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Determination of Total Phenolics, Total Flavonoids and Evaluation of DPPH Free Radical Scavenging Activity of Ashwagandha (*Withania somnifera* L.) Roots

SUSHEEL GULATI, V.K. MADAN*, SATYA SHREE JANGRA and I. S. YADAV

Medicinal, Aromatic and Potential Crops Section, Old IATTE Building, CCS Haryana Agricultural University, Hisar-125 004, India

*Corresponding author: E-mail: vikku60@gmail.com

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The present study was performed to estimate the total phenolics, total flavonoids and to evaluate the antioxidant activity of aqueous and ethanolic extracts of ashwagandha (*Withania somnifera* L.) roots of promising genotypes *viz.* HWS-08-14, HWS-08-18, HWS-1228, HWS-1229 and Selection-2B & varieties JA-20 and RVA-100. The results revealed that the extract yield of aqueous extracts (9.95 g/100 g) was higher than ethanolic extracts (3.96 g/100 g). Aqueous extracts contained the higher amount of total phenolics (3.58 mg GAE/g d.w.b.) than ethanolic extracts (2.19 mg GAE/g d.w.b.) whereas ethanolic extracts contained the higher amount of total flavonoids (1.16 mg CE/g d.w.b.) than aqueous extracts (0.82 mg CE/g d.w.b.). DPPH free radical scavenging activity of ashwagandha extracts varied widely and it increased with increase of concentration levels. Among aqueous and ethanolic extracts, aqueous extracts exhibited higher antioxidant activity. Hence, aqueous extracts of ashwagandha roots are better source of antioxidants in comparison to ethanolic extracts.

Keywords: *Withania somnifera*, Total phenolics, Total flavonoids, Antioxidant activity.

INTRODUCTION

Plant-based traditional medicine system continues to play a vital role in the health care system with about 60 % of the world inhabitants relying mainly on traditional medicines for their primary health care. Modern knowledge on medicinal plant research still contains at least 25 % drugs and many others, which are synthetic analogues, built on prototype compounds isolated from medicinal plants. The ongoing growing recognition of medicinal plants is due to escalating faith in herbal medicine [1]. The medicinal plant products, which are derived from plant parts such as stem, bark, leaves, fruits and seeds have been part of phytomedicine that produce a definite physiological action on human body. The most important of these natural bioactive constituents of plants are alkaloids, tannins, flavonoids and phenolic compounds [2]. Medicinal plants also contain large amounts of antioxidants, such as polyphenols, vitamin C, vitamin E, selenium, β-carotene, lycopene, lutein and other carotenoids, which play important roles in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides [3].

Among various medicinal plants, *Withania somnifera* is a popular Indian medicinal plant belonging to family Solanaceae and is also known as Ashwagandha, Indian ginseng and Winter cherry. It is an important herb in the ayurvedic and indigenous medical system. This plant grows widely in all drier parts of subtropical India *i.e.* in Madhya Pradesh, Uttar Pradesh, Punjab plains and North-Western part of India like Gujarat and Rajasthan. It is also found in Congo, South Africa, Egypt, Morocco, Jordan, Pakistan and Afghanistan. It has been reported that although all major parts of *Withania somnifera* such as the roots, fruits and leaves provide potential benefits for human health because of their high content of polyphenols and antioxidant activities but the roots are main part of the plant that are widely used as therapeutic agents [4]. The roots are reported to contain alkaloids, amino acids, steroids, volatile oil, starch, reducing sugars, glycosides [5]. The medicinal properties of *W. somnifera* is attributed to several classes of withanolides, a group of naturally occurring C-28 steroidal lactone triterpenoids, in which C-22 and C-26 are suitably oxidized to form a six membered lactone ring [6]. Major polyphenols in different parts *viz.* roots, fruits and leaves of ashwagandha include gallic acid, syringic acid, benzoic acid, *p*-coumaric acid and vanillic acid as well as catechin, kaempferol and naringenin [4].

Review of literature reveals that various field studies are being carried out on promising genotypes of ashwagandha but very scare information is available on phytochemical studies and antioxidant activity of ashwagandha roots. Therefore, the objective of present study was to estimate the phytochemicals in the promising genotypes of ashwagandha and also to evaluate the antioxidant activity of promising genotypes of ashwagandha.

EXPERIMENTAL

Ashwagandha (*Withania somnifera* L.) roots samples of promising genotypes (HWS-08-14, HWS-08-18, HWS-1228, HWS-1229 and Selection-2B) and two varieties (JA-20 and RVA-100) were procured from the experimental area of Medicinal, Aromatic and Potential Crops Section, Department of Genetics and Plant Breeding, Chaudhary Charan Singh Haryana Agricultural University, Hisar. Roots were shade dried. After drying, roots were cut into small pieces of 2-3 inches and were ground. For estimation of total phenolics, total flavonoids and evaluation of antioxidant activity, aqueous and ethanolic extracts were prepared by using Soxhlet apparatus. For aqueous and ethanolic extracts, 4 g of powdered samples of ashwagandha roots were placed in a filter paper (Whatman No. 1) thimble in a classical Soxhlet apparatus fitted with a 250 mL round bottom flask. The solvents (distilled water and ethanol) were added up to one and a half siphons that is approximately 150 mL. After the completion of first extraction step of 5 h, residue in thimble was again extracted twice (each extraction time 2 and 1 h, respectively) with suitable amount of respective solvents. Filtrates of each solvent from three extraction steps were pooled and their volumes were noted. These extracts were filtered and used for estimation of extract yield, total phenolics, total flavonoids and evaluation of antioxidant activity.

The commercially available chemicals from Merck, SRL (SISCO Research Labortories), Qualigens and Sigma-Aldrich, were used for various experimental procedures.

Estimation of total phenolics content: Total phenolics content of extracts was determined using Folin-Ciocalteu method [7]. Aliquots of 0.2 mL of extracts were mixed with 1 mL of 1 mol/L Folin-Ciocalteu reagent. After that, 2.0 mL of 20 % (w/v) sodium carbonate solution was added. The solutions were mixed and volume was made up to 10.0 mL with distilled water. The absorbance was measured at 730 nm using UV-VIS double beam Spectrophotometer Model 2203 (Systronics Co.). A calibration curve was prepared using gallic acid as standard. Results were expressed as mg GAE/g on dry weight basis.

Estimation of total flavonoids content: Total flavonoids content of extracts was estimated according to the colorimetric assay [8]. In 1 mL of extract, 4 mL of double distilled water and 0.3 mL of 5 % (w/v) $NaNO_2$ were added. After 5 min, 0.3 mL of 10 % (w/v) $AlCl_3$ was added. Immediately, 2 mL of 1 M NaOH was added and the volume was made up to 10.0 mL with double distilled water. The solution was mixed thoroughly and the absorbance was measured at 510 nm using UV-VIS double beam Spectrophotometer Model 2203 (Systronics Co.). A calibration curve was prepared using catechin as standard. Results were expressed as mg CE/g on dry weight basis.

DPPH free radical scavenging activity: The antioxidant activity of the extracts was evaluated by 2,2'-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity method [9]. Ethanolic and aqueous extracts of ashwagandha roots powder were dried up completely and the weight of dry mass was noted. The dry mass of ethanolic extracts was redissolved in appropriate amount of methanol to make the stock solution (5000 µg/mL). Since, the dry mass of water extract was not soluble in pure methanol, hence, it was redissolved in 50 % (v/v) methanol:water to make the stock solution. From stock solution, different concentrations (100 to 5000 µg/mL) were made by appropriate dilutions with respective solvents (*i.e.* methanol for ethanol extracts and with methanol:water for water extracts). For evaluation of antioxidant activity, in 0.2 mL of extracts (various concentrations), 3 mL of 2,2'-diphenyl-1-picrylhydrazyl radical (DPPH; 0.1 mM in 100 % methanol) was added and mixed thoroughly for 5 min. For antioxidant activity in water extracts (various concentrations), DPPH stock solution was prepared in 50 % (v/v) methanol:water and remaining procedure was same. A control was also made containing 0.2 mL of each solvent instead of extract. The absorbance of the sample as well as control was measured at 517 nm after 30 min of incubation in dark at room temperature using the UV-visible double beam spectrophotometer Model 2203 (Systronics Co.) against a blank containing respective solvent. Three replications were carried out for each sample. A graph was drawn by plotting percent DPPH free radical scavenging activity (y-axis) against extract concentration (x-axis). Then using the Microsoft Excel Software, a quadratic regression equation ($y = ax^2 + bx + c$) was obtained. By putting $y = 50$ % in the equation $y = ax^2 + bx + c$; it was converted to the form $ax^2 + bx + c = 0$. IC_{50} was calculated from the equation $ax^2 + bx + c = 0$ by using the formula:

$$x = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a}$$

where, $x = IC_{50}$ (µg/mL).

Calculation: The percentage of DPPH scavenged (% $DPPH^*_{sc}$) was calculated using:

$$DPPH^*_{sc}\ (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

where, $A_{control}$ is the absorbance of control and A_{sample} is the absorbance of the sample.

RESULTS AND DISCUSSION

Extract yield: Extract yield of ashwagandha roots of various genotypes/varieties varied widely and is shown in Table-1. Amongst solvents, the mean value of extract yield of aqueous extracts (9.95 g/100 g) was higher than ethanolic extracts (3.96 g/100 g). Our finding is in agreement with previous investigation which reported that aqueous extracts of ashwagandha roots had higher extraction yields (9.51 %) than ethanolic extracts (9.08 %) [10].

TABLE-1
EXTRACT YIELD (g/100 g) OF AQUEOUS AND ETHANOLIC EXTRACTS OF ASHWAGANDHA ROOTS OF VARIOUS GENOTYPES/VARIETIES

Genotypes/ varieties	Extract yield (g/100 g)		
	Aqueous	Ethanolic	Mean
HWS-08-14	11.48 ± 0.07	4.83 ± 0.05	8.16
HWS-08-18	13.65 ± 0.09	4.84 ± 0.06	9.24
HWS-1228	12.20 ± 0.06	3.52 ± 0.05	7.86
HWS-1229	9.00 ± 0.06	3.85 ± 0.07	6.42
Selection-2B	7.48 ± 0.05	4.35 ± 0.09	5.92
JA-20 (C)	7.32 ± 0.10	2.66 ± 0.08	4.99
RVA-100 (C)	8.50 ± 0.06	3.65 ± 0.05	6.08
Mean	9.95	3.96	

Total phenolic content: Total phenolic content in ashwagandha roots of various genotypes/varieties varied widely and is shown in Table-2. On dry weight basis, total phenolics content in aqueous extracts ranged from 2.41 to 5.37 mg GAE/g d.w.b. and genotype HWS-08-14 has highest total phenolics content (mg GAE/g) *i.e*. 5.37 followed by HWS-08-18 (4.25), HWS-1228 (3.71), HWS-1229 (3.45) and Selection-2B (3.20) in comparison to control varieties RVA-100 (2.67) and JA-20 (2.41). In ethanol extracts, total phenolics content ranged from 1.54 to 3.36 mg GAE/g d.w.b. and genotype HWS-08-14 has highest total phenolics content (mg GAE/g) *i.e*. 3.36 followed by Selection-2B (2.49), HWS-1229 (2.46), HWS-08-18 (1.83) and HWS-1228 (1.54) in comparison to control varieties RVA-100 (1.96) and JA-20 (1.70). Amongst solvents the mean value of total phenolics content (mg GAE/g) in aqueous extracts (3.58) was higher than ethanol extracts (2.19). Our results are in agreement with other research workers, who reported 0.94 mg/g phenols in ethanolic extracts of *Withania somnifera* (L.) roots variety-Poshita under *in vivo* conditions and 1.27 mg/g under *in vitro* conditions [11].

TABLE-2
TOTAL PHENOLICS (mg GAE/g) IN AQUEOUS AND ETHANOLIC EXTRACTS OF ASHWAGANDHA ROOTS OF VARIOUS GENOTYPES/VARIETIES ON DRY WEIGHT BASIS

Genotypes/ varieties	Total phenolics (mg GAE/g)		
	Aqueous	Ethanolic	Mean
HWS-08-14	5.37 ± 0.35	3.36 ± 0.08	4.37
HWS-08-18	4.25 ± 0.15	1.83 ± 0.10	3.04
HWS-1228	3.71 ± 0.16	1.54 ± 0.05	2.63
HWS-1229	3.45 ± 0.16	2.46 ± 0.09	2.96
Selection-2B	3.20 ± 0.25	2.49 ± 0.02	2.85
JA-20 (C)	2.41 ± 0.02	1.70 ± 0.09	2.06
RVA-100 (C)	2.67 ± 0.08	1.96 ± 0.06	2.32
Mean	3.58	2.19	

Total flavonoids content: Total flavonoids content in ashwagandha roots of various genotypes/varieties varied widely and is shown in Table-3. On dry weight basis, total flavonoids content in aqueous extracts ranged from 0.64 to 1.10 mg CE/g d.w.b. and genotype HWS-08-14 has highest total flavonoids content (mg CE/g) *i.e*. 1.10 followed by HWS-08-18 (0.86), Selection-2B (0.82), HWS-1229 (0.74) and HWS-1228 (0.64) in comparison to control varieties RVA-100 (0.80) and JA-20 (0.76). In ethanol extracts, total flavonoids content ranged from 0.96 to 1.59 mg CE/g d.w.b and genotype HWS-08-14 has highest total flavonoids content (mg CE/g) *i.e*. 1.59 followed by HWS-08-18 (1.30), HWS-1229 (1.20), Selection-2B (1.07) and HWS-1228 (0.96) in comparison to control varieties RVA-100 (1.07) and JA-20 (0.96). Amongst solvents the mean value of total flavonoids content (mg CE/g) in ethanolic extracts (1.16) was higher than aqueous extracts (0.82). Our finding is in agreement with previous investigation which reported 0.38 mg/g flavonoids content in water extracts of ashwagandha roots [12]. Total flavonoids content ranged from 2.87 to 4.98 mg/g d.w.b. in field grown conditions and 6.42 mg/g d.w.b. in *in vitro* roots of *Withania somnifera* [13].

TABLE-3
TOTAL FLAVONOIDS (mg CE/g) IN AQUEOUS AND ETHANOLIC EXTRACTS OF ASHWAGANDHA ROOTS OF VARIOUS GENOTYPES/VARIETIES ON DRY WEIGHT BASIS

Genotypes/ varieties	Total flavonoids (mg CE/g)		
	Aqueous	Ethanolic	Mean
HWS-08-14	1.10 ± 0.00	1.59 ± 0.08	1.35
HWS-08-18	0.86 ± 0.03	1.30 ± 0.07	1.08
HWS-1228	0.64 ± 0.04	0.96 ± 0.01	0.80
HWS-1229	0.74 ± 0.02	1.20 ± 0.05	0.97
Selection-2B	0.82 ± 0.01	1.07 ± 0.07	0.95
JA-20 (C)	0.76 ± 0.02	0.96 ± 0.01	0.86
RVA-100 (C)	0.80 ± 0.05	1.07 ± 0.03	0.94
Mean	0.82	1.16	–

DPPH free radical scavenging activity of aqueous extracts of ashwagandha roots of various genotypes/varieties: 2,2'-Diphenyl-1-picrylhydrazyl (DPPH) is a stable free radical (purple colour) and it transforms to non-radical form (yellow colour) by abstracting one electron and hence, it is widely used as a measure for the electron donation capacity of antioxidants under assay conditions. In present studies, DPPH free radical scavenging activity (%) of the aqueous extracts of ashwagandha roots increased with increase of concentration levels (Table-4). Amongst ashwagandha roots of various genotypes/varieties, DPPH free radical scavenging activity (%) of aqueous extracts of genotype HWS-08-14 was highest ranging from 10.40 to 90.34, followed by HWS-08-18 (6.72 to 87.39), HWS-1229 (7.40 to 76.30), Selection-2B (7.23 to 75.49) and HWS-1228 (2.09 to 77.64) in comparison to both varieties JA-20 (2.82 to 87.32) and RVA-100 (4.47 to 77.27) at 100 to 5000 µg/mL concentration levels.

The IC_{50} values (µg/mL) of genotypes HWS-08-14 (1140.5), HWS-08-18 (1385.7) and HWS-1229 (1497.8) was lowest in comparison to both varieties JA-20 (1578.6) and RVA-100 (2615.9) thereby showing that genotypes HWS-08-14, HWS-08-18 and HWS-1229 exhibited higher activity in comparison to both varieties JA-20 and RVA-100. Other research workers

TABLE-4
DPPH FREE RADICAL SCAVENGING ACTIVITY (%) OF AQUEOUS EXTRACTS OF ASHWAGANDHA ROOTS OF VARIOUS GENOTYPES/VARIETIES

Genotypes/varieties	DPPH free radical scavenging activity (%)						IC_{50} (µg/mL)
	5000 µg/mL	2500 µg/mL	1000 µg/mL	500 µg/mL	250 µg/mL	100 µg/mL	
HWS-08-14	90.34	79.65	51.83	29.58	15.23	10.40	1140.5
HWS-08-18	87.39	71.52	40.57	23.93	12.25	6.72	1385.7
HWS-1228	77.64	53.55	24.62	10.04	3.89	2.09	2463.2
HWS-1229	76.30	67.13	41.84	24.60	14.93	7.40	1497.8
Selection-2B	75.49	59.12	33.25	20.86	14.88	7.23	1856.5
JA-20 (C)	87.32	69.30	33.52	18.31	10.00	2.82	1578.6
RVA-100 (C)	77.27	49.66	25.44	15.56	7.98	4.47	2615.9

have also reported similar findings. Antioxidant activity of aqueous extract of *Withania somnifera* roots have been reported to range from 22.52 to 72.40 % at concentrations ranging from 10 to 250 µg/mL [14]. DPPH free radical scavenging activity of aqueous extract of *Withania somnifera* ranged from 13.80 to 61.44 % at concentrations levels from 100 to 1000 µg/mL [15].

DPPH free radical scavenging activity of ethanolic extracts of ashwagandha roots of various genotypes/varieties: In present studies, DPPH free radical scavenging activity (%) of the ethanolic extracts of ashwagandha roots increased with increase of concentration levels (Table-5). Amongst ashwagandha roots of various genotypes/varieties, DPPH free radical scavenging activity (%) of ethanolic extracts of genotype HWS-1229 was highest ranging from 5.98 to 72.84, followed by HWS-08-14 (1.98 to 85.11), HWS-08-18 (2.73 to 76.59), Selection-2B (1.54 to 72.80) and HWS-1228 (1.40 to 68.01) in comparison to both varieties JA-20 (2.48 to 82.27) and RVA-100 (1.42 to 72.22) at 100 to 5000 µg/mL concentration levels.

The IC_{50} values (µg/mL) of genotypes HWS-1229 (1507.1), HWS-08-14 (1533.9) and HWS-08-18 (1627.9) was lowest in comparison to both varieties JA-20 (2384.1) and RVA-100 (3307.9) thereby showing that genotypes HWS-1229, HWS-08-14 and HWS-08-18 exhibited higher activity in comparison to both varieties JA-20 and RVA-100. Other research workers have also reported similar findings. Antioxidant activity of ethanolic extract of *Withania somnifera* roots have been reported to from 83.51 to 99.62 % at concentrations ranging from 10 to 250 µg/mL [14]. DPPH free radical scavenging activity of hydroalcoholic extract of *Withania somnifera* ranged from 26.58 to 67.38 % at concentrations levels from 100 to 1000 µg/mL [15]. Graphical representation of DPPH free

TABLE-5
DPPH FREE RADICAL SCAVENGING ACTIVITY (%) OF ETHANOLIC EXTRACTS OF ASHWAGANDHA ROOTS OF VARIOUS GENOTYPES/VARIETIES

Genotypes/varieties	DPPH free radical scavenging activity (%)						IC_{50} (µg/mL)
	5000 µg/mL	2500 µg/mL	1000 µg/mL	500 µg/mL	250 µg/mL	100 µg/mL	
HWS-08-14	85.11	66.05	39.59	20.74	7.00	1.98	1533.9
HWS-08-18	76.59	66.64	35.55	19.49	10.11	2.73	1627.9
HWS-1228	68.01	46.51	21.47	9.87	3.18	1.40	2648.1
HWS-1229	72.84	64.93	38.36	23.97	13.68	5.98	1507.1
Selection-2B	72.80	44.18	23.16	13.18	7.60	1.54	2632.3
JA-20 (C)	82.27	53.06	22.43	8.74	4.17	2.48	2384.1
RVA-100 (C)	72.22	38.89	14.86	7.62	5.56	1.42	3307.9

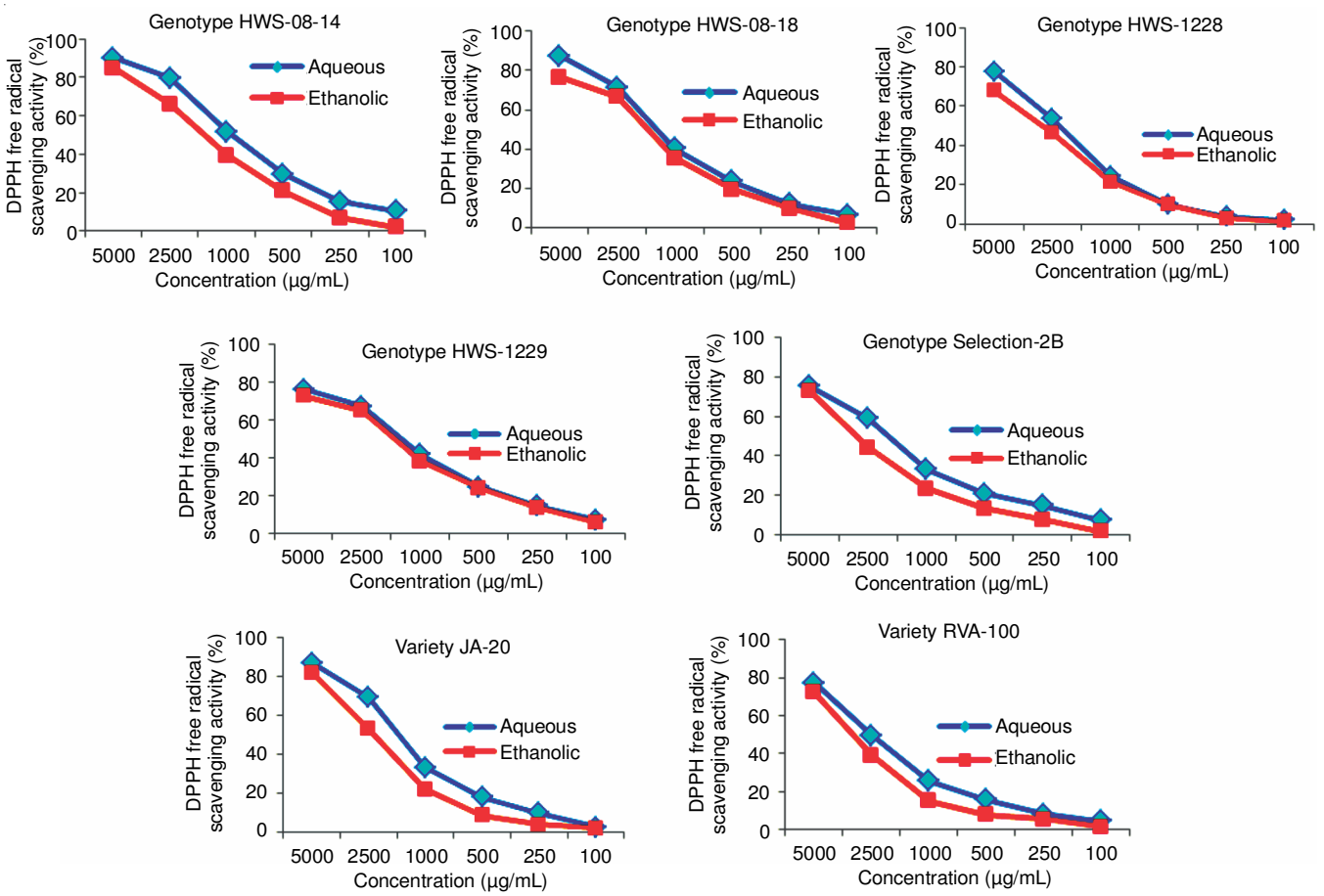

Fig. 1. DPPH free radical scavenging activity (%) of aqueous and ethanolic extracts of ashwagandha roots of various genotypes/varieties

radical scavenging activity (%) of aqueous and ethanolic extracts of ashwagandha roots of various genotypes/varieties at different concentration levels is given in Fig. 1.

Conclusion

The present study revealed the presence of total phenolics and total flavonoids in the aqueous and ethanolic extracts of roots of *W. somnifera*. Among aqueous and ethanolic extracts, aqueous extracts contained higher amount of total phenolics and also exhibited higher antioxidant activity while flavonoid content was found to be higher in ethanolic extracts. Hence, aqueous extracts of ashwagandha roots are better source of antioxidants in comparison to ethanol extracts.

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