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Research Article

COMPARATIVE ANALYSIS OF ANTIOXIDANT ACTIVITY AND PHYTOCHEMICAL CONTENTS IN ETHANOLIC LEAF EXTRACTS OF *IN VITRO* AND FIELD GROWN *WITHANIA SOMNIFERA*

PRABHJOT SINGH JASSAL[1]*, GAGANDEEP KAUR[2]

[1]Department of Biotechnology and Biosciences, Lovely Professional University, Phagwara - 144 411, Punjab, India.
Email: prabh_jyot2006@yahoo.co.in

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ABSTRACT

Objective: The present study was planned to compare antioxidant activity *in vitro* and field grown *Withania somnifera* was assessed using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and nitric oxide (NO) assays. Medicinal plants are a major source of phytochemicals used for the treatments of human diseases. *W. somnifera* has anti-inflammatory, antioxidant, and antimicrobial properties.

Methods: Antioxidant activity and phytochemical contents in *W. somnifera* were determined spectrophotometrically.

Results: The results of antioxidant activity of field grown ethanolic leaf extract of *W. somnifera* showed maximum inhibition of 72.08% and 77.85% in DPPH (50 µg/ml) and NO (100 µg/ml) scavenging assays, respectively. Field grown ethanolic leaf extract of *W. somnifera* showed maximum concentrations of phenolics, flavonoids, and carotenoids, as active phytochemicals, determined spectrophotometrically, which were found as 676.5 µg/ml, 557.5 µg/ml, and 469 µg/ml, respectively, as compared to *in vitro* plant extracts.

Conclusions: This study demonstrated that antioxidant activity and phytochemical contents of field grown ethanolic leaf extract of *W. somnifera* were found to be comparatively higher than *in vitro* plant extracts. Leaf extracts of *W. somnifera* are a potential source of antioxidants and could prevent many free radical-related diseases.

Keywords: Carotenoids content, 1-diphenyl-2-picrylhydrazyl scavenging assay, Flavonoids content, Nitric oxide radical scavenging assay, Phenolic content.

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INTRODUCTION

In recent years, much attention has been devoted to natural antioxidants and their association with health benefits [1]. Nature has been a source of medicinal agents for thousands of year, and an impressive number of modern drugs have been made from natural resources [2]. Plant produces various antioxidative compounds to counteract reactive oxygen species (ROS) to survive [3]. ROS, which includes free radicals such as superoxide anion radicals, hydroxyl radicals, and non-free-radical species such as hydrogen peroxide and singlet oxygen, is various forms of activated oxygen. These molecules are exacerbating factors in cellular injury and aging process [4]. In foods, ROS can cause lipid peroxidation, which leads to the deterioration of the food [5]. The oxidative deterioration of the lipid-containing food is responsible for the rancid odors and flavors during processing and storage, consequently decreasing the nutritional quality and safety of foods, due to the formation of secondary, in fact potentially toxic compounds. The addition of antioxidant is a method for increasing the shelf life of food products [6].

Medicinal plants consist of secondary metabolites such as alkaloids, glycosides, steroids, and flavonoids which are the important sources of drugs [7]. Plant phenolics are commonly found in both edible and non-edible plants and have been reported to have multiple biological effects including antioxidant activity. The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers. In addition, they have a metal chelation potential [8].

The phenolic compounds are increasingly of interest in the food industry because they retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food [9]. The importance of natural phenolic compounds from plant materials is also raising interest among scientists, food manufacturers, and consumers due to functional food with specific health effects [10].

Withania somnifera is an ayurvedic herb and belongs to the family of *Solanaceae*, which has been used for centuries in India to improve overall health, vitality, and longevity. It is an Indian medicinal plant and also known as Ashwagandha, Ginseng, and Winter cherry [11]. Ashwagandha may be capable of providing excellent defensive effects on the nervous system and used for the treatment of degenerative diseases such as Alzheimer's and Parkinson's diseases [12]. This plant has proven powerful antioxidant properties that destroy the free radicals that have been concerned in aging and numerous disease states. Even more remarkable evidence suggests that *W. somnifera* has anticancer properties [13]. The leaves of *W. somnifera* are reported to contain different phytochemical constituents such as 5 unidentified alkaloids, glycosides, 12 withanolides, chlorogenic acid, condensed tannins, many free amino acids, glucose, and flavonoids [14]. Withaferin A is a steroidal lactone, and it is the most significant withanolide isolated from the leaf and dried root extracts of *W. somnifera* [15].

Another important fact related to the cultivation of plant has high dormancy, as germination of this plant is very poor. The seed viability of *W. somnifera* is limited to 1 year [16]. The explants directly collected from the field can have higher percentage of contamination than explants taken from *in vitro* for cultivation purposes. The study is conducted to develop a method for the better cultivation of plant from various explants (axillary nodes, young leaves, and internodes of *W. somnifera*) *in vitro* condition. The technique of micropropagation is applied with the objective of enhancing the rate of multiplication of plant species under study and to increase the content of various phytochemicals and other bioactive compounds in *in vitro* raised cultures of *W. somnifera*.

In view of this, the present investigation will be an attempt for the comparative analysis of field grown and *in vitro* cultivated *W. somnifera* in context to its antioxidant activity by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and nitric oxide (NO) radical scavenging methods and also

to determine the concentration of their phytochemical contents in ethanolic leaf extract of *W. somnifera*.

METHODS

Collection of plant materials

Young leaves of *W. somnifera* were collected from Botanical Garden of Lovely Professional University, Punjab, and the leaves were taken for further analysis.

Chemicals

DPPH, curcumin, and gallic acid were obtained from MP Biomedicals. Sodium carbonate was obtained from HiMedia Laboratories Pvt. Ltd., India. Folin–Ciocalteu reagent was obtained from Merck, Mumbai, India. Sodium nitroprusside (SNP) and quercetin were obtained from Sisco Research Laboratories Pvt., Ltd., Mumbai, India. Griess reagent, sulfanilamide solution, naphthyl ethylenediamine dihydrochloride, potassium acetate, aluminum chloride, and methanol were obtained from Loba Chemie, Mumbai, India.

In vitro propagation of plants from explants

In vitro cultivation of *W. somnifera* plants were done according to the standard method given by Viji *et al.* [17].

Soxhlet extraction

Ethanol extract

The dried leaves were grinded in coarse powder using high capacity grinding machine. The powdered plant material (20 g) was successively extracted in a Soxhlet extractor at elevated temperature using 200 ml of ethanol, and the extract was filtered through filter paper and poured on Petri dishes to evaporate the solvents from the extract to get dry extracts. After drying, crude extracts were weighted and stored in stock vials and kept in refrigerator (0-4°C) for further use [18].

In vitro and *in vivo* antioxidant and free radical scavenging activity

DPPH radical scavenging assay

Free radical scavenging capacity of *in vitro* and field grown ethanolic leaf extracts of *W. somnifera* was determined using DPPH assay method. Freshly prepared DPPH solution was taken in test tubes, and extracts were added followed by serial dilutions (5-50 µg/ml) to every test tube so that the final volume was 5 ml. After 30 minutes, the absorbance was read at 517 nm using a spectrophotometer. Gallic acid was used as standard. Control sample was prepared, containing the same volume without any extract and standard, and the absorbance was read at 517 nm using a spectrophotometer [19].

NO radical scavenging assay

At physiological pH, NO generated from SNP aqueous solution interacts with oxygen to produce nitrite ions measured by Griess reagent [20]. NO scavenging assay was carried using SNP and determined by the use of the Griess reagent [21]. 2 ml of 10 mM SNP in 0.5 ml phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of extract/sub-fraction at various concentrations and the mixture was incubated at 25°C for 150 minutes. From the incubated mixture 0.5 ml was taken out and added into 1.0 ml sulfanilamide solution (0.33% in 20% glacial acetic acid) and further incubated at room temperature for 5 minutes. Finally, 1 ml naphthyl ethylenediamine dihydrochloride (0.1% w/v) was mixed and maintained at room temperature for 30 minutes. The absorbance was measured at 546 nm. A typical blank/control solution contained the same solution mixture without plant extract or standard. Curcumin was used as standard. The absorbance of the blank/control solution was measured at 546 nm. The percentage inhibition was calculated according to the following equation:

$$\% \text{ inhibition} = [(1-A_1/A_0)] \times 100$$

Whereas A_1 = Absorbance of the extract or standard; A_0 = Absorbance of the control.

Phytochemical analysis

Quantification of total phenolic content

Total phenolic contents in *in vitro* and field grown ethanolic leaf extract of *W. somnifera* were determined by the Folin-Ciocalteu reagent method, according to the standard procedure described by Demiray *et al*. [22].

Quantification of total flavonoids content

Total flavonoid content in *in vitro* and field grown ethanolic leaf extract of *W. somnifera* was determined by aluminum chloride colorimetric method, according to the standard method given by Jiao and Wang [23].

Quantification of total carotenoids content

Carotenoids contents *in vitro* and field grown ethanolic leaf extract of *W. somnifera* were estimated according to the method given by Mahadevan and Sridhar [24].

Statistical analysis

The statistical analysis was performed to calculate the IC50 values using Microsoft Excel. IC50 was calculated from linear equation relationship, i.e., y = mx + c, Whereas y=% of inhibition and x = Concentration of compound. For IC50 value, y=50 and × value was obtained as inhibitory concentration (IC50: Half maximal inhibitory concentration).

RESULTS

In vitro propagated plants from explants of field grown *W. somnifera*

Explants from field grown *W. somnifera* were selected after pruning and used for *in vitro* propagation under controlled conditions. Since numerous plants can be grown under *in vitro* conditions as the explants suitable for *in vitro* propagation can be obtained throughout the year, large number of plantlets were obtained, when culture tubes were kept in the growth room at 25±2°C, with a photoperiod of 16 hrs daylight and 8 hrs night breaks under the cool white fluorescent light after the incubation was observed.

Antioxidant activity of *in vitro* and field grown *W. Somnifera*

DPPH radical scavenging assay

The free radical scavenging activity of ethanolic extract of *W. somnifera* was studied by its ability to reduce the DPPH, a stable free radical and any molecule that can donate an electron or hydrogen to DPPH, can react with it and thereby bleach the DPPH absorption [19]. DPPH is a purple color dye having absorbance at 517 nm and after showing reaction with a hydrogen donor, its purple color fades or disappears due to its conversion to a colorless compound when neutralized, resulting in decrease in absorbance. The results of antioxidant activity for field grown ethanolic leaf extract of *W. somnifera* showed the maximum activity of 72.08% (Fig. 1a) as compared to *in vitro* (Fig. 1b) at concentration of 50 µg/ml, whereas Gallic acid (Fig. 1c) exhibited 77.87% inhibition at the same concentration. *In vitro* extracts of *W. somnifera* were observed to have the least free radical scavenging activity as compared to field grown ethanolic leaf extracts.

NO radical scavenging assay

NO radical is playing an important role in various types of inflammatory process. The production of NO radical at sustained levels, result in direct tissue toxicity, whereas chronic expression of NO radical is associated with various inflammatory conditions including juvenile diabetes, multiple sclerosis, arthritis, and ulcerative colitis [25]. NO, generated from SNP, is an unstable species and reacting with oxygen molecule produce stable nitrate. *W. somnifera* extracts also moderately inhibit nitrite formation, by directly competing with oxygen to react with NO. The results of antioxidant activity for field grown ethanolic leaf extract of *W. somnifera* showed the maximum activity of 77.85% (Fig. 2a) as compared to *in vitro* (Fig. 2b) at 100 µg/ml, whereas curcumin (Fig. 2c) exhibited 85.15% inhibition at the same concentration. *In vitro* extracts of *W. somnifera* were observed to have the least free radical scavenging activity as compared to field grown ethanolic leaf extracts.

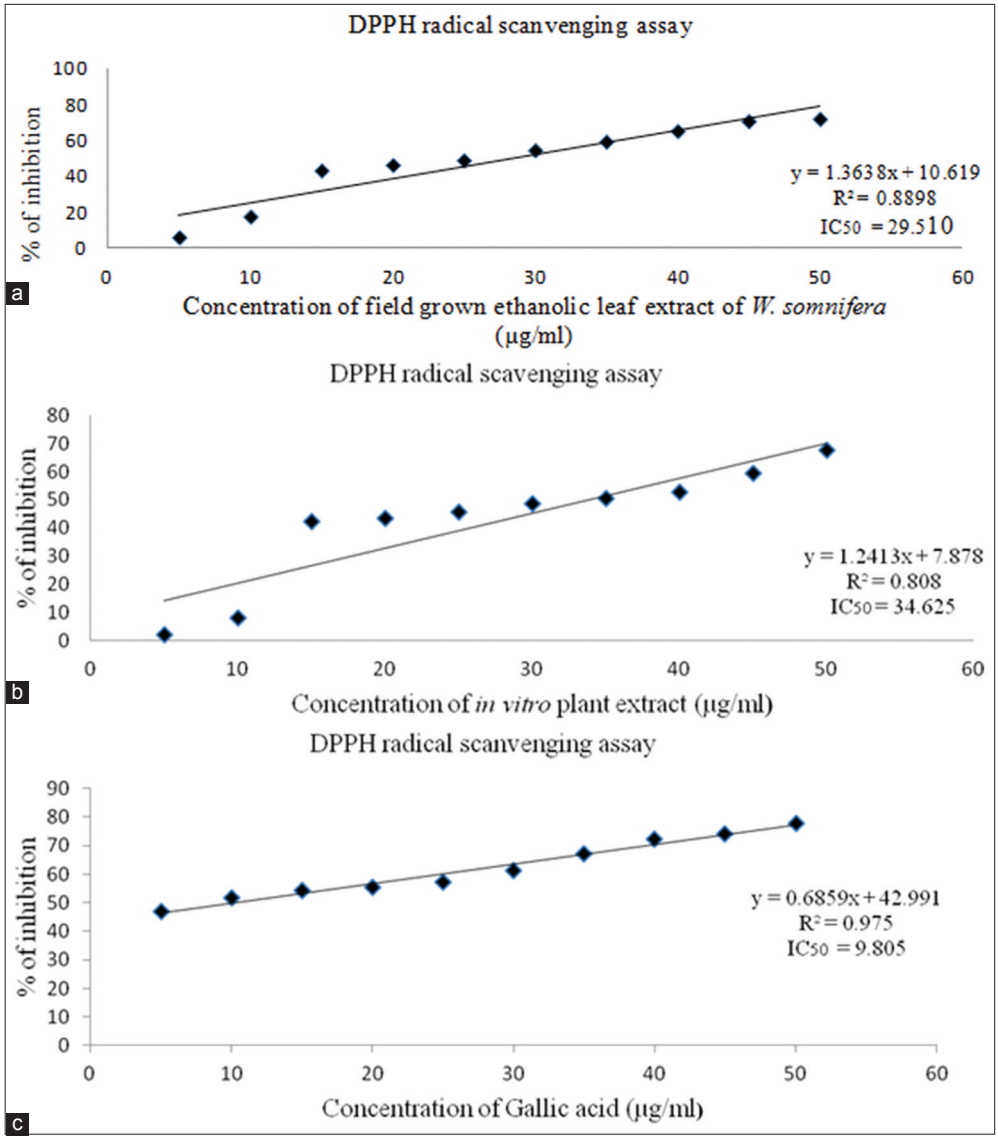

Fig. 1: 1, 1-diphenyl-2-picrylhydrazyl scavenging activity of (a) Field grown ethanolic leaf extract of *Withania somnifera*, (b) *In vitro* extracts of *Withania somnifera,* and (c) Gallic acid used as standard

Phytochemical analysis

Quantification of total phenolic content

Comparative analysis of total phenolic contents in *in vitro* and field grown ethanolic leaf extracts of *W. somnifera* was determined using the Folin–Ciocalteu reagent. The total phenolic contents of the test fractions were calculated using the standard curve of quercetin (y=0.002×+0.136) at different concentration of 100-1000 µg/ml at 720 nm. The results of total phenolic content in field grown ethanolic leaf extract of *W. somnifera* were obtained as maximum at concentration of 676.5 µg/ml at 1.489 nm when compared to *in vitro* extract (Fig. 3). Literature reveals that antioxidant activity of plant extract is mainly due to the presence of phenolic compounds, which act as free radical scavengers and metal ion chelators.

Quantification of total flavonoids content

The comparative analysis of total flavonoids content in *in vitro* and field grown ethanolic leaf extract of *W. somnifera* was determined using the aluminum chloride colorimetric method. The concentration of total flavonoids content was calculated using the standard curve of quercetin (y=0.002x–0.243) at different concentration of 100-1000 µg/ml at 430 nm. The results of total flavonoids content in field grown ethanolic leaf extract of *W. somnifera* were obtained as maximum at concentration of 557.5 µg/ml at 0.912 nm when compared to *in vitro* plant extract (Fig. 4).

Quantification of total carotenoids content

The total carotenoid contents in *in vitro* and field grown ethanolic leaf extract of *W. somnifera* were calculated using the standard curve of quercetin (y=0.001x+0.065) at different concentrations of 100-1000 µg/ml at 450 nm. The maximum concentration of total carotenoids content in field grown ethanolic leaf extract was obtained as 469 µg/ml at 0.534 nm as compared to *in vitro* plant extract (Fig. 5).

DISCUSSION

Based on the results of this study, it can be discussed that field grown ethanolic leaf extract of *W. somnifera* showed maximum antioxidant activity of 72.08% than *in vitro* extract at 50 µg/ml, in DPPH radical scavenging assay. In DPPH radical scavenging assay, chloroform, petroleum ether, and methanol extracts of *W. somnifera* showed maximum activity of 75.37%, 63.61%, and 54.16%, respectively, at 250 µg/ml, as reported by Shahriar *et al*. [18]. Shah *et al*. reported that the antioxidant activity of extracts was increased, with increase

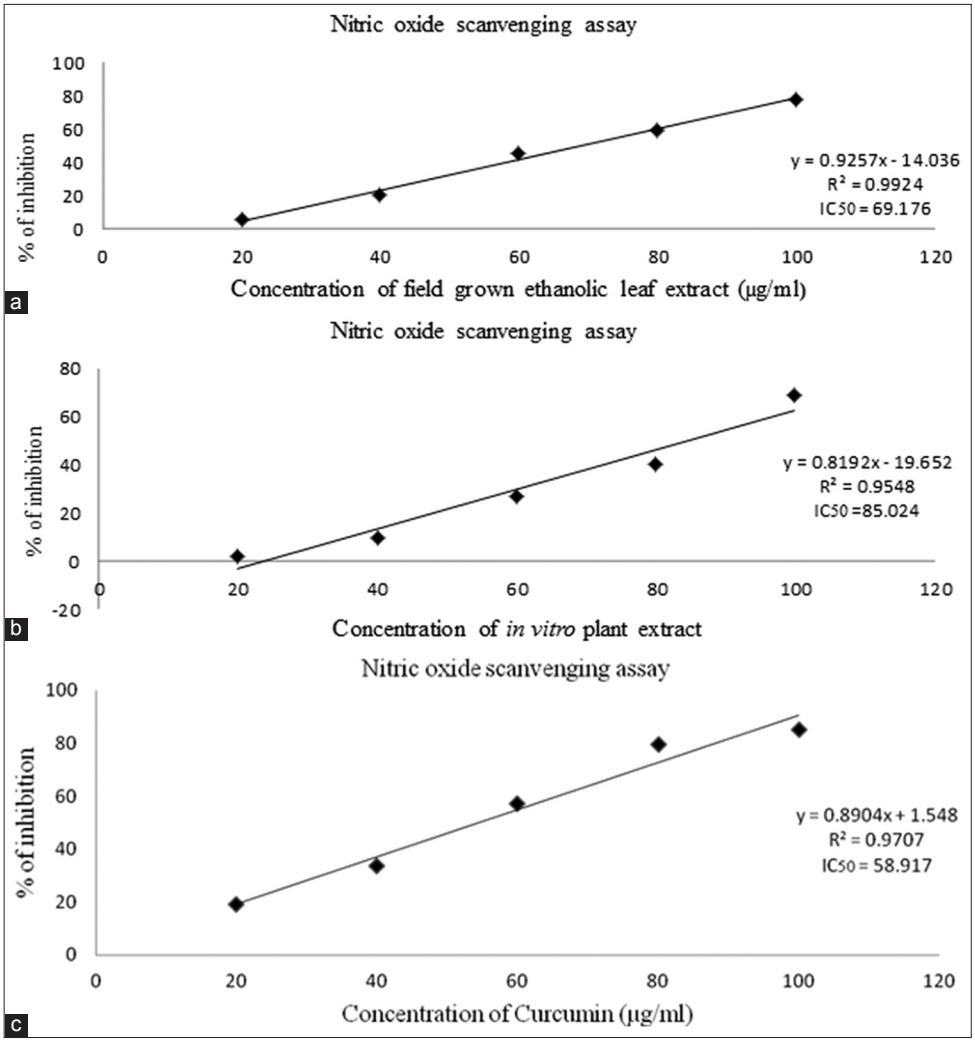

Fig. 2: Nitric oxide scavenging activity of (a) Field grown ethanolic leaf extract of *W. somnifera*, (b) *In vitro* extracts of *W. somnifera*, and (c) Curcumin used as standard

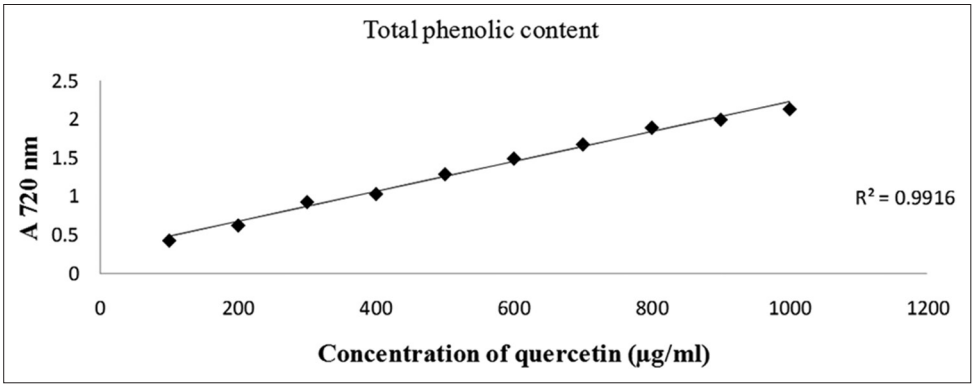

Fig. 3: Evaluation of total phenolic contents in *in vitro* and field grown ethanolic leaf extract of *W. somnifera* from standard curve of quercetin

in concentration of *W. Somnifera* [26]. According to the literature of Sharma and Sharma, the maximum value of IC50 in extract of *W. somnifera* as per DPPH and NO scavenging assay was obtained as 352±22 and 378±29 µg/ml, respectively, when compared to ascorbic acid (taken as standard) [27].

The present study proved that field grown ethanolic leaf extract showed maximum antioxidant activity of 77.85% when compared to the literature of Shahriar *et al*. at concentration of 100 µg/ml in NO radical scavenging assay method [18]. Maximum concentration of total phenolic, flavonoids, and carotenoids contents in field grown ethanolic leaf extract of *W. somnifera* was obtained as 676.5 µg/ml, 557.5 µg/ml, and 469 µg/ml, respectively, when compared to the literature of Chaudhuri *et al*. [28]. Nandi *et al*. (2011) reported that bioactive compounds separated from aqueous extract of *W. somnifera* are a potential source of antibacterial compounds with antioxidant

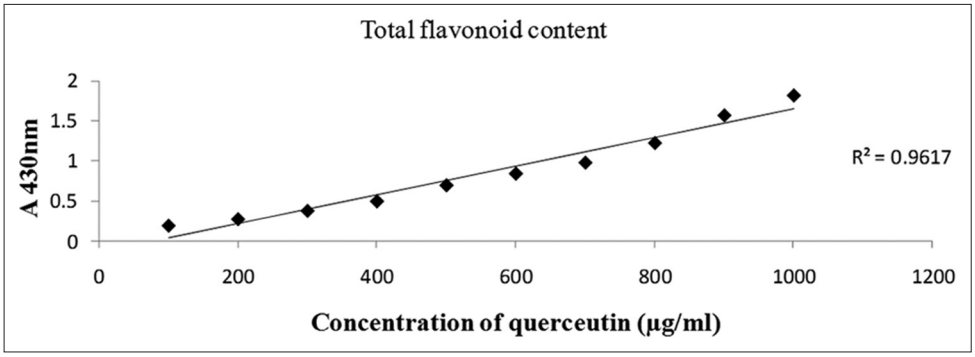

Fig. 4: Evaluation of total flavonoid contents in *in vitro* and field grown ethanolic leaf extract of *W. somnifera* from standard curve of quercetin

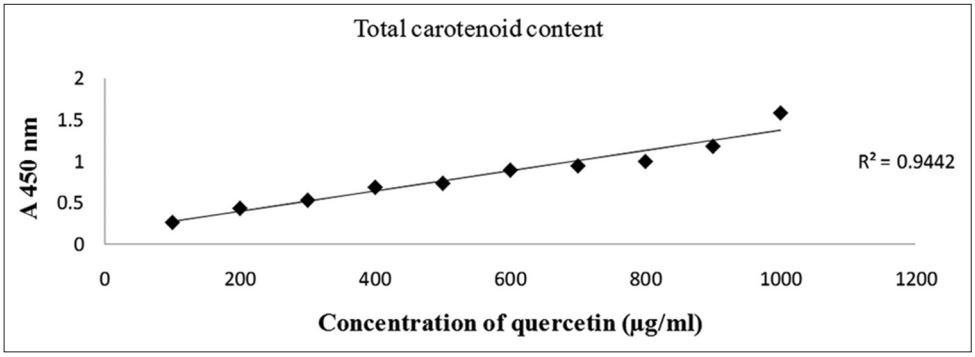

Fig. 5: Evaluation of total carotenoid contents in *in vitro* and field grown ethanolic leaf extract of *W. somnifera* from standard curve of quercetin

property [29]. Phenolic compounds are the most abundant secondary metabolites present in plant origin, which is the most important source for both human and animal diets. Phenolic content is more potent antioxidant than carotenoids because phenolics could act as hydrogen donors, quencher of singlet oxygen, and reducing agents [30,31], and thus it prevent the stress-induced oxidative stress [32].

IC50 indicates the potency of scavenging activity described by Shahriar *et al*. [18]. The IC50 values of *in vitro* and field grown ethanolic leaf extract of *W. somnifera*, gallic acid (standard) were obtained as 34.652 µg/ml, 29.510 µg/ml, and 9.805 µg/ml, respectively, in DPPH radical scavenging method. The IC50 values of *in vitro* and field grown ethanolic leaf extract of *W. somnifera*, curcumin (standard) were obtained as 85.024 µg/ml, 69.176 µg/ml, and 58.917 µg/ml, respectively, in NO radical scavenging method. According to the literature of Chaudhuri *et al*. [28], the results of IC50 values of *in vitro* root extract of *W. somnifera* and ascorbic acid (standard) were found as 650.37±107.18 µg/ml and 5.29±0.28 µg/ml, respectively, in DPPH radical scavenging method. The IC50 values of *in vitro* plant extract and curcumin were obtained as 405.91±145.84 µg/ml and 90.82±4.75 µg/ml, respectively, in NO radical scavenging method. Sharma and Sharma, (2014), have reported that multiple antioxidants are able to reduce inflammatory symptoms in inflammatory joint disease, acute and chronic pancreatitis, and adult respirator syndrome [27].

CONCLUSIONS

In the present study, we found that field grown ethanolic leaf extract of *W. somnifera* has excellent antioxidant activity to destroy the free radicals. This study also reveals the maximum concentration of total phenolic contents in field grown ethanolic leaf extract of *W. somnifera* as compared to *in vitro* extract. Antioxidant activity of plant extract is mainly due to the presence of phenolic compounds which are more potent antioxidant than other compounds because phenolics could act as a hydrogen donors, quencher of singlet oxygen, and reducing agents. From this study, we conclude that *W. somnifera* has effective radical scavenging activity, implying their potential use in pharmacological and food industries. Further studies are required to isolate the active principles and antioxidant activity of individual extracts of different parts. The pharmacological validation in terms of modern medicine will be of great medicinal importance in future.

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