 ml of 0.1 M phosphate buffer and 0.3 ml of catechol solution (0.01 M). The spectrophotometer was set at 495 nm. The enzyme extract (0.2 ml) was added to the same cuvette and the change in absorbance was recorded every 30 seconds up to 5 minutes.

Enzyme unit = K x (ΔA/ minutes)

where,

K for catechol oxidase = 0.272

K for laccase = 0.242

Appendix 10

Estimation of Superoxide dismutase

(Misra and Fridovich, 1972)

Principle

Superoxide dismutase uses the photochemical reduction of riboflavin as oxygen generating system and catalyses the inhibition of nitro blue tetrazolium (NBT) reduction, the extent of which can be assayed spectrophotometrically at 600 nm.

Reagents

1. Potassium phosphate buffer (500 mM, pH 7.8)
2. Methionine (45 µM)
3. Riboflavin (5.3 µM)
4. Nitro blue tetrazolium NBT (84 µM)
5. Potassium cyanide (20 µM)

Procedure

The incubation medium contained a final volume of 3.0 ml, 300 µl of all reagents 50 mM potassium phosphate buffer (pH 7.8), 45 µM methionine, 5.3 mM riboflavin, 84 µM nitro blue tetrazolium (NBT) and 20 mM potassium cyanide. The amount of homogenate added to this medium was kept below one unit of enzyme to ensure sufficient accuracy.

The tubes were placed in an aluminum foil-lined box maintained at 25ºC and equipped with 15W fluorescent lamps. After exposure to light for 10 minutes, the reduced nitro blue tetrazolium was measured spectrophotometrically at 600 nm. The maximum reduction was observed in the absence of enzyme giving a 50 % inhibition of the reduction of Nitro blue tetrazolium.

The SOD activity can be calculated using the formula,

$$\text{SOD activity } \frac{(A - B) - (C - D)}{(A - B)} \times 10$$

Where:

A: Absorbance of control,

B: Absorbance of Blank

C: Absorbance of sample

D: Absorbance value of No XO. (If No XO control is not required, use “B” (Blank) instead.)

Appendix 11

Estimation of Glutathione S-transferase Activity

(Beutler, 1984)

Principle

Glutathione S- transferase conjugates Glutathione with 1-chloro-2,4-dinitrobenzene (CDNB) and the extent of conjugation is used as a measure of enzyme activity from the proportional change in the absorption at 340 nm.

Reagents

1. TRIS hydrochloride
2. Potassium hydrogen phosphate (K_2HPO_4) buffer (0.5 ml, 0.5 M, pH 6.5)
3. 1-chloro-2,4-dinitrobenzene CDNB (25 mM)
4. 20 mM glutathione

Procedure

Ground about 5.0 g of the sample in a medium and made upto 20 ml with the medium containing 50 mM Tris hydrochloride (pH 7.2, 0.4 M sorbitol and 10 mM Sodium chloride). Centrifuged the homogenate at 2000 rpm for 10 minutes and used the supernatant for the assay. Potassium hydrogen phosphate buffer (0.5 ml, 0.5 M, pH 6.5) was taken in the tube and 0.1 ml of 1-chloro-2,4-dinitrobenzene (25 mM) and 8.8 ml of distilled water were added. Incubated the tubes at 37˚C for 10 minutes. Then 0.5 ml of 20 mM glutathione was added to the reaction mixture. Followed by 0.2 ml of enzyme extract. A blank like test was run without the addition of enzyme measured the absorbance at 340 nm. Glutathione S-transferase activity in the extract was expressed as µmoles of CDNB-GSH conjugate / minutes /mg protein.

Appendix 12

Estimation of Ascorbic Acid

(Nirmal *et al.*, 2008)

Principle

Ascorbate is converted to dehydroascorbate by treatment with activated charcoal or bromine. Dehydroascorbic acid then reacts with 2, 4 – dinitrophenyl hydrazine to form osazones, which dissolve in sulphuric acid to give an orange coloured solution. The coloured product can be measured spectrophotometrically at 540 nm.

Reagents

1. Trichloroacetic acid (4 %)
2. Sulphuric acid (9 N)
3. 2, 4 dinitrophenyl hydrazine reagent (2 % in 9 N sulpuric acid)
4. Thiourea solution (10 %)
5. Sulphuric acid (85 %)
6. Standard ascorbate solution: 100 mg ascorbate in 100 ml of 4 % Trichloroacetic acid.
7. Working standard solution: Diluted 10 ml of the stock solution to 100 ml with 4 % Trichloroacetic acid.

Procedure

The plant sample of 1 g was taken and homogenized with 4 % trichloroacetic acid to extract the ascorbate and the final volume was made up to 10 ml with 4 % trichloroacetic acid. The supernatant obtained after centrifugation at 2000 rpm for 10 minutes was treated with a pinch of activitated charcoal, shaken well and kept for 10 minutes. Centrifugation was repeated once again to remove the charcoal residue. The volume of the clear supernatants obtained was noted. Two different aliquots of the supernatant were taken for the assay (0.5 ml and 1.0 ml). The assay volumes were made upto 2.0 ml with 4 % trichloroacetic acid. A range of 0.2 to 1.0 ml of the working standard solution containing 20-100 g of ascorbate respectively were pipetted into clean dry test tubes, the volumes of which were also made upto 2.0 ml with 4 % trichloroacetic acid and 2, 4 dinitrophenyl hydrazine reagent (0.5 ml) was added to all tubes. Followed by 2 drops of 10 % thiourea solution. The osazones formed after incubation at 37˚C for 3 hours, were dissolved in 2.5 ml of 85% Sulphuric acid in cold condition, to avoid an appreciable rise in temperature. To the blank alone, 2, 4 dinitrophenyl hydrazine reagent and thiourea were added after the addition of Sulphuric acid. After

incubation for 30 minutes at room temperature, the samples were read at 540 nm and calculated the content of ascorbic acid in the sample using a standard graph.

Appendix 13

Estimation of α - tocopherol

(Emmerie- Engel 1938 Method described by Rosenberg, 1942)

Principle

The estimation of tocopherol can be done using Emmerie- Engel reaction, based on the reduction of ferric to ferrous ions by tocopherols, which forms a red colour with 2, 2' – dipyridyl. Tocopherols and carotenes are first extracted with xylene and read at 460 nm to measure carotenes. A correlation is made for this after adding ferric chloride and then read at 520 nm.

Reagents

1. Absolute alcohol
2. Xylene
3. 2,2'- dipyridyl (1.2 g in 1 litre of n- propanol)
4. Ferric chloride (1.2 g in 1 litre of ethanol stored in brown bottle)
5. Standard solution
6. Sulphuric acid (0.1 N)

Dissolved 10 mg /10 ml of α-tocopherol, 10 mg / 1 L in absolute alcohol. (91 mg of α-tocopherol is equivalent to 100 mg of tocopherol acetate).

Procedure

The plant sample (2.5 g) were homogenized in a small volume of 0.1 N sulphuric acid and the volume was finally made upto 50 ml by adding 0.1 N sulphuric acid slowly, without shaking and the contents were allowed to stand overnight. The contents of the flask were shaken vigourously on the next day and filtered through Whatman No.1 filter paper. Aliquots of the filtrate were used for the estimation of tocopherol. To the test and blank, added 1.5 ml of ethanol and to the standard, added 1.5 ml of water. Then added 1.5 ml of xylene to all the tubes stoppered, mixed well and centrifuged. After centrifugation, the xylene layer was transferred into another tube, taking care not to include any ethanol or protein. To 1.0 ml of xylene layer, 1.0 ml of 2, 2' dipyridyl reagent was added, stoppered and mixed. This reaction mixture was taken in the spectrophotometric cuvettes and the

extinction of the test and the standard were read against the blank at 460 nm. In turn, beginning with the blank, 0.33 ml of ferric chloride solution was added, mixed well and exactly after 15 minutes, the test and the standard were read against the blank at 520 nm. The level of tocopherol can be calculated using the formula,

$$\text{Amount of tocopherols in } \mu g = \frac{\text{Reading at 520 nm} - \text{Reading at 460 nm}}{\text{Reading of standard at 520 nm}} \text{ X } 0.29 \text{ X } 15$$

The results are expressed as µg tocopherol /g of sample.

Appendix -14

Estimation of Flavonoids

(Cameron *et al.*, 1943)

Reagents

1. Methanol
2. Chloroform
3. Vanillin reagent - (1 % vanillin in 70 % Sulphuric acid)
4. Catechin standard - (110 µg/ml)

Procedure

A portion of the sample was weighed out and the extraction was carried out in two steps, first, with methanol: H_2O (9:1) and second, with methanol: H_2O (1:1). In each step, sufficient solvent was added to make a liquid slurry and the mixture was left for 6-12 hrs. Filtration to separate the extract from the sample was carried out rapidly by using a glass wool or cotton wool plug in the neck of a filter funnel. The two extracts were then combined and evaporated to about 1/3 the original volume or until most of the methanol had been removed. The resultant aqueous extract was cleared off the contaminants such as fats, terpenes, chlorophylls and xanthophylls by extraction (in a separatory funnel) with hexane or chloroform. This was repeated several times and the extracts combined. The solvent-extracted aqueous layer containing the bulk of the flavonoids was then concentrated. An aliquot of the extract was pipetted into a test tube and evaporated to dryness. Then added 4.0 ml of vanillin reagent (1 per cent vanillin in 70 per cent sulphuric acid) and heated for 15

minutes in a boiling water bath. The standard (catechin 20-100 μg) was also treated in the same manner. After that the absorbance was measured at 360 nm.

Appendix 15

Estimation of phenols

(Malick and Singh, 1980)

Principle

Phenols react with phosphomolybdic acid in Folin -Ciocalteau reagent in alkaline medium and produce blue coloured complex (molybdenum blue), that is measured at 650 nm.

Reagents

1. 80 % ethanol
2. Diluted Folin-Ciocalteau reagent
3. 20 % Sodium carbonate
4. Stock solution:

   100 mg of catechol was made up to 100 ml with distilled water

5. Working standard:

   10 ml of stock standard was diluted to 100 ml. 1.0 ml of this contains 100 μg of catechol.

Procedure

1g of sample was homogenized using 20 ml of 80 % ethanol. The homogenate was centrifuged at 10,000 rpm for 20 minutes. The supernatant was saved. The residue was reextracted with 10ml of 80 % ethanol, centrifuged and collected the supernatant and evaporated to dryness. The residue was dissolved in a known volume of distilled water (50 ml) and 2.0 ml was taken for the experiment. A working standard of 0.5 – 2.5 ml catechol solution corresponding to 50 – 250 μg of catechol were pipetted out into a series of test tubes. The volume was made upto 2.5 ml with water. To all the tubes added 0.5 ml of diluted Folin–Ciocalteau reagent. After 3 minutes, added 2.0 ml of 20 % Sodium Carbonate solution to each tube and mixed thoroughly.

The tubes were placed in a boiling water bath for exactly one minute. Cooled and measured at 650 nm against a reagent blank. Constructed a standard graph by plotting the concentration of catechol on X-axis and absorbance on Y-axis.

From the graph, the amount of polyphenols present in the sample was estimated and expressed as mg of polyphenols per g of the sample.

Appendix 16

Estimation of reduced glutathione

(Moron *et al.*, 1979)

Principle

Reduced glutathione (GSH) is measured by its reaction with 5, 5-dithio- 2 -nitrobenzoic acid (DTNB) (Ellman's reaction) to give a compound that absorbs at 412 nm.

Reagents

1. 5, 5-dithio- 2 -nitrobenzoic acid (DTNB)
2. 5 % Trichloroacetic acid (TCA)
3. 0.2 M Sodium phosphate buffer
4. Phosphate buffer (0.2 M)
5. Standard Glutathione (GSH) (10 nmoles/ml in 5 % TCA)

Procedure

1 g of the sample was homogenized in 5 % trichloroacetic acid to give a 20 % homogenate. The precipitated protein was centrifuged at 1000 rpm for 10 minutes. The homogenate was cooled on ice and 0.1 ml of supernatant was taken for the estimation. The volume of the aliquot was made upto 1.0 ml with 0.2 M sodium phosphate buffer (pH 8.0), 2 ml of freshly prepared 5, 5-dithio- 2 -nitrobenzoic acid solution (0.6 mM) in 0.2 M phosphate buffer (pH 8.0), was added to the tubes and intensity of the yellow colour formed was read at 412 nm in a spectrophotometer after 10 minutes.

A standard curve of Glutathione was prepared using concentration ranging from 2 to 10 nmoles of Glutathione in 5 % Trichloroacetic acid.

Appendix 17

Column Chromatography

(Bajpai *et al.*, 2016)

Principle

When the mobile phase alongwith the mixture that needs to be separated is introduced from the top of the column, the movement of the individual components of the mixture is at different rates. The components with lower adsorption and affinity to stationary phase travel faster when compared to the greater adsorption and affinity with the stationary phase. The components that move fast are removed first, where as the components that move slow are eluted out last.

The adsorption of solute molecules to the column occurs in a reversible manner. The rate of the movement of the components is expressed as:

Rf = the distance travelled by solute/ the distance travelled by solvent

Rf is the retardation factor.

Procedure

The stationary phase was made wet with the help of the solvent as the upper level of the mobile phase and the stationary phase should match. The mobile phase or eluent is either solvent or mixture of solvents. In the first step, the compound mixture that needs to be separated was added from the top of the column without disturbing the top level. The tap was turned on and the adsorption process on the surface of silica was begun.

Without disturbing the stationary phase, the solvent mixture was added slowly by touching the sides of the glass column. The solvent was added throughout the experiment as per the requirement. The tap was turned on to initiate the movement of compounds in the mixture. The movement was based on the polarity of molecules in the sample. The non-polar components moved at a greater speed when compared to the polar components. For example, a compound mixture consisting of three different compounds viz red, blue, green then their order based on polarity will be as follows blue>red>green. As the polarity of the green compound was less, it moved first. When it arrived at the end of the column it was collected in a clean test tube. After this, the red compound was collected and at last blue compound was collected. All these were collected in separate test tubes.

Appendix 18

Thin Layer Chromatography (TLC)

(Smith *et al.*, 1997)

The hydroethanolic extract of the leaves of *Camellia sinensis* was analysed by thin layer chromatography. Thin layer chromatography technique is easy to perform and requires a simple apparatus. The mixture of compounds to be separated was placed near one end of the Thin Layer Chromatography plate and allowed to dry. The plate was then placed with this end dipping in the solvent mixture, taking care that the sample spot was not immersed in the solvent. As the solvent moved towards the other end of the plate, the test mixture separated into various components. The plate was removed after an optimal development time, dried and the spots are detected using a suitable location reagent. The silica gel acted as an inert support, the interstices of which held the more polar phase of the solvent mixture which thus acted as the stationary phase, the less polar phase acted as the mobile phase. Separation resulted from differences in partition equilibrium of the components in the mixture. However, the silica gel interacted with the components and these affected the separation. 10 µl of the plant extract was applied on (E.Merck) Aluminum plate pre-coated with Silica gel 60 F254 of 0.2 mm thickness using Hamilton syringe and CAMAG LINOMAT 5 instrument. The plate was developed in chloroform: methanol (1:9 v/v). After air drying, the plate was visualized in visible light. The results of the Thin Layer Chromatography profile (Rf) and Thin Layer Chromatography image were taken.

The Rf value of a compound is equal to the distance traveled by the compound divided by the distance traveled by the solvent front (both measured from the origin).

Appendix 19

Vanillin Assay

(Price *et al.*, 1978)

The vanillin reaction involves reaction of an aromatic aldehyde, vanillin, with the meta- substituted ring of flavanols to yield a red adduct. Catechin is commonly used to standardize the vanillin reaction, but there are problems with interpreting the meaning of catechin equivalents. Under normal conditions for the vanillin assay (methanol solvent), tannins (proanthocyanidins) and catechin both react with vanillin, but the rates of reaction of

the polymer and the monomer are quite different. In general, the colour yield is lower for the monomer than for the polymer. Although the absorbances obtained from running the vanillin reaction in methanol on an unknown tannin- containing sample can be converted to catechin equivalents the complexities of the system make it difficult to interpret the meaning of those equivalents at the molecular level.

Reagents

- 1 % vanillin in methanol (1.0 g vanillin up to 100 ml with absolute methanol). Store in a dark bottle at 4°C
- 8 % concentrated hydrochloric acid in methanol (8.0 ml concentrated Hydrochloric acid brought to 100 ml with absolute methanol).
- 4 % concentrated hydrochloric acid in methanol (4.0 ml concentrated Hydrochloric acid brought to 100 ml with absolute methanol).
- Constant temperature water bath set at 30ºC. (If this is not available, there will be temperature- dependent variation in the data).
- 0.3 mg/mL catechin (3.0 mg catechin brought to 10.0 ml with absolute methanol). Stored in a dark bottle at 4° C for up to three days.

Preparation of Working reagents

The working vanillin reagent must be prepared daily from the solutions described above. One part of the 1 % vanillin solution was mixed with one part of the 8 % hydrochloric acid solution. The working vanillin reagent and the 4 % hydrochloric acid solution are brought to 30°C in the water bath before starting the analysis each day**.**

Analysis of Standards

1. 0 to 1.0 ml aliquots of the catechin standard and extract were dispensed into two sets of culture tubes and each sample was brought to 1.0 ml, by the addition of absolute methanol. Tubes were then incubated in the water bath.
2. 5.0 ml of the working vanillin reagent was added at 1.0 minutes intervals to one set of standards, and 5.0 ml of 4% hydrochloric acid solution at 1.0 minutes intervals to the second set of standards.
3. The samples were left in the water bath for exactly 20.0 minutes, and then removed and the absorbance read at 500 nm.

Because the color develops over time, any sample has to conform to the precise 1.0 minute intervals for reading that were utilized in the reagent addition. The absorbance of the blank (vanillin reagent with no catechin) was subtracted from the absorbance of the corresponding vanillin- containing sample. A standard curve was constructed (Abs vs. mg catechin) and the linear portion of the curve was extrapolated to produce the standard curve. The value obtained was compared to the standard curve to obtain catechin equivalents.

Appendix 20

High Performance Thin Layer chromatography (HPTLC)

Khushboo *et al.*, 2009

Principle

Separation may result due to adsorption and partition depending upon the nature of absorbent used on plates and solvent system used for the development.

Procedure

Test solution preparation

The plant sample (50 mg) was weight in an electronic balance (Afcoset) accurately, dissolved in 1 ml of methanol and centrifuged at 3000 rpm for 5 minutes this solution was used as test solution for HPTLC analysis.

Sample application

2µl of test solution and 2 µl of standard solution were loaded as 6 mm band length in the 3 x 10 cm Silica gel $60F_{254}$ TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument.

Spot development

The sample loaded plate was kept in TLC twin trough developing chamber (after saturation with solvent vapour) with respective mobile phase and the plate was developed in the respective mobile phase up to 90 mm.

Photo-documentation

The developed plate was dried in a hot air oven to evaporate the solvents from the plate. The plate was kept in the photo-documentation chamber (CHMAG REPROSTAR 3) and captured the images in visible light, UV 254 nm and UV 366 nm.

Derivatization

The prepared plate was dried in a hot air oven at 100^{o}C after being sprayed with the appropriate spray reagent. The plate was photo-documented in visible light and UV 366 nm mode using the photo-documentation (CAMAG REPROSTAR 3) chamber.

Scanning

After derivatization, the plate was fixed in the scanner stage (CAMAG TLC SCANNER 3) and scanning was done in visible light (500 nm). The peak table, peak display and peak densitogram were noted. The software used was win CATS 1.3.4 version.

Appendix 21

Fourier Transform-Infrared Spectroscopy (FTIR)

(Nasir*et al.*, 2006)

Principle

Fourier Transform-Infrared Spectroscopy is very much helpful in examining the peak variation of amino groups and carboxylic groups. Some of the infrared radiation is absorbed by the sample and some of it gets passed through (transmitted). The resulting spectrum represents the molecular absorption and transmission, creating a molecular fingerprint of the sample, which corresponds to the frequencies of vibrations between the bonds of the atoms making up the material. Because each different material is a unique combination of atoms, no two compounds produce the exact same infrared spectrum. In addition, the size of the peaks in the spectrum is a direct indication of the amount of material present

Procedure

Infrared Spectroscopy Shimadzu Corporation of model IR prestige 21 was used. A drop of each extract was applied on a sodium chloride cell to obtain a thin layer. The cell was mounted on the IR region from 400 to 4000 cm-1 and scanned through the IR region.

Appendix 22

Nuclear Magnetic Resonance spectroscopy (NMR)

(Moore and Dalrymple, 1997)

NMR is concerned with the magnetic properties of certain atomic nuclei, notably the nucleus of the hydrogen atom-the proton-and that of the carbon-13, an isotope of carbon.

Studying a molecule by NMR spectroscopy enables us to record differences in the magnetic properties of the various magnetic nuclei present and to deduce in large measure whether the positions of these nuclei are within the molecule and also which atoms are present in neighboring groups. It can measure how many atoms are present in each of these environments Two-dimensional heteronuclear multiple quantum coherence transfer (2D HMQCT-NMR) spectra were recorded on a Bruker DRX500 NMR instrument operating at 500 MHz, for 6 hours at room temperature. The region from 0 to 12 ppm for 1H and 0– 200 ppm for 13º C was employed for scanning. Signals were referred to tetramethyl silane to within ± 0.01 ppm. About 30 mg sample dissolved in 0.5 ml DMSO was used for recording the spectra.

Appendix 23

Gas Chromatography- Mass Spectrometry (GC-MS) Analysis (Maciejewicz *et al.*, 2001)

Principle

As the name implies, it is actually two techniques that are combined to form a single method of analysing mixtures of chemicals. Gas chromatography separates the compounds of a mixture and mass spectroscopy characterizes each of the compounds individually. By combining the two techniques, a solution can be studied both qualitatively and quantitatively. Containing a number of chemicals.

Procedure

Chromatographic analysis of derivatized extracts were performed with a GC-MS Hewlett Packard 6890 Series II instrument equipped with an on-line injection system a mass selective detector Model HP5973A. A capillary column HP-J fused silica column (30 m x 0.25 mm, column thickness 0.25 m (5 %)-biphenyl-(95 %) dimethylsilioxane copolymer) was used. The carrier gas was at 0.21 ml/minutes constant flow. The compounds were separated by following method: isothermal at 100˚C for 15 minutes, 250˚C at 15˚C /minutes and finally isothermal at 280˚C for 10 minutes. Mass spectra were obtained in electron impact ionization at 70 eV. The injection volume was 1 µl (split ratio 60:1). The identification of compounds was accomplished using computer searches in commercial libraries.

Appendix 24

Liquid Chromatography Mass spectrometry (LC-MS)

(Bouhafsoun *et al.*, 2018)

LC–MS/MS analysis of the phenolic compounds was performed by using a Nexera model Shimadzu UHPLC coupled to a tandem MS instrument. The liquid chromatography was equipped with LC-30AD binary pumps, DGU-20A3R degasser, CTO-10ASvp column oven and SIL-30AC auto sampler. The chromatographic separation was performed on a C18 reversed-phase Inertsil ODS- 4 (150 mm × 4.6 mm, 3 μm) analytical column. The column temperature was fixed at 40 °C. The elution gradient consisted of mobile phase A (water, 5 mM ammonium formate and 0.1% formic acid) and mobile phase B (methanol, 5 mM ammonium formate and 0.1% formic acid). The gradient program with the following proportions of solvent B was applied t (minutes), B%: (0, 40), (20, 90), (23.99, 90), (24, 40), (29, 40). The solvent flow rate was maintained at 0.5 ml/minutes and injection volume was settled as 4 μl.

MS instrumentation

MS detection was performed using Shimadzu LCMS 8040 model triple quadrupole mass spectrometer equipped with an ESI source operating in both positive and negative ionization modes. LC–MS/MS data were collected and processed by Lab Solutions software (Shimadzu, Kyoto, Japan). The multiple reaction monitoring (MRM) mode was used to quantify the analytes: the assay of investigated compounds was performed following two or three transitions per compound, the first one for quantitative purposes and the second and/or the third one for confirmation. The optimum ESI conditions were determined as interface temperature; 350° C, DL temperature; 250° C, heat block temperature; 400° C, nebulizing gas flow (nitrogen); 3 L/minutes and drying gas flow (nitrogen); 15 L/minutes.

Appendix 25

Synthesis of Zinc oxide nanoparticles

(Gnanasangeetha and Thambavani, 2014) Slightly modified method

Zinc acetate solution of 0.01 M was taken and plant extract was added. The pH 12 of the mixture was maintained and the solution was stirred continuously for 2 h. A white

precipitate resulted which was then dried at 60ºC overnight. Prior to drying, the precipitate was centrifuged at 15,000 rpm for 5 minutes and washed twice with sterile de-ionized water. Complete conversion to ZnO nanoparticles took place during drying. However, the synthesis conditions were optimized for the current reaction by varying various parameters involved in synthesis. Various concentrations of zinc acetate, from .0025 M to .02 M, were used as substrates. Plant extract was added to 50 ml of zinc acetate solution in volumes ranging from 0.25 ml to 2 ml. The mixture was stirred continuously using a magnetic stirrer and was maintained at increasing pH values of 9, 10, 11, 12 and 13 using 2 M sodium hydroxide solution (2-3 ml). Finally, reaction temperature was maintained at 60, 70, 80 and 90º C. The same temperature at which synthesis was carried out was used for overnight drying of the precipitate obtained.

Appendix 26

Antioxidant capacity

(Rajeshkumar *et al.*, 2018)

Antioxidant capacity was measured by reduction of the absorbance of DPPH (2,2-diphenyl -1-picrylhydrazyl) radical in the presence of the synthesized zinc oxide nanoparticles. The deep violet color of DPPH turns yellow in the presence of an antioxidant compound. When DPPH is mixed with a hydrogen donor substance, free radicals are reduced and a color change occurs. The different volume of the samples was added to 1 ml of 0.1 mM DPPH solution in methanol. The solution mixture was incubated for 30 minutes at room temperature in the dark. The absorbance was measured at 517 nm after the incubation period to estimate the reduction in DPPH free radical number. Methanol solution mixed with DPPH was used as a control, vitamin C was used as the standard and methanol plus samples solution was used as a blank. All the experiments were performed in triplicate. DPPH free radical scavenging activity was calculated by the following formula;

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control} - \text{ Absobance of Sample Absorbance of contro}}{\text{Absorbance of control}} * 100$$

Appendix 27

Acetylcholinesterase inhibition activity

(Ingkaninan *et al.*, 2003)

3 ml of 50 mM Tris–HCl buffer (pH 8.0), 100 µl of sample solution at different concentrations (3 mg/ml, 1.5 mg/ml, 0.75 mg/ml) and 20µl AChE (6 U/mL) solution were mixed and incubated for 15 minutes at 30ºC, a 50 µl volume of 3 mM 5, 50-dithiobis-(2-nitrobenzoic acid) (DTNB) was added to this mixture. The reaction was then initiated by the addition of 50 µl of 15 mM acetyl thiocholine iodide (AChl). The hydrolysis of this substrate was monitored at precisely 405 nm wavelength. At this wavelength, the formation of yellow 5-thio-2-nitrobenzoate anion was noticed as the result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine iodide. The enzymatic activity was calculated as a percentage of the velocities compared to that of the assay using buffer instead of inhibitor (extract), based on the formula:

$$\mathbf{EA} = \frac{E-S}{E} \mathbf{X\ 100}$$

Where E is the activity of the enzyme without test sample and S the activity of the enzyme with test sample.

Appendix 28

Preparation of synthesized zinc oxide nanoparticle-capped catechin

(Arasoglu *et al.*, 2017)

10 mg of CAT were dissolved in 5 ml of ethanol and 100 mg of zinc oxide nanoparticle dissolved in 5 ml of 0.01N HCl. 10 mg of catechin was added to 5 ml of ethanol under stirring until it was dissolved. After that 5ml of zinc oxide nanoparticle solution was added drop wise into 20ml of catechin and zinc oxide nanoparticle mixture under stirring. Then, the mixture solution was brought to pH 5.0 by addition of aqueous sodium hydroxide. For, emulsification, 120s of sonication (80% amplitude) and 30minutes at 24,000 rpm at room temperature were applied respectively. The Synthesized zinc oxide nanoparticle-capped catechin was collected by centrifugation at 16,000 g for 30 minutes and freeze dried at -50ºC for 24hr.

Appendix 29

Determination of Entrapment efficiency and Loading capacity (Arasoglu *et al.*, 2017).

The entrapment efficiency and loading capacity of synthesized zinc oxide nanoparticle-capped catechin were determined as follows: synthesized zinc oxide nanoparticle-capped catechin suspensions were centrifuged at 16,000 × g for 30 minutes. The free catechin in the clear supernatant was measured in triplicates as described previously (Arasoglu *et al.,* 2017) with some modifications. The total volume of reaction medium was fixed at 10 ml, comprising of 1 ml of the supernatant, 4.5 ml of 1% (w/v) vanillin (prepared freshly in methanol), and 4.5 ml of 9 M HCl solution. The mixture was vortexed for 1 minute and then allowed to react for 30 minutes at room temperature. Afterwards, its absorbance was recorded at 500 nm with a UV-1800 spectrophotometer (Shimadzu Corporation in Japan), and the amount of free catechin in the supernatant was calculated by comparison with a standard curve of catechin ranging from 2 to 7 μg/ml ($y = 0.013$ x $R^2 = 0.989$). The entrapment efficiency and loading capacity were determined according to the following formula:

Entrapment Efficiency (%) = (T –F) / T x 100 …… (1)

Loading Capacity (%) = (T –F) / W x 100 …… (2)

Where T is the total amount of catechin in the solution, F is the amount of free catechin in the supernatant after ultrafiltration, and W is the mass of synthesized zinc oxide nanoparticle-capped catechin measured after freeze-drying.

Appendix 30

In vitro release

(Derman *et al.*, 2015)

The *in vitro* release of synthesized zinc oxide nanoparticle-capped catechin were conducted to determinate the release of catechin from the synthesized zinc oxide nanoparticle-capped catechin according to a previously published dissolution method (Derman *et al.,* 2015). Lyophilized nanoparticles were suspended in the release medium,

phosphate-buffered saline with 0.01% sodium azide, and incubated at 37° C in a shaking incubator (60 rpm), from which at predetermined time intervals up to 120 hrs (1, 2, 3, 4, 12, 24, 48, 72, 96, and 120 hrs) medium was fully removed and analyzed for catechin contents. The catechin concentration in the supernatant was measured with UV-Vis spectroscopy at 374 nm by comparing the concentration to a previously constructed standard calibration curve.

Appendix 31

TLC with bioassay detection for AchE inhibition

(Machado *et al.*, 2015)

The AChEI activity of sample was detected by using a thin layer chromatography (TLC) autographic assay. Aliquots of 100µg of each dried seaweed extract and 0.3 µg of Physostigmine (Sigma, used as positive control) were dissolved, spotted on TLC layers (Silica gel 60 F254, 10 × 10 cm, layer thickness 0.2 mm, E. Merck, Germany), which were developed with mobile phase hexane: ethylacetate: methanol (2:7:1 v/v/v), and then dried. Next, the plates were sprayed with the enzyme solution (6.66 U/ml) (Electric eel AChE type V, product no. C 2888, 1000 U – Sigma–Aldrich), thoroughly dried, and incubated in a humid atmosphere at 37◦C for20 minutes. Afterwards, the plates were sprayed with 0.25% of 1-naphthylacetate in ethanol (5 ml) plus 0.25 % of aqueous Fast BlueB salt solution (20 ml). The spots corresponding to potential acetyl-cholinesterase inhibitors were identified as clear zones against a purple background. Retention factor values (R_f) of bioactive compounds were determinate and employed for their preparative scale isolation by thin-layer chromatography (20 cm × 20 cm, layer thickness1.5 mm, 60 F 254, Sigma–Aldrich). Extract samples of 80 mg were applied on preparative plates which were developed under identical conditions to those used at analytical scale essays. After elution, the AChE positive regions were removed from the plates and extracted with DCM/MeOH (90:10 v/v).

Appendix 32

MTT (dimethylthiazolyl-20, 50-diphenyl-2-H-tetrazolium bromide) Dye Reduction Assay (Zheng *et al.*, 2018)

Principle

Living cells convert MTT into its formazon derivative. The number of surviving cells can be determinate by the amount of MTT formazon produced, which is measured in a microtiter plate reader after solubilization with a suitable solvent.

Reagents

1. Phosphate-buffered saline (PBS)
2. MTT (5mg/ml in PBS)
3. Dimethyl sulfoxide (DMSO)

Procedure

The dose dependent inhibition of cell viability assessed by MTT (dimethylthiazolyl-20, 50-diphenyl-2-H-tetrazolium bromide) assay based on the presence of mitochondrial reductase enzyme in live cell, which converts the MTT into purple color formazan crystals. The cells were plated at $1x10^4$ in 96 well plate. Cells were treated with different concentration of rivatigmine and synthesized zinc oxide nanoparticle-capped catechin (2-25µg) for 24h time point. After treatment period 100µl of MTT solution (0.5mg/ml) was added to each well and kept at CO_2 incubator for two hour. MTT containing solution was removed and then 100µl of DMSO added to each well to dissolve formazan crystals and kept in the dark for one hour. Formazan crystals dissolved in DMSO and produced a purple color. The intensity of the color was measured using a microplate reader (BioTek, USA) at 570 nm. The percentage cell viability was measured using the formula:

$$\text{Cell viability} = \frac{\text{O.D of treated cells}}{\text{O.D of control cells}} \times 100$$

Appendix 33

Lactate dehydrogenase (LDH) assay

(Kumar *et al.*, 2018)

LDH is a stable cytosolic enzyme present in the cytosol with intact cell membrane but it released once the cell loses membrane integrity or damage and it can be measured quantitatively by LDH assay. For LDH leakage assay, the same MTT assay treatment protocol was used and the conditioned medium alone was taken for the assay. 0.1 ml of condition media was added to 1 ml of buffered substrate, and kept in water bath at 37˚C. Then NAD+ (0.2 ml) solution was added and mixed, after 15 minutes incubation at 37˚C 1ml of 2, 4-Dinitrophenylhydrazine solution was added and incubated for 15 minutes. Lastly, 0.4 N of sodium hydroxide (10 ml) was added and incubated for 1–5 minutes and the absorbance was measured at 440 nm. Sodium pyruvate was used as standard to prepare the standard graph.

$$\text{LDH activity} = \frac{\text{OD of unknown}}{\text{OD of known} \times \text{standard concentration} = \mu\text{g of Lactate} \frac{\text{liberated}}{\text{ml}} \text{of conditioned media}}$$

Appendix 34

Intracellular ROS measurement and Oxidative damage

(Chen *et al.*, 2019)

The intracellular ROS level in treated neuro 2a cells was analyzed by DCFDA staining. The cells were grown in 24 well plate and treated with rivatigmine and synthesized zinc oxide nanoparticle-capped catechin (10-20 µg) for 24 h time point After SDF treatment period, the cells were incubated with 200 µl of DCFDA (10 µM) working solution at 37° C for 20 minutes. Following drug treatment, cells were washed three times with PBS, incubated with 10 μM DCFH-DA for 30 minutes at 37° C in the dark and washed three times with PBS to remove the extracellular DCFHDA and examined the intracellular ROS level under fluorescence microscope.

Appendix 35

Assay of Superoxidase Dismutase

(Zhao *et al.*, 2016)

Superoxidase Dismutase content measurement neuro 2a cells cultured for 24 h in 6-well plates (4×10^4 cells/well) were treated with vehicle or 100 μg/ml rivatigmine and synthesized zinc oxide nanoparticle-capped catechin (10-20 µg) for 8 h. They were digested with trypsin and centrifuged at 1000 rpm for 5 minutes. Thereafter, the cells were suspended in 500 μl PBS and lysed by sonication in the presence of a protease inhibitor, followed by centrifugation at 4000 rpm for 15 minutes. The supernatant was collected for analysis, according to the assay kits (Jian Cheng Biology, China) manufacturer instructions. Superoxidase Dismutase in the cell homogenates were determined by colorimetry at 550 nm. The activities of these enzymes were expressed as U/mg protein.

Appendix 36

Assay of Acetylcholinesterase

(Marinelli *et al.*, 2017)

Acetylcholinesterase activities in the cultures were analyzed colorimetrically using the Acetylcholinesterase Assay Kit from Abcam® (Cambridge, MA, USA). According to the manufacturers instructions, 50 µl of supernatant was transferred to a new plate and 50 µl of the master mix was added and stored for 30 minutes at room temperature protected from light. Finally, each plate well was measured at 410 nm wavelength by a micro plate reader.

Appendix 37

Cell cycle analyses by flow cytometer

(Valdiglesias *et al.*, 2011)

The cells (1×10^6 cells/per plate) were cultured in 100-mm culture plates containing growth medium. After starvation, the cells were treated with 10 µg/ml of rivatigmine and synthesized zinc oxide nanoparticle-capped catechin for 24 h, then the cells were harvested with 0.25 % trypsin and centrifuged at 3000x g for 5 minutes. The cells were washed with PBS. After centrifugation, the cells were fixed in 100 % ice-cold methanol overnight at -

20°C. The cells were then incubated in 50 μg/ml of propidium iodide in PBS and 1 mg/ml of ribonuclease in PBS for 30 minutes. Cell cycle analyses were performed on a BD FACS Canto^{TM}II (Becton and Dickinson Biosciences, Mountain View, CA, USA), and the data were analysed using BD FACS Canto clinical software.

Avinashilingam Institute for HomeScience and Higher Education for Women
(Deemed to be University Estd. u/s 3 of UGC Act 1956, Category 'A' by MHRDRe-accredited with A++ Grade by NAAC. CGPA 3.65/4, Category I by UGC
Coimbatore - 641 043, Tamil Nadu, India

Appendix L2
(Item No 5 of Check List)
Details of Research Publications

S.No	Article	Journal	Other Details Vol/No/Page No/ Year	Published in UGC-CARE / Scopus Indexed/ Web of Science
1	Molecular Docking and Therapeutic Targets of Flavanol Compounds from *Camellia Sinensis* on Alzheimer's Disease	The Indian Journal of Nutrition and Dietetics	2022 , 59 (2), 152-158 ISSN: 0022-3174; eISSN: 2348-621X	UGC-CARE
2	*In vitro* antioxidant and acetylcholinesterase activities of catechin-loaded green fabricated zinc oxide nanoparticles	Journal of Applied Biology and Biotechnology	Vol. 11(6), pp. 178-184, Nov-Dec, 2023 ISSN 2455-7005, eISSN: 2347-212X	Scopus

*Proof of list of Journals from Internet to be attached along with copies of reprints.

Scholar : Nandhini.B

Supervisor : 3/1/2024

Checked By:

HoD/Dean of Respective School

The scholar Miss. Nandhini, B (IMPHBCF002) has published her article in the following journals:

1. The Indian Journal of Nutrition and Dietetics - indexed and active in UGC care Grp. I from January 2021 to present.
2. Journal of Applied Biology and Biotechnology - is indexed and active in Scopus from 2019 to present.

This may be considered.

03.01.2024.

The Indian Journal of Nutrition and Dietetics
ISSN: 0022-3174; eISSN: 2348-621X
www.ijndonline.org

DOI:10.21048/IJND.2022.59.2.29907

Molecular Docking and Therapeutic Targets of Flavanol Compounds from *Camellia Sinensis* on Alzheimer's Disease

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Abstract

Alzheimer's disease is a progressive neurodegenerative disorder with neuropsychiatric symptoms and several cognitive functions and is biochemically characterized by a significant decrease in the brain neurotransmitter acetylcholine. The current study is performed to investigate possible interaction of the active components identified from Camellia sinensis (Green tea) with acetyl cholinesterase (AChE) through docking studies using Schrödinger software (Maestro V: 11.8 Schrödinger_suite-2019). In silico study results clearly showed that catechin and epicatechin-3-gallate binds effectively with AChE through strong hydrogen bonding.

Keywords: *Alzheimer's disease, acetylcholinesterase, catechin, epicatechin-3-gallate, docking, Camellia sinensis*

Introduction

Alzheimer's disease is a progressive neurodegenerative disorder in the elderly population with a predicted incidence of 115.4 million cases by 2050. It is characterized by pathological inclusions of extracellular ß-amyloid (Aß) plaques and intracellular neurofibrillary tangles[1]. Neuroprotective activity of natural flavonoids encompasses multiple effects within the brain, including their efficacy to shelter against neurotoxin-induced neuronal injury, to endorse learning memory, cognitive function which and to suppress the neuronal inflammation Dietary consumption of flavonoid rich foods such as cocoa, green tea and berries grasp the efficacy to attenuate neurodegeneration and averts or reverses the age-dependent deteriorations of cognitive function[2]. Inhibition of Acetylcholinesterase is considered a promising strategy for the treatment of some diseases caused by the

too low level of AChE, such as glaucoma, myasthenia gravis, gastric motility, and Alzheimer's disease[3]. Currently available many acetylcholinesterase inhibitors have been approved for the treatments for AD (donepezil, rivastigmine, galantamine, and memantine) which are symptomatic and do not decelerate or prevent the progression of the disease but they cause headache, bradycardia, nausea, vomiting, anorexia, and diarrhea, dizziness, insomnia, constipation, mild allergies, and auditory hallucination side effects. The enhancement of novel anti AD treatment is in high demand. The use of medicinal plants in the treatment of a variety of diseases has become increasingly widespread in recent years[4]. In the *in silico* study, investigated the AChE protein target with flavanol compounds against the molecular docking software and its possible ligand-protein interaction.

Materials and Methods

Protein target preparation

The 3D structure of acetylcholinesterase (AChE) was retrieved from the RCSB PDB database (http://www.rcsb.org/pdb/home/home.do). PDB code is 5EIE. They were saved as a Brookhaven protein data bank file.

Ligand generation

The 2D structure of epicatechin, catechin, epicatechin-3-gallate, epigallocatechin, epigallocatechin-3-gallate and gallic acid were retrieved from the PubChem online database.

Maestro

Maestro is the graphical user interface for all the Schrödinger products such as Combi Glide, Epik, Glide, Impact, Liasion, LigPrep, Phase, and Maestro model, Prime, QikProp and Qsite. Maestro is a powerful and versatile molecular modelling environment and the portal to the most advanced science in computational chemistry.

QikProp 3.0

QikProp 3.0 module of Schrödinger is a quick, accurate, easy-to-use absorption, distribution, metabolism, and excretion (ADME) prediction program. It predicts pharmaceutically relevant properties and physically significant descriptors of organic molecules. The ADME properties of all the ligands were detected by QikProp.

Ligand preparation 3.6

The preparation of the ligand was done using LigPrep 3.6, a module on the Maestro window of Schrödinger it is a robust quality, all-atom 3D structure for a large number of drug-like molecules, starting with 2D or 3D structure in Maestro format.

GLIDE grid-based ligand docking with energetics

Molecular docking was done using Glide (Schrödinger Module Version 9.4).

This searches for favorable interactions between one or more ligands and a receptor molecule usually a protein. The ligand poses that are generated by GLIDE pass through a series of hierarchical filters that evaluate the ligand interaction with the receptor.

Protein preparation wizard

Schrödinger Suite of programs, the bulk of receptor preparation is carried out with the protein preparation wizard. The protein preparation wizard aggregates automates and integrates the most frequently used tools and techniques in structure preparation, without shoehorning the researcher into a single inflexible process. In this study, the Glide Xtra precision (XP) docking procedure is used. The docked complex structures were viewed with Glide Pose Viewer. The Glide XP mode uses a more stringent scoring function than the Standard Precision (SP) mode. Glide XP was designed to remove false positives, the good ligand poses that were generated Glide SP docking was used as input for Glide XP docking.

Results and Discussion

Molecular docking study

Three dimensional (3D) structure of acetylcholinesterase (PDB ID: 5EIE) was obtained from Protein Data Bank (https://www.rcsb.org/pdb). A protein-ligand interaction study has been performed through Schrödinger software.

Table I shows the ADMET properties of catechin and epicatechin-3-gallate the drug-like activity of the ligand molecule. The bioavailability was found to be less than 70% and the predicted LD 50 value. The probability of ADMET Hepatotoxicity was 0. Based on several drug parameters,

TABLE I

ADMET Properties of Catechin and Epicatechin-3-gallate

Property	ADMET properties	Epicatechin-3-gallate	Catechin
Absorption	Water solubility	-2.911	-3.117
	$CaCO_2$ permeability	-0.264	-0.283
	% of human oral absorption	62.094	68.829
Distribution	BBB permeability	-1.847	-1.054
Metabolism	CYP2D6 substrate	NO	NO
	CYP1A2 Inhibitor	NO	NO
Excretion	Total Clearance	0.169	0.183
Toxicity	AMES toxicity	NO	NO

TABLE II

Binding Interactions of Acetyl Cholinesterase

Compound Name	Natural compounds binding interaction with acetyl cholinesterase			
	Hydrogen bond Protein…. Ligand	Amino acid	Distance	Docking score
Rivestimine	OH…N	TRP86	2.47	
	OH…N	TYR337	3.01	-7.104
	OH…N	HIP447	2.03	
Epicatechin	OH…N	PHE295	3.45	
	OH…O	SER125	2.74	-7.204
	OH…O	ASP74	3.06	
Catechin	OH…O	ASP74	2.75	
	OH…O	TYR72	2.74	-8.775
	OH…O	GLN202	2.70	
Epicatechin-3-gallate	OH…O	TRP86	2.98	
	OH…O	GLN202	2.77	-9.612
	OH…O	SER125	2.83	
	OH…O	ASP74	2.56	
Epigallocatechin	OH…O	SER293	3.02	
	OH…O	TYR341ASP7	3.28	-7.009
	OH…O	4	2.64	
Epigallocatechin gallate	OH…O	ARG296	2.27	
	OH…O	SER293	2.94	-7.842
Gallic acid	OH…O	GLN202	1.00	-7.80

the epicatechin-3-gallate and catechin can be suggested as good ligands with the least toxicity. Epicatechin-3-gallate and catechin are known to influence metabolism, cell permeation and bioavailability. Almost all the predicted properties of the tested compound were in the range as predicted by QikProp for 62.094 and 68.829% of known oral drugs and also satisfy Lipinski's rule of five.

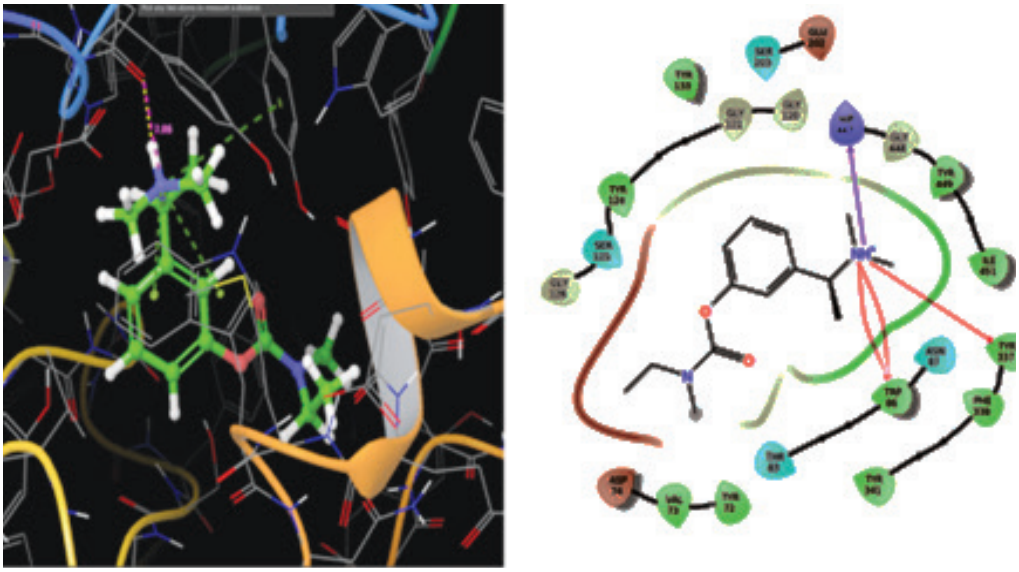

Figure 1
Binding of rivestimine in the active site of AChE

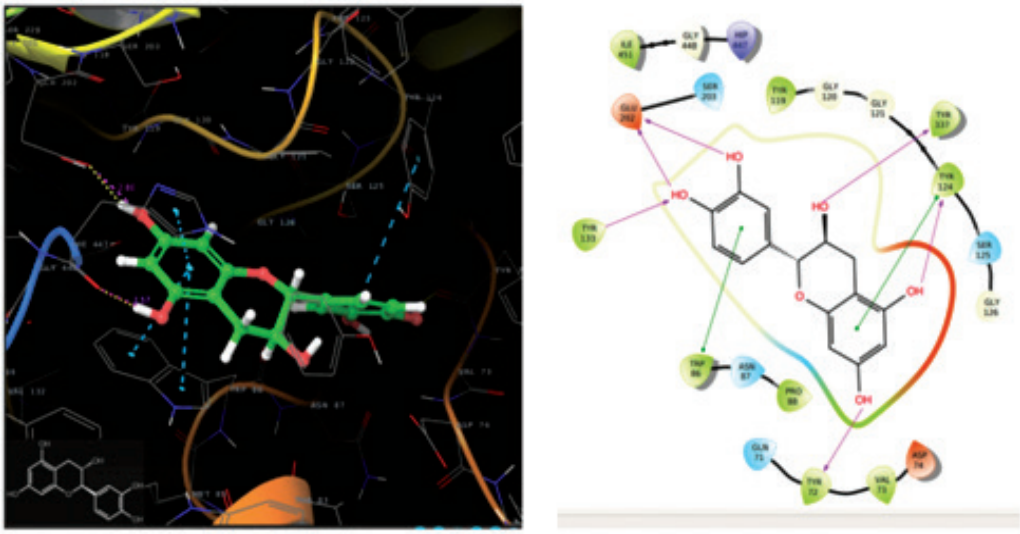

Figure 2
Binding of catechin in the active site of AChE

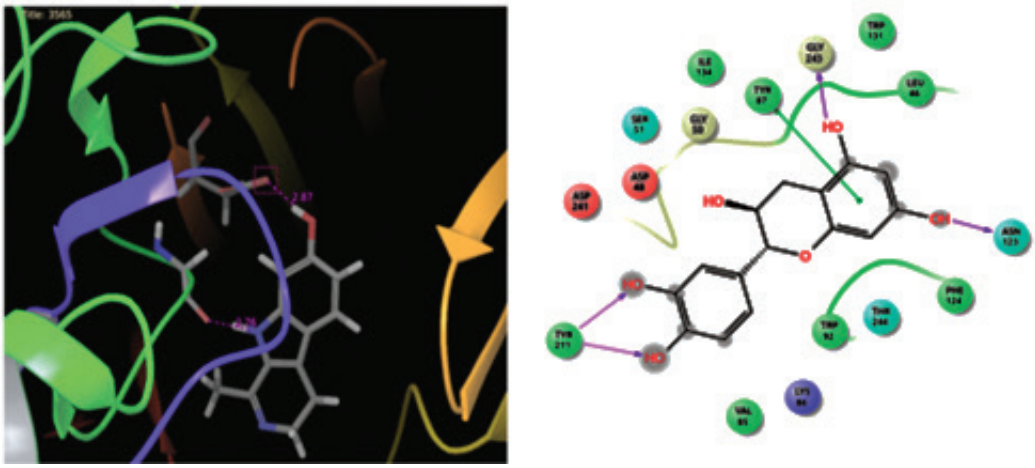

Figure 3
Binding of Epigallocatechin-3-gallate in the active site of AChE

The docking interaction results of AChE protein presented in Table II catechin showed bond interaction with ASP74, TYR72 and GLN202 and -8.775 kCal/mol binding affinity and epicatechin-3-gallate has shown four hydrogen bond interactions with TRP86, GLN202, SER125 and ASP74 by means of -9.612 k Cal/mol binding affinity. Epicatechin has shown two hydrogen bond interactions with SER125 and ASP74 amino acid with -7.204 binding affinity respectively. Epigallo catechin interacted with AChE with site -7.009 kCal/mol binding affinity amino acid present in an active site showed hydrogen bonding. Epigallocatechin gallate has -7.842 kCal/mol binding affinity and two hydrogen bond interactions with the active site of AChE. Gallic acid has shown one hydrogen atom with an active site. Rivastigmine has shown four hydrogen bond interactions with TRP86, TYR337, and HIP447 by means of -7.104 k Cal/mol binding affinity. Catechin and epicatechin-3-gallate have the least binding affinity and are considered the best drug compared to rivastigmine. The molecular visualization of molecular docking poses of rivastigmine, catechin and epicatechin-3-gallate in the active site of AChE is represented in Figures 1, 2 and 3.

Discussion

Identification of drug interaction atoms with specific amino acid residues of the therapeutic target has played an important role in drug synthesis and the function of identification of proteins using mutational studies. Drug interactions with

the active target of amino acid residues of protein target have given solutions to scientists for inhibition of target using *in silico* approaches[5].

The higher docking score is higher binding efficiency between the enzyme and the ligand also reported compound identified from the methanolic extract of *Grewia tiliaefolia* performed vitexin was observed with a high dock score of 68.106 indicating strong protein-ligand interaction[6]. Thus in the present *in silico* study, an attempt made to obtain natural flavonol compound against ACHE enzyme showed efficient hydrogen binding with high docking score compared with a synthetic drug. Hence catechin and epicatechin 3-gallate can bind actively to AChE. The results presented herewith provided a comparative docking analysis revealed catechin and epicatechin-3-gallate as the most potent anti-acetylcholinesterase compounds with strong binding energy. Thus the catechin and epicatechin-3-gallate were confirmed as the best inhibitor AChE targets.

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In vitro antioxidant and acetylcholinesterase activities of catechin-loaded green fabricated zinc oxide nanoparticles

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ABSTRACT

Catechin is an antioxidant, secondary metabolite found in *Camellia sinensis* that has unique qualities such as antioxidant activity, acetylcholinesterase activity, and neuroprotective benefit. In addition to having poor biological efficacy due to low stability, nanotechnology can enhance or improve stability. The aim of this study is to synthesize catechin-coated zinc oxide nanoparticles (CAT-ZnONPs) using *C. sinensis* leaf extract by the nanoprecipitation method and investigate their *in vitro* antioxidant and acetylcholinesterase activities. The ultraviolet-visible spectrophotometer and the Fourier transform infrared spectrophotometer were used to characterize the CAT-ZnONPs; the particle had a consistent distribution and no agglomeration of nanoparticles was observed using field emission-scanning electron microscopy. Based on X-ray diffraction (XRD) analysis, the average size of CAT-ZnONPs is between 50 and 60 nm, as measured using the intensity of XRD. *In vitro,* the antioxidant activity of CAT-ZnONPs using the DPPH technique showed 50% scavenging at a dosage of 35 µg/mL. *In vitro* acetylcholinesterase inhibition activity of CAT-ZnONPs with a IC_{50} concentration of 1.25 µg/mL was demonstrated. Thus, the obtained results revealed the first study to demonstrate CAT-ZnONPs as a therapeutic agent, which could be a promising drug delivery system and provide a novel process for curative intervention in neurological disorders in future research.

1. INTRODUCTION

Nanotechnology is a branch of interdisciplinary research that attracts researchers from many fields, including medicine, electronics, and biomaterials. Nanomaterials can be made with dimensions as small as 10–100 nm by a variety of methods, such as biological, chemical, and physical [1]. Nanotechnology is used in a wide variety of fields. In the food industry, nanocomposites are used to determine the amount of tartrazine (TRT) in food samples, and some carbon nanotubes (CNT), graphene (GR) are used in an electrochemical sensing system to detect the azo dye contaminations in food samples [2,3]. In biomedical applications, nanoparticles have a significant role in cancer therapy through targeted drug delivery and provide new approaches for diagnosis and biosensing [4]. In the biosensor application used to detect glutathione in body fluids, glutathione is used as an antioxidant to prevent the cells from oxidative damage caused by free radicals [5]. In environmental remediation photocatalytic application, nanocomposite is a good photocatalyst for degrading the organic compound nitrobenzene under light irradiation [6]. In waste water treatment, nanocomposite significantly improves its adsorption character for the deduction of metal ions, pharmaceutical waste, pesticides, organic pollutants, and heavy metals [7,8]. Nanomaterials have attracted the attention of scientists due to their unique properties, such as their high surface area, small size, thermal conductivity, shape, surface morphology, charge, zeta potential, and crystal structure [9]. These properties have led to their incorporation into the biotechnological and biomedical fields, especially in the treatment of fatal diseases such as cancer and Alzheimer's [10,11]. The green synthesis is a preferred method for nanoparticle synthesis due to its low cost, environmental friendliness, biocompatibility, ease of use, and rapid synthesis methods [12]. Different kinds of organisms, such as cyanobacteria, fungi, actinomycetes, bacteria, algae, and plants, are capable of synthesizing nanoparticles. Green synthesis has allowed for the synthesis of a wide variety of nanoparticles with diverse biological activities, including silver, copper, gold, zinc oxide, selenium oxide, and copper oxide [13-16]. Zinc is a vital mineral and an essential component for human development. Zinc deficiency can result in a wide variety of symptoms, including growth retardation, premature death, and problems with both male and female reproduction. ZnO-NPs are used in cosmetics and sunscreens because of their powerful ultraviolet (UV) absorption characteristics [17]. In addition to investigating their *in vitro* cytotoxic ability against cancer cells, researchers also tested ZnO-NPs for their potential to treat Alzheimer's disease and diabetes [18].

The plant *Camellia sinensis,* from the family Theaceae, is used to make the green, black, that are so popular around the world. However, green tea consumption has been shown to have the most dramatic effects on

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human health [19]. Tea leaf production is widespread throughout the world, with the majority consumed in Asia, some regions of North Africa, the United States, and Europe [20]. Catechins, flavanols, flavanones, phenolic acids, glycosides, and aglycones of plant pigments are just some of the phytochemicals found in *C. sinensis* [21]. Catechins have excellent antioxidant properties and are found in abundance in fresh tea leaves; they can account for up to 30% of the dry weight of the leaves [19]. The anti-cancer, anti-heart disease, anti-Alzheimer's, and anti-aging properties of *C. sinensis* are well documented [22]. The phenolic compounds served as a good reducing agent to reduce metal ions for the green synthesis of nanoparticles and had a stronger antioxidant capacity than other types of phytochemicals. *C. sinensis* have more consisted of proteins, lipids, and amino acids which is the influence to nanoparticle growth and reduced agglomeration [23].

This study will focus on the synthesis of zinc oxide nanoparticles (CAT-ZnONPs) using *C. sinensis* leaf extract and coated with catechine, which is enhance the bioactive potential and promise as antioxidants and acetylcholinesterase inhibitors. Analytical characterization of the green-synthesized CAT-ZnONPs using various techniques such as–visible spectrum analysis, scanning electron microscopy (SEM) with energy dispersive X-ray spectroscopy (EDAX), elemental mapping, Fourier transform infrared spectroscopy (FTIR), and X-ray diffraction (XRD). All the above characterizations are used to analyze the nanoparticle's topography, morphology, composition, and crystalline structure.

2. MATERIALS AND METHODS

2.1. Materials

Zinc acetate dihydrate $Zn(CH_3COO)_2.2H_2O$, Catechin and Ellman assay kit for AChE activity were procured from Sigma-Aldrich Chemical, Coimbatore and all Other Chemicals procured from HiMedia and were of analytical quality.

2.2. Synthesis of ZnONPs

According to Gnanasangeetha and Thambavani [24], the biosynthesis method was used to synthesize zinc oxide nanoparticles (ZnONPs) with some modifications. Zinc acetate dihydrate and sodium hydroxide were used as the precursors that were employed in the synthesis of nanoparticles. The 200 mM zinc acetate dihydrate solution was dissolved in 20 mL of distilled water and vigorously stirred in a magnetic stirrer. Following the addition of 5 mL aqueous extracts of *C. sinensis* leaf with zinc acetate solution, and then the addition of 2M of NaOH to make pH-12, the mixture was stirred in for 2 h at 60°C. Then, other elements were removed from the final precipitation using centrifugation at 1400 rpm for 5 min and washed with distilled water and ethanol. The precipitate was dried at 60°C overnight, then the dried white precipitate was powdered using an agate mortar, and the final ZnONPs were stored in an airtight container.

2.3. Preparation of CAT-ZnONPs

The catechin was coated on the surface of zinc oxide nanoparticles by the nanoprecipitation method with modifications according to Arasoğlu *et al.*, Liu *et al.* [25,26]. In a brief procedure, 100 mg of ZnONPs were dissolved in 5 mL of 0.01 N HCl and 10mg of CAT were dissolved in 5mL of ethanol with continuous stirring. 5 mL CAT solution was added drop by drop to the ZnONPs. During stirring, NaOH was added to the ZnONPs and CAT mixture for precipitate formation, and the pH was adjusted to 5.0. The obtained precipitate was centrifuged at 16,000 g at room temperature for 30 min, and the CAT-ZnONPs have been dried using freeze drying at −50°C for 24 h.

2.4. Characterization of CAT-ZnONPs

CAT-ZnONPs' Fourier transform-infrared spectra were captured by a Shimadzu Fourier-transform infrared (FT-IR) 8400 spectrophotometer in the 600–4000 cm^{-1} range, and UV–visible spectrum analysis was carried out using a Shimadzu 1800 spectrophotometer. Morphological identification of nanoparticles was analyzed by field emission FE-SEM, EDAX was used to analyze purification of nanoparticle and elements containing nanoparticles. An elemental mapping analysis was used to study the shape, structure, and composition of the CAT-ZnONPs. Particle size distribution and crystallinity of nanoparticles were analyzed using XRD.

2.5. DPPH radical scavenging activity of CAT-ZnONPs

CAT-ZnONPs's ability to scavenge the free radicals was investigated using the 2, 2-diphenyl-1-picrylhydrazile (DPPH) technique, as described in Rahman *et al.*, Najafabadand and Jamei [27,28] with some modifications. Different concentrations of CAT-ZnO NPs, from 25 µg/mL to 45 µg/mL were added to a 96-well plate containing a 1 mM DPPH solution. Ascorbic acid (Vitamin C) is used as the standard solution, followed by 30 min of incubation at room temperature in the dark. In methanol, DPPH appears purple but fades to yellow when neutralized by antioxidants. After incubation, the decolorization of DPPH may be observed, denoting the CAT-ZnONPs' ability to donate hydrogen atoms as antioxidants. The scavenging ability was measured at 517 nm absorbance. The experiment has been demonstrated in triplicate and calculated with a mean ± standard deviation. The scavenging capacity was estimated using the following equation:

$$\%\,\text{DPPH radical scavenging activity} = \frac{(A0\text{-}A1)}{A0} \times 100\%$$

Whereas, A0–OD value of blank, A1–OD value of Standard/CAT-ZnO NPs treated. The IC_{50} was calculated by a graph plotting the concentration of CAT-ZnO NPs against % of inhibition.

2.6. Thin-layer chromatography (TLC) with bioassay detection for AChE inhibition

The TLC with bioassay detection for AChE inhibition was altered from the study of Rhee *et al.* [29]. The stationary phase was a 25 mm F254 no. 5554 silica gel plate. Two mobile phases were used: dichloromethane: ethanol:water (4:4:0.5 v/v/v) and chloroform: methanol (9:1 v/v). On the plate, 3 µL of plant extracts in methanol were administered at a concentration of 5 mg/mL. Once the plate had been produced, it was allowed to dry at ambient temperature before being sprayed with 20 mM DTNB and 30 mM ATCI. The plate was dried at room temperature for 45 min before being sprayed with 10.17 U/mL AChE.

2.7. Acetylcholinesterase inhibition activity

The Ellman assay kit method was used to measure the acetylcholinesterase inhibition activity. A 96-well microtiter plate was filled to a volume of 100 L with assay buffer as a control without enzyme, and 100 L of AChE enzyme was added into the wells, followed by different concentrations of the nanoparticles with the enzyme: 0.25, 0.5, 0.75, 1.0, 1.25, 1.50, and 1.75 L. The plate was incubated for 5 min at room temperature. After that, 180 µL of Ellman's reagent was added to each well. The change has been observed in 412 nm absorbance. The experiment has been demonstrated in triplicate. The AChE activity was calculated as a percentage based on the formula.

$$\text{Enzyme activity } (\%) = \frac{E - S}{E} \times 100$$

Where E represents the AChE activity without a CAT-ZnONPs and S represents the enzyme's activity when a CAT-ZnONPs is present.

3. RESULTS AND DISCUSSION

3.1. UV-visible spectrum of (CAT-ZnONPs)

In the present study, we investigated the synthesis of zinc oxide nanoparticles coated with catachin (CAT-ZnONPs) using nanoprecipitation methods. Figure 1 shows the UV spectra of CAT-ZnONPs measured at 318 nm absorbance. The reaction stimulated for 1 h at a temperature of 26°C was fast and also used up minimal energy. In earlier research, we had established that catechin-AuNPs had been successfully synthesized [23].

3.2. FE-SEM and EDAX analyses of CAT-ZnONPs

FE-SEM is used to evaluate the surface morphology of the obtained CAT-ZnONPs, as shown in Figure 2. The distribution of nanoparticles has similar size and shape in the solution, which has the flower shape revealed by FE-SEM. The particle surface appeared to be smooth and agglomerated, which proves the catechin was coated on ZnONP. In the earlier study, quercetin-loaded PLGA nanoparticles were observed in FE-SEM images, and the hydrophobic properties of the morphological changes were investigated without the use of a solvent [24,30].

Only the peaks of the elements zinc, oxygen, chlorine, and potassium are found in the EDAX spectrum of CAT-ZnONPs, as shown in Figure 3. In the EDS limit, it is apparent that the ZnONPs prepared are completely free of impurities. According to the EDAX spectrum, Zn and O have very high atomic weights, and other components such as chlorine and potassium are present in very small amounts. As a result of the results, Zn and O were found in high concentrations in the nanoparticles, and the nanoparticles' high purity was demonstrated by the EDAX spectrum [Figure 3].

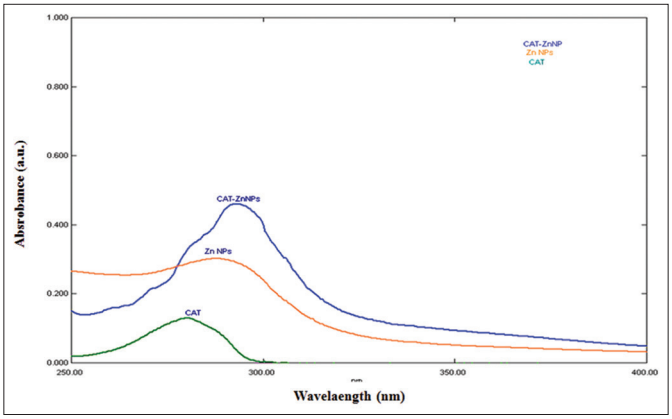

Figure 1: Ultraviolet-visible spectrum of catechin-coated zinc oxide nanoparticles.

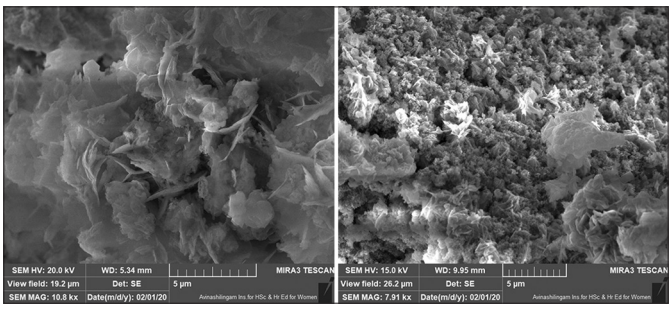

Figure 2: Field emission-scanning electron microscopy analysis of catechin-coated zinc oxide nanoparticles.

3.3. Elemental Mapping analysis of CAT-ZnONPs

To further support the EDAX spectrum's elemental investigation of CAT-ZnONPs. The elemental mapping only shows the zinc and oxygen peaks, as shown in Figure 4. These results showed a high signal from Zn atoms (75.03%) and O atoms (16.42% suggesting that catechin was successfully coated on the surface of ZnONPs. The visible peaks for other elements such as C atoms (8.13%) and K atoms (0.45%) were observed to be much lower. In a previous report, Tet-1 peptide successfully coated the surface of EGCG@Se, as shown by the elemental composition mapping study [31] which showed a strong single from Se along with C, N, and O atoms.

3.4. XRD patterns of CAT-ZnONPs

XRD spectroscopy confirmed the metallic Zn's crystalline structure in [Figure 5]. Peaks of intense diffraction were seen in the face-centered (100), (111), (101), (102), (110), (103), (112), (004), and (311) planes,

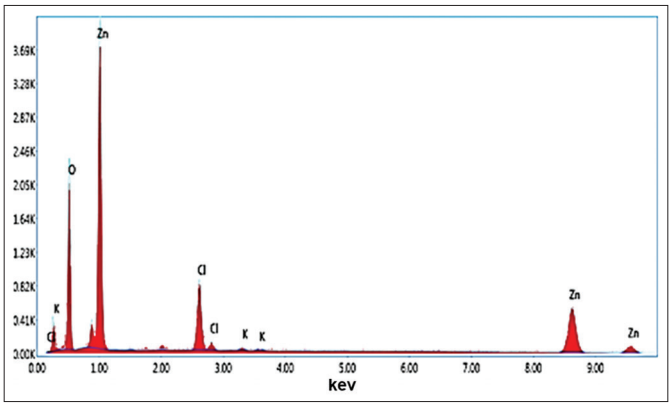

Figure 3: Energy dispersive X-ray spectroscopy spectrum of catechin-coated zinc oxide nanoparticles.

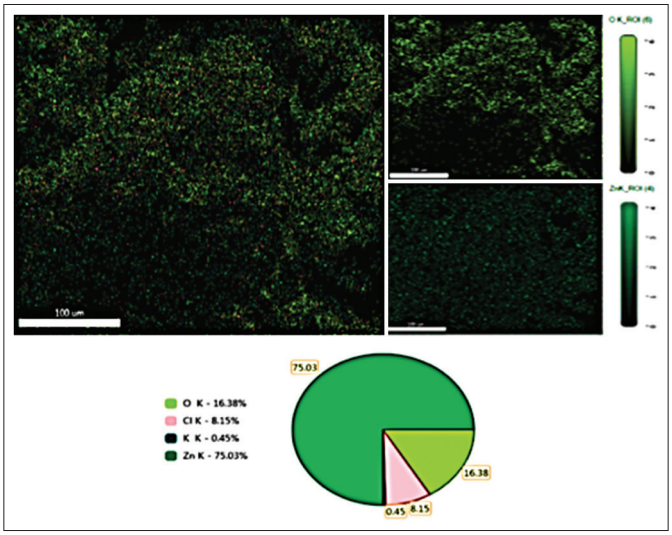

Figure 4: Elemental mapping analysis of catechin-coated zinc oxide nanoparticles.

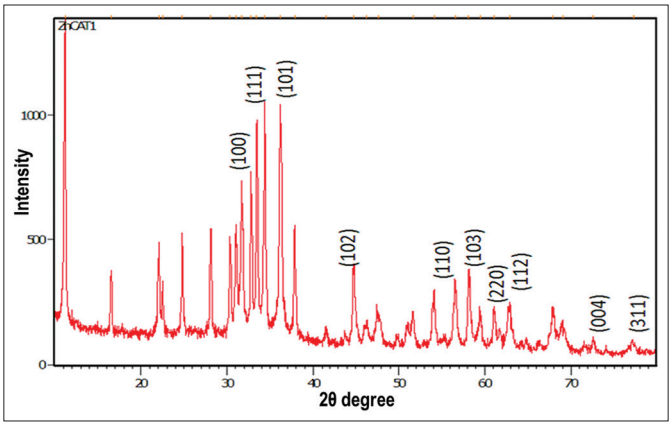

Figure 5: X-ray diffraction patterns of catechin-coated zinc oxide nanoparticles 2θ.

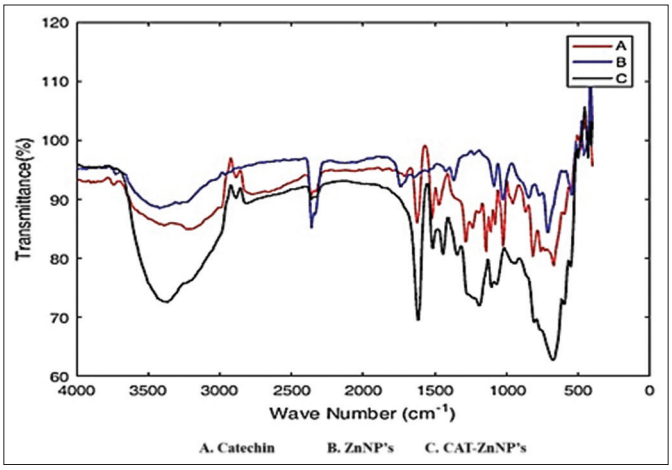

Figure 6: Fourier-transform infrared spectrum of catechin-coated zinc oxide nanoparticles.

respectively, at 31.9, 34.05, 36.02, 45.0, 56.86, 58.0, 61.0, 63.0, 72.28, and 77.7 (fcc). The strongest peak appeared at 34.05, indicating that the (111) plane was the most frequent orientation. Using the intensity of diffraction from Debye-Scherrer equation, the average size of CAT-ZnONPs is between 50 and 60 nm and was measured [32].

3.5. FT-IR spectrum of CAT-ZnONPs

FT-IR was used to further characterize CAT-ZnONPs to establish the existence of the chemical bonds depicted in Figure 6. The presence of the OH group was indicated by a broad, strong band in the FT-IR spectrum of the CAT-ZnONPs that ranged from 3446 cm^{-1}. 1620cm^{-1} peak responsible for the alkenyl C=C stretch. In addition, a weak band at 1442cm^{-1} was observed as a result of the methyl C-H bend vibration. A narrow band at 1519cm^{-1} is formed by the sample's -CO group and the fraction of catechin-coated zinc oxide nanoparticles. The band at 3387cm^{-1} is responsible for OH stretching strong bond As a result, the OH group's presence in the extract is confirmed. Zhang *et al.* [33] reported that the FT-IR spectrum of Tet-1 EGCC@Set was examined to investigate possible interactions to confirm the presence of Tet-1 on the surface of SeNPs.

3.6. Antioxidant activity of CAT-ZnONPs

Figure 7 depicts the DPPH scavenging activity of CAT-ZnONPs. The percentage inhibition of scavenging activity of CAT-ZnONPs was increasing in a dose-dependent manner that was comparable to that of standard ascorbic acid at the same concentration given as Supplementary Table1. The IC_{50} value of the CAT-ZnONPs was determined based on the 50% of free radicals scavenged by 35 µg/mL. Earlier, it was reported by Mathew *et al.* [34] that the attachment of Tet-1 to the curcumin-PLGA nanoparticles did not produce any change in their antioxidant activity. The point to be noted is that the CAT-ZnONPs do not destroy the antioxidant activity of catechin. Phenolic compounds and catechins found in *C. sinensis* is backbone of efficient antioxidant activity in the 96% of ethanolic extract has reported in past research [19]. In early reported antioxidant activity of *C. sinensis* extract with a concentration of IC_{50} 70.25 ± 2.85 µg/mL [22]

3.7. TLC qualitative acetylcholinesterase inhibition (AChEI) assay

An investigation of autographic assay using the TLC for qualitative examination of acetylcholinesterase inhibition (AChEI) [Figure 8].

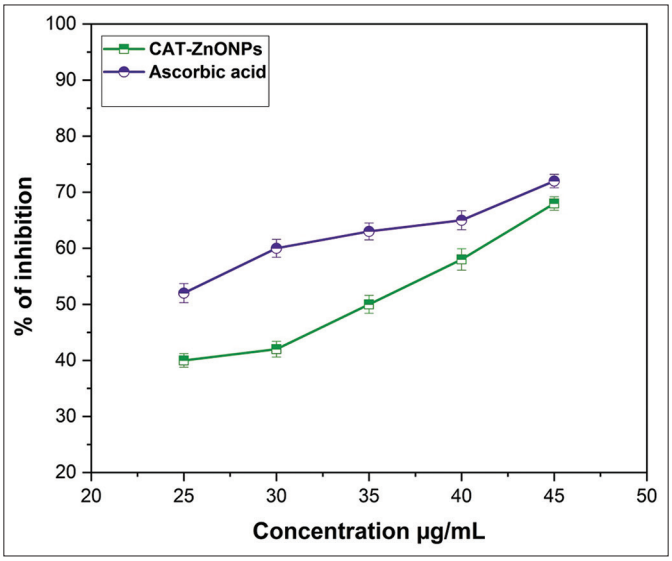

Figure 7: Antioxidant activity of catechin-coated zinc oxide nanoparticles.

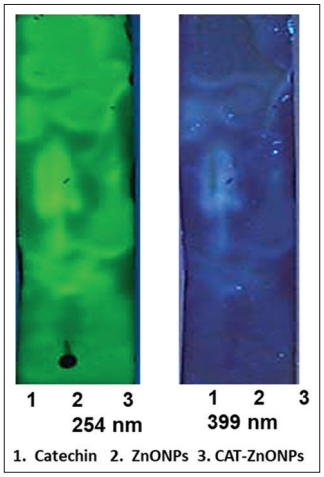

Figure 8: Acetylcholinesterase inhibition qualitative thin layer chromatography (TLC) assay, TLC elution system: Dichloromethane: Ethanol:water (4:4:0.5 v/v/v).

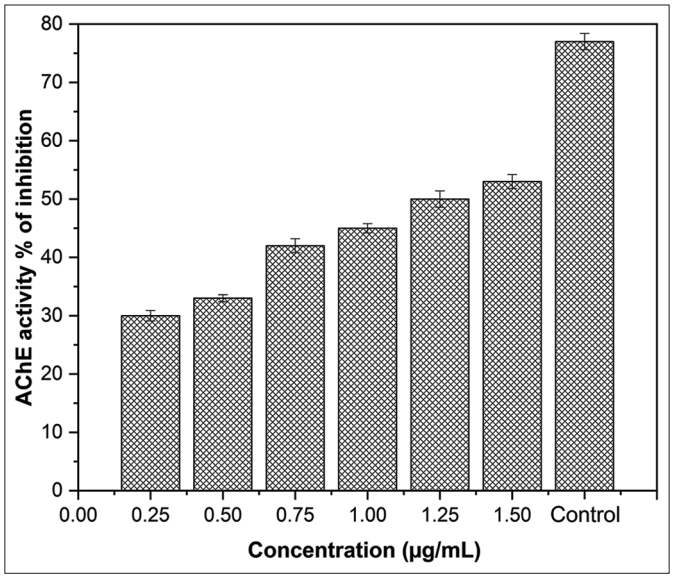

Figure 9: Acetylcholinesterase inhibitory activity of catechin-coated zinc oxide nanoparticles's.

Data of the autographic assay for catechin, ZnONPs, and CAT-ZnONPs are shown: The Retention factor (Rf) was determined by TLC for the catechin's observed in 0.63, ZnONP's has been observed in 0.58, and CAT-ZnONP's was in 0.61. *Ochtodes secundiramea* therapeutic potential is supported by the moderate AChEI activity and minimal off-target effects of their plant extracts, which were characterized in previous report [35] as moderate inhibitors.

3.8. Acetylcholinesterase (AChE) inhibitory activity

The concentrations of CAT-ZnONPs (0.25, 0.50, 0.75, 1, 1.25, and 1.5 µg/mL) and corresponding percentage inhibition values are shown in [Figure 9]. The *in vitro* analysis of CAT-ZnONPs' AChE inhibition potential was demonstrated in a dose-dependent manner with increasing concentrations of CAT-ZnONPs for its potential to inhibit AChE activity given as Supplementary Table 2. The minimum percentage of AChE inhibition activity was observed at a low concentration of CAT-ZnONPs (0.75 µg/mL). The high concentration of CAT-ZnONPs 1.5 µg/mL showed the maximum percentage of AChE inhibition activity. About 50% of AChE inhibition activity has been found in 1.25 µg/mL of the CAT-ZnONPs, so the IC_{50} dosage of nanoparticles with a concentration of 1.25 µg/mL was demonstrated.

In a related investigation, *Withania somnifera* lowered AChE activity by 30% when used at a concentration of 12.50 µg/mL, and reached roughly 50% inhibition at 50 µg/mL [36]. A previous study in oral treatment of ZnONPS and ZnCAP for brain abnormalities caused by retinone RTNE has significantly higher inhibition activity of AChE [37]. Previously reported, the highest AChE inhibitory activity (IC_{50} = 336.885.52 µg/mL) was observed in methanol extract of pericarp; additionally, green tea and tea polyphenols have potentially inhibited AChE with IC_{50} concentration of 30 µg/mL and 248 µg/mL, respectively [21].

4. CONCLUSION

Camellia sinensis was used to biosynthesize CAT-ZnONPs. The outcomes of the CAT-ZnONPs characterization examination using UV-VIS, FTIR, FE-SEM, EDAX, and XRD demonstrate that the material fits the criteria for an excellent nanodrug. The potential for the CAT-ZnONPs' high AChE inhibitory activity was there. *In vitro* studies of CAT-ZnONPs could be conducted in the future, as they have been shown to be an effective medicine for treating neuroprotective effects *in vivo*.

5. ACKNOWLEDGMENTS

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6. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS

This study does not involve experiments on animal or human subjects.

10. DATA AVAILABILITY

The data that are supporting the findings of the study are available within the article.

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SUPPLEMENTARY MATERIALS

Supplementary Table 1: DPPH radical scavenging activity.

S. No.	CAT-ZnONPs		Ascorbic acid	
	Concentration (µg/mL)	% of scavenging	Concentration (µg/mL)	% of scavenging
1.	25	40	25	52
2.	30	42	30	60
3.	35	50	35	63
4.	40	58	40	65
5.	45	68	45	72

CAT-ZnONPs: Catechin-coated zinc oxide nanoparticles

Supplementary Table 2: AChE inhibition activities.

S. No.	CAT-ZnONPs	
	Concentration (µg/mL)	% of AChE inhibition
1.	0.25	30
2.	0.5	33
3.	0.75	42
4.	1	45
5.	1.25	50
6.	1.5	53
7.	1.75	77

AChE: Acetylcholinesterase, CAT-ZnONPs: Catechin-coated zinc oxide nanoparticles

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दिनांक/Date: 26 August 2019

पौधे प्रमाणीकरण प्रमाणपत्र / PLANT AUTHENTICATION CERTIFICATE

The plant specimen brought by you for authentication is identified as *Camellia sinensis* (L.) Kuntze (= *Thea sinensis* L.) - THEACEAE. The identified specimen is returned herewith for preservation in their College/ Department/ Institution Herbarium.

26/08/2019

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