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# *In vitro* screening of *Ashwagandha* root extracts for the maximum functional components

**Ashok Kumar Yadav and Dinesh Chandra Rai**

**Abstract**
In the present study, the most effective solvent (among ethanol, water, methanol and acetone) for extracting the major antioxidant compounds especially polyphenolics from *Ashwagandha* root using various *in vitro* tests including 1, 1-diphenyl-2-picryl-hydrazil (DPPH) free radical scavenging, metal ion chelating, hydrogen peroxide scavenging, superoxide anion radical scavenging, and ferric thiocyanate reducing ability were identified. Ethanolic extract of *Withania somnifera* had significant ($p$<0.01) (DPPH) free radical scavenging (82%), metal ion chelating (78.88%), hydrogen peroxide scavenging (91.36%), superoxide anion radical scavenging (67.66%) and significant ($p$<0.05) ferric thiocyanate reducing activities. Therefore ethanol extract of *Ashwagandha* root have been found to have maximum functional components e.g. polyphenols and antioxidants.

**Keywords:** *Ashwagandha*, 1, 1-diphenyl-2-picryl-hydrazil, hydrogen peroxide, ferric thiocyanate, polyphenols, antioxidants

## Introduction
*Withania somnifera* (Family. *Solanaceae*) is a herbal drug from the Indian system of medicine and commonly known as *Ashwagandha. Ashwagandha* has anti-infective, antitumor, anti-stress, antioxidant, mind-boosting, rejuvenating, immunomodulatory and anti-ageing properties. It contains flavonoids and withanolides. The antioxidant effect is due to natural antioxidants, superoxide dismutase, catalase and glutathione peroxidase.

- It has anti-stress, adaptogenic, aphrodisiac, sedative, diuretic, antispasmodic, germicidal, antinflammatory action (Rajasankar *et al*. 2009) [17].
- It is a nervine tonic
- It enhances immunity and endurance
- It is a natural nutrient for insomnia
- It is good hypnotic in alcoholism
- It stimulates thyroid activity
- Enhances anti-peroxidation of liver

The roots of *Ashwagandha* have a lot of functionally important active constituents that are helpful in tumor treatment, immunomodulation, memory enhancing capacities, neuroprotective power etc. Thus this work was undertaken with the objective to find out the best solvent to extract the maximum functional components from the root of *Ashwagandha*.

## Materials and Methods
The roots of *Withania somnifera* (Family: *Solanaceae*) were collected from Department of Rasa Shastra, Faculty of Ayurveda, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India. Dried and powdered roots of *W. somnifera* (1mm size) were percolated four times with different solvents (Ethanol, Methanol, Acetone and Water) at room temperature. The combined extracts were filtered, centrifuged and concentrated to 1/6th of the original volume under reduced pressure in a vacuum dryer at 50 ± 5 °C. Finally the extracts were completely dried under vacuum in the desiccators for further analysis.
Total solids were determined by drying different solvent extracts of *Ashwagandha* root at 100 °C in hot air oven. The oven drying was done in triplicate to find out accurate result. Antiradical activity was determined by antioxidants of *Ashwagandha* root extract against two radicals DPPH and ABTS (2, 2- Azinobis- 3- ethylene benzoline-6 sulphonic acid). The percentage inhibitions of these two radicals were directly proportional to the presence of antioxidants in the extracts of different solvents.

DPPH inhibition in *Ashwagandha* root extract was determined by using the protocol of Brand-Williams *et al.* (1995) [2] with some modifications. ABTS inhibition in *Ashwagandha* root extract was determined by using the protocol of Miller *et al.* (1996) [12] with some modifications. Total phenolic content in *Ashwagandha* root extract was determined by using the protocol of Huang *et al.* (2005) [7] with some modifications. The ferrous ions chelating by the Ethanolic Extract of *Withania somnifera* (EEWS) and standards were estimated by the method of Dinis (1994) [3]. The ability of EEWS to scavenge hydrogen peroxide was determined according to the method of Ruch (1989) [18]. Measurement of superoxide anion radicals scavenging activity of EEWS was based on the method described by Liu (1997) [11]. Total reduction capability of EEWS was estimated by using the method of Oyaizu (1986) [14].

**Statistical Analysis**

Experimental results were mean ± SEM of three parallel measurements. Analysis of variance was performed by ANOVA followed by Newmans-Keul multiple comparison test. p values < 0.05 were regarded as significant.

**Results and discussion**

**Yield (Total soluble solids) of *Ashwagandha* root**

Table 1 shows that the highest yield was obtained in Ethanol extract of *Ashwagandha* as 0.28 mg/g of *Ashwagandha* root. The ability to extract soluble solids from *Ashwagandha* root was as follows.

Ethanol > Methanol > Water> Acetone

The higher solubility and extractability of the phenolic antioxidants in ethanol envisages the suitability of ethanol as an ideal solvent for deriving nutraceuticals from *Ashwagandha*.

**Table 1:** Yield of different *Ashwagandha* root extracts.

| Solvent | Yield in mg /g of *Ashwagandha* root extract |
|---|---|
| Water | 0.160 ± 0.002[a] |
| Acetone | 0.075 ± 0.003[b] |
| Ethanol | 0.285 ± 0.001[c] |
| Methanol | 0.195 ± 0.002[a] |

Each value is the mean ± SD of experiments performed in triplicate. Values in column with different superscripts are significantly different ($p$<0.05)

**Anti-radical activity**

Ethanol extract of *Ashwagandha* root was having the highest anti-oxidant activity as it showed near about 80.00% DPPH inhibition at 2mg/ml concentration (Fig. 1) and 21.52% ABTS inhibition (Table 2). Maximum antioxidant activity was achieved by *Ashwagandha* root extract i.e., 21.52 % ABTS inhibition and different extracts were in the sequence as follows:

Ethanol > Methanol > Acetone > Water

Antioxidant activity depends on the presence of amount of total polyphenolic compounds. As discussed earlier, highest extractability of polyphenolic compounds was achieved by ethanol, which showed maximum antiradical activity also.

**Table 2:** ABTS inhibition of different solvent extracts.

| Solvent | % ABTS inhibition of *Ashwagandha* root extract |
|---|---|
| Methanol | 20.97 ± 0.65[a] |
| Ethanol | 21.52 ± 0.29[a] |
| Acetone | 19.94 ± 0.85[a] |
| Water | 19.54 ± 0.35[a] |

Each value is the mean ± SD of experiments performed in triplicate. Values in column with different superscripts are significantly different ($p$<0.05)

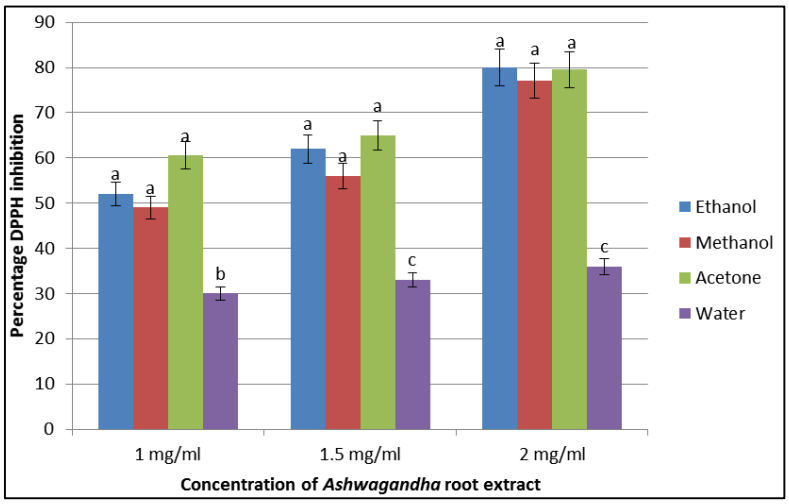


**Fig 1:** % DPPH Inhibition of different extracts of *Ashwagandha* root extract.

**Total phenol content**

Phenolic components are present in huge amount in *Ashwagandha* root. Table 3 showed that ethanolic extract of *Ashwagandha* root exhibited highest phenolic content i.e. 58.77 mg /100 g of dry sample in terms of gallic acid equivalent. The ability to extract polyphenols was as follows:

Ethanol > Methanol > Acetone> Water

Acetone and methanol have different capacity for the extraction of polyphenolic substances. It was reported that methanol is the best solvent for catechin extraction, whereas a better yield for procyanidins was obtained with 70% acetone. Oki *et al.* (2002) [13] detected a value of this variable 3 times higher when extracts of red-hulled rice were obtained using methanol rather than water.

Arts *et al.* (2001) [1] reported that both acetone and methanol (although methanol is more agreeable to work with) give similar maximum catechin yields, but the extraction was influenced by concentration and type of the solvent, which

affect the yield of catechins. Yet methanol was not as good as ethanol in obtaining total soluble solids but it showed great extractability as compared to ethanol and water.
Variations were justified by the well-known tendency of phenols to combine themselves through polymerization reactions; due to the more significant area of charge delocalization, oligomers exerted a higher antiradical activity than the original monomers (Pinelo *et al*. 2004 and Hagerman *et al*. 1998) [15, 5].

**Table 3:** Total phenolic content of *Ashwagandha* root extracts.

| Solvents | Total phenolic content in mg gallic acid equivalents/ 100 g of dry sample |
|---|---|
| Methanol | 52.811 ± 1.02[a] |
| Ethanol | 58.770 ± 1.15[a] |
| Acetone | 50.036 ± 1.01[a] |
| Water | 35.507 ± 0.85[b] |

Each value is the mean ± SD of experiments performed in triplicate. Values in column with different superscripts are significantly different ($p$<0.05)

Antioxidant potential of Ethanolic Extract of *Withania somnifera* (*Ashwagandha*) (EEWS) were also studied by various *in vitro* tests including 1, 1-diphenyl-2-picryl-hydrazil (DPPH) free radical scavenging, metal ion chelating, hydrogen peroxide scavenging, superoxide anion radical scavenging, and ferric thiocyanate reducing ability.

**Free radical scavenging activity by DPPH method**
A significant ($p$<0.01) decrease in the concentration of DPPH radical was observed due to the scavenging ability of the EEWS and standards (Fig 2). The standards used were BHA, BHT α-tocopherol and l- ascorbic acid. The scavenging effect of the EEWS and standards on the DPPH radical decreased in the order of EEWS > BHA > l-ascorbic acid > BHT > α tocopherol and were 82, 65, 63, 62 and 61% at the concentration of 50 µg/ml, respectively, which were statistically significant ($p$<0.01) compared to the control. The effects of EEWS at 50 µg/ml reached a plateau as shown in Fig 2. Free radical scavenging activity increased with increasing concentration.

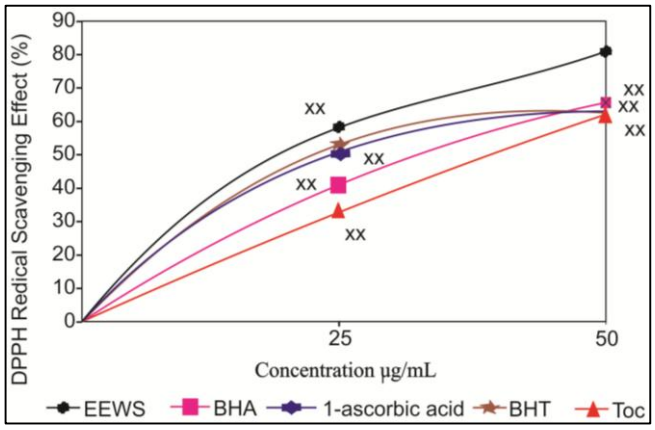


**Fig 2:** Free radical scavenging activity of different concentrations of standards and EEWS on DPPH radical.
Anova followed by Newmans Keul multiple comparison test.
n =3, **$p$<0.01 compared to control

**Ferrous metal ion chelating activity**
The formation of $Fe^{2+}$ ferrozine complex is not completed in the presence of EEWS and standards which indicate that EEWS chelate the iron (Fig 3). The absorbance of $Fe^{2+}$ ferrozine complex was dose dependent and linearly decreased (from 25 to 50 µg/ml). The difference between EEWS and the control was statistically significant ($p$<0.01). The percentage of metal chelating capacity of 50 µg/ml concentration of EEWS, α-tocopherol, l- ascorbic acid, BHA and BHT were found to be 78.88, 61.92, 70.02, 76.82 and 64.62 %, respectively. The effects of EEWS reached a plateau in the 50 µg/ml concentration. The metal scavenging effect of EEWS and standards decreased in the order of EEWS > BHA > l-ascorbic acid > BHT > α-tocopherol.

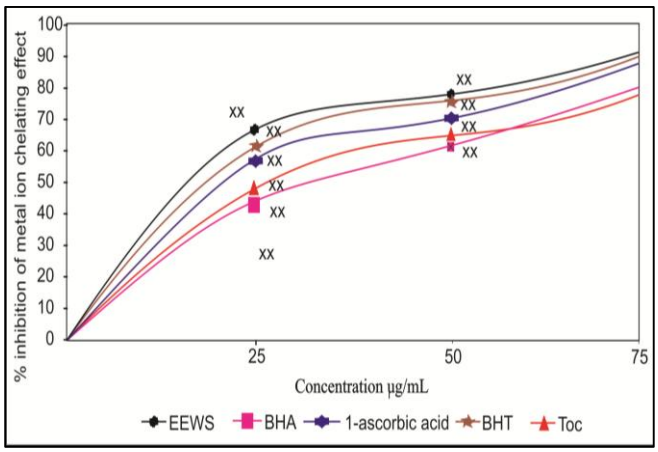


**Fig 3:** Ferrous ions chelating effect of different concentrations of EEWS Anova followed by Newmans Keul multiple comparison test. n =3, **$p$<0.01 compared to control

**Scavenging of hydrogen peroxide**
25 µg/ml EEWS had strong $H_2O_2$ scavenging activity in comparison with the same doses of BHA, BHT, l- ascorbic acid and α-tocopherol. The percentage of $H_2O_2$ scavenging activity by same concentration (25 µg/ml) of EEWS, BHA, BHT, l-ascorbic acid and α-tocopherol were found to be 91.36, 90.1, 72.56, 83.65 and 72.28 % respectively, which were statistically significantly different ($p$<0.01) compared to the control (Fig. 4). These results showed that EEWS had effective $H_2O_2$ scavenging activity and it was in the following order: EEWS > BHA > l-ascorbic acid > BHT > α-tocopherol.

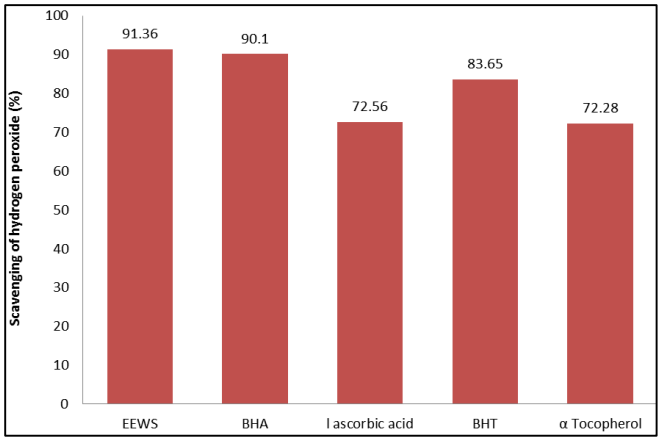


**Fig 4:** Scavenging of hydrogen peroxide of EEWS and standards Anova followed by Newmans-Keul multiple comparison test.

**Superoxide anion radical scavenging activity**
Percentage inhibition of superoxide radical generation at 25 µg/ml concentration of EEWS was determined and compared with same doses of BHA, BHT, α- tocopherol and l- ascorbic acid. EEWS had strong superoxide radical scavenging activity

comparable to that of BHA but lesser than BHT, α-tocopherol and l-ascorbic acid (Fig. 5). The percentage inhibition of superoxide generation by 25µg/ml concentration of EEWS was found to be 67.66 % whereas of BHT, α-tocopherol, l-ascorbic acid, BHA were found to be 81.56, 73.14, 70.21 and 68.83%, respectively, which were statistically significant ($p$<0.01) from the control. Superoxide radical scavenging activity of these samples followed the order: BHT > α-tocopherol > l- ascorbic acid > BHA > EEWS.

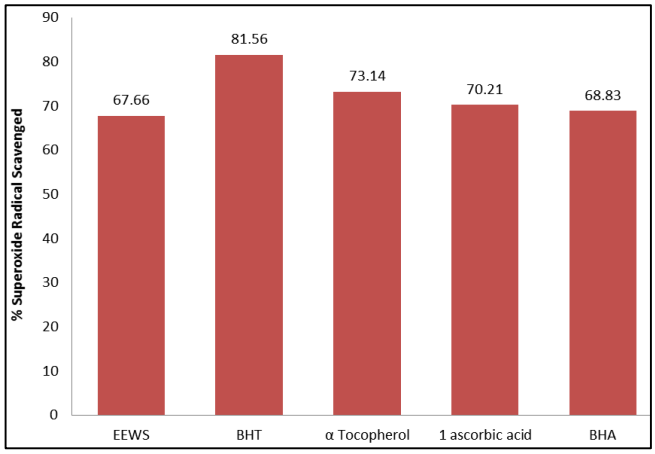


**Fig 5:** Superoxide anion radical scavenging activity of EEWS and standards Anova followed by Newmans-Keul multiple comparison test.

**Total reductive capability by potassium ferricyanide reduction method**

The reducing power of EEWS increased with increasing concentration (Fig. 6). All the concentration of EEWS showed higher activities than BHT, α-tocopherol and l-ascorbic acid and these differences were statistically significant ($p$<0.05). EEWS and BHA had statistically similar reducing power (p>0.05). Reducing power of EEWS and standard compounds followed the order: BHA > EEWS > α-tocopherol > l-ascorbic acid > BHT.

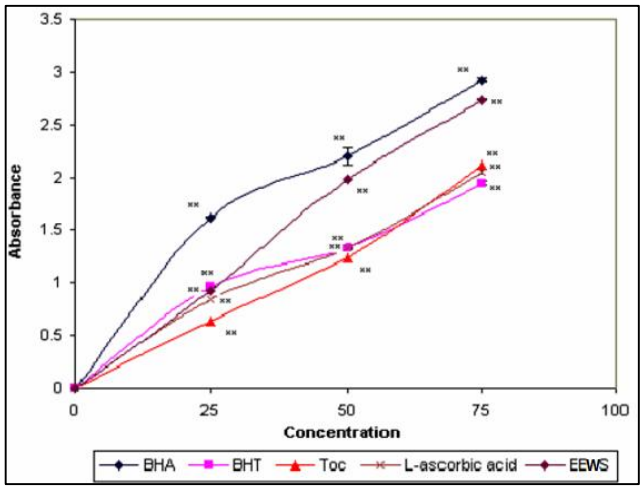


**Fig 6:** Total reductive capability of EEWS and standards. ANOVA followed by Newmans-Keul multiple comparison test. n =3, **$p$<0.01 compared to control

Prompted by these findings it was decided to evaluate the antioxidant ability of ethanolic extract of *Withania somnifera* (EEWS) by *in vitro* antioxidant assays. There are numerous antioxidant methods for evaluation of antioxidant activity. For *in vitro* antioxidant screening, (DPPH) free radical scavenging, metal ion chelating, hydrogen peroxide scavenging, superoxide anion radical scavenging and Ferric thiocyanate reducing activities are most commonly used. However, the total antioxidant activity of an antioxidant cannot be evaluated by using one single method, due to oxidative processes. Therefore, at least two methods should be employed in order to evaluate the total antioxidant activity (Ilhami *et al*. 2005) [8].

The model of scavenging the stable DPPH radical is a widely used method to evaluate antioxidant activities in a relatively short time compared with other methods. The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen donating ability. DPPH is a stable free radical and accepts an electron on hydrogen radical to become a stable diamagnetic molecule (Soares *et al*. 1997) [19]. The decrease in absorbance of DPPH radical caused by antioxidants, because of the reaction between antioxidant molecule and radical progresses, results in the scavenging of the radical by hydrogen donation. It is visually noticeable as a discoloration from purple to yellow. Fig. 2 indicates noticeable effect of EEWS on scavenging of free radicals. These results revealed that the EEWS is free radical inhibitor or scavenger acting possibly as primary antioxidants, which can be correlated with previous studies reported by Jeng-Leun Mau *et al*. (2001) [9].

Iron can stimulate lipid peroxidation by the Fenton reaction and also accelerates peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals that can themselves abstract hydrogen and perpetuate the chain reaction of lipid peroxidation (Halliwell 1991) [6]. In the presence of chelating agents, the complex formation is disrupted with the result that the red color of the complex is decreased. Measurement of color reduction therefore allows the estimation of the chelating activity of the coexisting chelator. In this assay, the EEWS and standard antioxidant compounds interfered with the formation of ferrous-ferrozine complex, suggesting that they had chelating activity and captured ferrous ion before ferrozine. The absorbance decreased linearly which indicated that the formation of $Fe^{2+}$ ferrozine complex was not completed in the presence of EEWS and the EEWS chelated the iron (Gulcin *et al.* 2003) [4]. Metal chelating capacity was significant since it reduced the concentration of the catalyzing transition metal in lipid peroxidation.

It was reported that chelating agents, which form σ bonds with a metal are effective as secondary antioxidants because they reduce the redox potential thereby stabilizing the oxidized form of the metal ion. The data obtained from Fig. 3. Reveals that EEWS demonstrated a marked capacity for iron binding, suggesting its action as peroxidation protector may be related to its iron binding capacity.

$H_2O_2$ is highly important because of its ability to penetrate biological membranes. $H_2O_2$ itself is not very reactive, but it can sometimes be toxic to cell because it may give rise to hydroxyl radical in the cells. The results showed that EEWS had an effective $H_2O_2$ scavenging activity. Superoxide anion radicals are produced endogenously by flavoenzymes like xanthine oxidase, which converts hypoxanthine to xanthine and subsequently to uric acid in ischemia-reperfusion. Superoxide is generated *in vivo* by several oxidative enzymes, including xanthine oxidase. In the PMS-NADH-NBT system, superoxide anion derived from dissolved oxygen by PMS-NADH coupling reaction reduces NBT (Ilhami *et al*. 2005) [8].

The decrease of absorbance at 560 nm with EEWS and antioxidants indicates the consumption of superoxide anion in the reaction mixture. For the measurements of the reductive ability, the $Fe^{3+}$, $Fe^{2+}$ transformation in the presence of EEWS was investigated and found to have significant reducing ability. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The antioxidant activity of an antioxidant compound has been attributed to various mechanisms among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging (Ilhami *et al.* 2005) [5]. Generally flavonoids are the important class of antioxidants; hence the medicinal plants containing flavonoids and phenolic compounds are repeatedly screened for antioxidant activity. In addition to flavonoids and phenolic compounds, some of the alkaloids, saponins and triterpenoids are reported to possess antioxidant activity eg. *Nelumbo nucifera* seeds (Rai *et al*. 2006) [16]. The presence of flavonoids, alkaloids and triterpenoids in alcoholic extract of *Withania somnifera* has been reported (Khan *et al*. 2003) [10] and the results of preliminary phytochemical investigation in the present study also further substantiates this. Hence, the observed *in vitro* antioxidant activity may be because of these phytoconstituents.

When the functional activities of extracts obtained were compared, Ethanol extracts showed the highest values in all four solvents except for DPPH Inhibition. Ethanol extracts showed maximum antioxidant activities and phenolic compounds whereas acetone showed maximum percentage of DPPH Inhibition. Yet acetone *Ashwagandha* root extracts possessed high functional properties in terms of percentage of DPPH Inhibition, it cannot be incorporated into *Shrikhand* as it is toxic in nature. Ethanol extracts exhibited highest functional properties than any other solvent extracts and were comparable with standard antioxidants viz. BHA, BHT, Ascorbic acid and α-Tocopherol.

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