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Research article

Influence of phytochemical composition on *in vitro* antioxidant and reducing activities of Indian ginseng [*Withania somnifera* (L.) Dunal] root extracts

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ABSTRACT

Background: Roots of *Withania somnifera* (WS) are a celebrated medicinal ingredient in Ayurvedic and many other indigenous systems of medicine. The present study investigates the effect of the phytochemical composition of the extracts on their antioxidant and reducing activities.
Methods: WS roots were extracted with water, acetone, aqueous methanol (1:1), and methanol:-chloroform:water (1:1:1) to obtain aqueous, acetone, hydro-methanolic, and methanol–chloroform–water extracts. Thereafter, phytochemical constitution and antioxidant and reducing activities of the extracts were compared using different qualitative and quantitative tests.
Results: Maximum extraction recovery was obtained with 50% aqueous methanol whereas extraction with acetone yielded the poorest recovery. Methanol–chloroform–water extract had the highest content of phytochemical constituents, except tannins, and also exhibited the highest antioxidant and reducing activities.
Conclusion: Phytochemical composition and antioxidant and reducing activities of the extracts were positively associated with the use of organic solvents during the extraction process. Alkaloids and flavonoids were the most important contributors in the antioxidant and reducing activities of the extracts.

1. Introduction

Withania somnifera (Linn.) Dunal, commonly known as Indian Ginseng or Indian winter cherry, is recognized as the Queen of Indian herbs and has received similar admiration in Unani, Siddha, and Chinese systems of medicine [1]. The roots are the most commonly used part and find enormous medicinal use. The powder of the roots is the key ingredient of almost any antioxidant, antistress, and antiaging formulation, human or veterinary, in India. The root powder and its preparations are consumed extensively as a functional food for promoting vitality and virility. The plant is cultivated in the states of Madhya Pradesh, Uttar Pradesh, Punjab, Gujarat, and Rajasthan. The plant also grows at large, wildly or commercially, in Congo, South Africa, Morocco, Egypt, Israel, Jordan, Pakistan, and Afghanistan [2,3]. The roots are reputed to promote health and longevity by augmenting defense against disease, arresting aging, revitalizing the body in debility, increasing resistance to adverse environmental factors, and creating a sense of well-being [1,4]. Extraction is an important step in the itinerary of phytochemical processing for the discovery of bioactive constituents from plant materials [5]. In the present study, qualitative and quantitative methods were used for comparing the phytochemical composition and *in vitro* antioxidant and reducing activities of different extracts of *Withania somnifera* roots. The effect of the varying concentration of phytochemicals in the extracts on their antioxidant and reducing activities was determined.

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Table 1
Physicochemical composition of WS roots

Physicochemical composition	
Analyte	Amount (%)
Total lipid	1.38
Total protein	4.27
Total sugars	7.17
Loss on drying	2.08
Total ash	4.33
Acid insoluble ash	0.65
Water soluble ash	1.85
Alcohol soluble extractive value	17.36
Water soluble extractive value	66.64

All values are expressed as % of air-dried weight
WS, *Withania somnifera*

2. Materials and methods

2.1. Withania somnifera roots

Roots were procured through a professional herb vendor and got authenticated from the Medicinal Plant Research and Development Centre, Pantnagar (Fig. S1). They were cut into smaller pieces, dried under hot circulating air at 40°C for 3–4 days, and ground to a fine powder. The powder was subjected to compositional tests for ascertaining pharmacognostic standards [6]. Total lipid, total protein, and total sugar were estimated according to standard methods [7]. Loss on drying, total ash, acid insoluble ash, water soluble ash, alcohol soluble extractive value, and water soluble extractive value were determined as described in the Ayurvedic Pharmacopoeia of India [8].

2.2. Extraction and spectral analysis

Two hundred grams of root powder was soaked in 1,000 mL of either water, methanol:chloroform:water (12:5:3), acetone, or aqueous methanol (1:1) for 48 hours at 40°C in a shaking incubator to obtain aqueous (Aqu), methanol–chloroform–water (MCW), acetone, and hydro-methanolic (HM) extracts, respectively; this step was repeated thrice. The resulting extracts were first filtered through double-layered muslin, and then through Whatman paper no. 42 (Whatman, GE Healthcare; India). The final extracts were obtained by drying the filtrate to a constant weight at 40°C. The yield was expressed as the mass of extract obtained per 100 g of roots. 0.1% solutions of the dried extracts were subjected to spectral analysis for evaluation of composition.

2.3. Qualitative and quantitative phytochemical analysis

The extremely low yield of acetone extract precluded its further use in the study. Small portions of each of the remaining three extracts viz. Aqu, MCW, and HM were subjected to qualitative tests for different phytochemical compounds as per standard methods [9,10]. Thereafter, alkaloids, total phenolic content (TPC), flavonoids, flavonols, proanthocyanidins, and tannins were quantitated as described earlier by Shabbir et al. [10] whereas withanolide content was determined as per Pati et al. [11].

2.4. In vitro antioxidant and reducing assays

Antioxidant assays were performed with minor modifications to standard methods as described elsewhere [10]. Further dilutions of the stocks were prepared in an appropriate medium/buffer as per the requirements of the individual assay. Where applicable, the median inhibitory concentration, IC_{50}, was also calculated.

2.5. Statistical analyses

Statistical inferences were drawn on the basis of analysis of variance followed by *post hoc* Tukey's test performed with Daniel's XL Toolbox 6.53. Multivariate analysis, *viz*. partial least square (PLS) regression analysis and principal component analysis (PCA), was performed in Multibase 2014 (www.numericaldynamics.com).

3. Results and discussion

According to the Ayurvedic Pharmacopoeia of India, the loss on drying, total ash, acid insoluble ash, and alcohol extractive values

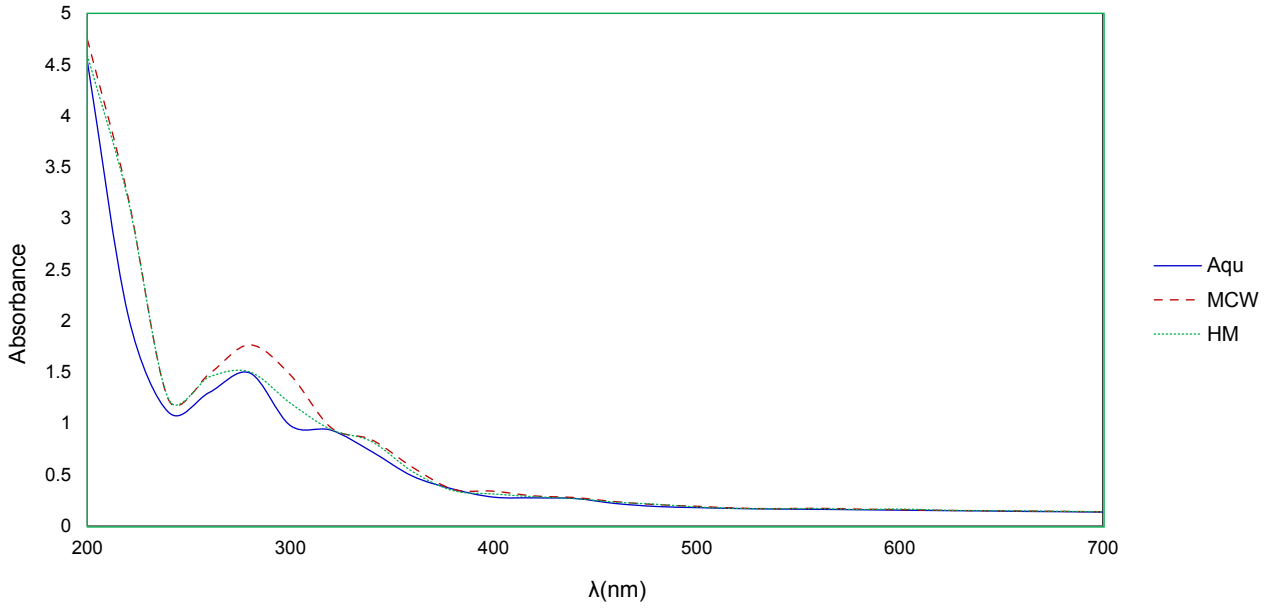

Fig. 1. Spectral analysis of WS root extracts. UV–Vis absorption characteristics of Aqu, HM, and MCW have been shown for comparison. Stronger absorbance suggests a higher concentration of phytochemicals. Aqu, aqueous; HM, hydro-methanolic; MCW, methanol–chloroform–water extract.

Table 2
Results of qualitative tests for phytochemical constituents of aqueous, hydromethanolic and methanol-chloroform-water extracts

Phytochemical	Aqueous	HM	MCW
Alkaloids	++	++	+++
Anthraquinones	–	–	–
Coumarins	+	+++	+++
Flavonoids	+	++	+++
Phenolics	++	+++	+++
Reducing sugars	+++	+++	+++
Glycosides	±	–	–
Saponins	+	+++	+++
Tannins	+++	++	++
Terpenoids	++	++	++

+, reaction in > 30 minutes; ++, reaction in 5–30 minutes; +++, reaction within 5 minutes; – no reaction up to 24 h; ± undefined color change; HM, hydromethanolic extract; MCW, methanol–chloroform–water extract

for WS roots should be ≤ 8%, ≤ 7%, ≤ 1%, and ≥ 15%, respectively [6]. The WS roots used in the present study satisfied these standards of pharmacognostic quality (Table 1).

Previously, comparison of six different solvents and solvent combinations found that the best extraction was achieved by acetone and MCW (methanol:chloroform:water, 12:5:3) [12]. This formed the basis for selection of acetone and MCW as solvents in the current study; water was also included as a third extraction solvent. Of these, acetone had a very poor yield (~0.162%); hence, it was excluded from further study on the basis of nonfeasibility, and the HM extract was included. Mean yields of Aqu, HM, and MCW extracts were 11.64%, 16.82%, and 14.39%, respectively. Maximum yield with aqueous methanol (1:1) may be due to optimum solvation of both hydrophilic and lipophilic components in the milieu. Previously, a mean yield of 15.40% was obtained upon hydroethanolic extraction of WS roots [13].

Spectral analysis showed increased absorbance by the extracts in the 240–360 nm range (Fig. 1) that can be attributed to a host of chemical constituents. Carbohydrates absorb strongly at 360 nm, whereas absorbance at 200 nm, 240 nm, 270 nm, and 300 nm reflect the presence of proteins and glycoproteins. Many alkaloids absorb strongly in the 250–260 nm wavelength range. Coumarins absorb strongly at 280 nm and 320 nm [14]. The MCW extract, though comparable to HM, exhibited the strongest absorbance of the three extracts. It must be noted that although spectral analysis gives a fair indication of the prominent phytochemical constituents, the results are not always reliable because the presence of many other compounds can also cause absorbance patterns. Thus, qualitative and quantitative estimation of the phytochemical constituents was performed. Qualitative phytochemical analyses (Table 2) also suggest a higher concentration of phytochemical constituents in the MCW extract.

Quantitative tests were performed to determine the concentration of the phytochemicals in the extracts. Alkaloids and withanolides were quantified using gravimetric methods. Both alkaloid and withanolide content of the extracts were in the order: MCW > HM > Aqu (Fig. 2) and the differences were highly significant ($p < 0.01$). The concentration of withanolides in the roots usually ranges from 0.001% to 0.5% of the dry weight. Total alkaloid content in the roots of Indian *W. somnifera* varies between 0.13%

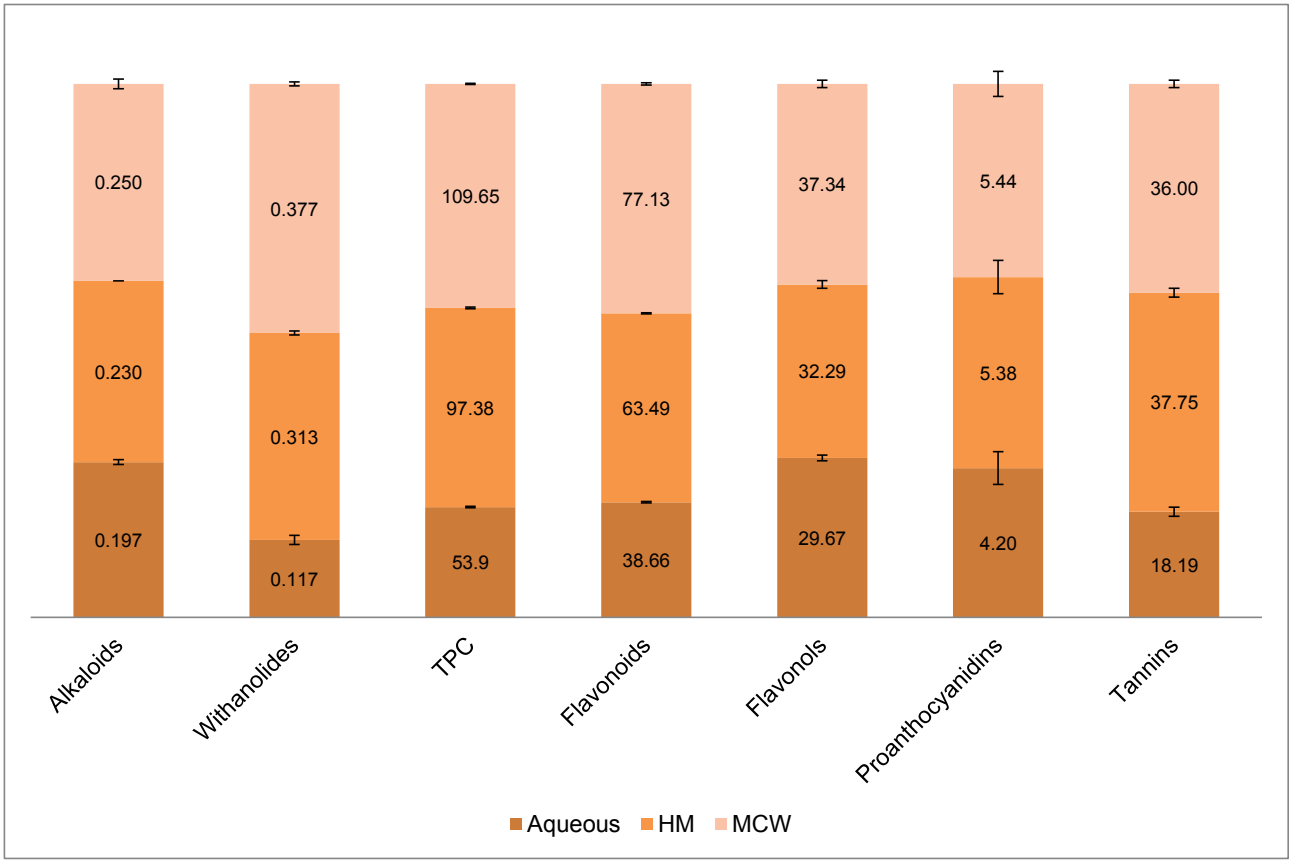

Fig. 2. Results of quantitative tests for phytochemicals in different extracts of WS roots. Mean ± SE values of different phytochemicals have been expressed as microgram equivalents of respective standards per milligram of extract; alkaloids and withanolides have been expressed as percent of dry weight. SE, standard error; TPC, total phenolic content.

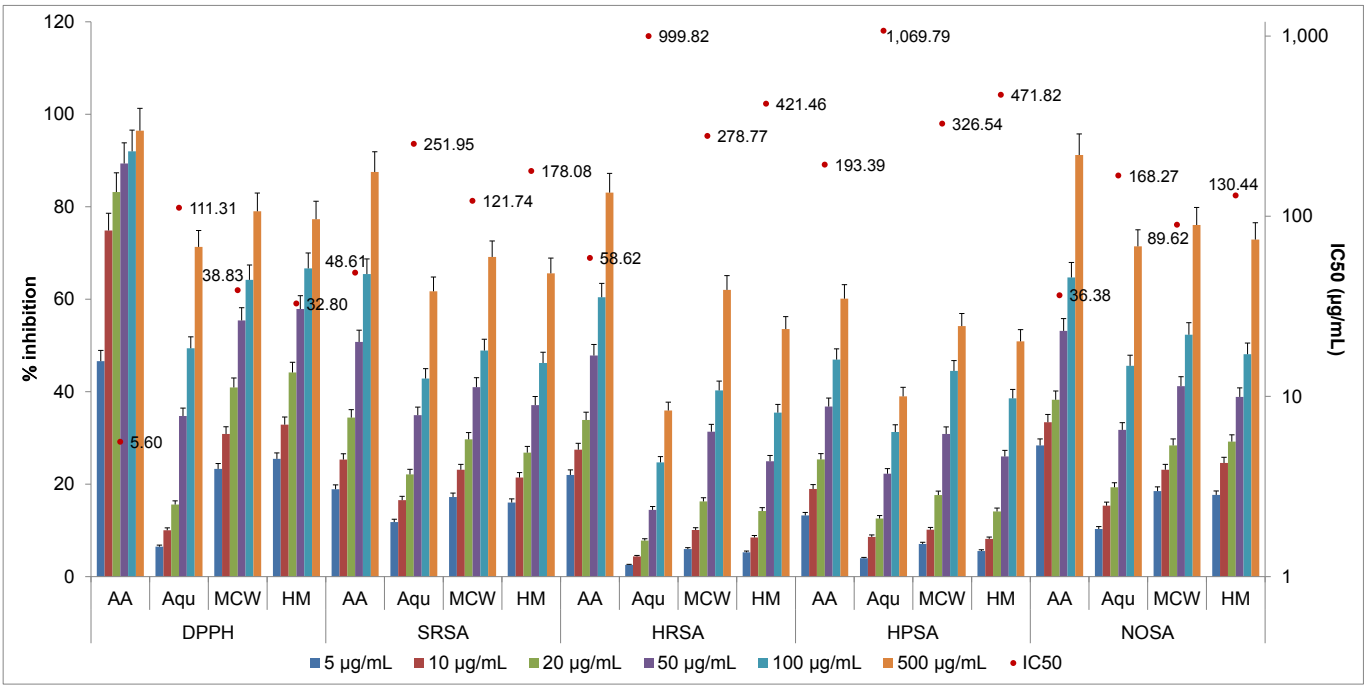

Fig. 3. Percent inhibition and median inhibitory concentration (IC_{50}) of ascorbic acid and different extracts of WS roots in antioxidant and reducing assays. AA, ascorbic acid; Aqu, aqueous; DPPH, DPPH radical scavenging assay; HM, hydro-methanolic extract; HPSA, hydrogen peroxide scavenging assay; HRSA, hydroxyl radical scavenging assay; IC_{50}, median inhibitory concentration; MCW, methanol–chloroform–water extract; NOSA, nitric oxide scavenging assay; SRSA, superoxide radical scavenging assay.

and 0.31%, though yields up to 4.3% have been recorded in other regions [15].

Total phenolic content (TPC) was determined on a gallic acid curve ($y = 0.0072x + 0.0472$; $R^2 = 0.9897$). TPC of the extracts was in the order: MCW > HM > Aqueous (Fig. 2); all differences being highly significant. Previously, the TPC of methanol, chloroform, and aqueous extracts have been reported at 42 μg, 66.72 μg, and 88.58 μg gallic acid equivalents (GAE)/mg extract, respectively. It was further suggested that most polyphenolics of WS were polar in nature [16]. Another study found the phenol content of the extracts in the order: chloroform > petroleum ether > methanol > ethanol > *n*-hexane; TPC of chloroform extract was 60.992 ± 0.367 μg GAE/mg [17]. In the present study, a higher TPC of MCW over HM and Aqu suggests a preferential extraction of phenolics in the order: chloroform > methanol > water, which appears to be in agreement with the latter study. Much lower TPC values for 80% methanolic extracts of WS roots have been reported from Sri Lanka [18].

Phenolic compounds are represented by flavonoids, tannins, and coumarins [14]. Flavonoid content was determined on a quercetin standard curve ($y = 0.012x + 0.0604$; $R^2 = 0.9918$). Flavonoid content of the extracts was in the order: MCW > HM > Aqu (Fig. 2), and differences between extracts were highly significant. Alam et al. [19] prepared WS fruit, root, and leaf extracts by using 80% methanol; 17.80 ± 5.80–32.58 ± 3.16 mg GAE phenolics and 15.49 ± 1.02–31.58 ± 5.07 mg catechin equivalent (CEQ) flavonoids were found per gram. Subsequently, 15.49 ± 1.02 mg GAE phenolics and 17.80 ± 5.80 mg CEQ flavonoids have been reported per gram of WS roots from Bangladesh [20]. Shahriar et al. [17] reported flavonoid content of methanol and chloroform extracts at 88.761 ± 1.032 mg and 122.094 ± 1.351 mg quercetin equivalents (QE)/g, respectively. Udayakumar et al. [21] reported 28.26 mg/g total phenolic compounds and 17.32 mg/g flavonoids in WS root extracts. Flavonol contents were determined on a quercetin standard curve ($y = 0.0103x + 0.3781$; $R^2 = 0.9858$); these were in the order: MCW > HM > Aqu (Fig. 3); the differences MCW versus HM and MCW versus Aqu were highly significant, whereas the difference HM versus Aqu was nonsignificant.

Tannins are chemically classified into hydrolysable tannins and condensed tannins, commonly called proanthocyanidins [14]. All differences in the proanthocyanidin content of the extracts (Fig. 2), determined on a gallic acid curve ($y = 0.0051x + 0.059$; $R^2 = 0.9905$), were nonsignificant. Tannin content was determined from a tannic acid curve ($y = 0.0061x + 0.0434$; $R^2 = 0.9895$). Tannin content of the extracts was in the order: HM > MCW > Aqu (Fig. 2); the differences HM versus Aqu and MCW versus Aqu were highly significant whereas the difference HM versus MCW was nonsignificant. The differences between the three extracts in their overall phytochemical composition were significant ($0.01 < p < 0.05$).

2,2-Diphenyl-1-picrylhydrazyl (DPPH) scavenging activity of MCW matched that of HM closely. However, none of the extracts fared as well as the ascorbic acid standard (Fig. 3). The statistical differences between the extracts were highly significant. Previously, Alam et al. [20] have reported much lower DPPH scavenging

Table 3
Correlation (R) between performance of different extracts in the radical scavenging assays

	DPPH	SRSA	HRSA	HPSA	NOSA
DPPH	1.000	–	–	–	–
SRSA	0.907	1.000	–	–	–
HRSA	0.980	0.960	1.000	–	–
HPSA	0.976	0.933	0.995	1.000	–
NOSA	0.871	0.996	0.932	0.899	1.000

DPPH, DPPH radical scavenging assay; HPSA, hydrogen peroxide scavenging assay; HRSA, hydroxyl radical scavenging assay; NOSA, nitric oxide scavenging assay; SRSA, superoxide radical scavenging assay

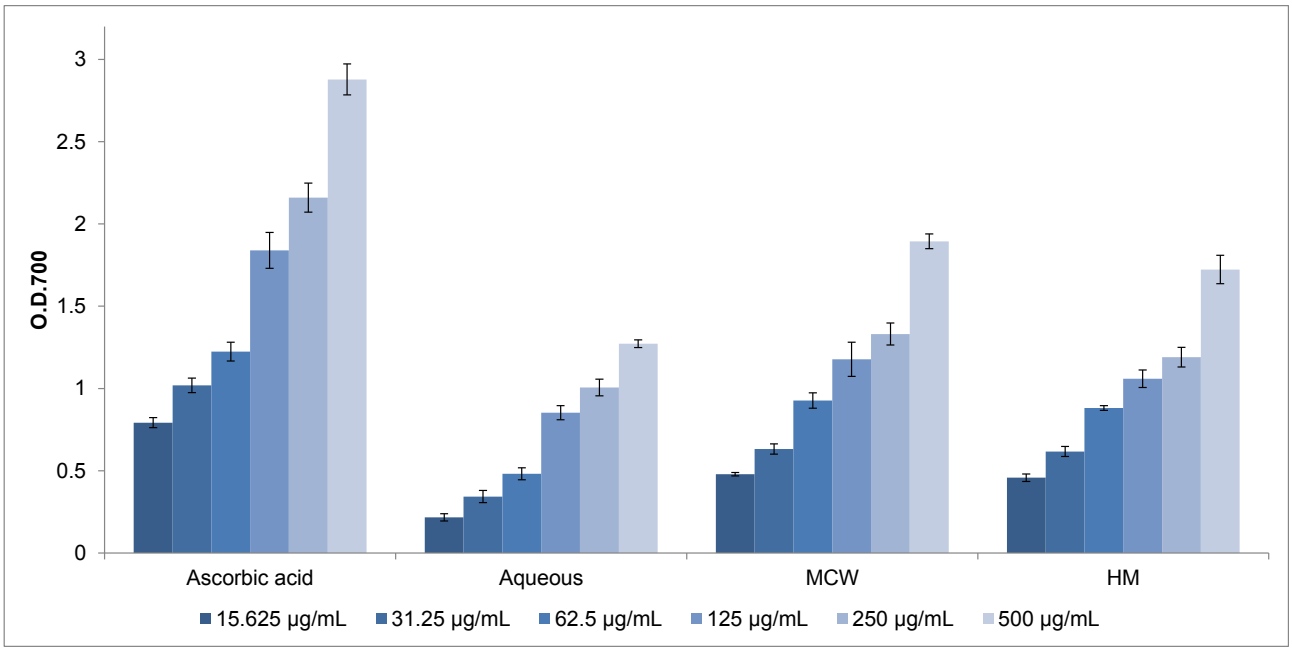

Fig. 4. Fe^{3+} reducing activities of ascorbic acid and different WS root extracts at varying concentrations. Higher absorbance at 700 nm is indicative of greater reducing activity. HM, hydro-methanolic extract; MCW, methanol–chloroform–water extract; WS, *Withania somnifera*.

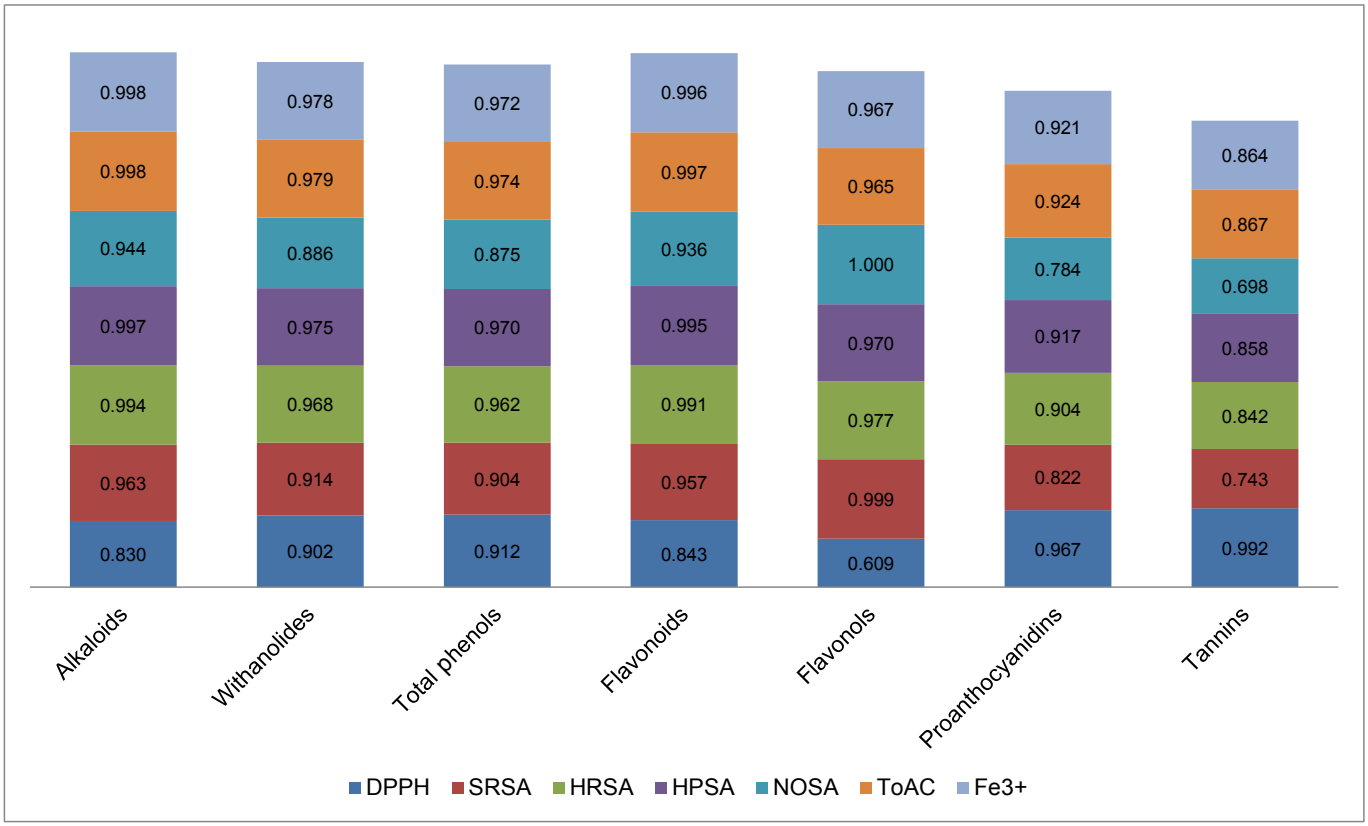

Fig. 5. PLS regression analysis of phytochemical constituents on antioxidant and reducing properties of WS root extracts. Values indicate the importance of a variable i.e., phytochemical concentrations in antioxidant/ reducing activity; total height of a column represents contribution in the overall antioxidant and reducing activities of extracts. DPPH, DPPH radical scavenging assay; HPSA, hydrogen peroxide scavenging assay; HRSA, hydroxyl radical scavenging assay; NOSA, nitric oxide scavenging assay; PLS, partial least square; SRSA, superoxide radical scavenging assay; ToAC, total antioxidant capacity; WS, *Withania somnifera*.

Table 4
Principal component analysis of the effect of phytochemical constitution on antioxidant and reducing activities of WS root extracts; contribution and eigenvalue of individual components

	Component		
	1	2	3
Contribution (%)	97.60	2.24	0.16
Accumulation of contribution (%)	97.60	99.84	100.00
Eigenvalue	1.95	0.04	0.00

activities of 80% aqueous methanol extract of WS root (IC_{50} = 801.93 ± 7.92 μg/mL). The ascorbic acid content of the roots remained indeterminate by the standard method as described by Alam et al. [20] (results not shown); previously, the ascorbic acid content of WS roots has been reported to be as low as 0.02%.

The superoxide radical scavenging activities of the three extracts and the standard (Fig. 3) closely matched at lower concentrations but differences became prominent at higher concentrations. Among the extracts, MCW showed the highest activity followed by HM and Aqu. Albeit graphical indifference, the statistical differences were highly significant. Our results are in partial agreement with former reports of IC_{50} of methanol, water, and chloroform extracts of WS root for superoxide radical at 94 μg/mL, 125 μg/mL, and 178 μg/mL, respectively [16].

Hydroxyl radicals are the most reactive and predominant radicals generated during aerobic metabolism [16]. The hydroxyl ion scavenging activity of ascorbic acid was prominently higher than any of the extracts (Fig. 3). Again, the statistical differences between the extracts and between concentrations within extracts were highly significant. Previously, IC_{50} of aqueous extract of WS root for hydroxyl radical was reported at ~800 μg/mL [16], which is comparable to our results (IC_{50} = 999.82 μg/mL).

Peroxide scavenging activities of the three extracts (Fig. 3) were similar at low concentrations but showed greater differences at higher concentrations. At a concentration of 100 μg/mL, both ascorbic acid and MCW exhibited nearly equal inhibition. The differences between the activities were highly significant. Although the IC_{50} of ascorbic acid for peroxide radical obtained by us in the present study is comparable to the previously reported value (164 μg/mL), the IC_{50} of Aqu for peroxide radical obtained in the present study (1,069.79 μg/mL) varied greatly from the previously reported value of 323 μg/mL [16].

Nitric oxide scavenging activity of ascorbic acid was much higher than any of the extracts (Fig. 3). The activities of MCW and HM were similar at lower concentrations whereas, at the highest tested concentration, the activities of all three extracts were similar. Yet, the differences between groups were highly significant. The performance of the different extracts in the different radical scavenging assays mostly showed good correlation with each other (Table 3).

Total antioxidant capacity (ToAC) was determined from an ascorbic acid standard curve ($y = 0.171\ ln(x) - 0.2106$; $R^2 = 0.9746$). ToAC of MCW was highest (83.354 ± 1.828) followed by HM (76.978 ± 2.210) and Aqu (68.439 ± 1.000). The difference between the ToAC of MCW and Aqu was highly significant whereas that between HM and Aqu was significant and that between MCW and HM was nonsignificant.

The ability of the extracts to reduce Fe^{3+} to Fe^{2+} was compared with that of ascorbic acid (Fig. 4). A higher absorbance value indicates higher activity. Fe^{3+} reducing activity of ascorbic acid was much higher than that of the extracts. Aqu exhibited the poorest activity of the three extracts, all differences between activities being highly significant.

PLS regression analysis (Fig. 5) revealed the importance of each phytochemical in the individual and overall antioxidant and reducing activities of the three extracts. The alkaloid content of an

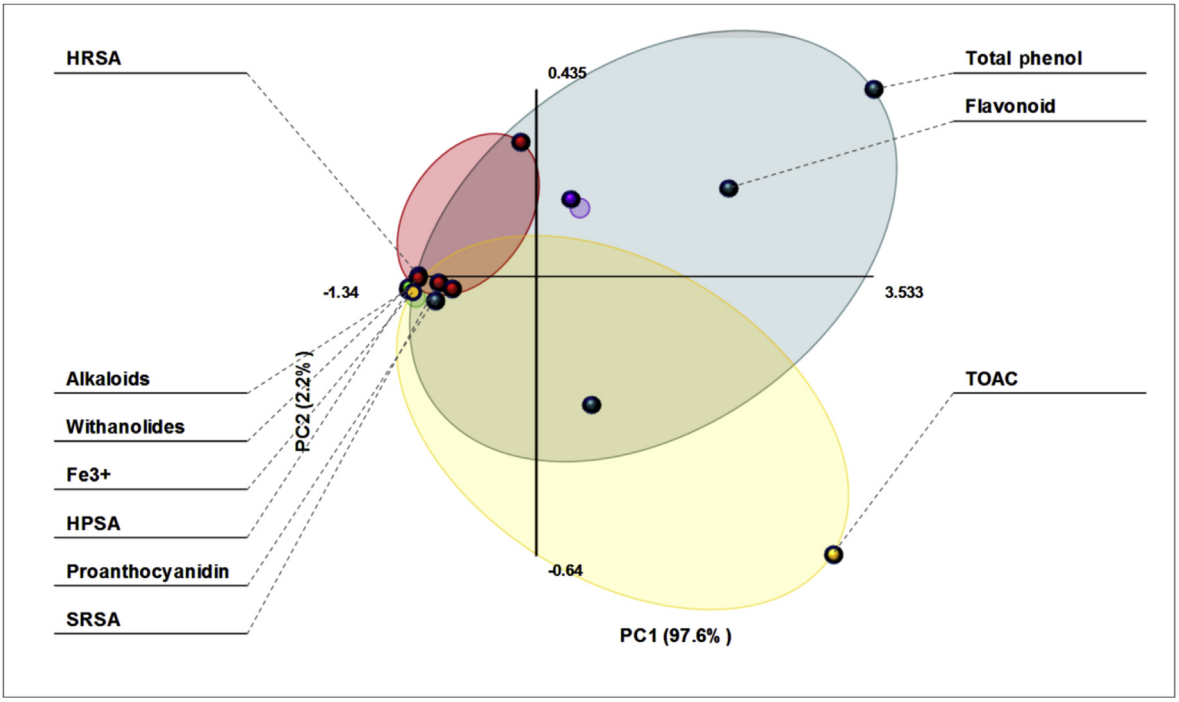

Fig. 6. Principal component analysis of the effect of phytochemical constitution of WS root extracts. PC1–PC2 biplot; red ellipse shows the distribution of scavenging assays, blue ellipse shows the distribution of phenolics (except tannins), yellow ellipse shows the distribution of reducing assays; purple ellipse shows tannins and green ellipse shows the distribution of alkaloids and withanolides. HPSA, hydrogen peroxide scavenging assay; HRSA, hydroxyl radical scavenging assay; SRSA, superoxide radical scavenging assay; ToAC, total antioxidant capacity; WS, *Withania somnifera*.

extract was the most important contributing variable in its hydroxyl radical scavenging, peroxide scavenging, total antioxidant, and Fe^{3+} reducing activities. Flavonol content appeared the most important determinant for superoxide and nitric oxide scavenging activities. Tannin content of an extract was most decisive of its DPPH radical scavenging activity; however, tannin content was also the least important variable in the overall antioxidant and reducing activities. Alkaloid content was the leading contributor to the overall antioxidant and reducing activities of the extracts, closely followed by flavonoids and withanolides.

PCA showed that MCW and HM were more similar to each other than to Aqu in terms of the phytochemical constitution as well as antioxidant and reducing activities. Also, the two extracts were more similar in terms of their Fe^{3+} reducing, hydrogen peroxide scavenging, and superoxide radical scavenging activities as compared with other parameters studied (Table 4; Fig. 6). It follows that the phytochemical composition and antioxidant and reducing activities of the extracts are positively influenced by the use of an organic solvent during the extraction process.

4. Conclusion

In the present study, a comparison of different solvent systems for extraction of the WS root powder showed maximum extraction recovery with aqueous methanol, whereas acetone extract had to be excluded from further studies due to exceptionally poor yield. Methanol–chloroform–water (MCW) extract exhibited the strongest absorbance and showed the highest content of all phytochemical constituents, except tannins. MCW also exhibited the highest antioxidant and reducing activities. The inclusion of an organic solvent in the extraction medium was found to positively influence the antioxidant and reducing activity of the extract. Multivariate analysis found alkaloids and flavonoids to be the most important contributors towards the overall antioxidant and reducing activities of WS root extracts. The MCW and HM extracts were more similar to each other than to aqueous extract, in terms of the phytochemical composition, and antioxidant and reducing activities, suggesting a favorable influence of organic solvents.

Conflicts of interest

The authors declare no conflicting interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jgr.2017.05.002.

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