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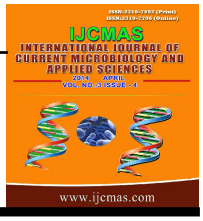

Original Research Article

Evaluation of phytochemical and in vitro anti-oxidant, anti proliferative activity of a polyherbal Siddha formulation *Vallarai nei*

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K e y w o r d s

Siddha;
Vallarai nei;
MTT assay

A B S T R A C T

In India, Cervical cancer occurrence in women is of high incidence rate that of 1, 30,000 patients are suffering among them 70,000-75,000 deaths occur annually. *Vallarai nei* (VN) a commonly known Siddha drug is prescribed by Siddha physicians for various ailments in clinical practice. In siddha literature *Vallarai nei* (VN) is indicated for *Yoni putru* (Cervical cancer).The objective of this study is to analyze scientific basis for the anti oxidant, anti proliferative property of VN on HeLa cell lines were evaluated *in vitro* by employing MTT assay. Preliminary phytochemical analysis was done to identify the presence of constituents. The crude extract exhibited cytotoxic effects on HeLa cell lines which correlates with the indication mentioned in the Siddha pharmacopeia. The IC 50 value obtained from the MTT assay was 51.6 ug/ml. The antioxidants activity assessed by different methods exhibited the presence of significant activity. The phytochemical screening revealed the presence of phenols and terpinoids which are useful in controlling the cancer cellular pathways. At the end the combined activities analyzed giving hope for prescribing the VN to the needy patients.

Introduction

Siddha system is one of the pioneer systems of medicine among traditional medicine practices in India (Sambasivam Pillai,1993). There are many formulations prescribed by the ancient Siddhars. Unique way of prescribing medicines by this system draws attention worldwide for keen research in drugs for reverse pharmacology manner. In this modern era there are number of new drug inventions are going day by day and substituting the previous generation drugs in order to empower the health systems. But the age old systems are blended with natural principles remains unchanged. The philosophy behind this system is bound with humane and nature of the universe. In Siddha system of medicine, the line of treatment goes with single drug therapy to compound drug formulations which are prescribed in classical literature as curative ailments for many diseases. WHO

reported the cervical cancer 275 000 deaths occurred worldwide in the year of 2008. About 70% of all cancer deaths occurred in low- and middle-income countries. Deaths from cancer worldwide are projected to continue to rise to over 13.1 million in 2030(WHO 2008 Cancer).

In India, Cervical cancer occurrence in women is of high incidence rate that of 1, 30,000 patients are suffering among them 70,000-75,000 deaths occurs annually (NCRP 2001). Some of the known causes are early age of marriage (<18 years), multiple sexual partners, multiple pregnancies, poor genital hygiene, smoking, use of oral contraceptives, religion, ethnicity, etc (Nair and Varalakshmi *et.al*, 2011). A ghee based poly herbal Siddha formulation *Vallarai nei* (VN) (Uthamarayan KS 1998) has been subjected for anti proliferative activity against HeLa cell lines, free radical scavenging activity and phytochemical analysis to establish its scientific basis as said in the literature.

Materials and Methods

Vallarai nei (VN) is purchased from a registered pharmacy. All the chemicals used in the present study were of analytical grade and purchased from a reputed laboratory. The alcoholic extracts at different concentrations were tested for percentage of cell viability against HeLa cell lies. The extract also subjected for Anti oxidant property by some of the specified methods. The same has been tested for phytochemical analysis for assessing its qualitative nature.

Cell lines and culture conditions

HeLa cell lines which are representing for human cervical carcinoma were purchased from NCCS Pune was maintained in Dulbecco's modified eagles media (HIMEDIA) supplemented with 10% FBS (Invitrogen) and grown to confluency at 37°c in 5 % CO2 (NBS, EPPENDORF, GERMANY) in a humidified atmosphere in a CO2 incubator. The cells were trypsinized (500µl of 0.025% Trypsin in PBS/ 0.5mM EDTA solution (HIMEDIA) for 2 minutes and passaged to T flasks in complete aseptic conditions.. Extracts were added to grown cells at a concentration of 10 µg, 50µg and 100µg from a stock of 10mg/mland incubated for 24 hours. The % difference in viability was determined by standard MTT assay after 24 hours of incubation

Preparation of *Vallarai nei* extract

The extracts are prepared by refluxing in ethanol for 72 hours followed by solvent recovery using rotary evaporator. The extracts are resuspended in 1% DMSO in a final concentration of 10mg/ml.

Cell viability assay (A*rung et al., 2009*)

Cell viability was determined by MTT assay performed according to the method described by Arung *el al.*, (2009) MTT is a colorimetric assay that measures the reduction of yellow 3-(4, 5dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilised with an organic solvent Dimethyl sulfoxide (HIMEDIA) and the released, solubilised formazan product was measured at 540nm. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells.The cell culture suspension was washed with

1x PBS and then added 30 µl of MTT solution to the culture (MTT -5mg/ml dissolved in PBS). It was then incubated at 37^0C for 3 hours. MTT was removed by washing with 1x PBS and 200µlof DMSO was added to the culture. Incubation was done at room temperature for 30 minutes until the cell got lysed and colour was obtained. The solution was transferred to centrifuge tubes and centrifuged at top speed for 2minutes to precipitate cell debris. Optical density was read at 540 nm using DMSO as blank.

(%) Viability = (OD of Test/OD of Control) × 100
Where OD = Optical density
The data were presented as percent of viable cells (%).

Total Cell concentration by Dye exclusion

Cells are loaded such that 5 X 10^6 cells /ml final density and grown to 60% con-fluency and was assessed by trypan blue cell exclusion assay (Strober W, 2001).

Observation of Cell morphological variations

It is viewed through phase contrast microscope and variations in morphology and photographs were taken (Figure 3).

Statistical analysis

The IC50 (median inhibition concentration) is the concentration of toxic compound that reduces the biological activity by 50 %. The IC50 value was obtained from the MTT assay and calculated using non-linear regression analysis in Microsoft Excel software. The value was expressed as a geometric mean. Differences were considered to be statistically significant when $p < 0.05$ and $p < 0.01$.

Determination of Anti oxidant activity

Anti oxidant activity of *Vallarai nei* is performed by using assay of Nitric oxide scavenging activity, Super oxide free radical scavenging activity and DPPH assay. The results were tabulated for further discussion.

Nitric Oxide Scavenging Activity

Nitric oxide (NO.) has also been involved in a variety of biological functions, including neurotransmission, vascular homeostasis, antimicrobial, and antitumor activities. Despite the possible beneficial effects of NO, its contribution to oxidative damage is also reported. This is due to the fact that NO can react with superoxide to form the peroxynitrite anion, which is a potential oxidant that can decompose to produce OH and NO. The procedure is based on the principle that, sodium nitro prusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions. Large amounts of NO may lead to tissue damage. Nitric oxide scavenging activity was measured spectrophotometrically. Sodium nitro prusside (5mmolL^{-1})in phosphate buffered saline pH 7.4,was mixed with different concentration of the extract (250-2500µg mL^{-1}) prepared in methanol and incubated at 25°C for 30minutes. A control without the test compound, but an equivalent amount of methanol was taken. After 30 minutes, 1.5mL of the incubated solution was removed and diluted with 1.5mL of Griess reagent (1%

sulphanilamide, 2% phosphoric acid and 0.1% N-1-naphthyl ethylene diaminedihydrochloride). Absorbance of the chromophore formed during diazotization of the nitrate with sulphanilamide and subsequent coupling with N-1 naphathyl ethylene diaminedihydrochloride was measured at 546nm and the percentage scavenging activity was measured with reference to the standard (Green *et al.*, 1982; Marcoci *et al.*, 1994a, b)

Super Oxide Free Radical Scavenging Activity

Super oxide is biologically important as it can form singlet oxygen and hydroxyl radical.

Overproduction of super oxide anion radical contributes to redox imbalance and associated with harmful physiological consequences. Super oxide anion are generated in PMS-NADH system by the oxidation of NADH and assayed by the reduction of NBT resulting in the formation of blue formazan product. 0.02ml of extracts, 0.05ml of Riboflavin solution (0.12mM), 0.2 ml of EDTA solution [0.1M], and 0.1 ml NBT (Nitro-blue tetrazolium) solution [1.5mM] were mixed in test tube and reaction mixture was diluted up to 2.64ml with phosphate buffer [0.067M]. The absorbance of solution was measured at 560nm using DMSO as blank after illumination for 5 min and difference in OD was determined after 30 minutes incubation in fluorescent light. Absorbance was measured after illumination for 30 min. at 560 nm on UV visible spectrometer. (Valentao *et al.*, 2002)

Calculation

% scavenging/Inhibition = [Absorbance of control - Absorbance of test sample/Absorbance of control] X 100

DPPH Assay (2, 2-diphenyl -1-picrylhydrazyl)

The radical scavenging activity of different extracts was determined by using DPPH assay according to Chang *et al* [2001].The decrease in the absorption of the DPPH solution after the addition of an antioxidant was measured at 517 nm. Ascorbic acid (10mg/ml DMSO) was used as reference.

Principle

1,1-diphenyl-2-picryl hydrazyl is a stable free radical with red colour which turns yellow when scavenged. The DPPH assay uses this character to show free radical scavenging activity. The scavenging reaction between (DPPH) and an antioxidant (H-A) can be written as,

DPPH + [H-A] →DPPH-H + (A)

Antioxidants react with DPPH and reduce it to DPPH-H and as consequence the absorbance decreases. The degree of discolouration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability.

Reagent Preparation

0.1mM DPPH solution was prepared by dissolving 4mg of DPPH in 100ml of ethanol.

Procedure

Different volumes (1.25-10µl) of plant extracts were made up to 40µl with

DMSO and 2.96ml DPPH (0.1mM) solution was added. The reaction mixture incubated in dark condition at room temperature for 20 minutes. After 20 minutes, the absorbance of the mixture was read at 517nm.3ml of DPPH was taken as control (Braca *et. al*, 2002).

Calculation

$$\% \text{ inhibition} = \frac{control - test}{control} X100$$

Phytochemical Analysis

Phytochemicals, chemical compounds that occur naturally in plants (phyto means "plant" in Greek), are responsible for color and biological properties. The term is generally used to refer to those chemicals that may have biological significance but are not established as essential nutrients. The following tests are used for the analysis of phytochemicals as described by a standard method (Harborne and Onwukaeme and coworkers, 1999). The following tests are used for the analysis of phytochemicals present in the alcoholic extract of the tested drug. Some of the tests are done based on the standard procedure to assess the presence of alkaloid, flavanoid, phenols, glycdosides, terpinoids, saponins and tannins. The inferences were listed in table 7.

Test for Alkaloids

Dragandroff's test

8g of Bi $(No_3)_3$. $5H_2O$ was dissolved in 20 ml HNO_3 and 2.72g of potassium iodide in 50 ml H_2O.These were mixed and allowed to stand. When KNO_3 crystals out, the supernatant was discarded off and made up to 100 ml with distilled water. The alkaloids were regenerated from the precipitate by treating with Na_2CO_3 followed by extraction of the liberated base with ether.To 0.5ml of alcoholic solution of extract added to 2.0 ml of HCl. To this acidic medium 1.0 ml of reagent was added. An orange red precipitate produced immediately indicates the presence of alkaloids.

Test for Flavanoids

Shinoda's test

In a test tube containing 0.5 ml of alcoholic extract 5-10 drops of dilute HCl and a small piece of $ZnCl_2$ or Mg were added and the solution was boiled for few minutes. In the presence of flavanoids reddish pink or dirty brown color was produced.

Test for Saponins

In a test tube containing 0.5 ml of aqueous extract, a drop of sodium bicarbonate was added. The mixture was shaken vigorously and kept for 3 minutes. A honey comb like froth was formed and it showed the presence of Saponins.

Test for Phenol

Ferric chloride test

To 2 ml of alcoholic solution of extract, 2 ml of distilled water followed by drops of 10% aqueous solution of $FeCl_3$ solution were added. Formation of blue or green indicates the presence of phenols**.**

Test for Glycosides

A small amount of alcoholic extract was dissolved in 1 ml of H_2O and the aqueous NaOH solution was added. Formation of yellow color indicates the presence of glycosides.

Test for Steroids

Salkowski test

To 2ml of chloroform extract 1ml of concentrated H_2SO_4 was added carefully along the sides of the test tube in the presence of sterols a red color was produced in the chloroform layer.

Test for Tannins

Ferric chloride test

To 1 -2 ml of aqueous extract, few drops of 5%aqueous ferric chloride solution was added. A bluish black colour, which disappears in addition of a few ml of sulfuric acid, formation of yellowish brown precipitate.

Test for Triterpenoids

Libermann Burchard test

The extract, 10 mg was dissolved in 1 ml of chloroform; 1ml of acetic anhydride was added following the addition of 2 ml of Conc.H2SO4. Formation of reddish violet colour indicates the presence of triterpenoids.

Results and Discussion

The results showed that there was a concentration dependent cytotoxic effect of crude extract of *Vallarai nei*. At the concentration increased from 10 to 100ug/ml, percentage of inhibition increased from64.83 % to 50.33%. At a concentration of 100 ug/ml there was a decrease in cell viability (Table 1, Figure 2). The IC50 value was obtained at 51.6 ug/ml (Figure 2a). The antioxidant activity observed in different methodologies showed that *Vallarai nei* is having significant anti oxidant activity (Figure 4). The total cell count of HeLa cells was decreasing with increase in concentration of the *Vallarai nei* extract indicating an inhibitory effect on the cancer cell line. Phytochemical analysis revealed that the presence of phenols and terpinoids (Table 2). Phenols can enhance the body's immune system to recognize and destroy cancer cells as well as inhibiting the development of new blood vessels (angiogenesis) that is necessary for tumour growth. They also attenuate adhesiveness and invasiveness of cancer cells thereby reducing their metastatic potential. Plant phenolics appear to have both preventive and treatment potential in combating cancer.

Antioxidants are slow down the oxidative damage of our body. Antioxidants act as a free radical scavengers. Preventing and repairing damages. Health problems such as Heart diseases, cancer and degenerative disorders are all exacerbated by oxidative damage. The Antioxidant activity of the drug was tested by DPPH, Nitric oxide scavenging activity, Super oxide free radicals scavenging activity.

The results suggested that the *Vallarai nei* extract inhibited the proliferation of human cervical cancer HeLa cells. Further studies are needed to explore the intracellular mechanism. The studies of anti proliferative, anti oxidant and phytochemical reveal that the VN is a good choice of drug as said in the literature. It can be combined with other drugs and administered to treat cervical cancer. Clinical Documentations on this line will give hope to the needy patients.

Table.1 Antiproliferative effect of *Vallarai nei* extract

Sample concentration (µg/ml)	OD at 540nm	% viability
Control	0.6034	100
10µg/ml	0.3912	64.83
50µg/ml	0.3037	50.32
100µg/ml	0.3027	50.33

Table.2 Phytochemical Analysis of *Vallarai nei*

Test	Observation	Inference
Alkaloid	No characteristic change was observed	Absence of alkaloid
Flavanoid	No characteristic change was observed	Absence of flavanoids
Phenols	Yellow precipitate was formed	Presence of phenols(+)
Glycosides	No characteristic change was observed	Absence of glycosides
Terpinoids	Red colour was formed	Presence of terpenoids(+++)
Saponins	No characteristic change was observed	Absence of saponins
Tannins	No characteristic change was observed	Absence of tannins

Fig.1 Ingredients of *Vallarai Nei*

Myristica fragrans

Myristica fragrans

Qurcus infectoria

Rhus succidinia

Piper cubeba

Woodfordia fruticosa

Syzygium aromaticum

Glycyrrhiza glabra

Eletteria cardamomum

Saccharum officinarum

Centella asiatica

Vallarai Nei

Fig.2 Percentage of viability at dose dependent manner of the tested drug

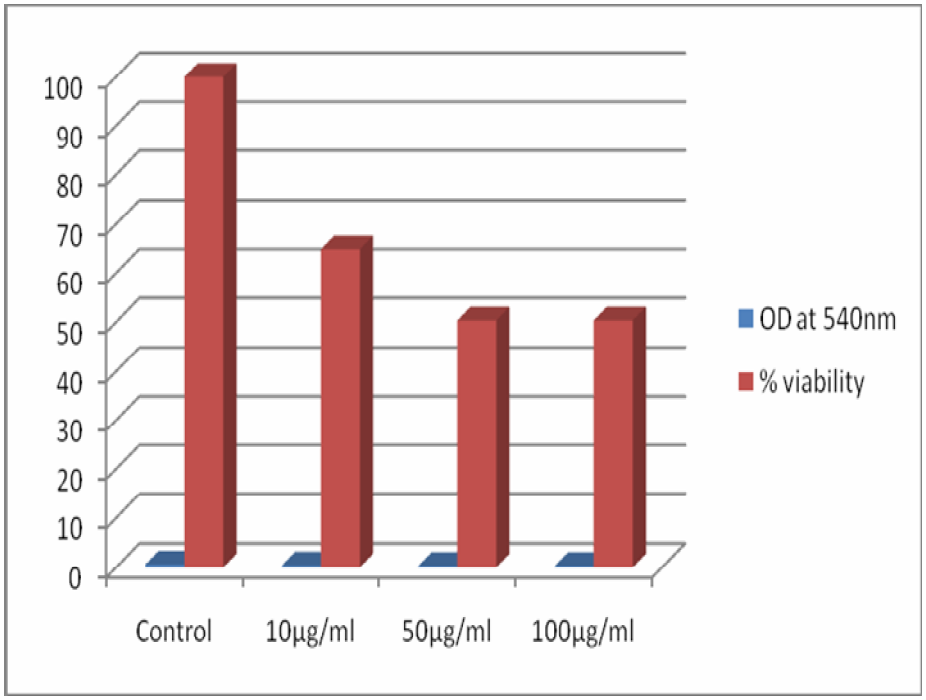


Fig.2a *Vallarai nei* at different concentration and the percentage of inhibition

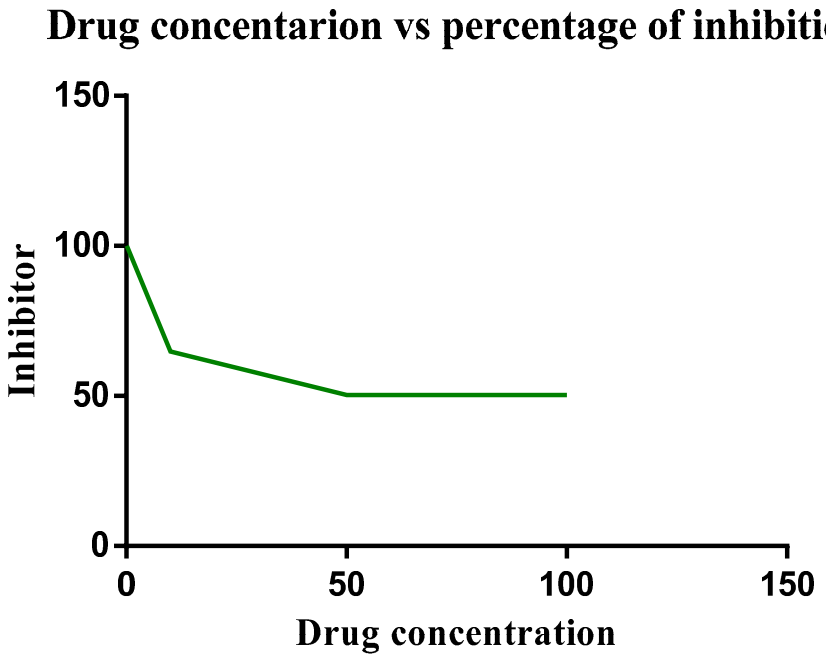
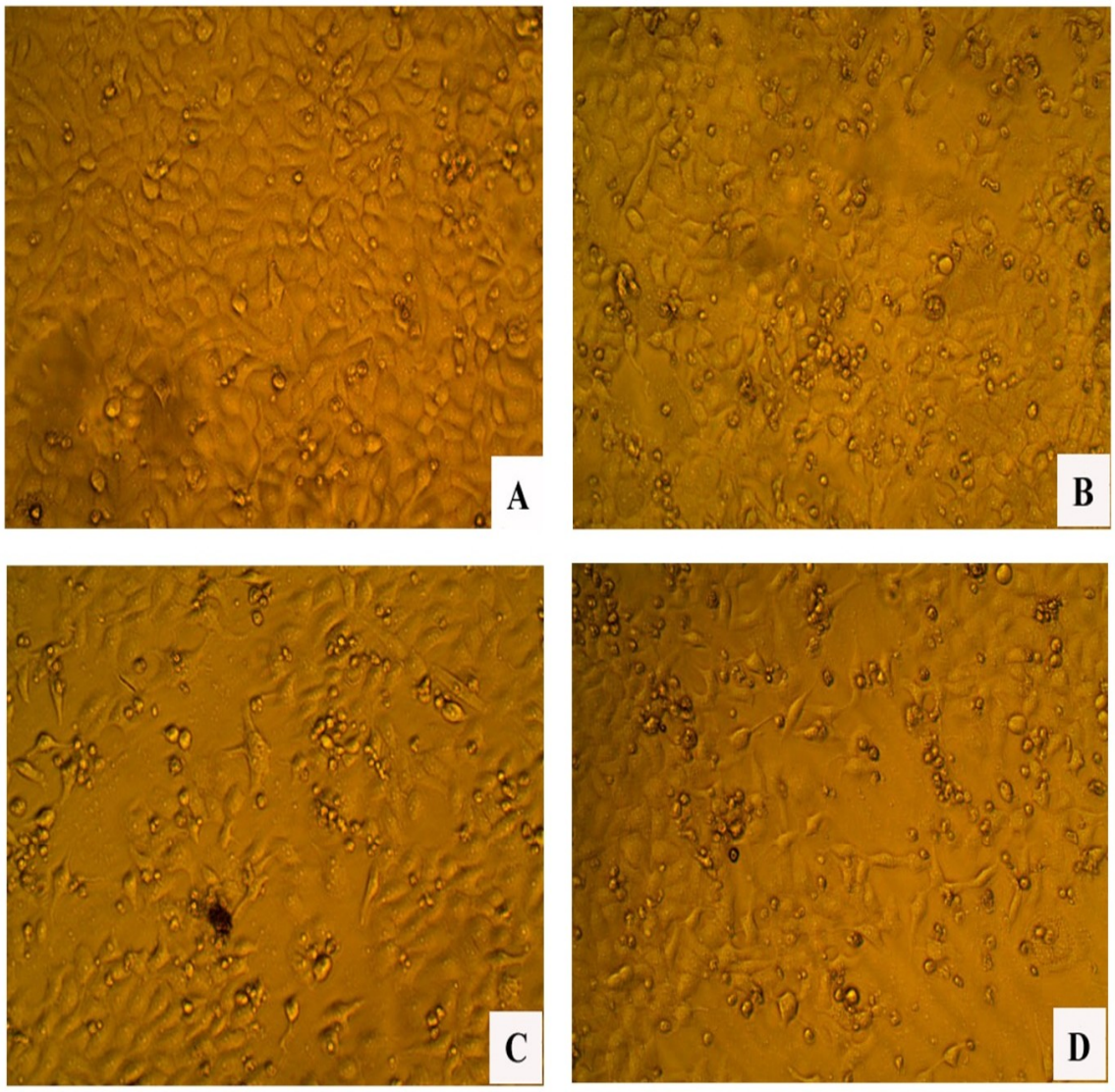
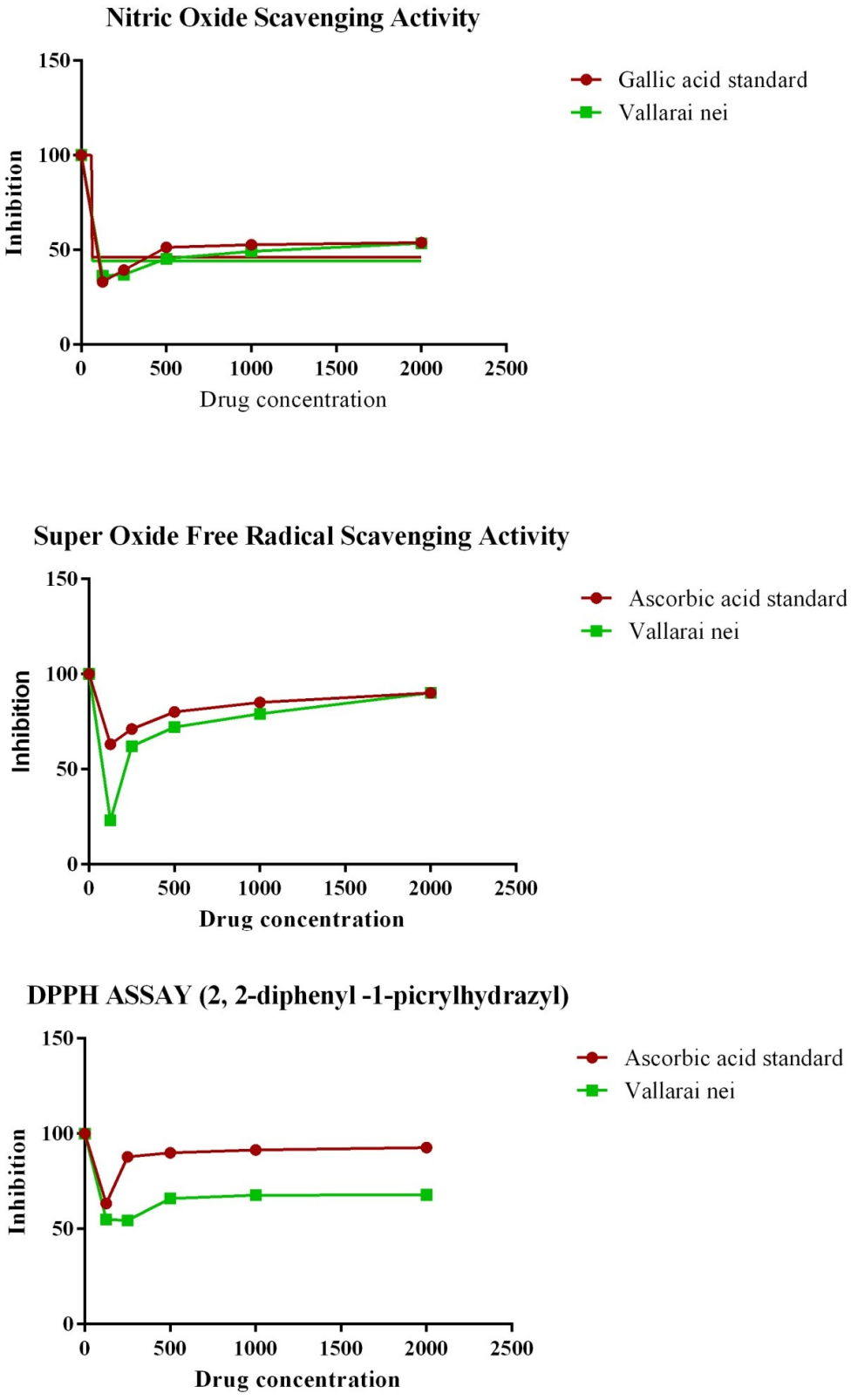


Fig 3 .HeLa Cell lines treated with *Vallarai nei* at different dosages



A - Control **B- 10 µg/ml** **C- 50 µg/ml** **D- 100µg/ml**

Fig.4 Anti oxidant activity of *Vallarai nei*



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