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Quantification of Total Phenolic and Flavonoid Contents, and Evaluation of Free Radical Scavenging Potential of *Vernonia cinerea*

Parveen Kumar Goyal[1,2], Santosh Kumar Verma[3], Anil Kumar Sharma[4*]

[1]Research Scholar, I.K.G. Punjab Technical University, Kapurthala, Punjab, India
[2]Department of Pharmacology, Hindu College of Pharmacy, Sonepat, Haryana, India
[3]Faculty of Pharmaceutical Sciences, Motherhood University, Roorkee, Uttrakhand, India
[4]Department of Pharmaceutical Sciences, CT Institute of Pharmaceutical Sciences, Jalandhar, Punjab, India
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ABSTRACT

The present manuscript is focused to quantify the total phenolic and flavonoid contents and to evaluate the free radical scavenging potential of a hydro-ethanolic extract of *Vernonia cinerea*. The Folin-Ciocalteau method was employed to estimate the total phenolic contents, while total flavonoid contents were determined by a colorimetric method using aluminium chloride. *V. cinerea* extract (VCE) was evaluated to scavenge several free radicals such as DPPH, superoxide and nitric oxide. The total phenolic and flavonoid contents in VCE were found to be 112.41 ± 1.56 mgGAE/g and 13.61 ± 1.82 mgQE/g of dried extract respectively. VCE significantly scavenged DPPH, superoxide, and nitric oxide radicals in a concentration-dependent manner. Ascorbic acid was used as the reference compound to compare the antioxidant potential of VCE. The IC_{50} value was used to express the free radical scavenging capacity. The IC_{50} values of VCE against DPPH, superoxide, and nitric oxide free radicals were found to be 429.94, 451.72, and 400.74 µg/ml respectively. Conclusively, VCE contained a considerable amount of phenolic and flavonoid contents that might contribute to its antioxidant potential against DPPH, superoxide, and nitric oxide free radicals.

Key Words: Flavonoid contents, Free radical, Nitric oxide, Phenolic contents, Superoxide, *Vernonia cinerea*

Introduction

Free radicals, generated as a result of interactions between oxygen and other molecules, are highly reactive and short-lived chemical species which have odd or unpaired electrons in their outer orbit. These are highly unstable, due to the presence of unpaired electrons, and have a constant tendency to get stabilized by reacting with some other molecules either by donating, extracting, or sharing electrons. They commonly react with various cellular components like lipids, proteins, DNA etc and damage them to attain a stable electronic configuration.

[*]Correspondence
Dr. Anil Kumar Sharma
Former Director
Department of Pharmaceutical Sciences,
CT Institute of Pharmaceutical Sciences
Jalandhar-144020, Punjab, India
E-mail: aksharma91@gmail.com
Present Address:
E-91, Naraina Vihar
New Delhi-110028, India

Most of the free radicals have centered oxygen (called Reactive Oxygen Species, ROS) like superoxide, peroxyl, hydroxyl, perhydroxyl, hydrogen peroxide, alkoxy, singlet oxygen, etc. or nitrogen (called Reactive Nitrogen Species, RNS) like nitric oxide, peroxynitrite, alkyl peroxynitrite, nitrogen dioxide, etc [1-3]. Oxidation is one of the vital metabolic reactions essential for living organisms including human beings and plays a significant role in energy production and other biological activities like detoxification, chemical signaling, immune responses etc [1, 4]. Therefore, production of free radical, as a result of oxidative reactions, is a constant and normal phenomenon during metabolic activities. In the human body, these free radicals are regularly controlled by some endogenous enzymes like glutathione peroxidase, catalase, superoxide dismutase etc. When due to continuous exposure to chemicals or contaminants, the overproduction of free radicals occurs and defense system of the body fails to combat, then irreversible oxidative damage to cells or cellular components

occurs that directly leads to several diseases or abnormalities. In human beings, free radicals are considered to contribute to many diseases like atherosclerosis, diabetes, hypertension, cirrhosis, cancer, Parkinsonism, Alzheimer's, arthritis, gastritis, AIDS, CNS injury, reperfusion injury of tissues, renal injury, cellular damage, etc [2, 5, 6]. The free radical scavengers or antioxidants help in treating or preventing such diseases by inhibiting oxidation reactions and by removing free radicals and their intermediates. In order to reduce the oxidative damages and to give protection against many diseases, antioxidants or free radical scavengers have indispensable place in the human diet as well as medicines. Many synthetic antioxidants like butylated hydroxyl toluene, butylated hydroxyl anisole etc which have long been used as preservatives by food industries are nowadays discouraged due to the carcinogenic effects. There is a constant need to explore some potential antioxidants of natural sources especially herbal which has less or no side effects and can be used to prevent the above-mentioned free radicals related diseases in human beings.

Vernonia cinerea (L.) Less. (Family: Asteraceae), commonly known as Sahadevi and Purple Fleabane is an important medicinal plant which chemically contains luteolin, vernolides, amyrins, sterols, terpenes, alkaloids, flavonoids, phenolic compounds, mucilages and amino acids [7-10]. It has traditionally been used as anodyne, anti-inflammatory, diaphoretic, diuretic, lithotriptic, stomachic, anti-periodic, etc. It has also been recommended for leucoderma, leprosy, psoriasis, chronic skin diseases, colic, dysuria and renal calculi [11, 12]. It has been scientifically substantiated for anti-inflammatory [13], antibacterial [14], antifungal [15], antidiarrhoeal [16], antidiabetic [17], antipyretic [18], anticancer [19], antiurolithiatic [20], hepato-protective [29], and nephroprotective potentials [22]. The present manuscript was aimed to quantify the total phenolic and flavonoid contents and to evaluate the free radicals scavenging potential of *V. cinerea* extract.

Materials and Methods

Plant materials

The dried whole plant of *Vernonia cinerea* (L.) Less., authenticated by the botanist Dr. H. B. Singh (Former Chief Scientist, Raw Materials Herbarium, and Museum, NISCAIR, New Delhi), was obtained as a gift sample from Aimil Pharmaceutical India Limited, New Delhi, (Specimen Ref. No. AIMIL/PD/2015). The specimen was deposited to Department of Pharmacognosy, Hindu College of Pharmacy, Sonepat, Haryana, India.

Chemical and reagents

Folin-Ciocalteau reagent was purchased from Qualigens Fine Chemicals, Mumbai. Gallic acid was obtained from Loba Chemie, Pvt. Ltd., Mumbai. Nitro blue tetrazolium (NBT), Riboflavin, DPPH (1,1-diphenyl-2-picryl hydrazyl), aluminum chloride, and sodium nitroprusside were purchased from HiMedia Laboratories Pvt. Ltd., Mumbai. Quercetin was purchased from Sigma-Aldrich, St. Louis, USA. Ascorbic acid was purchased from SD-Fine Chem Ltd., Mumbai. All other chemicals and reagents used were of analytical grade.

Preparation of hydroethanolic extract

The hydroethanolic extract of *V. cinerea* was prepared using the extraction procedure as previously followed in our laboratory [20]. Briefly, 100 g coarsely powdered *V. cinerea* was mixed with 2.5 L ethyl alcohol (60%) in a stoppered glass container and kept at 45°C for five days with occasional shaking. The contents were filtered and the filtrate was concentrated under vacuum. In order to obtain the dried extract, the concentrated contents were dried to constant weight at 45°C in a hot air oven. The dried extract was kept in a sterile glass container and labeled as VCE. The percentage yield of VCE was found to be 20.52% w/w.

Quantification of total phenolic contents

The phenolic contents in VCE were estimated by Folin-Ciocalteau methods [23] with some minor modifications [24-26]. The 0.2 ml ethanolic solution of extract (1.0 mg/ml) was mixed with 4.0 ml distilled water in a 10 ml volumetric flask and 0.5 ml Folin-Ciocalteau reagent was then added to it. After 10 minutes, the 2.0 ml sodium carbonate (20.0% w/v) solution was added to it and the total volume was made up to 10.0 ml. The contents were kept in dark for 60 minutes, and the absorbance was measured at 765 nm. The total phenolic contents were measured by the calibration curve. The varying concentrations (25, 50, 75, 100, 125, 150, 175, 200, 225, and 250 µg/ml) of Gallic acid, prepared from stock solution (1.0 mg/ml), were used for preparing calibration curve. Total phenolic contents were expressed as milligrams of the Gallic acid equivalent per gram of the dry extract. All the samples were analyzed in triplicate. Total phenolic contents were calculated by employing the formula:

$$T = (C \times V)/M$$

Where, T = Total phenolic contents in milligrams of Gallic acid equivalent per gram of the extract

C = Concentration of Gallic acid in mg/ml obtained from the calibration curve

V = Total volume of extract used in assay
M = Total weight of dry extract (in gram) used in assay.

Quantification of total flavonoid contents

The total flavonoid contents of VCE were determined by aluminum chloride colorimetric method [27, 28]. Quercetin was used as the reference standard to make a calibration curve. The varying concentrations (25, 50, 75, 100, 125, 150, 175, 200, 225, and 250 µg/ml) of Quercetin were obtained from 80% ethanolic solution (1.0 mg/ml). The 1.0 ml Quercetin solution of each concentration was separately mixed with 3.0 ml 95% ethanol, 0.2 ml aluminum chloride (10% w/v), 0.2 ml potassium acetate (1.0 M) and 5.6 ml of distilled water. After incubating the reaction mixture at room temperature for 30 minutes, the absorbance was measured at 415 nm. The amount of aluminum chloride was replaced by the same amount of distilled water in the blank. In order to determine the flavonoid contents, 1.0 ml ethanolic solution of each extract (1.0 mg/ml) was reacted with aluminum chloride and the similar protocol was followed. All the samples were analyzed in triplicate. The total flavonoid contents were obtained from the calibration curve of standard Quercetin solution and calculated by employing the following formula:

$$T = (C \times V)/M$$

Where, T = Total flavonoid contents in milligrams of Quercetin equivalent per gram of the extract; C = Concentration of Quercetin in mg/ml obtained from calibration curve; V = Total volume of extract used in the assay; and M = Total weight of dry extract (in gram) used in the assay.

Evaluation of free radical scavenging potential

DPPH radical scavenging assay

The assay is based on the fact that DPPH (1,1-diphenyl-2-picryl hydrazyl), a purple colored stable free radical, when mixed with free radical scavengers, is reduced and the color changes from purple to yellow, based on the efficacy of antioxidants which can be measured at 517 nm [3]. The assay was conducted as described by Blois with some minor modifications [29, 30]. Briefly, 1.0 ml methanolic solution (100 µM) of DPPH was mixed with 1.0 ml VCE solutions in different concentrations, i.e. 100, 200, 300, 400, 500, and 600 µg/ml. The reaction mixture was incubated at 37°C for 30 minutes in the dark and the absorbance was measured at 517 nm with the help of a double beam UV-Visible spectrophotometer. The percentage inhibition, as the DPPH radical scavenging potency, was calculated by using the formula:

$$\% \text{ inhibition} = [(A_c - A_t) / A_c] \times 100$$

Where, A_c = Absorbance of control; A_t = Absorbance of the sample.

The IC_{50} value, i.e. the concentration of VCE required to scavenge 50% of the DPPH radicals was determined and compared with that of standard drug ascorbic acid.

Superoxide free radicals scavenging assay

The method is based on the principle that photo-oxidation of riboflavin generates superoxide radicals that reduce the NBT (Nitro blue tetrazolium) to a blue colored formazan which can be measured at 560 nm [31, 32]. The antioxidant potential (i.e. capacity to inhibit the formazan formation by scavenging the superoxide radicals generated in riboflavin-light-NBT system) of VCE was calculated as the percentage of superoxide radicals scavenged and expressed in the form of IC_{50} values. Briefly, the reaction mixture containing 200 µl EDTA solution (0.1 M), 100 µl NBT (1.5 mM), 200 µl phosphate buffer (0.067 M) and 100 µl test samples (VCE solutions of varying concentrations, 100, 200, 300, 400, 500, and 600 µg/ml) was incubated for 5-8 minutes at 37°C. Then 100 µl riboflavin solution (0.12 mM) was added and the volume was made up to 3.0 ml using phosphate buffer. Then the contents were illuminated for 12 minutes by incubating in a lightbox and the absorbance was immediately measured at 560 nm against the blank [1]. Ascorbic acid was used as a reference standard. The percentage inhibition of free radical generation was calculated by using the formula:

$$\% \text{ inhibition} = [(A_c - A_t) / A_c] \times 100$$

Where, A_c = Absorbance of control; A_t = Absorbance of the sample.

Nitric-oxide (NO) radicals scavenging assay

The procedure is based on the principle that, sodium nitroprusside in aqueous solution at physiological pH 7.4 spontaneously generates nitric oxide, which interacts with oxygen (air) and produces nitrite ions. The nitrite ions diazotize with sulphanilic acid and couples with Griess reagent to produce a pink color which can be measured at 546 nm. NO scavengers compete with oxygen leading to decrease the production of nitrite ions. Briefly, the reaction mixture (3.0 ml) containing 2.0 ml of sodium nitroprusside (10 mM), 0.5 ml of standard phosphate buffer (pH 7.4) solution and 0.5 ml of test sample (varying concentration of 100-600 µg/ml VCE) or standard drug (ascorbic acid) was incubated at 25°C for 150 minutes. After incubation, 1.5 ml of reaction mixture and 1.5 ml of Griess reagent [1.0% sulphanilamide, 2.0% O-phosphoric acid and 0.1% of N-(1-naphthyl)

ethylenediamine dihydrochloride] were mixed and allowed to stand for thirty minutes at 25°C. The absorbance of the chromophore formed was measured at 540 nm by using a double beam UV-Visible spectrophotometer [33, 34]. NO radical scavenging activity was calculated using the following formula:

$$\% \text{ inhibition} = [(A_c - A_t) / A_c] \times 100$$

Where, A_c = Absorbance of control; A_t = Absorbance of the sample.

Data analysis

All values were expressed as mean ± standard error mean (SEM). Data were analyzed by employing One-way ANOVA followed by Dunnett's multiple comparison tests using GraphPad Prism version 5.00 (Trial) Software (GraphPad Software, Inc, La Jolla, USA). The values having $p < 0.05$ were considered as statistically significant.

Results and Discussion

Quantification of total phenolic and flavonoid contents

In the present study, the total phenolic contents in VCE were determined by Folin-Ciocalteau methods using Gallic acid as the standard phenolic compound. The total phenolic contents, calculated from the Gallic acids calibration curve ($y = 1.892 x + 0.007$, $R^2 = 0.996$) shown in Figure 1A, were found to be 112.41 ± 1.56 mgGAE/g (milligram of Gallic acid equivalent per gram of dry extract). The total flavonoid contents

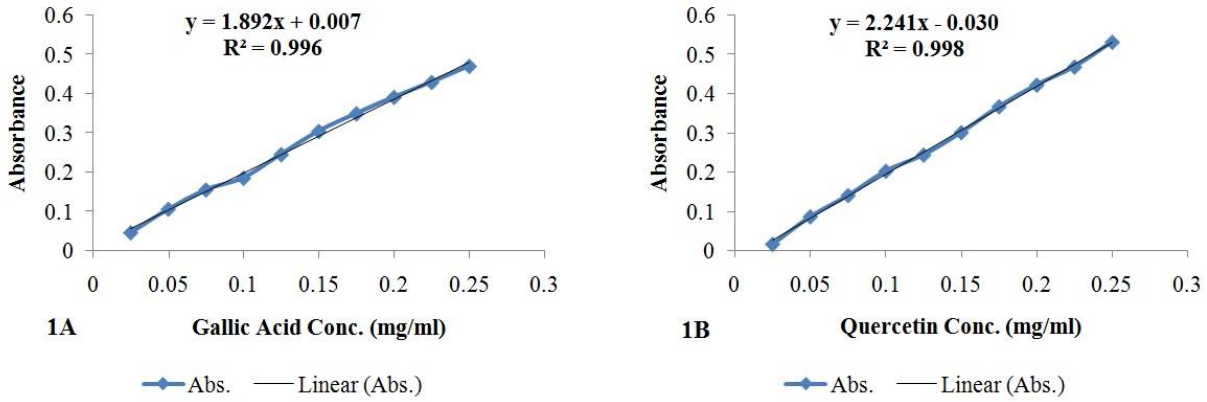

Fig 1: The calibration curve of Gallic acid (1A) and Quercetin (1B)

were determined by aluminum chloride method using Quercetin as the standard compound. The total flavonoid contents, calculated by Quercetin calibration curve ($y = 2.241 x + 0.030$, $R^2 = 0.998$) shown in Figure 1B, were found to be 13.61 ± 1.82 mgQE/g (milligram of Quercetin equivalent per gram of dry extract).

The phenolic compounds are the secondary plant metabolites which have one or more phenol molecules. The polyphenol contains at least two phenol units. The group of simple phenols (having one phenol unit) contains phenolic acids. Flavonoids are low-molecular-weight polyphenolic compounds which are biosynthesized by Shikimic acid and Acetic acid pathways. The phenolic compounds including flavonoids are responsible for a wide spectrum of biological activities like antioxidant, anti-inflammatory, antimutagenic, anticancer, etc [35, 36]. The polyphenolic compounds constitute a major class of antioxidants that terminate free radicals and their biological activities are chiefly concerned with their capacity to chelate metals, scavenge free radicals, activate antioxidant enzymes, inhibit hydrolytic and oxidative enzymes like phospholipase, lipoxygenase, cyclooxygenase, etc [6, 28, 32, 37]. The antioxidant potential of phenolic and flavonoid compounds depends on the presence of free hydroxyl groups, redox properties, and the presence of conjugated ring structures [1, 38]. The medicinal or food substances containing antioxidant compounds are mainly used to prevent or reduce the risks of free radicals associated diseases like diabetes, cancer, heart diseases, renal disorders, age-related diseases etc [39, 40].

Evaluation of free radical scavenging potential

DPPH radical scavenging potential

Scavenging of DPPH stable free radical is the general and widely used method to evaluate the antioxidant potential [41]. The extracts or compounds having antioxidant potential reduce DPPH free radicals and

change its purple color to yellow. In the present study, VCE significantly scavenged the DPPH radicals and the IC_{50} value was found to be 429.94 µg/ml as shown in Table 1. The IC_{50} value was calculated by using concentration vs mean percent inhibition of DPPH radical curve (y = 0.097x + 8.296, R^2 = 0.993) as shown in Figure 2A. The antioxidant potential of VCE was compared with that of ascorbic acid and the concentration vs mean percent inhibition curve is depicted in Figure 2B.

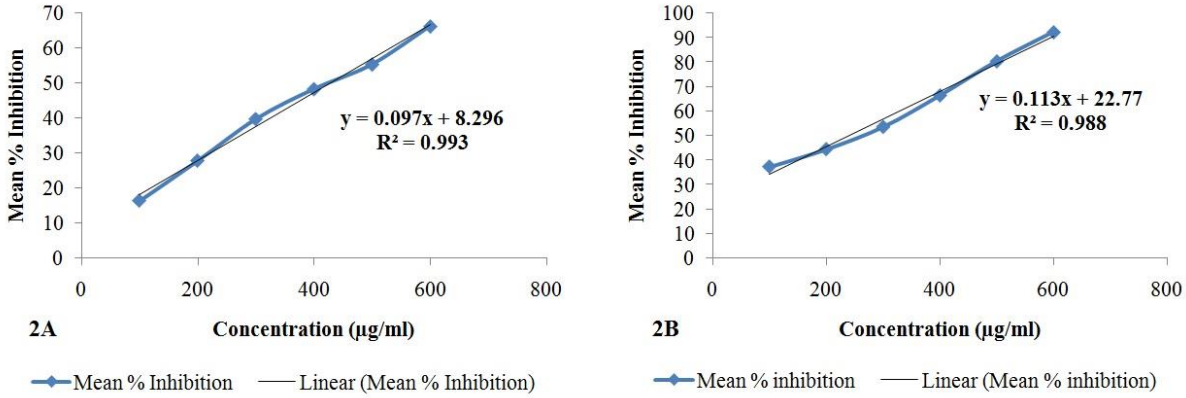

Fig 2: DPPH scavenging assay of VCE (2A) and Ascorbic acid (2B)

Table 1: Free Radicals Scavenging Potentials of VCE and Ascorbic acid

Treatment	Free Radicals	IC_{50} Value (µg/ml)	Percent Inhibition of Free Radicals (Mean ± SEM)					
			100 µg/ml	200 µg/ml	300 µg/ml	400 µg/ml	500 µg/ml	600 µg/ml
VCE	DPPH	429.94	16.41±0.27[a]	27.86±0.22[a]	39.76±0.48[a]	48.33±0.18[a]	55.37±0.18[a]	66.26±0.26[a]
AA	DPPH	240.97	37.23±0.17[a]	44.42±0.35[a]	53.54±0.42[a]	66.41±0.40[a]	80.39±0.38[a]	92.30±0.50[a]
VCE	SO	451.72	19.39±0.27[a]	25.64±0.32[a]	36.17±0.46[a]	46.13±0.46[a]	54.87±0.51[a]	62.86±0.24[a]
AA	SO	225.70	39.58±0.33[a]	47.83±0.35[a]	55.31±0.30[a]	65.31±0.46[a]	78.59±0.38[a]	89.69±0.36[a]
VCE	NO	400.74	17.82±0.34[a]	29.75±0.40[a]	41.50±0.50[a]	49.74±0.45[a]	60.24±0.54[a]	70.18±0.64[a]
AA	NO	269.91	31.28±0.67[a]	41.11±0.84[a]	53.94±0.67[a]	66.61±0.55[a]	76.00±0.64[a]	86.82±0.58[a]

AA: Ascorbic acid; SO: Superoxide; NO: Nitric oxide
All values were expressed as Mean ± SEM, n=3.
Data were analyzed by One-way ANOVA followed by Dunnett's Multiple Comparison Test.
All groups were compared with control group.
[a] $p < 0.001$.

Superoxide free radical scavenging potential

Superoxide anions are the most common type of free radicals observed in the conditions of oxidative stress and have the capacity to react with biological macromolecules. The superoxide radicals once generated either by auto-oxidation or enzymatic reactions lead to the production of other ROS such as hydroxyl radical, hydrogen peroxide, and singlet oxygen [1, 41, 42]. In the present study, the capacity of VCE to inhibit formazan formation by scavenging the superoxide radicals, generated in the riboflavin-light-NBT system, was expressed as the IC_{50} value and shown in Table 1. The IC_{50} value (451.72 µg/ml) was determined with the help of concentration vs mean percent inhibition of superoxide radical curve (y = 0.09x + 9.346, R^2 = 0.996) as shown in Figure 3A. The

concentration vs percent inhibition curve of reference drug ascorbic acid was shown in Figure 3B.

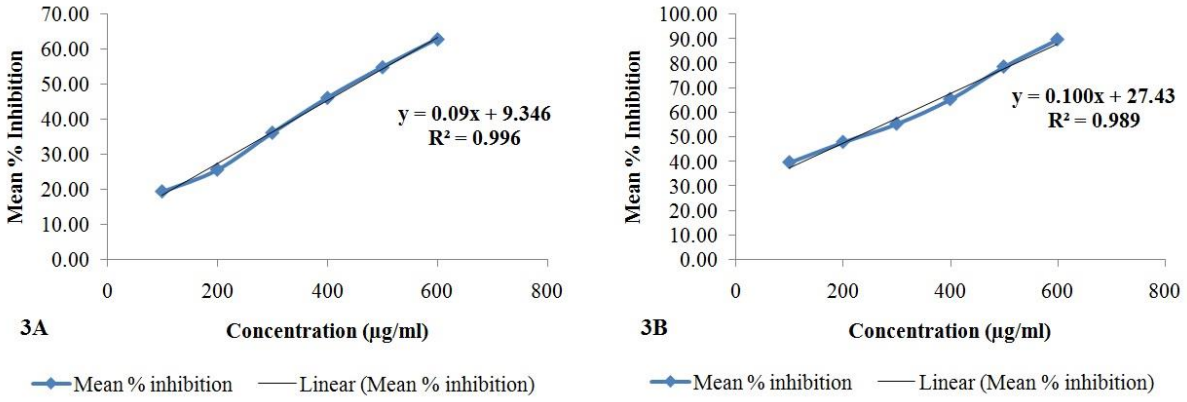

Fig 3: Superoxide scavenging assay of VCE (3A) and Ascorbic acid (3B)

Nitric-oxide (NO) radical scavenging potential

NO free radicals, produced in mammals from L-arginine amino acids by vascular endothelial cells, certain brain cells, and phagocytes, is considered to be involved in different physiological processes [3, 6, 43]. The overproduction of NO is associated with several diseases [44]. NO reacts with superoxide to form peroxynitrite free radicals and show inflammatory responses by releasing prostaglandins [3, 45]. It is also considered that repeated infection throughout life causes sustained overproduction of NO which is directly toxic to different body tissues and results in several inflammatory conditions and age-related diseases such as Alzheimer's disease, diabetes, and cardiovascular disorders [3, 46, 47]. In the present study, VCE showed a significant NO scavenging activity in a concentration-dependent manner and expressed as the IC_{50} value (400.74 µg/ml) shown in Table 1. The IC_{50} value was determined with the help of concentration vs mean percent inhibition of NO curve ($y = 0.103x + 8.724$, $R^2 = 0.997$) as shown in Figure 4A. Ascorbic acid was used as the reference compound and IC_{50} value was found to be 269.91 µg/ml and the concentration vs percent inhibition curve was expressed in Figure 4B.

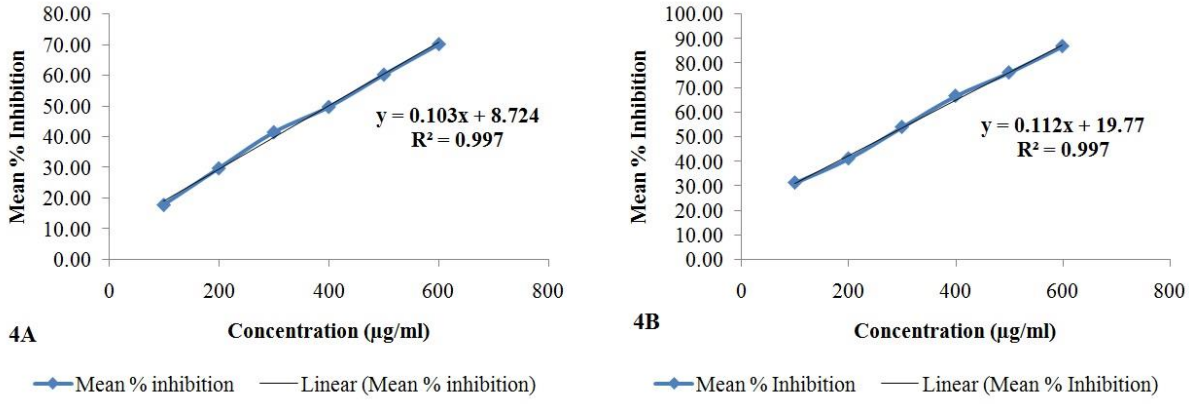

Fig 4: Nitric oxide scavenging assay of VCE (4A) and Ascorbic acid (4B)

Conclusion

The hydro-ethanolic extract of *V. cinerea* was found to contain a considerable amount of total phenolic and flavonoid contents. The extract exhibited significant free radical scavenging effects.

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