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Antioxidant and Chromatographic Properties of *Withania somnifera*

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Abstract

Withania somnifera is locally known as Ginseng and Ashwagandha. Ashwagandha is one of the most valued medicinal plants having a number of pharmaceutical and nutraceutical applications. The leaves are known for their antimicrobial, antioxidant and other medicinal purpose. Then antioxidant activity was assessed using DPPH, OH radical, iron reducing power and molybdenum scavenging assay which both extracts showed antioxidant activity with methanol having higher scavenging assay. TLC analysis was done which gives Rf value of 0.66, 0.62, 0.52. HPLC analysis was carried to quantify withaferin A. Total flavonoids and phenolics were estimated which the methanol extract gave a higher concentration of both than the ethanol extract. From this study it can be concluded that the leaves of *W. somnifera* have antioxidant activity.

Keywords: Ashwagandha; Antioxidant; HPLC; TLC; Withaferin A

Introduction

Medicinal plants are used as alternative remedies with immense importance. They promote countries by providing affordable medicine to poor people, create income, employment. People who live in rural areas knew about medicinal properties of plants, especially those close to their homes, in open fields, water margins, land waste. To obtain the extract from the whole plant or parts of the plants such as leaves, roots, flowers or fruits plant materials are isolated fresh. Ashwagandha is a small, with woody shrub of 60-200 cm in height belonging to Solanaceae family. *W. somnifera* is known locally as Ginseng and Ashwagandha. Leaves are insect repellent [1]. Ashwagandha, belongs to medicinal plants of great value with pharmacological [2]. Studies has shown that ashwagandha is a source of antioxidant such as vitamin E, vitamin C, phenolic acids, flavonoids, catechins, and anthocyanins that serves as dietary factors [3]. *W. somnifera* was found to show hydroxyl radical scavenging activity, reducing power and superoxide radical scavenging activity more than Aloe vera, but Aloe vera possesses more chelating power than *W. somnifera*. However both plants possesses almost the same hydrogen peroxide scavenging activity and therefore the total antioxidant capacity was found much higher in *W. somnifera* than Aloe vera [4]. It might be concluded that based on this study findings 70% methanol extract of *W. somnifera* root has free radical scavenging as well as iron chelating properties *in vitro* which contributed due to the presence of carbohydrates, phenolics, alkaloids flavonoids, and tannins, contents present in the plant [5]. The methanolic extract of Ashwagandha leaf was effective in scavenging DPPH radical, metal chelation, hydroxyl radical, super oxide radical and inhibition of lipid peroxidation [6]. Sitoindosides VII-X and withaferin A (glycol withanolides) that are found in ashwagandha serve as the active components for antioxidant activity which are tested using Superoxide Dismutase (SOD), Catalase (CAT), and glutathione peroxidase levels in frontal cortex and striatum of rat brain. Any decrease in the activity of any of the enzymes above may cause accumulation of toxic oxidative free radicals and cause degeneration of cells [7]. Chromatography is important for the analysis and isolation of herbal drugs. TLC (Thin Layer Chromatography) was performed *in vitro* which suggested 5 unidentified steroids and Diosgenin, cholesterol, Stigmasterol, sitosterol. HPTLC (High Performance Thin Layer Chromatography) was also done *in vitro* and scanned at different wavelength and absorption spectra of each substance were prepared and some of the substance was confirmed [8]. The aqueous extract of *W. somnifera* roots extract was separated using TLC which 2 bioactive spots were identified. Spot 1 turned orange when spraying the TLC plate with dragendroff reagent indicating the presence of alkaloids, again when sprayed with phosphomolybdic acid spot 2 only turned blue which indicates the presence of essential oils and then when sprayed with ferric chloride spot 2 turned to dark brown indicating the presence of phenolics compound [9]. The presence of withaferin A and withanolide A was revealed using TLC which were then quantified using High Performance Liquid Chromatography. The study shows that withaferin A is higher in leaf than root while withanolide A is higher in root than leaf [10]. Withaferin A was isolated from leaves of ashwagandha and was purified using HPLC. The compound after running in TLC gave Rf value of 0.65 which is as the same as the standard withaferin A and the compound was comfirmed [11].

Methods

Sample preparation

0.5 g of the powdered extract was weighed. The weighed powder was transferred to a centrifuge tube. The powder was dissolved with 4 mL of the respective solution (ethanol or methanol). The centrifuge tube was allowed in a shaker for 2 h. Then the centrifuge tube was allowed to stand at room temperature for 24 h. Then the solution was centrifuged at 1000 rpm for 15 min. The supernatant was transferred to a petri plate using a micropipette. The supernatant on the petri plate was allowed to dry in an oven dryer. The residue was subsequently diluted with the same solution and the process was repeated. The supernatant was collected 3 times after which the dried residue will be

diluted again with the solution and collect the solution in a microfuge tube. Then solution in the microfuge tube was then used for the analysis.

Thin layer chromatography

Thin layer chromatography was performed using a silica gel. The solvents chloroform:methanol at the ratio of 9:1 was used as the mobile phase. 10 µL of the sample was applied towards the edge of the plate. The silica gel was dipped in the mobile phase and covered with glass. The setup was kept at room temperature to allow the mobile phase to travel along with the sample [11].

The Rf value was calculated using the formular below:

Rf=Distance travelled by solute/Distance travelled by solvent

High performance liquid chromatography

HPLC (High Performance Liquid Chromatography) analysis was carried out using a Shimadzu LC-20AD pump system which is equipped with a Shimadzu SPD-20AT UV-Visible detector and it was detected at a wavelength of 230 nm and 20 µL Rheodyne injector loop. A reversed-phase column (with Luna C18 4.6 mm×260 mm particle size 5 µ) with a solvent {acetonitrile: 1% Glacial Acetic acid–6:4 (V/V)} was eluted at a speed of 1.0 mL/min. Preparation of sample was carried out in HPLC grade methanol [11].

Antioxidant Activity

Molybdenum scavenging

Various concentration of the extract was made from 20-120 µg/mL. 1 mL of the reagents was added. Reagents composition (4 mM NH_4 mb, 28 Mm $NaPO_4$ and 0.6 m H_2SO_4). Then incubated in water at 95°C for 90 min. The absorbance was read at 695 nm.

DPPH scavenging activity

Various concentration of the extract was added from 20-120 µL. Then methanol was added to the various extract from 980-880 µL to make 1 L each. Then 1 mL of DPPH reagent was added to the various solutions, then allowed for incubation at 30 min in dark at room temperature. Then finally absorbance was taken at 517 nm [12].

OH radical scavenging activity

Various concentration of extract was added in a test tube from 20-120 µg/mL 1 mL of iron-EDTA solution was added (0.13% ferrous NH_4SO_4 and 0.26% EDTA). 0.5 mL EDTA solution was added (0.018%). 1 mL DMSO (0.85% v/v). 0.5 mL ascorbic acid (0.22%), then incubated at 90°C for 15 min. Then ice cold TCA (17.5% w/v) was added. Then 3 mL of Nash reagent was added, and then incubated for 15 min at room temperature. Then take absorbance at 412 nm [6].

Fe^{3+} iron reducing power

Various concentration of the extract was added (20-120 µg/mL). 1 mL Phosphate buffer (0.2 M, pH 6.6) was added to the extract. 1 mL of potassium ferricyanide $K_3(Fe(CN)_6)$, then incubated at 50°C in water bath for 20 min. Then added 500 µL of 10% TCA. Then 500 µL of freshly prepared iron chloride ($FeCl_3$) was added. The absorbance was read at 700 nm [12].

Detection of flavonoids (ferric chloride test)

Few drops of neutral ferric chloride solution were added dropwise to 1 mL each of the extracts solution. Formation of blackish red colour suggests flavonoids are present [13].

Detection of phenols (Ferric chloride test)

1 mL each of the extracts was dissolved in alcohol or water. This solution was treated separately with a few drops of neutral ferric chloride solution. A change in colour confirms that phenols are present [13].

Determination of total phenolic content

Total phenolic content in both extract of the plant used in this study was determined using the Folin-Ciocalteu reagent method. 1 mL of the plant extract was mixed with 5 mL of Folin-Ciocalteu reagent (diluted with water 1:10 v/v). Then 4 mL of 7.5% sodium carbonate was added. The tubes were vortex for some few seconds. The tubes were allowed to stand for 30 min at 20°C for colour formation. Absorbance was taken at 765 nm [12].

Determination of total flavonoid content

The total flavonoids content in the two extracts of the plant used in this study was determined by aluminium chloride colorimetric method. 1 mL of the plant extract was mixed with 3 mL of methanol in a test tube. Then 0.2 mL of aluminium chloride. Then 0.2 mL of 1 M potassium acetate. Then finally 5.6 mL of distilled water. It was kept at room temperature for 30 min. Then absorbance was measured at 415 nm [12].

Results

TLC

Figure 1: Results for the TLC analysis of ashwagandha plant extracts.

The figure below is a TLC sheet showing some spots which might indicates the presence of some compounds. A clear spot of Rf 0.44 was obtained (Figure 1).

HPLC

HPLC analysis was done on *W. somnifera* leaves extracts which gave the following chromatogram as shown below (Figures 2 and 3).

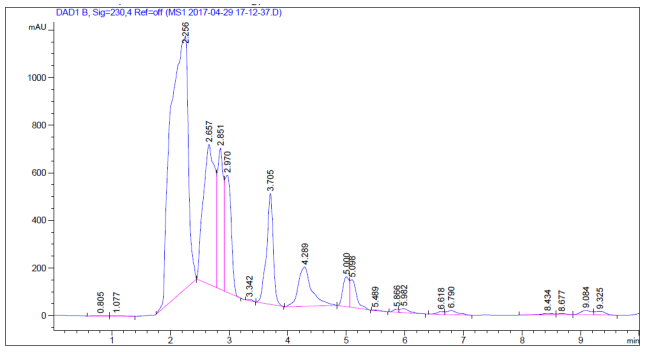

Figure 2: Chromatogram for methanol extract.

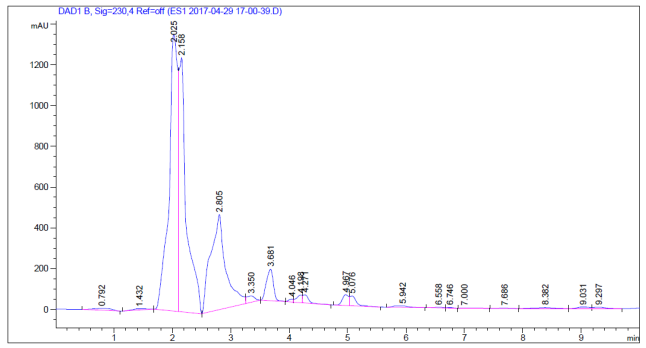

Figure 3: Chromatogram for ethanol extract.

Antioxidant

DPPH

Concentration	Percent inhibition	
	Methanol	Ethanol
20	65.5 ± 0.01	5.37 ± 0.02
40	70.96 ± 1.23	16.4 ± 0.02
60	78.4 ± 0.01	37.63 ± 1.91
80	79.5 ± 0.02	50.53 ± 3.23
100	84.9 ± 1.93	61.2 ± 0.01
120	91.3 ± 0.00	63.4 ± 0.02

Table 1: Results of DPPH.

The results of DPPH scavenging activity of the plant extracts is given in the table above (Table 1).

Values are express in mean ± standard deviation. Values with the same superscript indicates significant difference at $p<0.05$.

The table above shows that the methanol extract has a higher percent inhibition than the ethanol extracts which increases as the concentration increases (Figure 4).

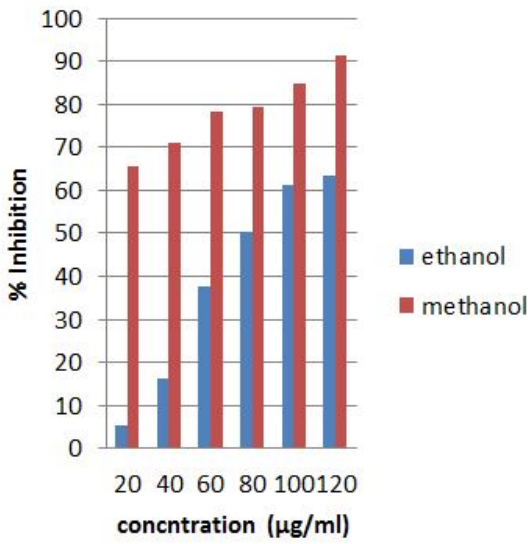

Figure 4: Relationship between the various concentrations of the 2 extracts against the % inhibition.

Iron reducing power

The results of the iron reducing power of the extracts as well as the standard used are shown below (Table 2).

Conc (µg/mL)	Extracts		Standard
	Methanol	Ethanol	Ascorbic acid
20	0.56 ± 0.02	0.11 ± 0.01	0.48 ± 0.02
40	0.54 ± 0.02	0.51 ± 0.01	0.9 ± 0.02
60	1.38 ± 0.01	0.68 ± 0.02	1.26 ± 0.01
80	1.61 ± 0.03	1.18 ± 0.03	1.84 ± 0.01
100	1.74 ± 0.0	1.44 ± 0.02	2.34 ± 0.02
120	1.86 ± 0.04	1.59 ± 0.03	2.54 ± 0.03

Table 2: Absorbance of extracts with standard (Ascorbic acid).

Values are express in mean ± standard deviation. Values with the same superscript indicates significant difference at $p<0.05$ (Figure 5).

In the table above it shows that the methanol has high absorbance than the ethanol and it progresses as the concentration increases and compared to the standard ascorbic acid which also increases.

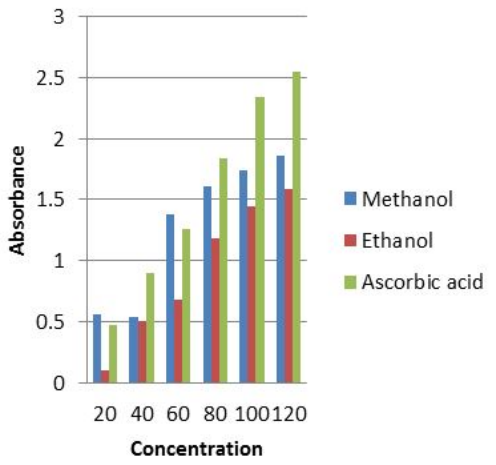

Figure 5: Graph of absorbance against concentration (iron).

The figure above shows the relationship of the various concentrations of the 2 extracts of iron reducing power activity against absorbance.

OH radical scavenging activity

Concentration	% Inhibition of extracts	
	Ethanol	Methanol
20	23.53 ± 3.92	54.91 ± 0.00
40	29.41 ± 0.00	62.75 ± 3.92
60	39.22 ± 3.00	68.63 ± 3.92
80	49.02 ± 3.92	70.59 ± 1.96
100	54.91 ± 3.92	74.51 ± 3.92
120	62.75 ± 1.96	78.43 ± 0.00

Table 3: Percent inhibition of extracts.

Values are express in mean ± standard deviation. Values with the same superscript indicates significant difference at $p<0.05$.

In the table above the methanol extract has higher percent inhibition than the ethanol extract and the inhibition increases with increase in the concentration of the extract thereby increase in OH radical scavenging assay (Table 3).

The results of hydroxyl radical scavenging activity of the plant extracts is given in the table below (Figure 6).

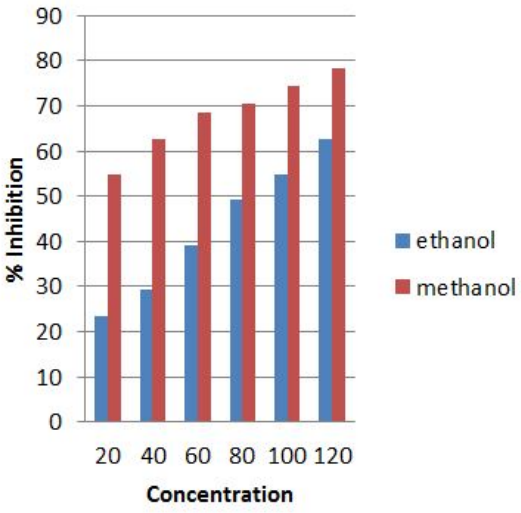

Figure 6: Graph of % inhibition against concentration (OH radical).

The figure above shows the graphical relationship OH radical scavenging activity of the various concentrations of the extracts against percent inhibition.

Molybdenum scavenging assay

The results of the molybdenum scavenging assay of the plant extracts are given in the table below (Table 4).

Concentration	Absorbance of extracts	
	Methanol	Ethanol
20	0.43 ± 0.02	0.19 ± 0.02
40	0.99 ± 0.01	0.37 ± 0.02
60	1.08 ± 0.01	0.61 ± 0.01
80	1.32 ± 0.02	0.76 ± 0.02
100	1.66 ± 0.02	0.79 ± 0.02
120	1.73 ± 0.00	0.92 ± 0.01

Table 4: Absorbance rate of extracts.

Values are express in mean ± standard deviation. Values with the same superscript indicates significant difference at $p<0.05$.

The table above shows that the methanol extract has a higher absorbance than the ethanol extract which increases with increase in the concentrations of the extract (Figure 7).

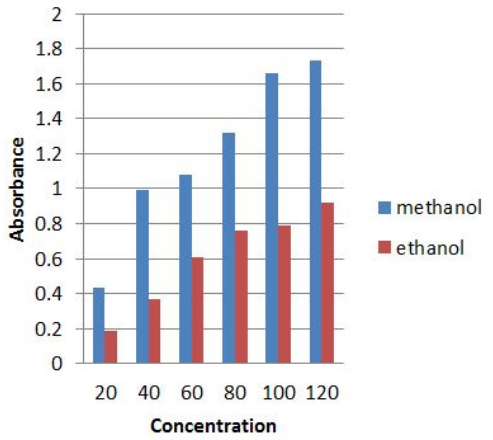

Figure 7: Graph of absorbance against concentration (molybdenum).

The figure above shows the graphical relationship of the molybdenum scavenging activity of the various concentration of the extracts against absorbance.

The table below shows that the methanol extract has a higher amount of both phenolics and flavonoids than the ethanol extract due to the high absorbance of the extract (Table 5).

Compounds	Extracts absorbance	
	Methanol	Ethanol
Total phenolic	1.37	0.67
Total flavonoids	0.26	0.24

Table 5: Total flavonoids and phenolics.

Discussion

The antioxidant ability of the methanol and ethanol extract of ashwagandha was evaluated by its reaction to DPPH. This is a stable free radical which can accept and react with hydrogen or electron from any donor molecule thereby resulting in the bleaching of DPPH absorption. DPPH is a dye which is purple in colour and absorbs maximum at 517 nm. This dye when reacted with hydrogen the purple colour disappears or reduces as a result of conversion of DPPH to 2, 2-diphenyl-1-picryl hydrazine resulting in the decrease in its absorbance [14,15]. A dose dependent change in the scavenging activity of the plant was observed in both extracts. However, the methanol extract was found to be effective in DPPH scavenging activity than ethanol. This finding is also supported with the findings of Ajay et al. [6] which found that the methanol extract showed better scavenging assay. Also, Mohammad et al. found that the methanol and ethanol extract of roots of *W. somnifera* progresses as the findings of this research. However, the percent inhibition of the leaves extract obtain in this research is more than the percent inhibition obtain in the research [16]. This indicates that the leaves may have more antibacterial capacity than the root.

The reducing ability of both extracts of the leaf (methanol and ethanol) increases with increase in sample concentration. High absorbance suggests high antioxidant activity of the extract. The reducing capability of the extract was associated with the transformation of Fe^{3+} to Fe^{2+} *via* electron transforming ability indicating significant antioxidant ability. The reducing power of different extracts of ashwagandha was compared with standard ascorbic acid. The results suggest that the extracts contain phenolic compound which offer great reducing power. This might be accepted since the methanol extracts having higher phenolic compounds tends to have more reducing power activity. This study is in accordance with the study of Mohammad et al. [15] who reported also that the methanol extract has high absorbance than the ethanol extracts of the roots. However, the absorbance is much higher in this study which might be suggested that the leaves may have high reducing potential.

The presence of hydrogen peroxide (H_2O_2) in the cells leads to oxidative damage of DNA, therefore the continues eradication of hydrogen peroxide is very vital for the survival of the cells. This study had shown that methanolic extract had a high hydroxyl radical scavenging activity which was also reported [17]. Ajay et al. showed that the methanol extract showed a bitter scavenging assay than the rest of the extracts been used. The scavenging activity of the extract increases with decrease in the absorbance of the extract and increase in percent inhibition of the extract.

The scavenging activity of molybdenum is based on the fact that in the presence of an antioxidant molybdenum VI is been converted to molybdenum V forming a green coloured complex of phosphomolybdate V [18]. The scavenging assay of molybdenum is regarded as the total antioxidant activity and the higher the absorbance the higher the antioxidant activity hence the higher the total scavenging activity of the given extracts. Based on the results the extracts showed an increase in the scavenging assay as the concentration increases, therefore the methanol extracts with higher absorbance showed more total antioxidant activity compared with the ethanol extracts. This in accordance with the study of Mohammad et al. [16] who reported that the methanol extract has high scavenging ability than the ethanol extract.

From the result obtained in Figure 1 there is a presence of a spot with Rf value of 0.44. Rf value of 0.65 for withaferin A was obtained in the study done by Keesara and Jat. Also the study done by Monika found spots with Rf value of 0.61 for cholesterol, 0.91 for stigmasterol, and so on. Also study by Keesara and Jat found some spots with one having Rf value of 0.66 which corresponds to the compound cholesterol.

HPLC is a method used to isolate, quantify and validate β-sitosterol, glucoside and withaferin A [14]. As observed from the chromatogram above both methanol and ethanol extract shown almost the same chromatogram with methanol having a peak area of 2.256 and ethanol having a peak area of 2.025. By comparing with a standard graph of area against concentration of withaferin A, the concentration in both cases was found to be 0.00027 mg/mL. In the study done by Keesara and Jat the concentration of withaferin A was found to be 1 mg/mL.

The extracts showed the presence of phenolics and flavonoids due to the colour changes. This is in accordance with most of the previous literature which shows the presence of both phenols and flavonoids in the extract as seen in the research [19].

These compounds were estimated explore its relationship with antioxidant activity of the plant extract. Most plants contains phenols

as their constituents due to the presence of OH given them their radical scavenging activity. This polyphenols may have an effect on carcinogenesis and mutagenesis [20,21]. As observed from the results in table the methanol extracts possess more phenolic contents than ethanol suggesting that it has more radical scavenging ability. The difference in the contents of phenolics is due to the polarity of the extract. The concentration of phenolics in methanol is 75 µg/mL and in ethanol is 25 µg/mL which was obtained by comparing with the standard calibration curve of phenolics. A maximum radical scavenging activity was found in the methanol extract which also has the high amount of phenolic content as also reported [6]. The study done by Ajay et al. obtained a much lesser concentration of phenolics than in this present study and hence maybe responsible for the more antioxidant activity obtained in this study.

Flavonoids are another class of secondary phenolics which also has a powerful antioxidant property and it was also observed in this study that the methanol extracts has high amount of flavonoids than the ethanol extracts. The methanol has 20 µg/mL and ethanol has 16 µg/mL of flavonoids. The study by Ajay et al. also obtained a higher concentration of flavonoids in methanol extract than in the ethanol extract.

Conclusion

This study shows that the antioxidant activity of the extract can be attributed by the presence of flavonoids and phenolics. Therefore, as the methanol extract shows a greater concentration of phenolics and flavonoids than the ethanol extract, hence it can be concluded that the methanol extract might be a better solvent for extraction for medicinal purpose.

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