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ANTIOXIDANT ACTIVITY AND FREE RADICAL SCAVENGING CAPACITY OF IN VITRO AND IN VIVO REGENRATD LEAF, STEM AND ROOT TISSUES OF WITHANIA SOMNIFERA- POSHITA VARIETY

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ABSTRACT

Many medicinal plants contain large amounts of antioxidants such as secondary metabolites, which can play an important role in absorbing and neutralizing free radicals. In the present study antioxidant activity and free radical scavenging efficacy of *in vitro* and *in vivo* regenerated plant extracts of *Withania somnifera*- Poshita variety was carried out. Among the various parts tested for SOD and catalase activity, the *in vitro* regenerated root possessed more activity when compared to the *in vitro* regenerated stem and *in vitro* regenerated leaves. But peroxidase, glutathione peroxidase and glutathione S-transferase showed more activity in the *in vitro* regenerated leaf possess when compared to *in vitro* regenerated stem and *in vitro* regenerated root. The content of the non-enzymic antioxidants were significantly higher ($P>0.05$) in the *in vitro* regenerated leaves. The methanolic extract of *Withania somnifera*- Poshita variety leaves showed minimum concentration and more effective in scavenging DPPH, nitric oxide, hydrogen peroxide and hydroxyl radicals. This radical scavenging activity might be due to the active antioxidants present in the methanolic extract of *Withania somnifera*- Poshita variety leaves.

Key words: Medicinal plants; *in vitro*, *in vivo*, Antioxidant activity, Free radical scavenging capacity.

INTRODUCTION

Plant-derived drugs remain an important resource, especially in developing countries, to combat serious diseases. Approximately 60% to 80% of the world's population still relies on traditional medicines for the treatment of common illness [1]. Medicinal plants are also cheaper and more accessible to most of the population in the world [2]. Hence, many pharmacognostical and pharmacological investigations are carried out for the development of novel therapeutic agents for the treatment of human ailments such as cancer and infectious diseases [3]. Medicinal plants have regained a wide recognition due to an escalating faith in herbal medicine in the last few decades contributed by its lesser side effects compared to allopathic medicine[4].

MATERIALS AND METHODS

The seeds of *Withania somnifera* -poshita cultivar were collected from Central Institute of Medicinal Aromatic Plants (CIMAP) and cultivated in Avinashilingam deemed University Campus Tamil Nadu, India. 5 month old *in vivo* regenerated *Withania somnifera*- Poshita variety leaf, stem and root were collected, washed with fresh water and dried under at room temperature. The leaves were powdered and stored in sterile containers for further use. 50 g of dried powdered samples were taken. The treated samples were dissolved in 150 ml of chloroform, ethyl acetate and methanol respectively. All the preparations were kept in shaker for 3 days. Then the solvents were filtered through filter paper to remove free extractable substances. The filtrate was concentrated by drying at room temperature for several days till dried leaves sample were obtained. For extract of plants grown *in vitro,* 5 months old plants maintained in MS hormone free medium were used. The same method has done for the *in vitro* regenerated 5 month old leaf, stem and root plant parts and prepared the extracts.

Determination of antioxidant potential

Antioxidants are vital substances which possess the ability to protect the human body from damage by free radical-induced oxidative stress [5]. The anti oxidative system includes both enzymatic and non enzymatic systems.

Enzymatic antioxidants

The enzymatic antioxidants Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GSH-Px), Glutathione S - transferase (GST) and Peroxidase (Px) were found to limit the cellular concentration of free radicals and prevent excessive oxidative damage[6]. Superoxide dismutase activity was estimated by the method of Misra and Fridovich[7].The

activity of catalase was assayed spectrophotometrically following the method of Luck[8]. The method proposed by Reddy *et al* [9]. was adopted for assaying the activity of peroxidase. The method for estimation of glutathione peroxidase proposed by Rotruck *et al.*[10]. The method of Beutler [11] was followed to determine the glutathione S-transferase.

Non-enzymatic antioxidants

The natural defense of the human against free radicals is not always sufficient mainly due to the significant exposition to free radicals from external sources in the modern world. The dietary intake of antioxidants plays an important role in the protection of the humans against free radicals. Many clinical and epidemiological studies demonstrate the relationship between the antioxidant activity of the substances present in the diet and the prevention from diseases such as cardiovascular diseases or carcinogenesis[12]. Ascorbic acid content was estimated by the method of Roe and Kuether [13]. The levels of α-tocopherol was estimated spectrophotometrically by the method of Rosenberg [14]. Carotenoids were analyzed by the method of Zakaria *et al* [15]. The content of polyphenols was determined by the method of Mallick and Singh[16].The procedure for the determination of flavonoids by the method explained by Zhishen *et al.*[17].

Determination of free radical scavenging activity

Reactive oxygen species (ROS), include free radicals such as hydroxyl (OH radical), superoxide (O_2 radical), nitric oxide (NO radical), peroxyl (RO_2 radical), and non-free radical species namely hydrogen peroxide (H_2O_2), singlet oxygen, ozone (O_3), which are different forms of activated oxygen [18]. The procedure for the determination of various free radical scavenging activity was given below: The 1,1-diphenyl 1-2-picryl hydrazyl (DPPH) scavenging activity was determined by the method described by Mensor *et al.*[19]. The procedure proposed by Re *et al.*[20] was followed for determining 2,2'-azinobis (3-ethyl benzothiazoline-6-sulfonic acid ammonium salt) (ABTS) scavenging effect. The method for analysis of hydrogen peroxide scavenging activity proposed by Ruch *et al.* [21]. Inhibition of hydroxyl radical generation was done by the method proposed by Elizabeth and Rao[22]. Inhibition of superoxide generation was determined by the method described by McCord and Fridovich [23]. The method of Green and Hill [24] was followed for the determination of inhibition of nitric oxide generation.

RESULTS

The enzymic antioxidants analyzed were SOD, catalase, peroxidase, glutathione peroxidase and glutahione S-transferase and the activities obtained are presented below (Table 1). The activities of superoxide dismutase, catalase, peroxidase, glutathione peroxidase and glutathione S-transferase were assessed in *in vitro* and *in vivo* regenerated leaf, stem and root samples. The results revealed that all the plant samples possessed considerable activities of all enzymes. In all the assays performed, the activities of enzymic antioxidants such as SOD, catalase, peroxidase, glutathione peroxidase and glutathione S-transferase were predominantly higher in *in vitro* regenerated leaf, stem and root samples when compared to *in vivo* grown plant samples of *Withania somnifera* (L.) Poshita variety. Among the various parts tested for SOD and catalase activity, the *in vitro* regenerated root possessed more activity when compared to the *in vitro* regenerated stem and *in vitro* regenerated leaves. But peroxidase, glutathione peroxidase and glutathione S-transferase showed more activity in the *in vitro* regenerated leaf possess when compared to *in vitro* regenerated stem and *in vitro* regenerated root.

From the results reported by Sumathi and Padma [25]., who have screened the different parts of *Withania somnifera* (L.), namely the roots, leaves, stem, fresh tubers and dry tubers for their antioxidant potential, the leaves recorded significantly higher activities of enzymic antioxidants. Jaleel [26] reported that antioxidant enzymes like superoxide dismutase, ascorbate peroxidase, catalase, polyphenol oxidase and peroxidase have maximum activities in root tissues of *Withania somnifera* (L.) wild variety when compared with leaves. The results of the present study revealed that the leaves and roots were found to be potential sources of antioxidants. The body has an effective mechanism to prevent and neutralize the free radical induced damage. This is accomplished by a set of endogenous antioxidants such as ascorbic acid, α-tocopherol, carotenoids, polyphenols and flavonoids. Table 2 depicts the non-enzymic antioxidant levels in *Withania somnifera* (L.) Poshita variety. The major representatives of the non-enzymic antioxidants, namely ascorbic acid, α-tocopherol, carotenoids, polyphenols and flavonoids were estimated in the extracts of *in vitro* and *in vivo* regenerated leaf, stem and root of *Withania somnifera* (L.) Poshita variety. The content of the non-enzymic antioxidants such as ascorbic acid, α-tocopherol, carotenoids, polyphenols and flavonoids were significantly higher ($P>0.05$) in the *in vitro* regenerated leaves. Among the non enzymic antioxidants, the *in vitro* regenerated leaves showed higher levels when compared to *in vitro* stem and *in vitro* root.

Sumathi and Padma[27] screened the different parts of *Withania somnifera* (L.), namely the roots, leaves, stem, fresh tubers and dry tubers for their antioxidant potential. The leaves recorded significantly higher activities of non-enzymic antioxidants followed by the fresh tubers. Ascorbic acid is a key antioxidant, partially protecting lipids from peroxidative damage and it has many biological activities in the human body [28]. Tocopherol is used in combating free radicals and the most active form of vitamin E is present in the cellular membrane and acts as a protective lipid soluble agent, generating the poorly reactive tocopherol radicals [29].

Carotenoids have the capacity of quenching singlet oxygen and acting as free radical scavengers and antioxidants [30]. Carotenoids and other antioxidant pigments are involved in several physiological processes and signaling in animals that cannot synthesize them and therefore, must acquire them from food [31]. Flavonoids are also described as scavengers of reactive oxygen species, via inhibition of oxido-reductases [31]. Flavonoids and other phenolic compounds of plant origin have been reported as scavengers and inhibitors of lipid peroxidation [32]

Assessment of free radical scavenging effect of *Withania somnifera* (L.) Poshita variety

Various phytochemical components especially polyphenols (such as flavonoids, phenolic acids, tannins, etc.) are known to be responsible for the free radical scavenging and antioxidant activities of plants. Phenolic substances possess many biological effects. These effects are mainly attributed to their antioxidant activities in scavenging free radicals, inhibition of peroxidation and chelating transition metals [33].

In order to understand the chemical components present in the extract that contributes to the strongest antioxidant activity, the *in vivo* and *in vitro* regenerated leaf, stem and root of *W. somnifera* (L.) Poshita variety were extracted into solvents of differing polarity (chloroform, ethyl acetate and methanol). These extracts were then assessed for their radical scavenging effects against a sequence of oxidant moieties that included a stable radical DPPH, ABTS, H_2O_2, OH radicals, SO radicals and NO radicals. The results are presented in figures 1 to 6. Among the three extracts analyzed, the maximum radical scavenging effect was elicited by the methanolic extract of in vitro regenerated leaves, which showed a powerful scavenging against a group of radicals such as DPPH, ABTS, H_2O_2, OH , SO and NO radicals, followed by the chloroform extract and the least scavenging effect was from the ethyl acetate extract. The maximum extent of scavenging was mediated by the *in vitro* regenerated plant parts.

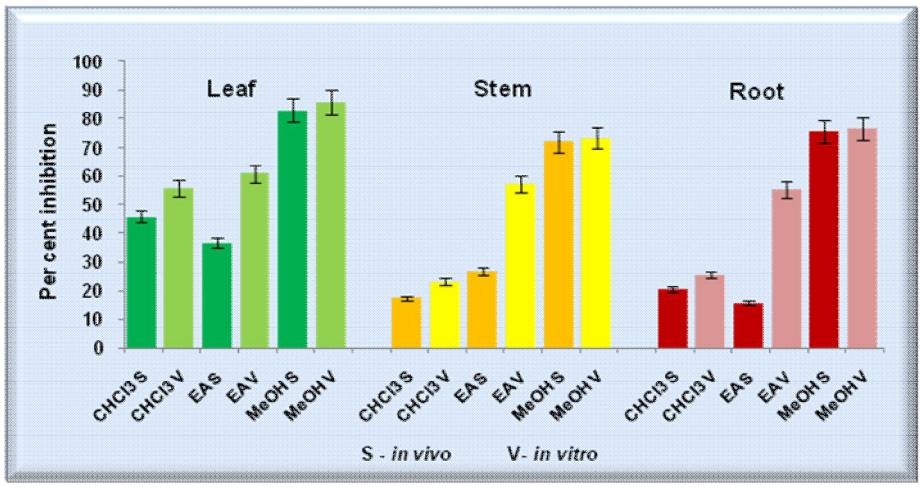

Figure 1 : ABTS radical scavenging effect of *Withania somnifera* (L.) Poshita variety

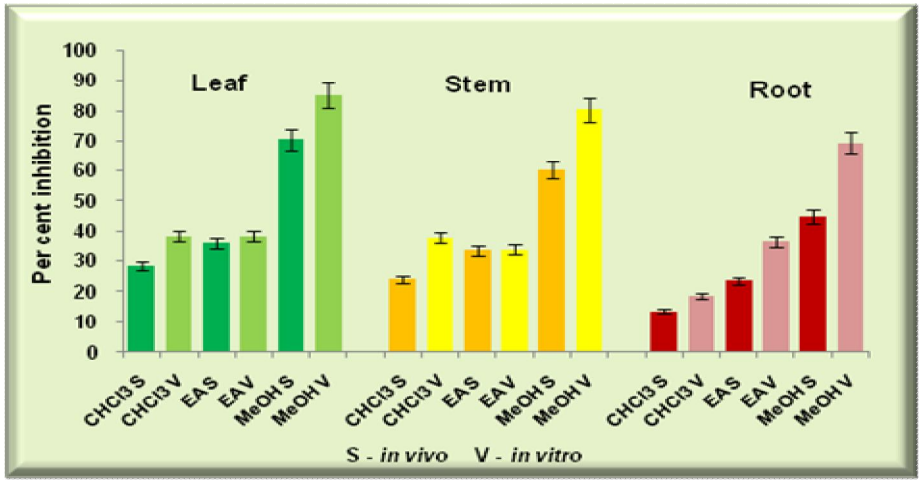

Figure 2: DPPH radical scavenging effect of W*ithania somnifera* (L.) Poshita variety

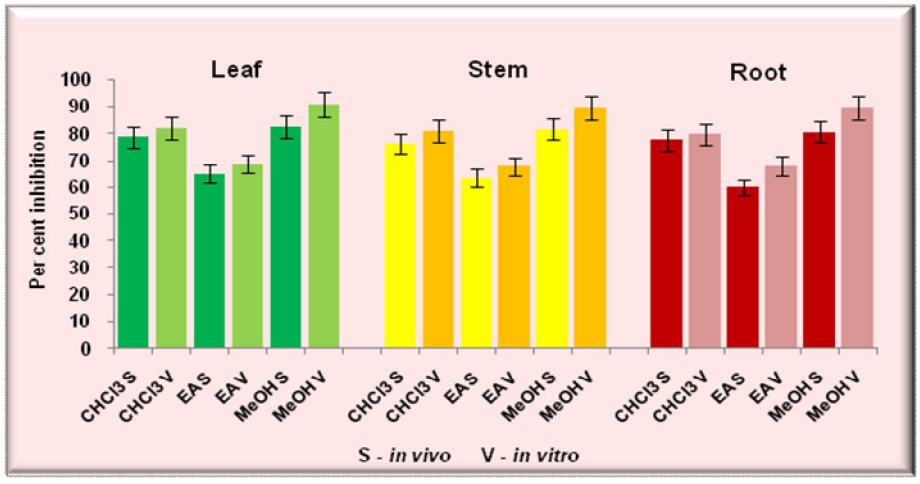

Figure 3 : H_2O_2 radical scavenging effect of *Withania somnifera* (L.) Poshita variety

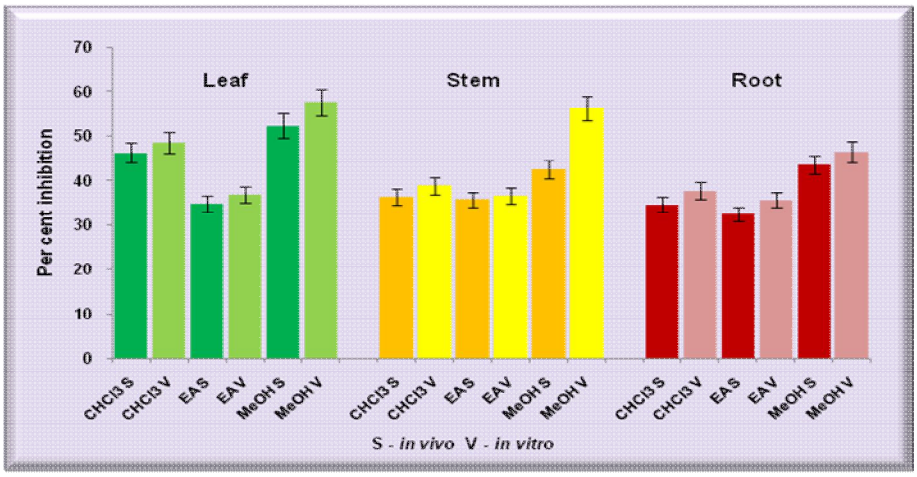

Figure 4: OH radical scavenging effect of *withania somnifera* (l.) poshita variety

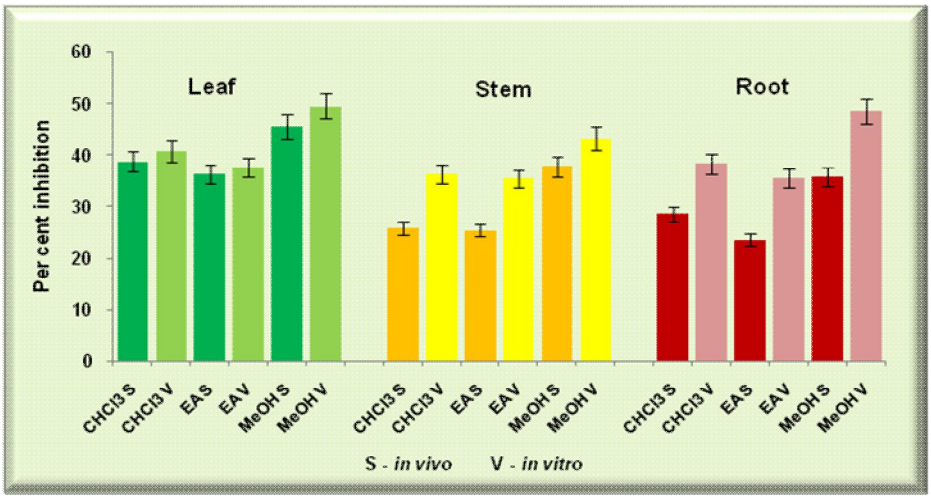

Figure 5: SO radical scavenging effect of *withania somnifera* (l.) Poshita variety

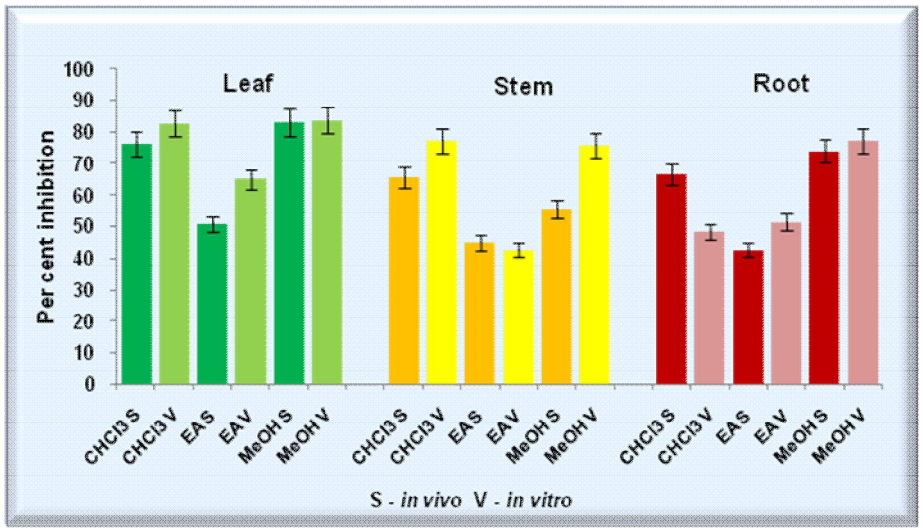

Figure 6: NO radical scavenging effect of *withania somnifera* (l.) Poshita variety

The methanolic extract of *Manikara zapota* showed strong activity in scavenging DPPH radical, which implicates an essential defence against the free radicals [34]. The aqueous, methanolic and ethanolic extracts of *Melissa officinalis*, *Matricaria recuttia* and *Cymbopogan citrates* were found to exhibit DPPH scavenging activity [35]. The methanolic extracts of leaves and flowers of *Lippia alba* exhibited very significant DPPH radical scavenging activity compared to the standard antioxidant ascorbic acid [36].

Gulcin *et al*. [37] have shown that ligustroside and oleuropein, isolated from the methanolic extracts of the root bark of *Chinonanthus virginicus*, exhibited good ABTS radical scavenging activity. Lopez- Giraldo *et al*. [38] reported that *Tecoma stans* possessed strong ABTS scavenging activity and the activity was attributed by its phenolic and flavonoid content. Gulcin *et al*.[39] have reported that the water and methanolic extracts of *Ocimum basilicum* had strong antioxidant activity and were effective in scavenging H_2O_2.

The methanol extract of *Lagerstroema speciosa* (L). showed higher hydroxyl radical scavenging activity when compared to the ethyl acetate, ethanol and water extracts [40] . The methanolic extract of *Picrasma quassiades* [41] and of the leaves of *Stachytarpheta angustifolia* inhibited hydroxyl radicals[42]

Superoxide radicals are one of the most important reactive oxygen free radicals constantly produced in living cells [43]. They have relatively weak chemical reactivity because they cannot penetrate lipid membranes and they are rapidly converted into H_2O_2 by superoxide dismutase [44] Many reports in the literature associate the SO radical scavenging of plants and their components with strong antioxidant activity. Anandjiwala [45] reported that the methanolic extract of *Bergia suffruticosa* showed inhibition of superoxide generation. Ogunlana *et al*.[46] showed that the crude extract of *Newboudia* leaves exhibited very high inhibition of superoxide generation. The crude methanolic bark extract of *Terminalia arjuna* was found to possess high phenolics, high reducing power and high free radical scavenging activity including nitric oxide radicals [47].

Thus, the present study found that *Withania somnifera* (L.) Poshita regenerated under *in vitro* and *in vivo* regenerated plant parts have effective antioxidant and radical scavenging activity, implying their use in pharmacological and food industries due to their antioxidant properties.

Table 1: Enzymic antioxidant activities of *in vivo* and *in vitro* regenerated leaf, stem and root of *Withania somnifera* (L.) *Poshita variety*

Enzymic antioxidants	Leaf		Stem		Root	
	in vivo	***in vitro***	***in vivo***	***in vitro***	***in vivo***	***in vitro***
SOD (U/g) #	23.80 ± 0.16	26.51 ± 0.532	34.73 ± 0.16	35.19 ± 0.47	36.20 ± 0.99	39.05 ± 0.64
Catalase (U/g)*	161.48 ± 0.29	163.83 ± 0.42	154.36 ± 1.93	173.64 ± 3.64	212.46 ± 2.30	226.74 ± 1.25
Peroxidase (U/g) $	56.87 ± 0.13	63.15 ± 1.19	48.27 ± 0.23	34.06 ± 1.27	56.88 ± 0.72	50.74 ± 0.25
Glutahione peroxidase (U/g) &	60.01 ± 0.42	79.68 ± 0.18	12.42 ± 0.32	50.16 ± 0.50	15.84 ± 0.55	36.71 ± 0.25
Glutahione S-transferase (U/g) +	0.30 ± 0.01	0.37 ± 0. 02	0.14 ± 0.01	0.23 ± 0. 01	0.28 ± 0.02	0.27 ± 0.01

Values are mean ± SD of triplicates
 # 1 Unit - Amount of enzyme that gives 50% inhibition of the extent of NBT reduction in 1 minute
** 1 Unit - Amount of enzyme required to decrease the absorbance at 240nm by 0.05 units / Minute*
$ 1 Unit - Change in absorbance / minute at 430nm
& nmoles of GSH consumed/ min/g sample
+ nmoles of CDNB-GSH conjugate/min/g sample

Table 2: Non-enzymic antioxidant activities of *in vivo* and *in vitro* regenerated leaf, stem and root of *Withania somnifera* (L.) *Poshita variety*

Enzymic antioxidants	**Leaf**		**Stem**		**Root**	
	in vivo	***in vitro***	***in vivo***	***in vitro***	***in vivo***	***in vitro***
Ascorbic acid (mg/g)	*0.52 ± 0.03*	***2.71 ± 0.03***	*0.32 ± 0.05*	***0.42 ± 0.02***	*0.24 ± 0.01*	***0.27 ± 0.02***
Tocopherol (µg/g)	*3.55 ± 0.08*	***5.51 ± 0.33***	*2.20 ± 0.09*	***2.88 ± 0.14***	*2.08 ± 0.84*	***3.70 ± 0.33***
Carotinoids (mg/g)	*6.52 ± 0.82*	***7.84 ± 0.21***	*4.58 ± 0.91*	***7.39 ± 0.28***	*2.44 ± 2.67*	***6.57 ± 5.84***
Polyphenols (mg/g)	*18.9 ± 0.02*	***29.5 ± 0.07***	*13.5 ± 0.04*	***14.09 ± 0.10***	*12.5 ± 0.04*	***18.70 ± 0.04***
Flavonoids (mg/g)	*6.49 ± 4.31*	***7.73 ± 3.46***	*5.71 ± 0.70*	***5.71± 1.14***	*5.02 ± 0.71*	***5.04 ± 1.45***

Values are mean ± SD of triplicates

CONCLUSION

The present study found that *Withania somnifera* (L.) Poshita regenerated under *in vitro* and *in vivo* regenerated plant parts have effective antioxidant and radical scavenging activity, implying their use in pharmacological and food industries due to their antioxidant properties. 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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