Antioxidant and Anticancer Activities of Roots of *Catharanthus roseus* (L.) G.Don

Keerthana S.[1], Mahalakshmi S.[1], Kavitha S.[1], Ramesh Babu N.G.[1], Sivaraj C.[2]* and Arumugam P.[2]
1. Department of Biotechnology, Adhiyamaan College of Engineering, Hosur-635130, Tamil Nadu, INDIA
2. Phytochemistry and Natural products Laboratory, Armats Biotek Training and Research Institute, Guindy, Chennai-600032, Tamil Nadu, INDIA
*****shivaraj27@gmail.com

Abstract
Antioxidant assays were performed to identify the antioxidant property of roots Catharanthus roseus. In the present study, in vitro antioxidant tests were performed for roots Catharanthus roseus for converting the radicals into non-radicals. Haemolysis inhibition assay was carried out to determine the toxicity of the root extract. Maximum antioxidant reduction was observed in phosphomolybdenum assay based on the reduction of Mo (VI) to Mo (V). Recent studies represent the significant use of Catharanthus roseus as an oral insulin substitute.

The GC-MS analysis was carried out to identify the volatile and semi-volatile compounds present in the root extract. The results of GC-MS analysis showed peak value for the compound Phenol, 2, 6-bis (1,1-dimethylethyl)-4-[(4-hydroxy-3,5dimethylphenyl) methyl]-. The anticancer activity of roots of Catharanthus roseus was studied against HepG-2 cell culture by MTT assay method. The results of the study indicate that the bioactive compounds present in the roots of Catharanthus roseus can be used for the treatment of cancer after certain clinical trials.

Keywords: *Catharanthus roseus* roots, Anti-oxidant activity, HepG-2 cell lines, MTT assay, GCMS.

Introduction

Cancer is one of the most fatal diseases that is confronting humanity right after cardiovascular diseases. Recent literature indicates the prevalence of cancer and its mortality rate. Plant-derived secondary metabolites have a therapeutic value in the treatment of cancers.

In the past few years, novel treatment methods have resulted in a considerable progress for the treatment of liver cancer. According to the 2019 survey conducted by the American Cancer Society, liver cancer was estimated to account for about 2.4% of all new cancer cases in the USA and for 5.2% of all cancer-related deaths. From the recent cancer patient registries, the risk factors include liver cirrhosis, hepatitis virus infections, metabolic syndrome and alcohol abuse[15].

Since cancer is one of the most common devastating diseases, there has been an intense search on various biological sources to develop a novel anti-cancer drug to combat this disease. The plant *Catharanthus roseus* is a flowering plant that belongs to the family Apocynaceae[1]. The plant is a rich source of alkaloids particularly, vincristine and vinblastine which are more specifically used in chemotherapeutic medications. The plant is traditionally used in the treatment of diabetes by local populations in India, Africa and China. The increasing death rate as a result of cancer has now led to search for novel drugs that can kill cancer cells without harming normal cells[10].

The substances of plant origin that exhibit antitumor properties belong to various groups of compounds such as alkaloids, diterpenes, diterpenoquinone, purine-based compounds, lactonic sesquiterpene, peptides, cyclic depsipeptide, proteins, macrocyclic polyethers[9]. The plant being a rich source of phytochemical components can be used in the treatment of various diseases like dyspepsia, asthma, tuberculosis, acne, allergy, Alzheimer's disease, cancer (particularly blood and lung cancer) and also in diabetes[4]. Free radicals are one of the major causes to oxidation of cells that result in creating imbalance between formation and neutralization of pro-oxidants that may result in oxidative stress in humans.

Plants are a rich source of antioxidants as they contain phenols, flavonoids, alkaloids, tannins, vitamins, terpenoids and many other phytochemical compounds that greatly help in the production of ethnomedicine[2]. The plant contains significant amounts of volatile and phenolic compounds including caffeoylquinic acids and flavonal glycosides which have potent antioxidant activity.

Recent research has proved that the ingestion of natural antioxidant can reduce the risk of cancer and chronic diseases. About 10% of the plant species are used as food by both humans and other animals. Hence, there is a possibility to use plants in medical practice and in remedial purposes. The current study aims to evaluate the possibility for novel pharmaceuticals since the plant is resistive to several bacterial species and is also used to treat cancer.

Material and Methods

Collection and handling of raw materials: *Catharanthus roseus* roots were collected in early December of 2019 from industrial estate in Hosur town, Krishnagiri district, Tamil Nadu, India. The roots of *Catharanthus roseus* were initially cleaned in running tap water and then washed with sterile distilled water. The cleaned roots were shade dried and cut into small pieces. About 150 g of the cut root samples were soaked in 200 mL of methanol for 72 hrs. The mixture was then filtered using filter paper and stored under sterile

conditions for future use. About 25 mL of plant sample was poured into sterile Petri plates and allowed to condense. This was later used for column chromatography.

***In vitro* antioxidant assays:** Spectrophotometric antioxidant assays were performed to identify the antioxidant property of methanolic root extract of *Catharanthus roseus.* Antioxidants are the components that either delay or inhibit the oxidation processes which occur under the influence of atmospheric oxygen or reactive oxygen species. These antioxidants are known as good stabilizers as they are used in the stabilization of cosmetics and pharmaceuticals[12]. Antioxidant assays were performed in order to prevent the deterioration of free radicals, fats and similar other food components.

DPPH˙ radical scavenging activity: The DPPH scavenging activity of methanol root extract of *Catharanthus roseus* was determined based on the reduction of purple DPPH to yellow coloured diphenylpicrylhydrazine. DPPH radical has an advantage of being unaffected by certain side reactions such as enzyme inhibition and metal ion chelation process unlike other free radicals[16]. The measurement of absorbance and colour changes was done at a wavelength of 517 nm (Systronics, Spectrophotometer 104). Ascorbic acid was used as the positive control. The percentage of inhibition was calculated by the following equation:

$$\%\ of\ inhibition = \frac{control - sample}{control} \times 100$$

Superoxide radical scavenging activity: Superoxide radical scavenging activity was carried out by following the method of Padh et al.[18] The result of superoxide radical scavenging activity was reported based on its ability to inhibit the formation of formazan upon the photochemical reduction of nitroblue tetrazolium (NBT). Generation of superoxide radicals is aided by the riboflavin-UV light system[18]. The decrease in absorbance was measured at 590 nm using UV-Vis spectrophotometer (Systronics, Spectrophotometer 104). Ascorbic acid was used as the positive control. The percentage of inhibition was calculated by the following equation:

$$\%\ of\ inhibition = \frac{control - sample}{control} \times 100$$

Ferric (Fe^{3+}) reducing power assay: FRAP assay was experimented by slightly modifying the method of Jayanthi et al.[14] Ferric reducing antioxidant power assay (FRAP) is used to determine the reducing power of methanol root extracts of *Catharanthus roseus*.

The FRAP assay measures the reduction of ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) by the antioxidants present in the methanol root extract[14]. The absorbance was measured at 700 nm. Ascorbic acid was used as a reference compound. The percentage reduction of ferric ions by the extract was calculated by the following equation:

$$\%\ of\ reduction = \frac{sample - contol}{sample} \times 100$$

Phosphomolybdenum reduction assay: Phosphomolybdenum reduction activity of methanol root extract of *Catharanthus roseus* was evaluated for its antioxidant capacity as explained by Prieto et al. The total antioxidant capacity of the sample was estimated based on the reduction of Mo (VI) to Mo (V)[3]. This method involves thermally generating auto-oxidation during prolonged incubation at higher temperature. Unlike other antioxidant assays, it remains complete irrespective of concentration of free metal ions present in the sample.

The absorbance of the reaction mixture was measured at 695 nm using UV-Vis Spectrophotometer (Systronics, Spectrophotometer 104). Ascorbic acid was used as the standard reference. The percentage of reduction was calculated by the following equation:

$$\%\ of\ reduction = \frac{(sample - control)}{sample} \times 100$$

Antihaemolytic assay: Anti-haemolytic activity of root extract of *Catharanthus roseus* was assayed using human venous blood sample. Human erythrocytes were collected from healthy adults in sterile EDTA vials. Anti-haemolytic activity of the methanol root extract of *Catharanthus roseus* was carried out under *in vitro* conditions. The decrease in absorbance was measured in UV-Vis Spectrophotometer (Systronics, Spectrophotometer 104) at 540 nm. The percentage of haemolytic inhibition was calculated by the following equation:

$$\%\ of\ \ inhibition = \frac{(control - sample)}{control} \times 100$$

Antibacterial activity: *In vitro* antibacterial activity of methanol root extract of *Catharanthus roseus* was studied against six bacterial strains by agar well diffusion method. Tetracycline (30 µL) was loaded as a standard and the other well was used the control[13]. The antimicrobial spectrum of the extract for the bacterial species was determined in terms of zone formed around each well. The diameter of zone of inhibition produced by the extract was compared with the zone produced by the standard tetracycline[7, 8].

Bacterial strains: The root extract of *Catharanthus roseus* was tested for its antibacterial activity against six bacterial strains namely *Bacillus subtilis, Staphylococcus aureus, Micrococcus luteus, Shigella flexneri, Escherichia coli* and *Proteus vulgaris.*

Analytical methods

Anti-cancer activity: The purpose of the MTT assay is to measure viable cells in relatively high throughput 96-well plates without the need for elaborate cell counting. The most common need for conducting MTT assay is to determine

cytotoxicity of several drugs at different concentrations. The principle of the MTT assay is marked with a constant mitochondrial activity and so it is liable to an increase or decrease in the number of viable cells for most viable cells[6]. The tetrazolium salt of MTT is converted into formazan crystals which are thereby reflected by the mitochondrial activities of the cells and it is finally solubilised for homogenous measurement.

Chemicals and reagents: MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) Invitrogen, USA. Acridine orange and other fine chemicals were obtained from Sigma, Aldrich, USA.

Cell culture: HepG2 cells obtained from NCCS (National Centre for Cell Science, Pune) were cultured in Rose-well Park Memorial Institute (RPMI) medium, supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin (250 U/mL), gentamicin (100µg/mL) and amphotericin B (1mg/mL) obtained from Sigma Chemicals, MO, USA. All cell cultures were maintained at 37˚C in a humidified atmosphere of 5% CO_2. Cells were allowed to grow on colonies for over 24 hrs before use.

Cell growth inhibition studies by MTT assay: Cell viability was measured with the conventional MTT reduction assay. HepG2 cells were seeded at a density of 5×10^3 cells/well in 96-well plates for 24 hrs, in 200 µL of RPMI with 10% FBS. Then culture supernatant was removed and RPMI containing various concentrations (5–160 µg/mL) of methanol root extract of *Catharanthus roseus* was added and incubated for 48 hrs. After treatment, the cells were incubated with MTT (10 µL, 5 mg/mL) at 37°C for 4 hrs and then with DMSO at room temperature for 1 hr. The plates were read at 595 nm on a scanning multi-well spectrophotometer. The viability of cells was calculated by the following equation:

$$cell\ viability = \frac{mean\ OD}{control\ OD} \times 100$$

Gas chromatography–mass spectrometry (GC-MS): GC-MS analysis was performed to identify the volatile and semi-volatile compounds present in the root extract of *Catharanthus roseus*. GC-MS analysis on the methanol root extract of *Catharanthus roseus* was carried out and the root extract was loaded into a HP-5 column (30 mm X 0.25 mm i.d with 0.25 µm film thickness), Agilent technologies 6890 N JEOL GC Mate II GC-MS model was used for carrying out GC-MS analysis[11].

Chromatographic conditions: Helium was used as a carrier gas of flow rate 1 mL/min, the injector was operated at a temperature of 200°C and column oven temperature was programmed as 50-250°C at a rate of 10°C/min injection mode[5].

Mass spectroscopy conditions: Ionization voltage of 70 eV, ion source temperature and interface temperature of 250°C with a mass range of 50-600 mass units were maintained[17].

Identification of components: The mass spectrum of GC-MS was analysed with the aid of the database from the National Institute of Standards and Technology (NIST). The mass spectrum of the components obtained from root extract of *Catharanthus roseus* was compared with that of spectrum of the known components which are stored in the NIST library.

Results and Discussion

In vitro antioxidant assays

DPPH˙ radical scavenging activity: Scavenging of 1, 1-diphenyl 2-picrylhydrazyl (DPPH) free radical is one of the prominent decolourization antioxidant assays. The maximum DPPH˙ radical scavenging activity of root extract of *Catharanthus roseus* was 80.68 ± 0.96% at 120 µg/mL concentration. The IC_{50} was found to be 57.39 µg/mL concentrations and it was compared with standard ascorbic acid with the IC_{50} 11.98 µg/mL concentration. The absorbance depends on the concentration of antioxidants present in the root extract. The root extract demonstrated high capacity for scavenging the free radicals by reducing the stable 1, 1-diphenyl-2- picrylhydrazyl (DPPH) radical to 1, 1-diphenyl-2-picrylhydrazine.

An odd electron in the DPPH is responsible for the visible deep purple color and also the absorbance reaction occurred at 517 nm. Once the DPPH is decolorized, the antioxidant ability of the DPPH radical is determined quantitatively by measuring the changes in absorbance. This concludes that the root extract has the ability to scavenge free radicals.

Table 1
DPPH˙ radical scavenging activity of root extract of *Catharanthus roseus*

Concentration (µg/mL)	% of Inhibition
20	25.04 ± 0.12
40	36.36 ± 0.42
60	52.27 ± 0.41
80	65.9 ± 0.24
100	73.86 ± 0.65
120	80.68 ± 0.96

Superoxide ($O_2^{\cdot-}$) radical scavenging activity: In superoxide radical scavenging activity, superoxide anion reduces the yellow dye (NBT^{2+}) to blue formazan. The maximum superoxide radical scavenging activity of *Catharanthus roseus* root extract was 92.12 ± 0.21% at 120 µg/mL concentration. The IC_{50} was found to be 20.37 µg/mL concentration and it was compared with the standard, ascorbic acid (IC_{50} = 9.65 µg/mL concentration). Later effects of $O_2^{\cdot-}$ anion are very harmful to cellular components. The consumption of superoxide anion in the reaction mixture is noted with the decrease in absorbance with antioxidants.

Antioxidants showcase the capacity to inhibit the formation of blue NBT formation. Studies have reported that flavonoids are effective antioxidants mainly because they scavenge superoxide anions. The results suggest that the *Catharanthus roseus* root extract is a more potent scavenger for superoxide radicals in a concentration dependent.

Table 2
Superoxide (O_2^-) radical scavenging activity of root extract of *Catharanthus roseus*

Concentration (µg/mL)	% of Inhibition
20	49.02±0.09
40	72.30± 0.59
60	83.58±0.06
80	88.09±0.12
100	90.77±0.18
120	92.12±0.21

Ferric (Fe^{3+}) reducing power activity: The ferric (Fe^{3+}) reducing power assay is carried out by the reduction of Fe^{3+} to Fe^{2+} by *Catharanthus roseus* root extract resulting in the formation of Ferro-ferric complex. Depending on the concentration of antioxidants, the colour changes from yellow to green or blue colour representing the reducing capacity of *Catharanthus roseus* root extract. The reducing capacity of a compound may serve as a potent indicator of its antioxidant activity. However, the activities of antioxidants can be determined by various mechanisms such as reducing capacity and radical scavenging, chain initiation prevention and the decomposition of peroxides.

The maximum Fe^{3+} reduction was found to be 76.28 ± 0.41% at 120 µg/mL concentration with the RC_{50} value of 57.79 µg/mL concentration. The RC_{50} of standard ascorbic acid was 7.72 µg/mL concentration. The reducing power of the *Catharanthus roseus* root extract showed that it has good antioxidant potential.

Table 3
Ferric (Fe^{3+}) reducing power activity of root extract of *Catharanthus roseus*

Concentration (µg/mL)	% of reduction
20	03.65 ± 0.24
40	34.96 ± 0.07
60	51.43 ± 0.67
80	64.38 ± 0.14
100	69.74 ± 0.13
120	76.28 ± 0.41

Phosphomolybdenum reduction activity: The total antioxidant activity of root extract of *Catharanthus roseus* was carried out by the phosphomolybdenum reduction method. At acidic pH, Mo (VI) was reduced to Mo (V) by the formation of green phosphate Mo (V) complex. The maximum phosphomolybdenum reduction was measured as 91.01 ± 0.22% at 120 µg/mL concentration with RC_{50} value of 18.08 µg/mL concentration. It was compared with the standard ascorbic acid (RC_{50} = 6.34 µg/mL concentration). The root extract has the ability to reduce radicals by donating electrons through phenolic compounds such as flavonoids, polyphenols, tannins andphenolic terpenes which determine the antioxidant effects of *Catharanthus roseus.*

The fundamental mechanism for a lot of human neurological disorders such as inflammation, viral infections, autoimmune pathologies anddigestive system disorders including gastrointestinal inflammation and ulcer, the causative agent is oxidative injury. According to the current study, it is clear that the *Catharanthus roseus* extract has significant antioxidants, through which it can yet reduce the exacerbation of free radicals.

Table 4
Phosphomolybdenum reduction activity of root extract of *Catharanthus roseus*

Concentration (µg/mL)	% of reduction
20	55.46 ± 0.24
40	81.05 ± 0.80
60	87 ± 0.05
80	89.42 ± 0.14
100	89.22 ± 0.61
120	91.01 ± 0.22

Antihaemolytic assay: The human red blood cells were determined for anti-haemolytic activity by incubating suspensions with serial dilutions by using PBS several times as they rinsed red blood cells until they reached the OD of the supernatant as control by centrifugation for 3 min at 3,000 *g*. The total numbers of RBCs were counted by a hemocytometer. The absorbance was measured at 570 nm and the decrease in absorbance was found to be 56.31± 0.47% at 120 µg/mL concentration. The results validate the non-toxic effect of methanolic root extracts of *Catharanthus roseus* and thereby paving the way towards possible pharmaceutical applications which benefits mankind.

Table 5
Anti-haemolytic activity of root extract of *Catharanthus roseus*

Concentration (µg/ml)	% of inhibition
20	23.01±0.07
40	34.49±0.37
60	40.01±0.14
80	45.12±1.13
100	49.82±0.35
120	56.31±0.47

Antibacterial activity: The effectiveness and the antimicrobial susceptibility of the *Catharanthus roseus* root

extract were analysed by measuring the diameter of the clear zone in cultures contained in Petri plates. The root extract showed maximum (18 mm) zone of inhibition against *Staphylococcus aureus* and minimum (13 mm) zone of inhibition against *Micrococcus luteus*. Due to the presence of alkaloids, the antibacterial activity may show the inhibition of bacterial growth. Based on the results, it can be concluded that the root may contain a powerful antibacterial constituent that has the ability to treat diseases caused by pathogens.

Analytical methods

Anti-cancer activity: The viability of HepG2 cells decreases with increase in concentration of the root extract. The MTT assay was carried out by varying drug concentration, time of exposure to drug, length of assay and cell density. The anti-proliferative activity of methanolic root extract of *Catharanthus roseus* was analysed by MTT assay method. Only viable cells have the ability to reduce MTT tetrazolium into a coloured formazan product. The cytoxicity of *Catharanthus roseus* root extract is expressed in IC_{50}, which is defined as the concentration at which 50% of the total toxicity of extract is reduced.

The IC_{50} was found to be 1.711 µg/ mL concentration. When the concentration of the sample is increased, it was observed that there is rapid decrease in cell proliferation and the cell-cell contact seemed to be reduced.

The secondary metabolites present in the *Catharanthus roseus* root extract inhibited the proliferation of HepG2 cells by increasing the rate of cell death. All these inhibition occurred under *in vitro* conditions in a dose dependent manner. From the results obtained, it can be concluded that decrease in the rate of malignancy of the cells causing liver cancer was due to the increase in concentration of the root extract.

Gas chromatography-mass spectrometry analysis: GC-MS analysis of root extract showed thirteen major peaks each representing a chemical compound. The results of gas chromatogram represent the retention time of eluted compounds in the root extract, while the results of the corresponding mass spectrum helped in determining the molecular mass of the compounds by comparing the spectra available in the NIST library. The results represent the presence of alcohols, esters, acids, steroids, nitrogen derived compounds and other secondary metabolites. The retention time, molecular weight and the bioactivity of the compounds are presented. The highest peak was obtained for the compound 2-Pentadecanone, 6,10,14-trimethyl- with retention time of 16.22.

Table 6
Antibacterial activity of *Catharanthus roseus* root extract

Bacteria	Zone of inhibition (mm)			
	100 µg/mL	**150 µg/mL**	**200 µg/mL**	**Standard Tetracycline (30 µg/mL)**
Staphylococcus aureus	14	16	18	20
Bacillus subtilis	10	12	14	26
Micrococcus luteus	10	12	13	18
Shigella flexneri	12	13	16	16
Proteus vulgaris	10	12	13	14
Escherichia coli	14	15	17	14

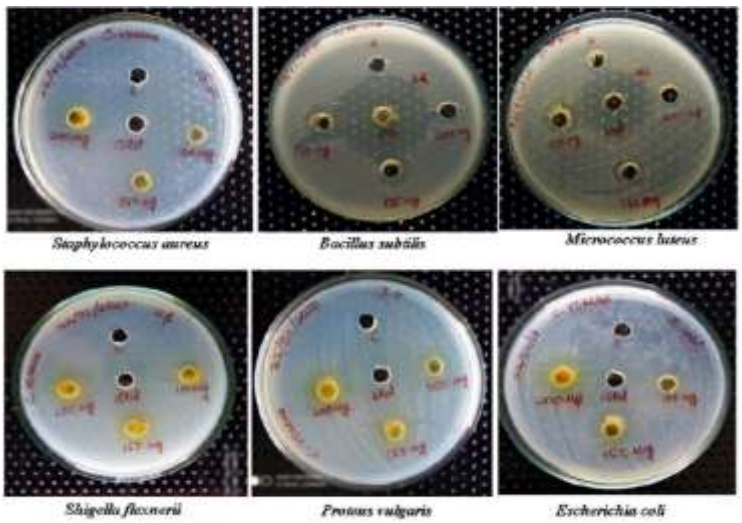


Fig. 1: Antibacterial activity of root extracts of *Catharanthus roseus*

Table 7
Percentage of cell death of *Catharanthus roseus*

Concentration (µg/mL)	% of Cell death
0.781	37.23±3.60
1.562	45.62±1.21
3.125	61.00±1.24
6.25	73.84±0.39
12.5	81.32±1.01
25	86.13±1.22
50	91.34±0.24
100	94.65±1.07

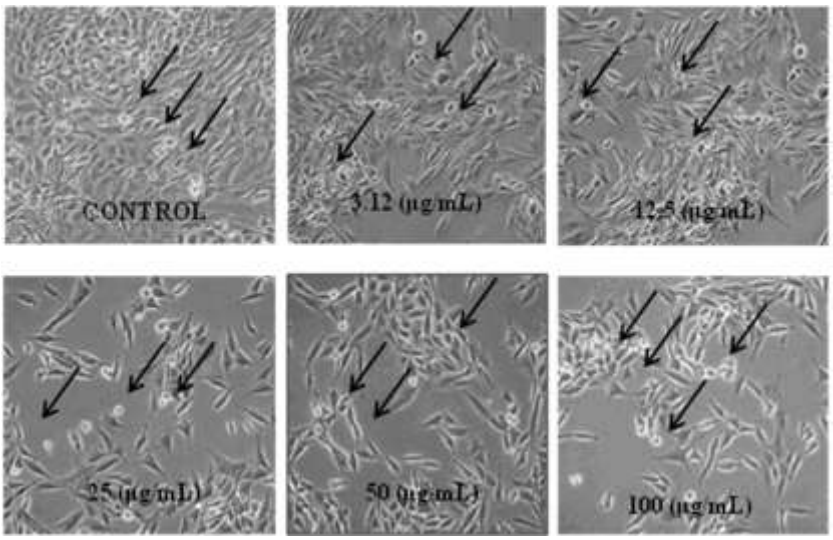


Fig. 2: Morphology of cell death at different concentration of root extracts of *Catharanthus roseus*

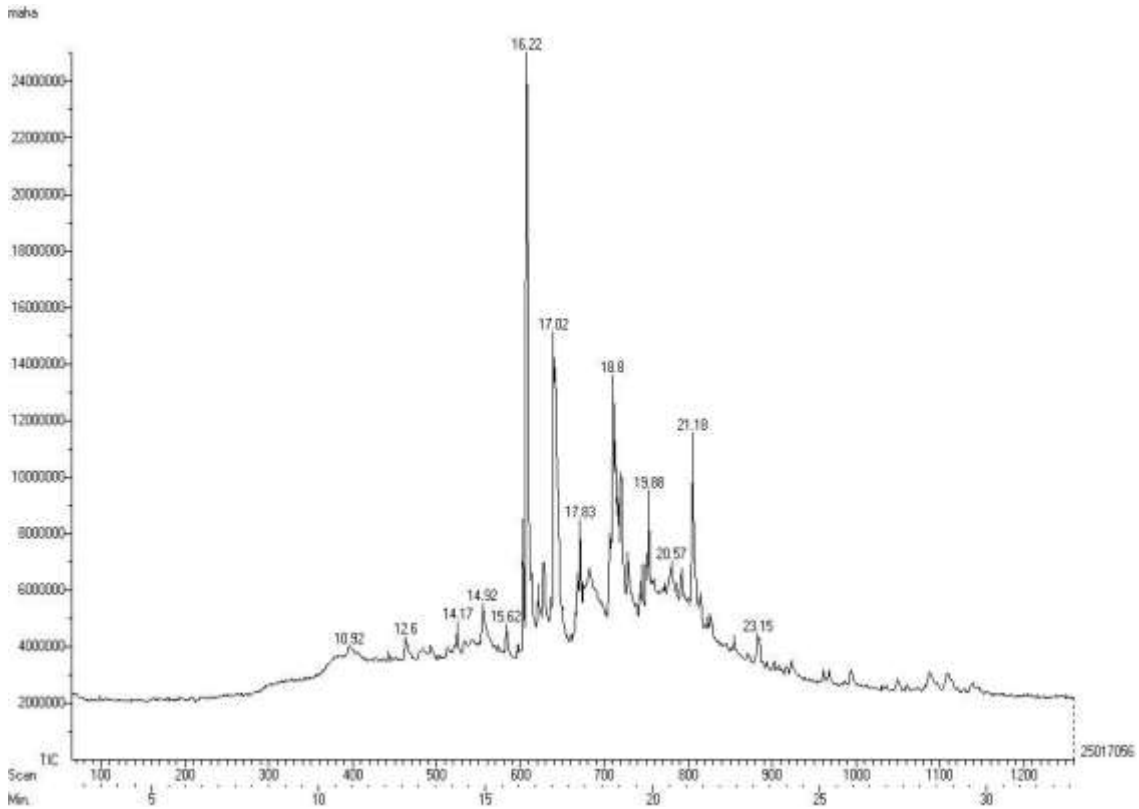
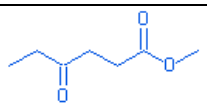
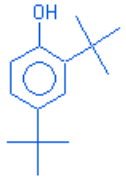
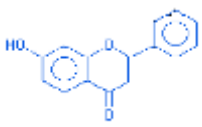
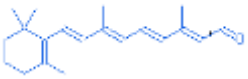
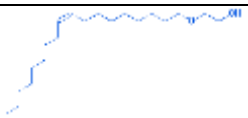
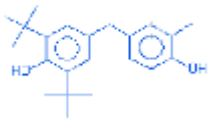
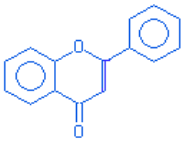


Fig. 3: GC- MS Chromatogram of *Catharanthus roseus* root extract

Table 8
Components isolated from *Catharanthus roseus* root extract by GC-MS analysis

Compound Name	Molecular Formula	RT (Retention Time)	Molecular Weight g/mol	Compound Structure	Biological Activity
Hexanoic acid, 4-oxo,methyl ester	$C_7H_{12}O_3$	10.92	144.168		-
Phenol, 2,4-bis (1,1-dimethylethyl)-	$C_{17}H_{30}OSi$	12.6	278.5		Anti-biofilm agent.
7-methoxy-2,2,4,8-tetramethyltricyclo[5.3.1.0(4,11)]undecane	$C_{16}H_{28}O$	14.192	236.39		Anti-fungal.
4H-1-Bezopyran-4-one,7-hydroxy-2-phenyl-	$C_{15}H_{10}O_3$	15.62	238.24		Hallucinogenic, Hematopoietic and Anti-HIV
2-Pentadecanone,6,10,14-trimethyl-	$C_{18}H_{36}O$	16.22	268.477		Anti-microbial and Hepatotoxic effect
Cyclopropaneoctanal, 2-octyl-	$C_{19}H_{36}O$	17.02	280.5		Anti-microbial, Anti-neuralgic and Hypoglycaemic
Rectinal, 9-cis-	$C_{20}H_{28}O$	17.83	284.4		Anti-oxidant
Phytol	$C_{20}H_{40}O$	18.8	128.170		Anti-cancer and Anti-spasmodic
Ethanol,2-(9-octadecenyloxy)-,[Z]-	$C_{20}H_{40}O_2$	19.88	312.42		Anti-cancer and Anti-gonorrhoeal
Z-5-Methyl-6-heneicosen-11-one	$C_{22}H_{42}O$	20.57	322.6		Anti-inflammatory, Anti-microbial and Increase in bioavailability of zinc
4,8,12,16-Tetramethylheptadecan-4-olide	$C_{21}H_{40}O_2$	21.18	324.5		-

Phenol,2,6-bis(1,1-dimethylethyl)-4-[(4-hydroxy-3,5-dimethylphenyl)methyl]-	$C_{23}H_{32}O_2$	23.15	340.434		Anti-microbial, Anti-oxidant and Anti-malarial
Flavone	$C_{15}H_{10}O_2$	14.17	222.24		Anti-diabetic, Anti-cancer, Anti-allergic and Anti-ulcer

Conclusion

The current study demonstrated that the methanolic root extract of *Catharanthus roseus* possesses different pharmacological activities as it acts as a carrier of several active biological components. The significant antioxidant, antibacterial, anti-haemolytic and anticancer results, evidently prove their potent pharmacological activity. Thus, it may be concluded that the root extract of *Catharanthus roseus* might be used as a potential source of anticancer agent after clinical trials.

Acknowledgement

We would like to thank IIT Madras for helping in GC-MS analysis.

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(Received 30th March 2020, accepted 31st May 2020)