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Phytochemical Profiling and Chromatographic Characterization of Bioactive Compounds in *Withania somnifera*(Ashwagandha)

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Abstract
Ashwagandha is an ancient Ayurvedic medicinal plant. Recently it has received quite a bit of attention for its potential health benefits and its application as a replacement to synthetic drugs by herbal treatment. Commonly known colloquially as an adaptogenic plant something that might assist the body in dealing with a wide variety of stressors. Ashwagandha has many bioactive chemical constituents. It is also a variety of active constituent's groups such as alkaloids flavonoids saponins and withanolides. It consists of the analysis of the phytochemical profile of Ashwagandha in relation to the established profile and major bio-actives reported from preliminary phytochemical and HPLC analyses. The compounds were organized into a chemical class of compounds and, chromatographically, withanolide compounds. In general, the result is consistent with the common use of Ashwagandha as a reservoir of biologically active compounds. But more evidence is needed to verify its effectiveness and safety.

Keywords
Ashwagandha, *Withania somnifera*, Phytochemical analysis, Withanolides, Ethanolic extract, HPLC, Medicinal plants, Bioactive compounds

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INTRODUCTION
Medicinal plants are believed to have been used for health for centuries the most commonly used of which is withania somnifera. This plant is very fascinating in both Ayurveda and current research as far as the health effects produced and the specific plant has been reported to yield numerous positive activities. Ashwagandha is widely practised in traditional medicine in order to help the body deal with stress[1]. This is called an adaptogen which assists to maintain balance in the physiological system during periods of stress. Thus it is utilized for anxiety, sleep disturbances and general fatigue[2][3]. The plant contains a lot of chemicals. They include alkaloids, flavonoids, saponins as well as more commonly withanolides which have been regarded as most vital molecules. Recent studies on such compounds are associated with most of the biological activity that has been studied in previous years in the field including anti-inflammatory and antioxidant activity [4][5][6]. Recently, the studies of these plants have been increasing in popularity mostly owing to their relatively abundant origin with fewer adverse effects than synthetic drugs [1]. More empirical research also confirmed that Ashwagandha could also exhibit beneficial sleep effects and lowered stress [7]. In that regard thus supports the objective of lab studies of Ashwagandha which involves determining its phytochemical characteristics for knowledge about its composition and utilization.

MATERIALS AND METHODS
Materials and Chemicals
Ashwagandha powder was obtained from a local supplier in Iraq. All chemicals and reagents used in this study were of analytical grade. Ethanol and methanol were purchased from Sigma-Aldrich (Germany). Hydrochloric acid (HCl), ferric chloride ($FeCl_3$), sulfuric acid (H_2SO_4), sodium hydroxide (NaOH), and other reagents were obtained from Merck (Germany). Distilled water was prepared in the laboratory.

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Equipment and Apparatus
The following instruments were used during the study:

- HPLC system (Shimadzu, Japan)
- UV detector (Shimadzu, Japan)
- Analytical balance (Sartorius, Germany)
- Water bath (Memmert, Germany)
- Hot plate (Stuart, UK)
- Micropipettes (Eppendorf, Germany)
- Glassware (Pyrex, USA)

Plant material preparation
Roots were air dried under shade at room temperature then ground to fine powder using a mortar and pestle. The powdered material was kept in airtight containers until further use.

Extraction Procedure
Ethanolic extraction was carried out by the Soxhlet apparatus. About 30 g of powdered plant material was extracted with 250 mL of 70% ethanol for 6 hours.

The extract was filtered and concentrated in a water bath at controlled temperature until a semi-solid crude extract was obtained. For later analysis the extract was stored at 4°C.

Qualitative Phytochemical Analysis.
Initial phytochemical screening was conducted to identify the major bioactive compounds.

Test for Alkaloids (Dragendorff's Test).
The extract was acidic with dilute HCl and filtered. A few drops of Dragendorff's reagent were added into the filtrate. The formation of an orange precipitate indicated the presence of alkaloids.

Test for Flavonoids (Shinoda Test)
A small amount of magnesium ribbon and a few drops of concentrated HCl were added to the extract. The appearance of a pink or red color indicated the presence of flavonoids.

Test for Saponins (Froth Test)
The extract was diluted with distilled water and shaken vigorously. The formation of a stable froth indicated the presence of saponins.

Test for Tannins (Ferric Chloride Test)
A few drops of 5% ferric chloride solution were added to the extract. A dark green or blue coloration indicated the presence of tannins.

Test for Glycosides (Keller–Killiani Test)
The extract was treated with glacial acetic acid and ferric chloride, followed by careful addition of concentrated sulfuric acid. The formation of a brown ring at the interface indicated the presence of glycosides.

Test for Terpenoids (Salkowski Test)
The extract was mixed with chloroform and concentrated sulfuric acid. The appearance of reddish-brown coloration indicated the presence of terpenoids.

Test for Steroids (Liebermann–Burchard Test)
The extract was treated with acetic acid followed by concentrated sulfuric acid. A green or bluish color indicated the presence of steroids.

HPLC Analysis
High-performance liquid chromatography (HPLC) analysis was performed using a Shimadzu system equipped with a UV detector. Separation was carried out using a C18 reverse-phase column.

The mobile phase consisted of a mixture of methanol and water (70:30 v/v). The flow rate was maintained at 1.0 mL/min, and the detection wavelength was set at 338 nm. The injection volume was 20 µL.

The retention times and peak areas of the sample were compared with those of the reference standard to confirm the presence of bioactive compounds.

Statistical Consideration
All experiments were performed under controlled laboratory conditions. The results of qualitative analysis were reported based on observable changes (color or precipitate formation). Chromatographic data were analyzed based on peak area and retention time.

RESULTS

Qualitative Phytochemical Analysis

The qualitative phytochemical screening of the ethanolic extract of *Withania somnifera* revealed the presence of several bioactive compounds. Alkaloids, flavonoids, saponins, terpenoids and steroids were detected, while tannins and glycosides were not observed under the experimental conditions.

These results indicate that the plant extract contains important classes of secondary metabolites known for their pharmacological activities.

Table 1: *Qualitative Phytochemical Analysis*

Phytochemical Class	Test	Result
Alkaloids	Dragendorff's Test	+
Saponins	Foam test	+
Terpenoids	Salkowski Test	+
Steroids	Liebermann–Burchard Test	+
Flavonoids	Shinoda Test	+
Flavonoids	Ammonia test	+
Tannins	Ferric chloride test	-
Glycosides	Keller-Killiani test	-

HPLC Analysis

The HPLC chromatogram of the standard showed three major peaks at retention times of about 2.694, 3.253, and 4.293 minutes, with the highest area at 3.253 minutes. Likewise, the chromatographic profiles of Ashwagandha extract showed peaks at retention times of nearly 2.637, 3.240, and 4.435 minutes. The similarity between the retention times of the sample and the standard confirms the presence of compounds structurally related to the reference standard, most probably containing withanolides. In addition, there are several peaks in the sample chromatogram indicating complex mixture of phytochemical constituents.

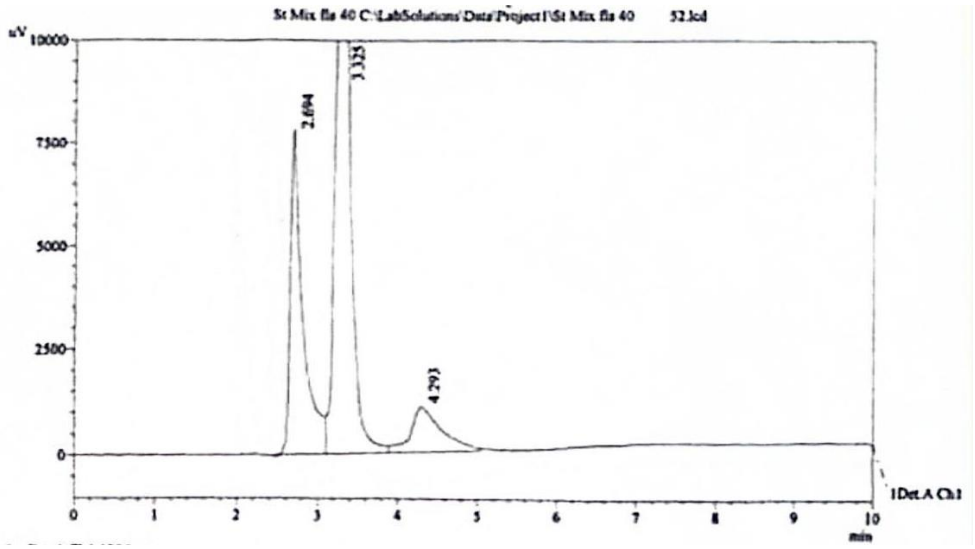
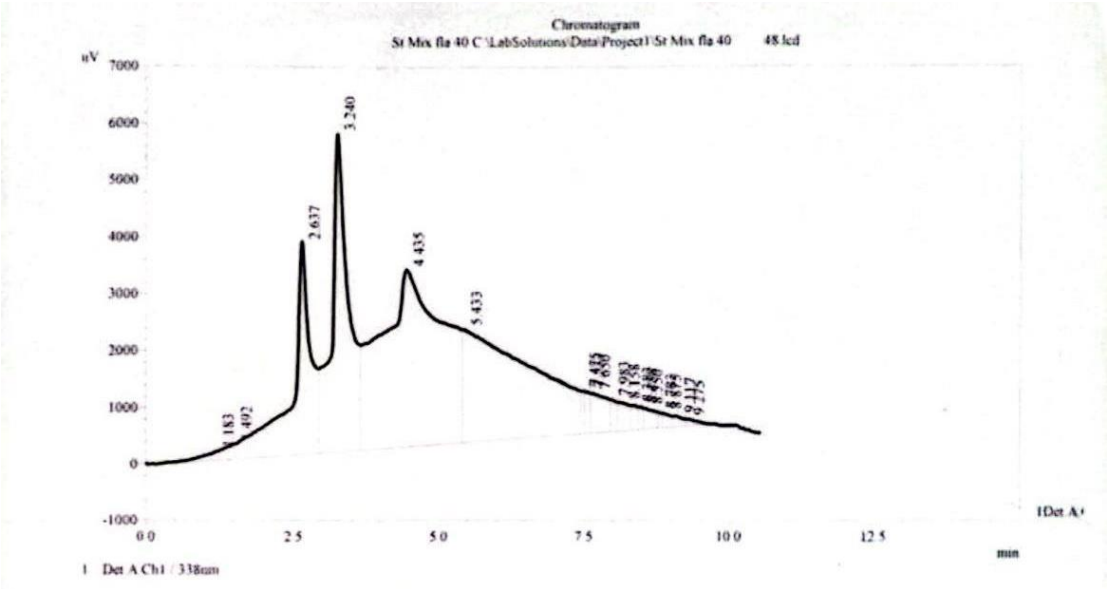


Figure 1. *HPLC chromatogram of reference standard showing major peaks at 338 nm detection wavelength*

Table 2: *Detector A Ch1338nm*

Peak	Ret. Time	Area	Height	Area %	Height %
1	2.694	85630	7798	30.102	37.19
2	3.325	164683	11695	57.299	56.823
3	4.295	30152	1088	10.599	5.287
Total	—	**284464**	**20581**	**100**	**100**



Figures 2. *Phytochemical Screening Results of the Sample*

Table 3: *Detector A Ch1 338nm*

Peak	Ret. Time	Area	Height	Area %	Height %
1	1.183	2675	160	0.406	0.792
2	1.492	4149	269	0.63	1.328
3	2.637	86412	3741	13.115	18.491
4	3.24	113107	5600	17.167	27.678
5	4.435	238190	3107	36.152	15.356
6	5.433	165188	1997	25.072	9.871
7	7.475	3045	766	0.462	3.786
8	7.533	4409	751	0.669	3.711
9	7.65	13554	713	2.057	3.522
10	7.983	3832	570	0.582	2.818
11	8.158	6744	500	1.024	2.473
12	8.383	3193	433	0.485	2.142
13	8.458	2156	401	0.327	1.98
14	8.55	4915	375	0.746	1.854
15	8.783	1205	285	0.183	1.411
16	8.875	2343	259	0.356	1.281

17	9.117	2181	180	0.331	0.89
18	9.275	1561	125	0.237	0.616
Total	—	**658861**	**20231**	**100**	**100**

DISCUSSION

Based on these results, the extract of Withania somnifera is composed of multiple substances. The HPLC results showed multiple peaks which indicate that the extract is not one compound but a combination of different compounds as is also expected from a plant extract. Alkaloids, flavonoids, saponins, terpenoids and steroids were detected by phytochemical tests. This is not surprising because prior studies on Ashwagandha also showed similar findings [10][11]. The different contributions of each group of compounds may differ. Flavonoids, for example, are often considered to be responsible for antioxidant activities whereas saponins are often linked to immune-related activities. The most well-known active compounds are believed to be withanolides, and they are attributed to most of the pharmacological activities of the plant [13][14]. It appeared to have been effective while extracting using ethanol due to its ability to detect multiple classes of compounds. So, extraction yields can depend on temperature, time and solvent concentration [15]. Some peaks in the sample did closely follow those that were observed in the standard according to HPLC results; thus, some compounds, and likely withanolides, may also exist [11][12]. Conversely, these results may differ not just depending on the source of the plant but also the environmental conditions and sometimes how the sample was handled beforehand [16]. All of these findings align with what has previously been described and contribute to the position that Ashwagandha contains biologically active compounds that may explain its traditional uses [17][18][19].

CONCLUSION

Therefore, the synthesis data of this study show that Withania somnifera has a wide range of bioactive constituents including alkaloids, flavonoids, saponins, terpenoids, and steroids. These findings are in line with other studies confirming the traditional medicinal application of Ashwagandha. HPLC also indicates the presence of compounds similar to withanolides, which are associated with various important pharmacological activities. These findings highlight Ashwagandha as a naturally occurring source of therapeutic agents and emphasize the need for further research.

RECOMMENDATIONS

Quantitative characterization of bioactive compounds should be conducted by LC-MS and NMR in the future, to improve the identification ability. Furthermore, by diversifying extraction and the solvents used, a greater quantity (and variety) of phytochemicals can also be obtained. More human in vivo and clinical investigations are also needed to determine safety, efficacy, and pharmacologic activities of Ashwagandha in humans. In the same way, a rigorous standardization of the extract is essential for continuity in quality and therapeutic effectiveness.

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