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PRELIMINARY PHYTOCHEMICAL SCREENING AND ANTIOXIDANT ACTIVITY NARDOSTACHYS JATAMANSI LEAVES EXTRACT

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ABSTRACT

Background: Nardostachys jatamansi DC is a Himalayan medicinal herb that has been described in various traditional systems of medicine for its use in cancer. In view of its traditional claims, and chemical constituents were evaluated in antioxidant properties. **Methods**: Petroleum ether (NJPE) and hydro alcoholic (NJHA) roots of N. jatamansi were prepared. Phytochemical investigation and antioxidant activities were determined using suitable methods. **Results**: NJH fractions exhibited prominent antioxidant activity with significant correlation between phenolic content and ABTS (IC50) scavenging ($R = -0.9988$, $P < 0.05$), and total antioxidant capacity ($R = 0.4396$, $P > 0.05$). Statistical analyses revealed NJHA and NJPE exhibited significantly higher ($P < 0.05$). Cell cycle analysis demonstrated that NJM, NJPE and NJEA caused G2/M arrest while NJDE caused oxidation cells. **Conclusion**: According to our findings, NJHA has important properties that are mediated via pro-apoptotic and cell cycle disruption. Additionally, this study emphasizes NJHA's antioxidant capacity, which is explained by the phenols it contains. Further mechanistic and phytochemical studies are being conducted to determine the active principles of NJPE, which was shown to be the most powerful fraction..

KEYWORDS: Antioxidant, cell cycle, apoptosis, and Nardostachys jatamansi.

1. INTRODUCTION

Superoxide, hydroxyl, and nitric oxide radicals are examples of reactive oxygen species (ROS) and reactive nitrogen species (RNS) that may damage DNA and cause lipid and protein oxidation in biological systems. The human body's antioxidant system can typically scavenge these radicals, maintaining the equilibrium between oxidation and anti-oxidation.

Plant polyphenols are an essential family of chemicals that suppress oxidative stress and DNA damage linked to mutagenesis, carcinogenesis, and premature aging. They also act on numerous cancer inflammation and reactive oxygen/reactive nitrogen species (ROS/RNS)-mediated pathways. Purines, pyrimidines, and chromatin proteins are all reacted with by free radicals, resulting in base modifications, unstable genomes, and genetic changes. Uncontrolled cell proliferation and tumor growth are the results of these transformed cells' changed amounts of cell cycle and apoptosis signaling molecules. As a result, stopping ROS/RNS from damaging cellular macromolecules has become a desirable cancer prevention technique.

A tiny, upright, hairy perennial herb that is threatened, Nardostachys jatamansi DC is a member of the valerianaceae family. Harvested across the Himalayas, the roots and rhizomes are sold from the mountainous areas to India's plains. Additionally, the plant grows in Afghanistan, Pakistan, South-West China, Nepal, and Bhutan. Since 1000–800 B.C., N. jatamansi has been used medicinally in Ayurvedic and Unani systems of medicine. Sesquiterpenoids, terpenic coumarins, flavonoids, alkaloids, phenols, lignans, and neo-lignans are abundant in the rhizomes.

The herb is used as a sedative, antidepressant, antiepileptic, antihysteric, hypotensive, antispasmodic, anti-inflammatory, and cardiotonic, according to traditional medical systems. The roots are used as stimulants, diuretics, emmenagogues, antispasmodics, deobstruents, aromatics, and bitter tonics. Additionally, the plant's roots were traditionally utilized to treat solid tumors and indurations. under several medical systems.

The cytotoxicity of N. jatamansi's alcoholic extract and nbutanol fraction in lung, liver, ovarian, and prostate cancer cell lines has been documented by Bhagat et al. Additionally, the cytotoxicity of the crude chloroform:methanol extract and two novel sesquiterpenoids produced from the roots and rhizomes of N. jatamansi have been investigated in lung, prostate, ER-positive breast cancer, and neuroblastoma cell lines.

To the best of our knowledge, this is the first study to examine the antioxidant qualities and cytotoxic activity of N. jatamansi's entire hydro acloholic and petroleum ether extract. We report for the first time the antioxidant activity of the extract and subsequent fractions of N. jatamansi by a variety of antioxidant assays. The antioxidant potential of the whole hydro alcoholic extract of N. jatamansi has been reported by DPPH, superoxide, hydroxyl radical scavenging, and total antioxidant capacity assays. In order to provide strong support for its use as an adjuvant to reduce oxidative stress in the advancement of cancer, a potential association between the antioxidant activity and the total phenolic and flavonoid content of the plant extract/fractions was also examined.

2. METHODS

2.1 Chemicals

We bought the following supplies from Sigma Chemicals Co. (St. Louis, MO, USA): Folin-Ciocalteu reagent, gallic acid, quercetin, ascorbic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 3-(4, 5-dimethylthiazolyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), crystal violet, and propidium iodide. The remaining chemicals and solvents were all analytical grade and acquired from standard suppliers.

2.2 Plant material

In September 2021, N. jatamansi roots and rhizomes were gathered from the Uttarakhand area of Garhwal. Dr. V.P. Bhatt, a scientist at the Herbal Research & Development Institute in Chamoli, Uttarakhand, verified the plant's authenticity. For future use, a voucher specimen (Ref number 320/ HRDI/21-1/2020-21) has been placed in our institute's herbarium, the Herbal Research & Development Institute.

2.3 Soxhlet extraction

After being dried and ground into a powder using petroleum ether, Nardostachys jatamansi was put in a Soxhlet apparatus thimble. Using a 70% ethanol (hydroalcoholic) solvent solution, the extraction was conducted for 8–10 hours at a temperature of 40–60 degrees Celsius on the heating mantle. Following the extraction procedure, the sample's extract was filtered and dried out. An airtight container was used to collect the extracts. All extracts' extraction yields were determined using the equation below.

Formula of Percentage yield = $\frac{\text{Actual yield}}{\text{Theoretical yield}} \times 100$

2.4 Qualitative Phytochemical Estimation of Extracts

Using conventional method, comprehensive phytochemical testing was conducted to determine if various phytoconstituents were present in Nardostachys jatamansi extracts. The following tests were performed on the extracts:

Tests for carbohydrates

- **Molisch test:** Two to three drops of alcoholic α-naphthol solution were added to one milliliter of extract. Along the test tube's side, concentrated sulfuric acid was applied. The presence of carbohydrates in the test samples is confirmed by the purple ring that forms at the intersection of two liquids.
- **Fehling's test:** Similar amounts of Fehling's solutions A and B were added to 1 milliliter of extract, and the mixture was heated for a few minutes in a water bath. A brick-red precipitate was seen to form.
- **Benedict's test:** A test tube containing an equal volume of Benedict's reagent and

extract was heated in a water bath for five to ten minutes. The amount of reducing sugar contained in the test solution determines whether the solution looks green, yellow, or red during the process.

- **Barfoed's test:** A test tube containing milliliters of extract and Barfoed's reagent was heated on a water bath for two minutes. The presence of monosaccharide is shown by the red color caused by the production of cupric oxide.

Test for alkaloids

Prior to filtering, each test extract was individually treated with dil. hydrochloric acid. The following tests were performed on the filtrate of each test extract:

- **Mayer's test:** Along the tube's sidewalls, a few drops of Mayer's reagent were added to two to three milliliters of filtrate. Alkaloids are present when a white or creamy precipitate forms.
- **Hager's test:** In a test tube, a few drops of Hager's reagent were added to 1-2 milliliters of filtrate. When a yellow-colored precipitate forms, alkaloids are present.
- **Wagner's test:** In a test tube, a few drops of Wagner's reagent were added to 1-2 milliliters of filtrate. Alkaloids are present when a reddish-brown precipitate forms.

Test for flavonoids

- **Lead acetate test:** A few drops of lead acetate solution were added to the extract. The presence of flavonoids may be indicated by the formation of a yellow precipitate.
- **Alkaline reagent test:** In a test tube, the extract was individually treated with a few drops of sodium hydroxide. The presence of flavonoids is shown by the formation of a bright yellow hue that fades when a few drops of diluted acid are added.
- **Shinoda test:** Five milliliters (95%) of ethanol were added to the extract. A small amount of magnesium turning was applied to the mixture, and then intense hydrochloric acid was added drop by drop. Flavonoids are present when a pink hue forms.

Test for glycosides

- **Borntrager's test:** Dilute sulfuric acid was added to 3 milliliters of extract, heated for 5 minutes, and then filtered. An equal volume of either chloroform or benzene was added to the cooled filtrate, and it was well shaken. Ammonia was added to the organic solvent layer after it had been separated. Anthraquinone glycosides are present when the ammonical layer becomes pink to crimson.

- **Legal's test:** Pyridine was used to dissolve 1 milliliter of the extract. After adding 1 milliliter of sodium nitropruside solution, 10% sodium hydroxide solution was used to make the mixture alkaline. The presence of cardiac glycosides is indicated by the formation of a pink to blood red hue.
- **Keller-Killiani test:** In a test tube, two milliliters of extract were mixed with three milliliters of glacial acetic acid and one drop of 5% ferric chloride. By the test tube's side, gently add 0.5 cc of concentrated sulfuric acid. Heart glycosides are present when a blue tint forms in the acetic acid layer.

Test for protein and amino acids

- **Biuret's test:** In a test tube, 1 milliliter of a 10% sodium hydroxide solution was added to the extract, and it was then heated. To the combination above, a drop of a 0.7% copper sulphate solution was added. The presence of proteins is indicated by the production of a violet or pink color.
- **Ninhydrin test:** For ten minutes, three milliliters of the extract and three drops of a 5% Ninhydrin solution were heated in a water bath. The presence of amino acids is shown by the formation of blue color.

Test for saponins

- **Foam test*:*** In a graduated cylinder, 1 milliliter of extract was dissolved in 20 milliliters of distilled water and then shacked for 15 minutes. A layer of continuous foam about 1 cm thick was seen to form.

Test for triterpenoids and steroids

- **Salkowski's test:** Chloroform was used to treat the extract before it was filtered. A few drops of strong sulfuric acid were added to the filtrate, agitated, and left to stand. Sterol is present if the bottom layers become crimson. Triterpenes are present when a golden yellow coating forms at the bottom.
- **Libermann-Burchard's test:** Chloroform was used to treat the extract. A few drops of acetic anhydride were added to this solution, which was then heated and allowed to cool. Through the test tube's sidewalls, concentrated sulfuric acid was introduced. The presence of steroids is shown by the creation of a brown ring at the intersection of two layers, whereas the presence of triterpenoids is indicated by the production of a deep red hue.

Test for tannin and phenolic compounds

- **Ferric chloride test:** Distilled water was used to dissolve a portion of the extract. Two milliliters of a 5% ferric chloride solution were added to this mixture. Phenolic chemicals are present when a blue, green, or violet hue forms
- **Lead acetate test:** Distilled water was used to dissolve a portion of the extract. A few drops of lead acetate solution were added to this mixture. Phenolic chemicals are present when a white precipitate forms.
- **Gelatin Test:** A certain amount of the extract was dissolved in the distilled water. Two milliliters of a 1% gelatin solution with 10% sodium chloride were added to this mixture. The formation of white precipitate indicates that phenolic chemicals are present.

2.5 (*In-vitro* Anti-oxidant Activity)

2.5.1 DPPH Radical Scavenging Activity

A)Preparation of DPPH reagent

0.1mM solution of 2,2-Diphenyl-1-picrylhydrazyl (DPPH) in methanol was prepared.

B) Preparation of Sample/Standard

A new extract/standard methanol solution containing 1 mg/ml was made. In a set of test tubes, varying volumes of extracts or standard (20–100 µl) were collected from the stock solution, and methanol was added to bring the volume down to 1 ml. After 30 minutes of dark incubation at room temperature, absorbance at 517 nm was measured after 2 ml of 0.1 mM DPPH reagent had been added and completely mixed.

C) Preparation of control

Three milliliters of 0.1 mM DPPH solution were used as a control, and they were incubated for thirty minutes at room temperature in a dark environment. At 517 nm, the control's absorbance was measured in comparison to methanol (as a blank).

Percentage antioxidant activity of sample/standard was calculated by using formula:

% Inhibition = [(Ab of control- Ab of sample/ Ab of control x 100]

2.5.2 Reducing power assay

Preparation of standard solution

Three milliliters of distilled water/solvent were used to dissolve three milligrams of ascorbic acid. Concentrations of 20, 40, 60, 80, and 100 µg/ml were achieved by diluting this solution

with distilled water.

Preparation of extracts

One milligram of dried extracts was dissolved in one milliliter of methanol to create stock solutions of extracts with a concentration of one milligram per milliliter. After then, samples with concentrations of 20, 40, 60, 80, and 100 µg/ml were made.

Protocol for reducing power

This procedure involved mixing 2.5 ml of (pH 6.6) phosphate buffer and 2.5 ml of (1%) potassium ferricyanide with aliquots of different quantities of ascorbic acid as a standard and extracts (20 to 100µg/ml) in 1.0 ml of deionized water. After cooling, the mixture was incubated for 20 minutes at 50°C in a water bath. After adding aliquots of 2.5 ml of 10% trichloroacetic acid, the mixture was centrifuged for 10 minutes at 3000 rpm. 2.5 ml of distilled water and 0.5 ml of freshly made (0.1%) ferric chloride solution were combined with the top layer of solution. Using a Systronic double beam-UV-2201 UV spectrometer, the absorbance was measured at 700 nm. Extract was not included to create a blank.

2.5.3 Ferric thiocynate assay

This test measures hydroperoxide indirectly by adding linoleic acid to the reaction mixture that has been oxidized by air throughout the experiment. Four milliliters of 0.05 mol/L phosphate buffer (pH 7.0), 1.95 milliliters of distilled water, 2.05 milliliters of 2.51% linoleic acid in 99.8% ethanol, and two milliliters of sample (or methanol (as blank) or ascorbic acid (as reference)) were put in an Erlenmeyer flask in a rotary incubator (150 r/min, 40 C) in a dark environment. A test tube was filled with 0.1 mL of the reaction mixture in order to quantify the antioxidant activity.

0.1 mL of 30% ammonium thiocyanate, 9.7 mL of 75% ethanol, and 0.1 mL of 2 × 10−2 mol/L ferrous chloride in 3.5% hydrochloric acid were then added. The absorbance at 500 nm was measured three minutes after ferrous chloride was added to the reaction mixture. Until the absorbance of the control reached its maximum value, measurements were made every 24 hours. As a negative control, this combination was also made without linoleic acid. As positive controls, ascorbic acid was employed. The following formula was used to determine antioxidant activity:

% inhibition = (Acontrol – Asample/ A control) × 100

Where $A_{control}$ is the absorbance of the control reaction and A_{sample} is the absorbance of the tested extract samples.

3. Statistical analyses

Graph Pad Prism 5 is used to analyze the data, which are shown as the mean ± standard error of mean (SEM) of three duplicate determinations. To ascertain the differences between the means, the post-hoc Tukeys test and one-way analysis of variance (ANOVA) were employed. The relationship between antioxidant activity and total phenolic and flavonoid content was ascertained using Pearson's correlation analysis. $P < 0.05$ was chosen as the threshold for statistical significance.

4. RESULTS AND DISCUSSION

4.1 Percentage yield

The solvent, color of extract, theorictical weight and yield in gram t and % yield of N. jatamansi are summarized in Table 1.

Table 1: Percentage yield of extracts.

S. No.	Plant name	Solvent	Color of extract	Theoretical weight (gm)	Yield (gm)	% Yield
1.	*Nardostachys jatamansi*	Petroleum Ether	Dark yellow to brown	80 gm	0.899	1.12
2.	*Nardostachys jatamansi*	70% Ethanol	Brown	80 gm	8.369	10.46

4.2 Qualitative Phytochemical Analysis of different extracts

Phytochemical analysis of total NJ hydro alcoholic (NJHA) extract revealed the presence of sterols, triterpenoids, phenols, flavonoids, alkaloids, saponins, tannins, fixed oils and fats.

Liebermann Burchard and Salkowski's test were positive for exhibiting the presence of sterols and triterpenoids. NJPE, showed a strong presence of phenols in the lead acetate test.

Dragendorff and Mayer's test were positive for NJPE.and NJHA suggesting the presence of alkaloids. The foam test exhibited the presence of saponins in NJHA. Phenols are an important class of antioxidants due to their ideal structural chemistry for free-radical scavenging activity.

They can act as reducing agents, metal chelators and free radical quenchers by donating an electron or hydrogen atom to free radicals.

Phytochemical stuidy of Pet. Ether Extract

Phytochemical stuidy of Hydroalcoholic Extract

Table2: Phytochemical analysis of *Nardostachys jatamansi* Extract.

S. No.	Experiment	Result	
		Petroleum ether	Hydroalcoholic
Test for Carbohydrates			
1.	Molisch's Test	-	+
2.	Fehling's Test	-	+
3.	Benedict's Test	-	+
4.	Bareford's Test	-	+
Test for Alkaloids			
1.	Mayer's Test	-	+
2.	Hager's Test	-	+
3.	Wagner's Test	+	+
4.	Dragendroff's Test	-	-
Test for Terpenoids			
1.	Salkowski Test	-	+
2.	Libermann- Burchard's Test	-	+
Test for Flavonoids			
1.	Lead Acetate Test	-	+
2.	Alkaline Reagent Test	-	+
3.	Shinoda Test	-	+
Test for Tannins and Phenolic Compounds			
1.	FeCl3 Test	-	+
2.	Lead Acetate Test	+	+
3.	Gelatine Test	-	+
Test for Saponins			
1.	Froth Test	+	+
Test for Protein and Amino acids			

1.	Ninhydrin Test	-	+
2.	Biuret's Test	-	+
3.	Million's Test	-	_
Test for Glycosides			
1.	Legal's Test	-	+
2.	Keller Killani Test	-	+
3.	Borntrager's Test	-	+

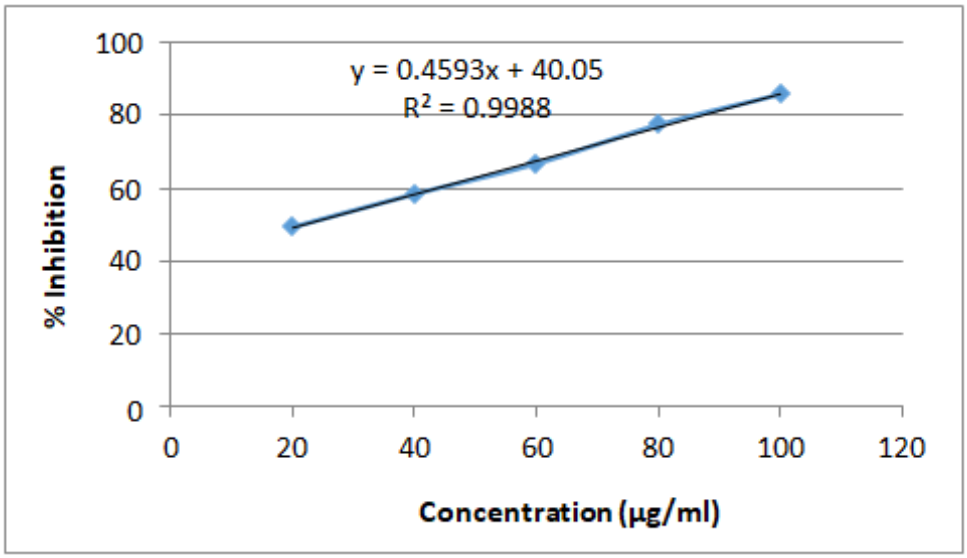
4.3 Anti-oxidant activity

4.3.1 DPPH Assay

NJHA extract showed dose-dependent scavenging activity of DPPH and ABTS free radicals. The highest free radical scavenging was observed for NJAH with IC5047.03844 in DPPH. Correlation analysis revealed a moderate correlation (R = 0.9988) between total phenolic content and DPPH scavenging activity. A strong and significant (R = −0.9988, P < 0.05) correlation was observed between total phenolic content and ABTS scavenging indicating that phenolic compounds are primarily responsible for ABTS radical scavenging activity (Table 3). The correlation coefficients between total flavonoid content and DPPH and ABTS radical scavenging assays were much lower as compared to total phenolic content. The total flavonoid content is a sum of flavones, flavonols and flavonones. However, the aluminium chloride method estimates only the content of flavones and flavonols therefore, the total flavonoid content could be underestimated resulting in lower correlation coefficients with free radical scavenging assays.

Table 3: DPPH radical scavenging activity of Ascorbic acid.

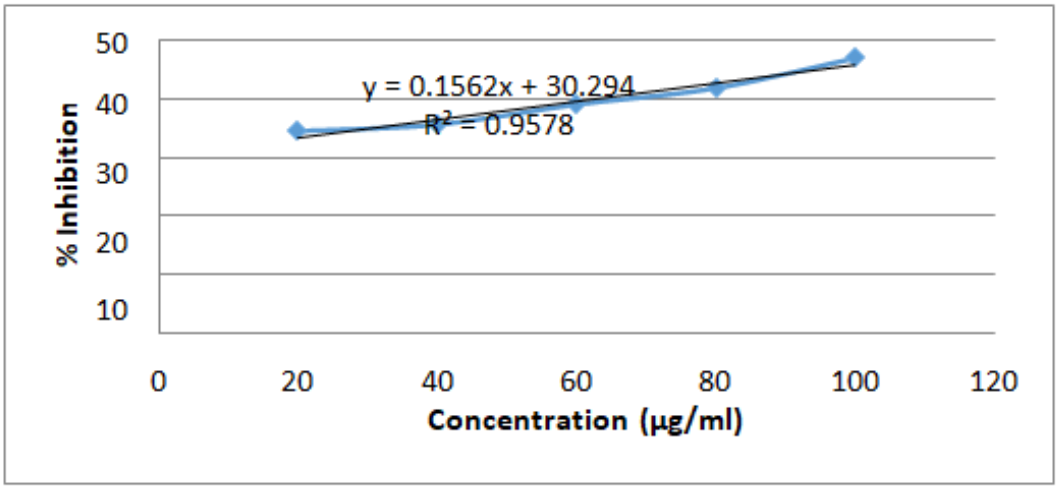
Concentration (µg/ml)	Absorbance	% Inhibition
20	0.5273	49.42391
40	0.4442	58.45652
60	0.3669	66.8587
80	0.2694	77.45652
100	0.1921	85.8587
Control	0.982	
IC50 21.677		



Graph 1: Graph represents the Percentage Inhibition *Vs* Concentration of Ascorbic acid

Table 4: DPPH radical scavenging activity of Hydroalcoholic extract of *Nardostachys jatamansi.*

Concentration (µg/ml)	Absorbance	% Inhibition
20	0.5376	34.54384
40	0.5270	35.68855
60	0.5045	38.11836
80	0.4877	39.93261
100	0.4219	47.03844
Control	0.926	
IC50 126.34		



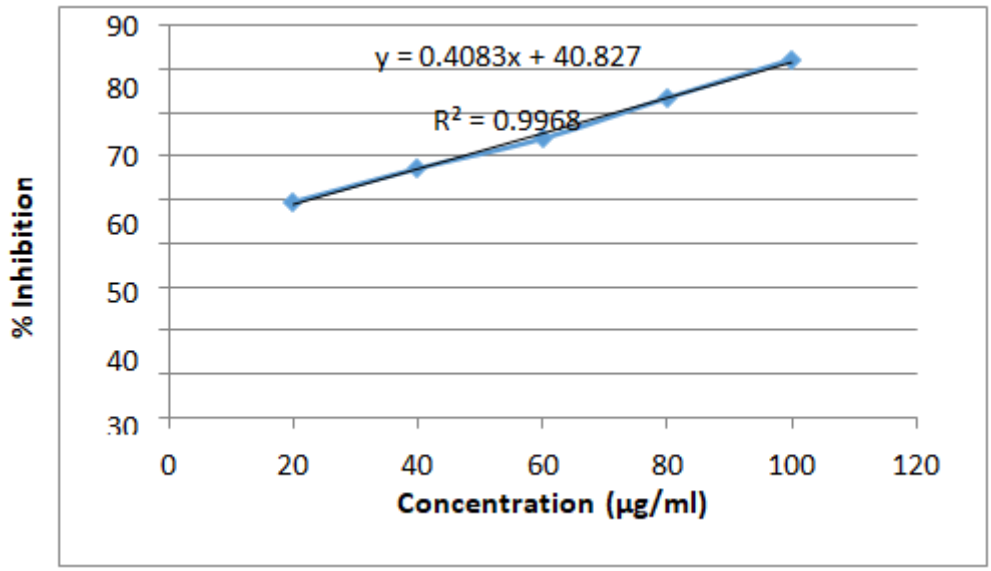
Graph 2: Graph represents the Percentage Inhibition *Vs* Concentration of Hydroalcoholic extract of *Nardostachys jatamansi.*

4.3.2 Ferric thiocynate assay

The degree of lipid peroxidation which was evaluated using the ferric thiocyanate method can be used to measure the antioxidant potential of compounds or extracts. Table 3 shows the results of the antilipid peroxide formation of the root extracts and constituents of G. involucrata. As depicted in Table 3, the NJHA extract inhibit peroxide formation by 46%, respectively, demonstrating their potential in preventing the formation of lipid peroxides.The results turned out to be comparable with those of ascorbic acid which inhibited the DPPH radical by 82%.

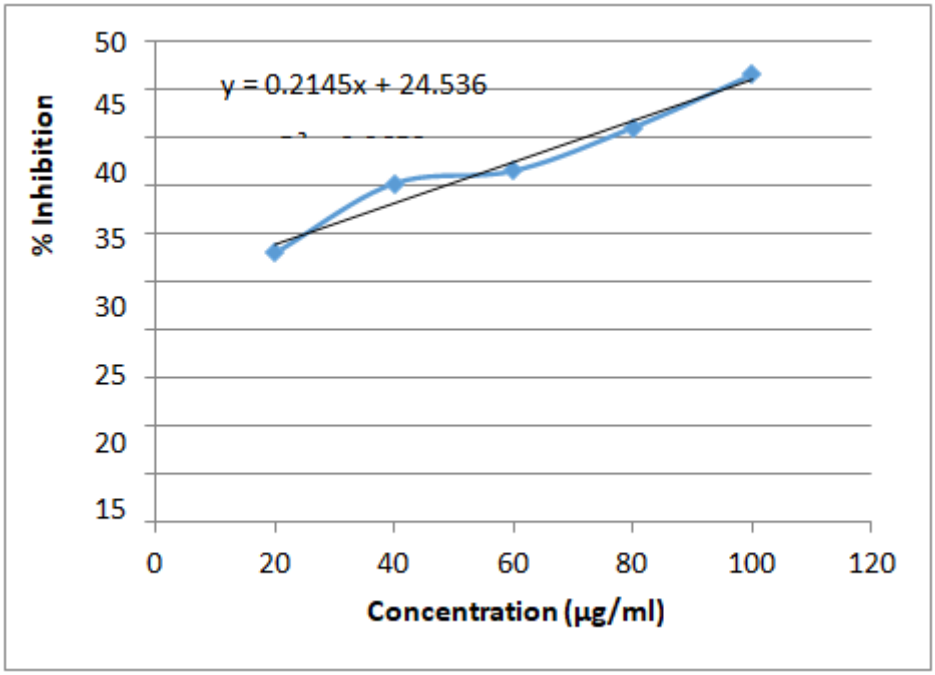
Table5: Ferric thiocynate activity of Ascorbic acid

Concentration (µg/ml)	Absorbance	% Inhibition
20	0.3798	49.55526
40	0.3098	57.2307
60	0.2469	64.12763
80	0.1620	73.43684
100	0.0813	82.28553
Control	0.912	
IC50 22.0		



Graph3: represents the Percentage Inhibition *Vs* Concentrationof Ascorbic acid Table 6 Ferric thiocynate activity of Hydroalcoholic extract of *Nardostachys jatamansi*

Concentration (µg/ml)	Absorbance	% Inhibition
20	0.5771	27.92149
40	0.511	35.16930
60	0.499	36.48509
80	0.458	40.98070
100	0.408	46.46316
Control	0.912	
IC50 119.018		



Graph4: Graph represents the Percentage Inhibition *Vs* Concentration of Hydroalcoholic extract of *Nardostachys jatamansi*

CONCLUSION

Our results suggest that N. jatamansi may serve as an excellent lead for the development of antioxidant agents. It is an accessible source of natural antioxidants with considerable health benefits. Further work to elucidate the mechanism of action of neurological potentials and to identify the active constituents is under study in our laboratory

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