MATERIALS AND METHODS

3.1 Collection of plant materials

Hybanthus enneaspermus (L). F. Muell. (Plate 3.1) was gathered from ABS medicinal garden, Karipatti, Salem District and the plants were upheld in the greenhouse of Sowdeswari College, Salem, Tamil Nadu, India for *in vitro* development.

Plate 3.1 Habit of *Hyabanthus enneaspermus* L. F.Muell

Systematic position

Kingdom: Plantae

Division: Angiosperms

Class: Dicotyledons

Sub-Class: Polypetalae

Order: Malpighales

Family: Violaceae

Genus: *Hybenthus*

Species: *enneaspermus* (L.) F.Muell.

3.1.1 Authentication

The plant was authenticated by the taxonomist Dr.E.G.Wesely, Department of Botany, Aringar Anna Arts College, Namakkal, Tamil Nadu, India.

3.2 Preparation of Stock Solution

The individual volume of all stock ingredients of the medium varied depending upon the requirements and concentration. The composition of MS medium (Murashige and Skoog, 1962) was given in Table 3.1. The stock solutions of macronutrients and micronutrients ($MgSO_4$, $CaCl_2$, KNO_3, NH_4NO_3, KH_2PO4, $MnSO_4$, $ZnSO_4$, H_3BO_3, Na_2MOO_4, $CuSO_4$, and $CaCl_2$ iron source, vitamins and aminoacids were prepared individually for MS medium. Stock solutions were kept in sterile amber brown bottles and stored under refrigeration at 4˚C and were used within 30-40 days from the date of preparation.

Preparation of growth regulator stock solutions was done as follows Cytokinin 6-Benzyl aminopurine (BAP) and Kinetin were dissolved in 0.1 N HCl and the final volume were prepared with distilled water. Auxins which includes Indole 3- butyric acid (IBA), 2,4-Dichlorophenoxy acetic acid (2,4-D) and Napthalene acetic acid (NAA) were dissolved in ethanol and then made to final volume with distilled water. All the stock solutions of the plant growth regulator were stored in the refrigerator at 4^oC.

Table 3.1. Composition of Murashige and Skoog (MS) medium (1962)

Constituent	Concentration (mg/l)
Macronutrients	
NH_4NO_3	1650
KNO_3	1900
$CaCl_2$ $2H_2O$	440
$MgSO_4$. $7H_2O$	370
KH_2PO_4	170
Micronutrients	
H_3BO_3	6.2
$MnSO_4$. $4H_2O$	22.3
$ZnSO_4$. $7H_2O$	8.6
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_45H_2O$	0.025
$CoCl_2$. $6H_2O$	0.025
Iron source	
Fe EDTA Na salt	40
Vitamins	
Nicotinic acid	0.5
Thiamine HCl	0.1
Pyridoxine HCl	0.5
Myo-inositol	100
Others	
Glycine	2.0
Sucrose	30000
Agar	8000
pH 5.8	

3.2.1 Selection and preparation of medium for callus induction

Different concentrations of the Murashige and Skoog (MS) culture media were used for this study to get the maximum callus production. The media was prepared by adding the required amount of macronutrients, micronutrients, iron source, vitamins and plant growth regulator and the last volume was prepared with distilled water. The pH of the medium was modified to 5.8 using 0.1 N HCl or 0.1 N NaOH, and the agar (0.8 w/v) was dissolved in the media by heating. The medium was distributed in culture tubes and plugged tightly with cotton plugs and autoclaved at 121˚C for 15 minutes. Antioxidants such as polyvinyl pyrolidine, activated charcoal, ascorbic acid and citric acid (25-100 mg/l) were added to the culture medium before autoclaving to prevent browning in explants.

3.3 Source of explants

Non meristematic explants like leaf explants were excised with sterile blade and gathered in a beaker. The excised leaf explants were exhaustively washed with flowing tap water for 5 to 10 minutes. Thereafter, the explants rinsed with cleanser (Teepol 2% v/v) solution for 3 minutes followed by fungicide (Bavistine 1% w/v) for 2 minutes and then drenched in 70% (v/v) ethanol for 30 seconds after the each treatment the explants were washed three times with distilled water. Further, sterilization treatments were finished under a laminar-flow chamber. The explants were lastly sterilized with 0.1% (w/v) $HgCl_2$ for 2 minutes and these explants were then exhaustively rinsed 3-4 times with pasteurized double distilled water to eliminate the hints of mercuric chloride, now the explants are prepared for inoculation on required medium.

3.3.1 Inoculation of explants

The leaf explants were inoculated on Murashige and Skoog (MS) basal medium supplemented with various concentration and blend of plant growth regulators (PGRs).

By methods of a long stainless steel forceps, one explant per tube was positioned. It was a routine procedure to flame the mouth of the test tube after unsealing and before recapitulating the tubes to decrease contamination. To facilitate planting, two forceps were utilized alternatively to permit sufficient time to cool, besides, to avert fiery the fingers and explants.

3.3.2 Culture condition

The cultures were preserved at 25 ± 2˚C under 16/8 h light/dark conditions of 80 µE $ms^{-2}s^{-1}$ irradiance delivered by fluorescent lamps (TL 40W/54 cool-day light) for callus induction and plantlets regeneration.

3.4 Callus induction

The leaf explants were cultured on Murashige and Skoog (1962) basal medium covering 3% (w/v) sucrose, 0.8% (w/v) agar and various concentrations of 2,4-D and NAA alone or in blend with BAP and Kinetin for optimal callus induction. Data were recorded at 10, 20 and 30 days after inoculation of explants on the regeneration medium. The calli were graded along with their colour in a symbol of B - Brown; G - Green; W - White; LG - Light green; GY -Geenish yellow; GW - Greenish white; GB - Greenish brown; LB - Light brown; WB - White brown. The nature of callus was estimated by the callus compactness and graded into three categories: compact (C), less compact (LC) and friable (F). Abundance of callus was evaluated through a transparent measuring ruler and graded along with their length scale: large (L) = 20 mm and above, medium (M) = 10 to 20 mm and small (S) = 10 mm below. The calli were sub cultured and maintained by *in vitro* conditions. The impacts of these quantitative characters with period of the time for regeneration were measured in percentage.

3.5 Shoot bud regeneration and multiplication

Dedifferentiated calli were moved to regeneration medium covering 3% (w/v) sucrose, 0.8% (w/v) gel and various concentrations of BAP (1.0 – 3.0 mg/l) and Kinetin (1.0 – 3.0 mg/l) in combination with different concentrations of NAA which were utilized for shoot buds differentiation. Following a month, the clumps of shoots were sub cultured on MS medium with appropriate growth regulators for multiplication and growing of the shoots. The level of shoots and plantlets were assessed based on of the growth of the plantlets from calli. Multiplied numerous shoots were partitioned into small clusters of 2 - 3 shoots. They were sub cultured on same medium for shoot elongation. After two weeks, shoots longer than 3.0 cm were calculated and shifted to rooting medium.

3.6 Root induction and transplantation

The longer shoots (3 cm length) were excised and moved to MS basal medium covering 3% (w/v) sucrose, 0.8% (w/v) agar and various concentrations of Indole-3-butyric acid (IBA at 0.1 – 2.0 mg/l) alone or in blend with BA (0.1 – 2.0 mg/l) / Kinetin (0.1 – 2.0 mg/l) for root induction. Rooting was monitored from two to three weeks. Plantlets with mature roots were isolated from the culture tubes and after rinsing their roots in flowing tap water, they were developed in the combination of red garden soil, river sand and saw dust in the proportion of 1:1:1 in paper cups for a month and consequently moved to pots. Potted plants were roofed with transparent polythene membrane to high dampness and watered three days in a week with half strength MS salts solution for two weeks. Plantlets were left for a seven days in the paper cups at the controlled temperature (25 ± 2˚C) with 60% relative dampness. The survival level was monitored in all the explants. After the commencement of new roots, they were kept in

the green house and developed for maturity. Samples were photographed at various phases of growth period.

3.7 Statistical analysis

During the experiment period the growth of callus were analyzed in terms of number of days for initiation of callus. Percentage of tubes responded and colour of callus were calculated. There were three replicates were done for every treatment and the tests were rehashed thrice. Data were taken after 3 weeks with two subcultures. The cultures were observed sporadically and the morphological vicissitudes were noted based on of visual perception. Statistical analysis was done and the data are specified as mean ± SE and mean separation was finished by utilizing Duncan's New Multiple Range Test (DMRT) means were contrasted with $P < 0.05$ at level of significance.

3.8 Preparation of extract

Principle

The bioactive principles present in the plant materials are extracted by utilizing organic solvents. Extraction depends on relative solubility of these components in the organic solvents. The organic solvents of varying polarities percolated into the plant material thereby causing dissolution of the active components. The extracts are filtered then the solvents are vaporized to obtain a concentrated form of extract.

Procedure

Hybanthus enneaspermus leaves were rinsed with distilled water to eliminate dirt and soil. These washed materials were shade dried for a 3 weeks, powdered in a mixer and kept in a tray under room temperature (37˚C) until further extraction. The powdered *in vivo* and *in vitro* leaf explant material was successively extracted with aqueous petroleum ether, chloroform, acetone and carbinol for 12-14 hours sequentially

using a Soxhlet apparatus. All the extracts were filtered continuously till the colour solution change to colourless. The process was rehashed thrice in a same manner and weighed to regulate the yield for all extracts .The solvents from various extracts were then concentrated in a rotary vacuum evaporator at reduced pressure at temperature below 40˚C. The yield of the extract of petroleum ether, chloroform, acetone, carbinol and water was 15, 21, 12.5 and 17% respectively. The dried extracts were stored at 4˚C.

3.9 Phytochemical analysis

3.9.1 Qualitative phytochemical analysis

The preliminary phytochemical analysis were carried out to find various phytochemicals present in both *in vivo* and *in vitro* leaf extract of *H. enneaspermus*. The following 12 qualitative chemical tests were executed on extracts extracted using petroleum ether, chloroform, aceton and carbinol as solvents and utilizing the standard procedure as described by Krishna *et al.,* 1995.

3.9.1.1 Test for alkaloids

1. Dragendorff's Test

Principle

A solution of potassium bismuth iodide when added to an alkaloid containing solution gives an orange red precipitate.

Materials required

Dragendorff's reagent (Potassium bismuth iodide solution)

Procedure

To 1 ml of extract, 1 ml of potassium bismuth iodide solution was added. The color change was monitored and recorded.

2. Mayer's test

Principle

A solution of potassium mercuric iodide added to of alkaloid containing solution gives whitish or cream colored precipitate.

Materials required

Mayer's reagent (Potassium mercuric iodide solution)

Procedure

To 1ml of extract, 1ml of potassium mercuric iodide solution was added and the colour change was monitored and noted.

3. Hager's test

Principle

A saturated aqueous solution of picric acid added to a solution containing alkaloid gives a yellow coloured precipitate.

Materials required

Hager's reagent (saturated aqueous solution of picric acid)

Procedure

To 1 ml of extract, 3 ml of saturated aqueous solution of picric acid was added. The colour change was monitored and noted.

4. Wagner's test

Principle

A solution of iodine in potassium iodide added to a solution containing alkaloid gives a reddish brown precipitate.

Materials required

Wagner's reagent (solution of iodine in potassium iodide)

Procedure

To 1 ml of extract, 3 ml of Wagner's reagent was added. The colour change was monitored and noted

3.9.1.2 Test for carbohydrates

1. Molisch's Test

To 2 ml of extract 2-3 drops of alpha naphthalene solution in alcohol was added, shaken for 2 mins and 1 ml of concentrated sulphuric acid was added, gradually from the sides of the test tube. A deep violet colour at the junction of two layers shows the occurrence of carbohydrates.

2. Fehling's Test

Materials Required

Fehling solution A: Copper sulphate (34.66 g) was dissolved and made up to 500 ml with distilled water.

Fehling solution B: Potassium sodium tartrate (173 g) and sodium hydroxide (50 g) was dissolved in distilled water and made up to 500ml.

Procedure

The extracts (100 mg) were dissolved in 5 ml of distilled water and filtered. 1 ml of filtrate was heated on water wash with 1 ml each of Fehling solution A and Fehling solution B. The formation of red colour precipitate specifies the occurrence of reducing sugar.

3. Anthrone Test

Principle

Carbohydrate attains dried when react with conc. H_2SO_4 to form furfural. This furfural reacts with anthrone to give bluish green coloured complex.

Procedure

To 2 ml of extract 2 ml of anthrone reagent was added, mix thoroughly and observe the colour change.

3.9.1.3 Test for Phytosterols

1. Liebermann's - Burchard test

Principle

Lieberman-Burchard is a reaction utilized in a colorimetric test to identify steroids, which provides a deep red colour. The colour is due to the hydroxyl group (-OH) of steroids reacting with the reagents and increasing the conjugation of the un-saturation in the adjacent fused ring.

Materials required

1. Acetic anhydride
2. Concentrated H_2SO_4
3. Chloroform

Procedure

To 1 ml of test sample, 1 ml of chloroform, 2-3 ml of acetic anhydride and 1 to 2 drops of concentrated H_2SO_4 were added. A red coloration shows the occurrence of steroids. The colour change was monitored and noted.

2. Phosphomolybdic acid Test

Principle

This test is utilized for the detection of reducing substances like steroids. Phosphomolybdic acid is used as a charring reagent for substance bound TLC plates resulting in fluorescent spots can be detected at 254 and 360 nm. Brown, grey or black spots indicate presence of steroids.

Materials required

1. 250 mg molybdatophosphoric acid in 50 mL ethanol
2. Thin Layer Chromatography (TLC) sheet

Procedure

The extracts were spotted on separate TLC sheets and sprayed with a solution of phosphomolybdic acid and heated in an oven at 120°C. The appearance of spots was observed and recorded.

3. Vanillin - sulphuric acid Test

Principle

The vanillin sulphuric acid reagent utilized with G (gypsum) binder plates will charr the polymer binders in the harder layer of the plates and give purple or violet blue colour for steroids.

Materials required

1. Vanillin - sulphuric acid reagent

   0.5g vanillin in 100ml sulphuric acid and ethanol in the ratio 40:10
2. TLC sheet

Procedure

The extracts were spotted on separate TLC sheets and sprayed with a solution of 1% solution of vanillin in conc. sulphuric acid and heated in an oven at 120°C. The appearance of spots was observed and recorded.

3.9.1.4 Test for saponins

1. Froth test

Principle

The honeycomb foam or froth formed by a compound on the water surface shows the occurrence of saponin.

Materials required

Distilled water

Procedure

To 10 mg leaves explant was extracted, 10 ml of sterile distilled water was added and boiled for 5 min and filtered. To 10 ml of distilled water taken in a test tube, 2.5 ml of the filtrate was added. The test tube was stopped and shaken forcefully for about 30 seconds. It was then permitted to stand for 30 minutes and the observations were recorded.

3.9.1.5 Test for flavonoids

1. Shinoda test

Principle

The Shinoda test includes a reductive conversion of colourless or pale yellow coloured flavones and flavonols into profoundly coloured products. Pink colouring of the solution shows the existence of flavonoids.

Materials required

1. Ethanol
2. Magnesium ribbon
3. Concentrated Hydrochloric acid (HCl)

Procedure

To a small quantity of test sample dissolved in ethanol, magnesium ribbon and 1 ml of concentrated HCl were added and the colour change was monitored and recorded.

3.9.1.6 Test for Proteins

1. Biuret Test

Principle

The biuret test confidently recognizes the existence of proteins (not less than two peptides). The reaction includes in this test are the complex formation of the proteins with Cu_2^+ ions in a powerfully alkaline solution.

Procedure

To 2 ml of extracts of *H. enneaspermus*, add 5 - 6 drops of dilute $CuSO_4$ (Fehling's solution A diluted 1/10 with water), add 3 ml 40% sodium hydroxide (NaOH) solution. Monitor the color change. If the protein sample is unsolvable in water, then follow the process specified below:

Measure 3 ml of acetone and 1.5 ml of water into a test tube. Add 1 drop of dilute NaOH and a little amount of extract to be tested. Boil constantly over a small flame for 2 min and allowed to cool. Then, add 0.5 ml of 40% NaOH and 2 drops of a 1/10 diluted Fehling's solution A. monitor the color change.

2. Millon's Test

Principle

Compounds comprising hydroxybenzene radical respond with Millon's reagent to form red complexes. The only amino acid having hydroxybenzene ring is tyrosine. Thus, this test is particular for the amino acid tyrosine and the protein comprising this amino acid. Tyrosine when reacted with acidified mercuric sulphate solution provides yellow precipitate of mercury-amino acid complex. On the addition of sodium nitrate solution and heating, the yellow complex of mercury-amino acid complex transforms to mercury phenolate which is in red color.

Materials required

1. Millon's reagent (Acidified mercuric sulphate)
2. 1 % sodium nitrite

Procedure

To 1ml of extract in dry test tube, add 1ml of Millon's reagent and mix well. Boil gently for 1 minute. Cool under tap water. Now add 5 drops of 1 % sodium nitrite. Heat the solution to some extent. Look for the progress of brick red precipitate.

3. Picric acid test

To 3 ml of extract solution in a test tube, add 2 ml of saturated picric acid solution, a yellow gelatinous precipitate shows the existence of proteins.

3.9.1.7 Test for Amino acids

1. Ninhydrin Test

Principle

Ninhydrin is a chemical utilized to identify ammonia or primary and secondary amines. At pH 4 amino acids react with ninhydrin and the reduction product attained

from ninhydrin then reacts with ammonia and provides a blue colored substance. This reaction gives an extremely sensitive test for amino acids.

Procedure

To 1 ml of numerous extracts of *H. enneaspermus* add 5 drops of 0.2% ninhydrin solution in acetone. Heat constantly over a water wash for 2 min and allow cooling then monitor the blue color formation.

3.9.1.8 Test for fixed oils (spot test)

A small quantity of extract was pressed between two filter papers. Oil stain on the paper shows the existence of fixed oils.

3.9.1.9 Test for phenolic compounds

1. Ferric chloride test

Principle

Phenol complex with Fe (III) and provides an intensely unfathomable bluish green coloured solution

Materials required

Ferric chloride solution

Procedure

To 2 ml of extract, 2 ml of ferric chloride solution ($FeCl_3$) was added and the colour change was monitored and noted. The development of dark green colour shows the existence of phenolic compounds.

2. Lead acetate test

To 2 ml of extract, 2 ml of 10% lead acetate solution was added and the colour change was witnessed and noted. The development of white precipitate shows the existence of phenolic compounds.

3.9.1.10 Test for tannins

About 0.5 mg of extract was heated in 20 ml of distilled water in a test tube and then filtered. Ferric chloride (0.1%) was added to the filtrate sample and the presence of green colour showed the existence of tannins.

3.9.1.11 Test for glycosides

1. Legal Test

To 2 ml of the extract, 1ml of pyridine and 1 ml of sodium nitroprusside were added. The change in colour pink or red shows the existence of cardiac glycosides

2. Keller-killiani Test

To the test tubes comprising 2 ml of extract 1 ml of glacial acetic acid, 3 drops 5% W/V ferric chloride and concentrated sulphuric acid were added and monitored, vanishing of reddish brown colour at the junction of two layers and bluish green in upper layer shows the existence of cardiac glycosides.

3. Borntrager's Test

To the test tubes comprising 2 ml of extract 2 ml of dilute sulphuric acid was added, heat for 5 min and filtered. To the filtrates, equal volumes of chloroform was added and mixed well. organic layers were divided and ammonia was added to this. Pinkish red colour of the ammonia layer showed the existence of anthraquinone glycosides.

3.9.1.12 Detection of Gum and Mucilage

100 mg of extract was dissolved in 10 ml of distilled water, to this 25 ml of absolute alcohol was added with constant stirring. White or cloudy precipitate shows the existence of gum and mucilage.

3.9.1.13 Test for Coumarins

To 1ml of the plant extract (0.1 g) was taken in a small test tube and roofed with filter paper moistened with 1 N NaOH. The test tube was positioned for few minutes in heating water. Then the filter paper was detached and observed in UV light for yellow fluorescence to show the existence of coumarins.

3.10 Gas chromatography mass spectrometry (GC-MS) analysis

For the identification of bioactive components in extract with greater antimicrobial activity, carbinol extract of *H. enneaspermus* was subjected to GC-MS analysis (Subashri and Pillai, 2014).

Procedure

GC-MS analysis was carried out on a GC-MS -5975C agilent system comprising an auto sampler and a gas chromatograph interfaced to a mass spectrometer (GC-MS) instrument, using the following conditions: column Elite-1 fused silica capillary column (30×0.25 mm ID × 1EM df, composed of 100% Dimethyl poly siloxane), functioning in electron impact mode at 70eV; helium (99.999%) was utilized as carrier gas at a continuous flow of 1.51 ml/min and an injection volume of 1 l was employed (split ratio of 10:1) injector temperature 2400°C; ion-source temperature 2000° C. The oven temperature was programmed from 700°C (isothermal for 2 min), with an upsurge of 100°C/min, to 3000°C/min, ends with a 9 min isothermal at 3000°C. Mass spectra were reserved at 70eV; with a scan range 40-1000 m/z. Solvent cut time

was 5 min; MS start time being 0 min; MS end time being 23 min; Ion source temperature set to 2000°C and interface temperature being 2400°C.

Identification of bioactive components

Interpretation of mass spectrum of GC-MS was carried out utilizing the database of National Institute of Standard and Technology (NIST) having above 62000 patterns. The mass spectrum of the unnamed component was contrasted with the spectrum of the recognized components stored in the NIST library. The name, molecular weight and structure of the component of the test materials were recognized.

3.11 Synthesis of zinc oxide (ZnO) and cerium oxide (CeO_2) nanoparticles

Preparation of plant extracts

The *in vitro* leaves of *H. enneaspermus* were washed exhaustively with distilled water and shade dried for 3 weeks. The dried leaves were powdered to fine particles by utilizing a mixer grinder. 10 g of leaf powder was dissolved in 100 ml of deionized water after that heated at 60°C for 10 min. The extract was permitted to cool, filtered (Whatman No.1 filter paper) and refrigerated until further utilization.

Materials required

1. 1 mM aqueous zinc (II) nitrate hexahydrate ($Zn(NO_3)_2. 6H_2O$)
2. 1 mM aqueous Cerium (III) nitrate hexahydrate ($Ce(NO_3)_3. 6H_2O$

Procedure

90 ml of 1 mM zinc (II) nitrate hexahydrate ($Zn(NO_3)_2. 6H_2O$) solution was mixed with 10 ml of aqueous extract of *H. enneaspermus* and permit to stand at room temperature until further colour change occurs. This solution was stirred continuously at a temperature of 120°C for 4-6 h. A white precipitate formed and then it turns into a yellowish brown in colour on constant stirring. Additionally, the precipitate was

calcined at 700°C for 5 h. Thus, ZnO nanopowder was obtained. The resulting dried ZnO nanopowder was stored in air tight container for further analysis.

90 ml of 1 mM Cerium (III) nitrate hexahydrate ($Ce(NO_3)_3$. $6H_2O$ solution was mixed with 10 ml of aqueous extract of *H. enneaspermus* and permit to stand at room temperature until further colour change occurs. This solution was stirred continuously at a temperature of 120°C for 4-6 h. A yellow precipitate formed and then it became a yellowish brown in colour on continuous stirring. Additionally, the precipitate was calcined at 700 ˚C for 5 h. Thus, a CeO_2 nanoparticle was obtained. The resulting dried CeO_2 nanopowder was stored in air tight container for further analysis.

3.12 Characterization techniques

Bio synthesized zinc oxide nanoparticles (ZnONP) and cerium oxide nanoparticles (CeO_2 NP) were characterized by UV visible spectroscopy, X-ray diffraction (XRD), Scanning Electron Microscopy (SEM), X- ray Photon Spectroscopy (XPS), Fourier transform infrared spectroscopy (FTIR), Energy dispersive X-ray analysis (EDX) and High resonance Transmission electron microscopy (HR-TEM).

UV visible spectroscopy

Bio-reduction of ZnO NP and CeO_2 NP was noted as a function of time using water as reference using UV-visible spectrophotometer at a scanning range of 0 - 900 nm.

Fourier Transform - Infrared Spectroscopy (FTIR)

The ZnONP and CeO_2NP were mixed with potassium bromide utilizing agate mortar and pestle to get a fine homogeneous powder of small particle size and made into pellets for recording the IR spectra. Transparent oxide semiconductor samples were prepared to form a disc of a few tenths of a millimetre in thickness with the

density between 10-100 mg/cm^3. The sample discs were formed by applying a pressure from a few hundred to tens of thousand pounds per square inch at ambient temperature under dry condition. The dried product extracts of *H. enneaspermus* were subjected to infrared spectral (IR) analysis to get the information on stretching modes of metal-oxygen groups. FTIR spectrophotometer (spectrum-2000 Perkin Elemer) was utilized to detect the stretching of metal oxygen bond.

X – Ray Diffraction (XRD)

An XRD is a non-destructive type of analytical technique which gives valuable insight into the lattice structure of a crystalline substance like unit cell dimensions, bond angles, etc. XRD is depends on the principle of valuable interference of x-rays and the sample concerned should be crystalline. X-Ray analysis was conducted via the PW 1148/89-based X-ray diffractometer employing manganese-filtered Cu Kα radiation wavelength (150.1542 nm) at 298K. The instrument was furnished with graphite monochromator and functioned at 40kV and 30mA.

X-Ray Photoelectron Specstroscopy (XPS)

XPS-measurements of the dried zinc oxide and cerium oxide nanoparticles were performed with an AXIS Ultra DLD photoelectron spectrometer manufactured by Kratos Analytical (Manchester, UK). XPS spectra were noted by utilizing monochromated aluminium K_α radiation for excitation, at a pressure of approximately 5×10^{-9} mbar. The electron emission angle was 0° and the source-to-analyser angle was 60°. The binding energy scale of the instrument was adjusted following a Kratos Analytical process which utilizes ISO 15472 binding energy data. Spectra were taken by setting the instrument to the hybrid lens mode and the slot mode provided that around a 300 × 700 μm^2 analysis area. Moreover, the charge neutraliser was used. The dried nanoparticles sample was also measured with EnviroESCA.

For characterisation of dried samples by XPS, the dispersion of the zinc oxide and cerium oxide nanoparticles in ethylene glycol was diluted with carbinol 1:50. A silicon wafer (100 crystal orientation) was purified using an UV Ozone Cleaner UVC-1014 (185 and 254 nm wavelength UV radiation source) manufactured by NanoBioAnalytics (Berlin, Germany) for 20 min. Finally, 2 μl of nanoparticle dispersion were deposited onto the wafer and heated up to 100°C for 15 min.

Field Emission - Scanning Electron Microscopy (FE-SEM) And Energy Dispersive X-Ray (EDX) Analysis

The zinc oxide and cerium oxide nanoparticles were characterized using high resolution FE-SEM analysis. The samples were prepared by simple drop coating of the suspension of ZnO NPs and CeO2 NPs separately on a carbon layered copper grid by essentially dropping a very small amount of the sample on the grid, with added solution being detached utilizing blotting paper. The film on the SEM grid was then permitted to dry under a mercury lamp for 5 min. EDX investigation was then executed utilizing the Hitachi S-3400N FE-SEM instrument furnished with a Thermo EDX attachment.

High Resonance Transmission Electron Microscopy (HR-TEM)

TEM Microscope yields information of materials regarding its particle morphology and crystallographic arrangements (i.e. crystal structure). The size, shape and alignment of the particles of the specimen and their affiliation to each other were determined on the scale of atomic diameters. Crystallographic information was obtained in terms of the alignment of atoms in the specimen and their degree of order, finding of atomic-scale flaws in areas contained in a few nanometres. To characterize the sample for its crystal lattice, lattice parameters, orientation relationship between two or possibly a large number of single crystal and the crystallographic orientation of defects of all kinds, dislocations, stacking faults, voids, boundaries etc., the electron

diffraction was performed. The size and morphology were studied by TEM, JEOL model JEM 3010 Electron microscope. Average size and size distribution of nanoparticles were assessed depends on TEM micrographs with the help of Sigma-Scan Pro software (SPSS Ins, Version 4.01.003).

Pholtoluminescence (PL)

The photoluminescence (PL) measurement was performed on a Perkin Elmer LS 45 spectrometer in the wavelength range of 350-550 nm.

3.13 *In vitro* Cytotoxic Activity

Anticancer activity of synthesized zinc oxide and cerium oxide nanoparticles of *H. enneaspermus* against human breast cancer (MCF-7) cells was analysed.

3.13.1 MTT Assay

The MTT assay is depends on the reduction on tetrazolium. The MTT assays will rely on number of cells present and mitochondrial activity per cell. The principle included is the cleavage of MTT into a blue coloured item (formazan) by mitochondrial enzyme succinate dehydrogenase (Denizot & Lang, 1986).

Procedure

ZnO NPs and CeO2 NPs from *H. enneaspermus* might have been tried for cytotoxic action against breast (MCF-7) cancer cell lines. MCF-7 cells were separately cultured in Minimal Essential Medium (MEM) supplemented with 10% inactivated Fetal bovine serum (Sigma-Aldrich), L- glutamine (3%), 100 µg/ml streptomycin and 100 U/ml penicillin G and grown at 37°C in a moistened atmosphere of 5% CO_2 in air. 200µl of 48hr monolayer culture of cells were individually sowed at densities of 5000 cells/well in a 96 - well microtiter plates. ZnO NPs and CeO2 NPs were added at a concentration ranges from 1, 10, 100 and 500µg/ml for MCF-7 cells, then cells were

incubated. Cellular morphology was perceived utilizing a phase contrast microscope (Leica, Germany) after 24 hours. 3-(4, 5-dimethyl thiazol-2yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay was executed to assess cytotoxic activity of ZnO NPs and CeO2 NPs. 200µl of 0.5% MTT was supplemented to each wells and incubated for 4 hours to obtain formazan crystals. It was dissolved in DMSO and the absorbance was read at 595 nm utilizing microplate reader (Bio-Rad, CA). Wells deprived of ZnO NPs and CeO2 NPs assisted as blank. Percentage inhibition of cell viability was measured utilizing the formula, $A_{control} - A_{sample} / A_{control} \times 100$ (Gu and Sivam, 2006).

3.13.2 Apoptosis - Dual AO/EB staining of ZnO NPs and CeO2 NPs

A human breast cancer cell line (MCF-7 cells), purchased from National Centre for Cell Sciences (NCCS), Pune in the logarithmic growth stage were absorbed with 0.25% trypsin. RPMI1604 culture medium containing 10% fetal calf serum was deposited in each well of a 96-well plate (100 µl/well). Cells were added to a final concentration of 5×10^5/ml and the plates were incubated. Cells were left untreated or treated with 10 µg/ml of ZnO NPs and CeO2 NPs synthesized from *H. enneaspermus*. The samples in a 96-well plate were divided into 3 groups (Control, ZnO NPs and CeO2 NPs), with 24 well samples in each group corresponding to different reagent concentrations. After being cultured for 24 h, 20 µl of trypsin was added into every cell. When cells had sloughed off, suspensions (25 µl) were moved to glass slides. Dual fluorescent discoloration solution (1 µl) comprising 100µg/ml AO and 100 µg/ml EB (AO/EB, Sigma) was added to every suspension and then roofed with a coverslip. The morphology of apoptotic cells was inspected and 500 cells were totalled within 20 min utilizing a fluorescent microscope (OLYMPUS, Japan). Dual acridine orange/ethidium bromide (AO/EB) discoloration way was rehashed 3 times at least. Acridine orange is

occupied by both viable and nonviable cells and discharge green fluorescence if interconnected into double stranded nucleic acid (DNA) or red fluorescence if bound to single stranded nucleic acid (RNA). Ethidium bromide is occupied only by nonviable cells and discharge red fluorescence by interconnected into DNA. We recognized four kinds of cells along with the fluorescence discharge and the morphological aspect of chromatin condensation in the discolored nuclei. Viable cells have uniform bright green nuclei with efficient structure. Apoptotic cells have orange to red nuclei with condensed or fragmented chromatin. Necrotic cells have a consistently orange to red nuclei with condensed structure (Baskic *et al*., 2006).

3.14 Antimicrobial Activity

Antimicrobial susceptibility test was done by utilizing the disc diffusion assay of the extracts that could inhibit the growth of bacteria or fungi.

Source of microorganisms

Strains of bacteria including *Escherichia coli* (MTCC 443), *Klebsiella pneumoniae* (MTCC 109), *Pseudomonas aeruginosa* (MTCC 1035), *Salmonella typhi* (MTCC 98), *Staphylococcus aureus* (MTCC 29213), *Proteus vulgaris* (MTCC 1771), *Streptococcus faecalis* (MTCC 0459), *Enterococcus faecalis* (MTCC 2729), yeast, *Candida albicans* (MTCC 183) and fungus *Cryptococcus neoformans* (MTCC 1346) were procured from Institute of Microbial Technology, Chandigarh.

Disc Diffusion Assay

Antimicrobial susceptibility tests estimate the capability of an antibiotic or other antimicrobial agent to inhibit bacterial or fungal growth *in vitro* which can be measured by the diffusion method.

Materials required

1. Nutrient Agar Medium

Glucose	- 5g
Beef extract	- 3g
Peptone	- 5g
Sodium chloride (Nacl)	- 5g
Agar	-15g
Distilled water	- 1L
pH	-7.0

2. Sabourad's Dextrose Agar Medium

Glucose	- 20g
Peptone	-10g
Agar	-15g
Distilled water	-1L
pH	- 6.5

3. Sterile Whatman No.1 filter paper disc
4. 1% Dimethyl sulfoxide (DMSO)
5. Chloramphenicol
6. Nystatin

Procedure

All bacterial strains were cultured on nutrient agar plates and incubated at 37°C while fungal strains were cultured on Sabourad's dextrose agar plates and incubated at 25°C. Overnight grown cultures of the chosen bacterial and fungal strains were inoculated in respective media and incubated at specified temperature for 24h and were utilized for the disc diffusion assay (Bauer *et al*., 1966). Sterile media plates were swabbed with 100 µl the overnight grown cultures of selected microorganism. Sterile What-man No.1 filter paper discs (6 mm diameter) were impregnated with 20 µl (100 µg) of acetone and carbinolic extracts of *H. enneaspermus* in dimethyl sulfoxide, air

dried and positioned on the seeded agar plates. The plates containing bacterial swabs were incubated at 37°C and those with fungal swabs were incubated at 25°C for 24h. Chloramphenicol and nystatin (30µg) were utilized as positive control for bacterial and fungal strains respectively while 1% DMSO was used as negative control. Triplicates were maintained and diameters of inhibition zone (DIZ) were measured for each of the organisms and expressed as mean ± S.D.

Statistical analysis

The results are expressed as Mean ± SD and Data were analysed by One - Way Analysis of Variance (ANOVA) followed by the Duncan's Multiple Range Test for comparisons using SPSS.