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Characterization of phenolics and biological activities of different solvent extracts from *Withania somnifera* fruit

[1]Tabassam, Q., [1,2]Mehmood, T., [1]*Anwar, F., [3]Saari, N. and [1]Qadir, R.

[1]*Institute of Chemistry, University of Sargodha, Sargodha 40100, Pakistan*
[2]*Institute of Biochemistry and Biotechnology, University of Veterinary and Animal Sciences, Lahore 54000, Pakistan*
[3]*Department of Food Science, Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia*

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Abstract

The present work studies the profiling of phenolic bioactive and *in vitro* biological (anticancer, antioxidant, and antimicrobial) activities of different solvent extracts from *Withania somnifera* fruit. Anticancer activity was performed using potato-disc assay and *Agrobacterium tumefaciens*. While antibacterial and antifungal evaluation was done by using disc diffusion method against bacterial (*Staphylococcus aureus, S. epidermidis, Escherichia coli,* and *Klebsiella pneumonia*) and fungal (*Aspergillus flavus* and *Fusarium oxysporum*) strains. Among different extraction solvents used, *n*-hexane extract exhibited the highest inhibition of tumour initiation (64%), whereas ethyl acetate (15%) was the lowest by using potato-disc assay. Highest total phenolic and total flavonoid contents were noted for methanolic (69.10 GAE mg/g DW%) and *n*-hexane (29.45 CE mg/g DW%) extracts, respectively. For antioxidant potential, 2,2,1-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging (IC_{50}) and reducing power EC_{50} were noted to be superior (0.6 and 2.0 mg/mL, respectively) for *n*-hexane extract. All the tested extracts showed considerable antibacterial and antifungal activity with the highest growth inhibition zones for *K. pneumoniae* (31.70 mm) and *A. flavus* (27.09 mm) were shown by *n*-hexane extract. High Performance Liquid Chromatographic (HPLC) analysis of individual phenolics (gallic acid, 2,288.48 mg/kg) indicated the highest contents of these compounds in *n*-hexane extract, which might explain the potent biological activities of this extract. Our findings revealed that the bioactive present in the tested fruit had significant potential as anticancer, antibacterial, and antifungal agents. Further studies are needed to elucidate the mechanism of actions of isolated bioactive against specific diseases such as cancer, especially in the case of *n*-hexane fraction.

Introduction

Medicinal plants are valuable natural resources that contain bioactive secondary metabolites with therapeutic potentials (Sharoba and Ramadan, 2011). Selected bioactive such as phenolics play physiological role in curing diseases through the modification of genetic pathways, inhibition of microbial growth, and by increasing antioxidant potential. In fact, plant phenolics play a vital role towards regulating and boosting the defence and immune functions in cells as an antioxidant, and thus controlling chronic and degenerative diseases by capturing the free radicals (Saha *et al.,* 2019). Currently, there is a revival of interest in the use of plant-based products as a source of food and medicine due to their safer nature, as compared to allopathic medicines which have side effects (Anwar *et al.,* 2019; Cordisco *et al.,* 2019).

Withania somnifera, commonly known as ginseng, ashwagandha, winter cherry, and auksin, belongs to *Solanaceae* family, and is an important medicinal herb in the Ayurveda folk medicine for over 3,000 years (Eroğlu *et al.,* 2018). It is a xerophytic plant, distributed in the dried parts of Pakistan, Italy, Afghanistan, Palestine, Egypt, Jordan, Morocco, Spain, Canary Island, Eastern Africa Congo, India, and Israel (Revuelta *et al.,* 2016). Chemical composition of *W. somnifera* comprises of alkaloids, withanolide, steroidal lactones, sitoindosides, and flavonoids, isolated from leaves and roots of the plant (Azgomi *et al.,* 2018).

Withania somnifera has multiple pharmacological activities due to the presence of natural antioxidants/reducing agents that quench singlet oxygen species. Various parts of this plant such as fruits are being used as nutritional food, and play important role in pharmaceutical. However, the fruits have rarely been explored for their chemical composition (Ramadan

*Corresponding author.
Email: fqanwar@yahoo.com

and Moersal, 2007). Furthermore, the plant extracts possess nootropic, cardio protective, anti-inflammatory, antitumor, antistress, antioxidant, immunomodulatory, and anti-Parkinson's activities (Ramadan and Moersal, 2007; Azgomi *et al.,* 2018).

All parts of the plant have medicinal properties; roots are used as an immune stimulator and to lessen aging process; while fruits are excellent source of vitamins, pectin, provitamins, and soluble sugars such as fructose that provides defence against various diseases (Ramadan, 2011). It has also been reported that the root extract from *W. somnifera* exerted anticarcinogenic effect when applied on a rat model (Turrini *et al.,* 2016). Leaves of *W. somnifera* contain phytochemicals such as withaferin A which induce apoptosis, angiogenesis, and metastasis in humans (Branton and Jana, 2017).

Being a promising candidate with nutraceutical properties (Ramadan and Mörsel, 2003), it would be interesting to characterise phenolics and explore anticancer, antioxidant, and antimicrobial activities of fruits harvested from wildly grown *W. somnifera* in Pakistan. In the present work, different solvents are used for the extraction of bioactive components. To the best of our knowledge, no earlier studies have been conducted on the nutra-pharmaceutical attributes of *W. somnifera* fruit in relation to different extraction solvents employed.

Materials and methods

The chemicals and reagents such as 1,1-diphenyl-2-picrylhydrazyl (DPPH), ascorbic acid, catechin, and gallic acid used were purchased from Sigma Aldrich. Solvents were of analytical grade, and purchased from Merck.

Plant material collection

Withania somnifera fruit samples were collected from Soon Valley, Sargodha district, Punjab, Pakistan. Following the identification and authentication by a botanist from the Department of Botany, University of Sargodha, Pakistan, the samples were washed and dried under shade. The dried whole fruits were ground into homogeneous powder.

Extraction of phytochemicals using various solvents

A series of extractions were performed with solvents of varying polarity such as pure 100% methanol, ethanol, *n*-hexane, ethyl acetate, and water on a shaker. After removal of excess solvent using rotary evaporator (1L/2L R-1001VN/R-1001LN, Shimadzu, Japan), the crude concentrated extracts were obtained in semi-solid form, and stored in refrigerator until further analyses. The percentage of extract yield was calculated using the formula reported by Ramadan and Moersel (2009).

Phytochemical investigation

Qualitative estimation of various phytochemicals such as tannins, phenols, flavonoids, proteins, carbohydrates, alkaloids, saponins, oxalic acid, steroids, inorganic acids, terpenes, and diterpenes was carried out using standardised colorimetric tests in all *W. somnifera* fruit extracts following the method of Kokate (2014).

Antioxidant determination

To check the antioxidant potential of *W. somnifera* fruit extracts, different assays were used i.e. DPPH, reducing power, and quantitative determination of total phenolic and flavonoids.

DPPH scavenging activity assay

The DPPH assay was carried out following Zhuang *et al.* (2012). BHA was used as a positive control, and IC_{50} of extracts was measured.

Estimation of reducing power

Withania somnifera fruit's reducing power was determined following Zhuang *et al.* (2012). Ascorbic acid was used as standard, and IC_{50} of extracts was measured.

Spectrophotometric analysis of total phenolic and flavonoids contents

Total phenolic contents (TPC) were estimated by Folin-Ciocalteu assay as described by Qadir *et al.* (2019). The results were expressed as gallic acid equivalent mg/g of dry weight. Total flavonoids contents (TFC) were determined by following the procedure as described in one of our recent study (Qadir *et al.,* 2018). The results were expressed as catechin equivalent (R^2 = 0.9911) mg/g of dry weight.

Antimicrobial activity

In order to check the antimicrobial effects of different extracts of *W. somnifera* fruit, selected bacterial (Gram-positive and Gram-negative) and fungal strains were used. Statistical analysis was also done to check the significance of the data generated ($p < 0.05$).

Antibacterial activity

Two Gram-positive (*Staphylococcus aureus* and *S. epidermidis*) and two Gram-negative (*Escherichia coli* and *Klebsiella pneumoniae*) bacterial strains were used. Antibacterial activity was evaluated by the

disc diffusion assay as described by Alam *et al.* (2014) with slight modification. The zone of inhibition was measured in mm. Control antibiotic disc containing streptomycin was used as a standard drug.

Antifungal activity

To check the antifungal activity of extracts, two fungal strains (*Aspergillus flavus* and *Fusarium oxysporum*) were used. For the calculation of zone of inhibition, fluconazole drug was used as a reference. The zone of inhibition was checked after 48 h against all extracts.

Anticancer activity

Potato disc assay was used to check the anticancer activity of different solvent extracts in which the inhibition of tumour growth and tumour initiation were assessed following the procedures described by Lellau and Liebezeit (2003).

HPLC analysis of phenolics

The separation of phenolic acids from extracts was accomplished by using a Spectra System SCM 1000 (Thermo Finnigan, California, USA) liquid chromatograph assembled with a diode array detector (DAD) system. Separation was done by a reverse-phase Hypersil Gold C_{18} (250 × 4.6 mm, 5 µ m) column (Thermo Corporation, USA), preceded by a guard column of the same packing material. The gradient mobile phase was solvents A and B, consisting of 0.1% formic acid (v/v) in methanol and water, respectively. The flow rate was set to 1 mL/min. The linear gradient was 5% (A) to 30% (A) for 25 min, followed by 30% (A) for 45 min, 100% (A) from 45.1 up to 52.0 min, then elution was brought back to 5% (A) up to 55 min for column equilibration. UV analysis was done by using the DAD, set at 270, 310, and 325 nm. Chromquest version 4.2 software was used for data interpretation. All phenolic compounds were individually confirmed by comparing their retention time (tR) with corresponding standards. The standard solutions with varying concentration ranged from 1 - 50 µg/mL were introduced into the HPLC-DAD system for the construction of standard calibration curve. The concentrations of the compounds were calculated from peak area in accordance with their respective standard curves (Nour *et al.,* 2013).

Statistical analysis

All experiments were done in triplicate. Data was analysed by using descriptive statistics and one-way ANOVA on SPSS software (Qadir *et al.,* 2018).

Results and discussion

The present work was carried out to evaluate the extraction efficacy of different solvents towards the recovery of crude bioactive components with potential antioxidant, antimicrobial, and anticancer activities, followed by the characterisation of phenolic bioactive in the extracts.

Extraction efficacy

Biological activities of plants extract depend upon the amount, nature, and concentration of bioactive components extracted in the extraction media/-solvents; thus, it is important to select the appropriate solvent to get the maximum amount of potent extractable compounds. By analysing the study of Ramadan *et al.* (2008) which used multiple solvents (aqueous and *n*-hexane) system for the recovery of various bioactives, in the present work, five solvents including ethanol, methanol, *n*-hexane, ethyl acetate, and water were employed to recover bioactive extracts from fruits of *W. somnifera*.

The maximum yield (95%) was obtained with ethanol followed by methanol (50%), water (47%), and *n*-hexane (40%) extracts. The minimum yield was obtained with ethyl acetate (30%). By looking in relative percentage of extract yield, it seems to be in favour of polarity as higher yields were obtained with polar solvents. Similar results were observed by Truong *et al.* (2019) when they evaluated different solvents to check the phytochemicals yield. They concluded that percentage yield is significantly dependent on polarity of the solvent used. The bioactivities of extracted components/extracts may differ due to nature of material, agro-climatic changes, and method followed to carried out the studies (Truong *et al.,* 2019).

Phytochemicals analysis

Phytochemicals are the plants bioactive which play vital role in disease prevention. In the present work, qualitative tests were performed to check important metabolites with regard to various solvent systems.

A list of phytochemicals is presented in Table 1. Phenols, flavonoids, tannins, and alkaloids were present in all extracts as indicated by the qualitative tests. The compounds such as phenolics are investigated for various pharmacological activities as they have a role in disease prevention, can suppress enzyme action, and control chronic diseases such as cancers, diabetes, and cardiovascular diseases (Sajid *et al.,* 2012; Nour *et al.,* 2013). The plants that have phenolic compounds can be used as

Table 1. List of phytochemicals detected in different solvent extracts from *Withania somnifera* fruit.

Phytochemical	Methanol	Ethanol	Ethyl acetate	*n*-hexane	Water
Phenols	+ve	+ve	+ve	+ve	+ve
Flavonoids	+ve	+ve	+ve	+ve	+ve
Tannins	+ve	+ve	+ve	+ve	+ve
Alkaloids	+ve	+ve	+ve	+ve	+ve
Coumarins	-ve	+ve	+ve	+ve	+ve
Carbohydrates	-ve	-ve	+ve	+ve	-ve
Deoxysugar	-ve	-ve	-ve	+ve	-ve
Saponins	-ve	-ve	-ve	+ve	-ve
Sterols	+ve	+ve	+ve	+ve	+ve
Terpenoids	+ve	+ve	+ve	+ve	-ve
Diterpenes	+ve	+ve	+ve	+ve	-ve
Triterpenes	+ve	+ve	+ve	+ve	-ve
Proteins	-ve	-ve	-ve	-ve	-ve

+ve = present; -ve = absent

anti-inflammatory agents (Ezeonu and Ejikeme, 2016). Flavonoids have the ability to play a role in platelet aggregation, hence they overcome platelet sickness disease (Okwu and Nnamdi, 2008). Tannins play effective role against bacterial diseases and have defensive function against microbial diseases. Alkaloids are known for their anticancer, antimicrobial, and hepatoprotective potentials (Fall *et al.,* 2019). Saponins were detected only in *n*-hexane extract in the present work. The current results agree with the findings of earlier researchers, who found saponins to be absent in methanol and ethanol extract of fruit seeds (Kamble *et al.,* 2016).

All the listed phytochemicals such as phenols, flavonoids, alkaloids, tannins, saponins, proteins, and carbohydrates were found in *n*-hexane extracts. While a low value was obtained in water extract, and protein was found to be absent in all extracts. Our findings are supported by the study of Ngo *et al.* (2017) who described that there is a significant effect of solvent on the yield of extracted compounds. Water is a highly polar solvent, thus it works well in combination with other organic solvents to extract highly polar compounds (Gull *et al.,* 2012).

Antioxidant potential

Antioxidants act as a scavenger of free radicals which cause health damaging effects. Their functions may vary from inhibitor, scavenger, decomposer, binder, and damage repairing. Various studies revealed that they have vital role in slowing the progression of cancer. Many plants are well known for their protective effects against oxidative stress because they have phytochemicals due to the antioxidant properties (Narváez-Cuenca *et al.,* 2014).

Fruits are considered rich sources of phenolics. Phenolics play vital role in antioxidant potential through their radical scavenging power. In the present work, total phenolic contents (TPC) were measured with Folin-Ciocalteu reagent assay which indicated interesting results. The levels of TPC were measured in extracts obtained with five different solvent systems (highly polar to non-polar) from fruit of *W. somnifera*. The values presented in Table 2 showed that TPC varied from 15.97 - 69.1 mg GAE/g. The solvent effects on TPC extraction were noted as *n*-hexane (69.42%) > methanol (65.48%) > ethanol (60.42%) > ethyl acetate (30.2%) > water (15.97%). The maximum TPC were extracted with *n*-hexane solvent while the minimum was with water.

The total flavonoid contents (TFC) also showed variation with regards to extraction solvent system. The distribution of TFC in relation to different extraction solvents was observed to be *n*-hexane extract (29.45 mg CE/g) > ethanol (24.39 mg CE/g) > ethyl acetate (26.46 mg CE/g) > methanol (20.82 mg CE/g) > water (17.3 mg CE/g). Again, *n*-hexane extract was found to be the best source of TFC as seen in the case of TPC. Our results are in agreement with Adhikari *et al.* (2020) who studied *W. somnifera*

Table 2. Antioxidant, antibacterial, and antifungal activities of various solvent extracts from *Withania somnifera* fruit.

Solvent extract	**TFC (CE mg/g DW)**	**TPC (GAE mg/g)**	**DPPH IC_{50} (mg/mL)**	**Reducing power EC_{50} (mg/mL)**	**Minimum inhibitory concentration (2 mg/mL) and zone of inhibition against bacteria strain**	***Escherichia coli* (Gram-ve) (mg/mL)**	***Klebsiella pneumoniae* (Gram-ve) (mg/mL)**	***Staphylococcus epidermidis* (Gram+ve) (mg/mL)**	***Staphylococcus aureus* (Gram +ve) (mg/mL)**	**Zone of inhibition diameter against fungal strains at the inhibitory concentration (2 mg/mL)**	***Aspergillus flavus***	***Fusarium oxysporum***
Methanol	20.82 ± 0.8	65.48 ± 1.21	13.1 ± 0.82	3.77 ± 1.0		6.30 ± 0.03	19.58 ± 0.01	17.09 ± 0.45	15.97 ± 0.17		19.64 ± 0.9	18.75 ± 0.41
Ethanol	24.39 ± 0.71	60.42 ± 0.43	12.00 ± 0.56	2.76 ± 0.3		7.30 ± 0.03	19.58 ± 0.01	10.0 ± 0.45	16.97 ± 0.17		17.64 ± 0.9	15.75 ± 0.41
Ethyl acetate	26.46 ± 0.72	30.2 ± 1.5	10.98 ± 0.21	3.53 ± 1.1		7.90 ± 0.1	13.7 ± 0.27	8.00 ± 0.45	15.66 ± 0.31		5.01 ± 0.07	4.34 ± 0.02
n-hexane	29.45 ± 0.84	69.10 ± 1.0	0.6 ± 0.01	2.0 ± 0.1		16.90 ± 0.1	31.70 ± 0.27	24.4 ± 0.45	29.66 ± 0.31		27.09 ± 0.04	19.45 ± 0.07
Water	17.3 ± 1.0	15.97 ± 0.9	15.7 ± 0.6	6.61 ± 0.03		13.73 ± 0.17	27.66 ± 0.16	14.43 ± 0.12	28.09 ± 0.20		9.01 ± 0.07	5.34 ± 0.02
					Streptomycin drug	40.50 ± 0.28	39.50 ± 0.20	38.50 ± 0.28	41.00± 0.38	**Fluconazole drug**	39.00 ± 0.05	39.00 ± 0.05

Values are means ± SD ($n = 3$) of three separate experiments. There was significant ($p < 0.05$) differences of means among tested extracts towards antioxidant, disease-causing bacteria, and fungal strains.

fruit extracts from different regions of India, and reported that the amount of extracted phenolics/flavonoids mainly depended on the nature of extraction solvent and geographical location of plants (Adhikari *et al.,* 2020).

DPPH free radical scavenging assay is one of the most common assays used to measure the scavenging activity of antioxidant species/extracts. In the present work, DPPH activity of plant extract was measured in relation to IC_{50} value. IC_{50} has an inverse relation toward antioxidant activity; the lower the IC_{50}, the more the antioxidant potential as it indicates the amount needed to decrease radical concentration by 50%.

In the present work, DPPH activity (IC_{50}) with respect to extraction solvents ranged from 0.6 - 15.7 mg/mL. The maximum activity was observed for *n*-hexane extract. Our investigations are in line with Khan *et al.* (2016) who investigated that higher DPPH activity mainly relates to higher contents of flavonoids and phenolics in plant *Datura alba*.

Reducing power assay is a direct measure for assessment of antioxidant potential of plants. Many researchers revealed in their investigation that reducing power assay is a reliable indicator to explore potential antioxidant capacity of plant materials. EC_{50} values for different solvent extracts analysed were found in the range of 2.0 to 6.61 mg/mL. EC50 value shows an inverse relationship relating to antioxidant potential, and maximum value was obtained for water extract (6.61 mg/mL) thus indicating its lowest antioxidant potential. Such variation in antioxidant behaviour can be observed due to difference in polarity of extraction solvent and nature of extractable components (Joseph *et al.,* 2017).

Antibacterial analysis

All the extracts tested showed their antimicrobial activity against selected microorganisms as shown in Table 2. The minimum inhibitory concentration (MIC) was 30 mg/mL. However, only 100 µL concentration (approximately 2 mg crude extract) from stock solution was applied towards the tested microorganisms. The zone of growth inhibition ranged from 7 - 28 mm for bacterial strains. The inhibitory value for *E. coli, K. pneumoniae, S. epidermidis,* and *S. aureus* were 6.30 - 13.73, 13.7 - 31.70, 8.0 - 24.4, and 15.66 - 29.66 mm, respectively.

The order of antibacterial activity of different extracts against *E. coli* was methanol > ethanol > ethyl acetate > *n*-hexane > water; for *Klebsiella pneumonia* was ethyl acetate > methanol > ethanol > water > *n*-hexane; for *S. epidermidis* was ethyl acetate > ethanol > methanol > water > n-hexane; and for *S. aureus* was ethyl acetate > methanol > ethanol > water > n-hexane.

Interestingly, *n*-hexane extract showed good behaviour in terms of zone of inhibition value against Gram-negative bacteria (*K. pneumonia*). These findings are in contrast with Hendra *et al.* (2011) who reported that plant extracts show more potent activity against Gram-positive bacteria (Hendra *et al.,* 2011). So, it can be assumed that antibacterial activity of plant materials not only vary depending upon plant origin, but also depending on specific bacterial strains. Moreover, antibacterial effectiveness of any plant extract varied as function of plant parts such as leaf and root, nature/polarity of extraction media, and concentration/dose of bioactive agents applied (Singariya *et al.,* 2012).

Antifungal analysis

The antifungal potentials of five different solvent extracts from *W. somnifera* fruit along with a standard drug, fluconazole, against *A. flavus* and *F. oxysporum* are depicted in Table 2. The diameter of growth inhibition zone was in the range of 5 - 27 mm for fungal strains. The growth inhibition zone for *A. flavus* varied from 5.1 to 27.09 mm. For *F. oxysporum*, these values were 4.34 - 19.45 mm with MIC value of 30 mg/mL. Among the tested extracts, methanol and *n*-hexane extracts gave sizable zone of inhibition with contribution of 19.64 ± 0.9 and 27.09 ± 0.04, respectively, against *A. flavus* and 18.75 ± 0.41 and 19.45 ± 0.07, respectively, against *F. oxysporum*. In a study from Tunisia, it was revealed that aqueous extract of *Withania* fruit showed higher potential against *F. oxysporum*. Such variations in activity might be linked to contents and nature of bioactive constituents which varied from region to region (Nefzi *et al.,* 2016). The antifungal activities of the extracts in the present work can be mainly linked to the presence of phenolic bioactive among other phytochemicals.

Anticancer activity

In the present work, different solvent extracts from *W. somnifera* fruit were tested by potato disc tumour assay, in which the inhibition of tumour growth and initiation was checked. All the solvent extracts showed inhibition of tumour growth except for ethyl acetate extract. Maximum activity against inhibition of tumour initiation was achieved by methanol and *n*-hexane extract as shown in Table 3.

It has been observed that *n*-hexane and ethanol fraction had maximum inhibition of tumour growth of 64 and 55%, respectively, while ethyl acetate had lower activity. In a previous study,

Table 3. Anticancer potential of different solvent extras from *Withania somnifera* fruit in terms of inhibition against tumour initiation and tumour growth.

Solvent extract	Inhibition of tumour initiation (%)	Inhibition of tumour growth
Methanol	47	+ve
Ethanol	55	+ve
Ethyl acetate	15	-ve
*n-h*exane	64	+ve
Water	33	+ ve

Wadhwa *et al.* (2013) studied anticancer activity of roots and leaves of *W. somnifera*; and the extracts were found to be highly active against breast cancer (Wadhwa *et al.,* 2013). It may be suggested for future research to find components and elucidate mechanism of actions revealing which particular active compounds are responsible for anticancer activity in order to explore maximum medicinal benefits of this plant.

HPLC analysis

In the present work, qualitative and quantitative profiling of phenolics in different extracts of *W. somnifera* fruit was made using HPLC, and the result are presented in Table 4, whereas related HPLC chromatograms are shown in Figure 1 (A-D).

Phenolic acids have multiple medicinal benefits including promising effect against cancers; they reduce the risk of stomach cancer by reduction of carcinogenic nitrosamine (Sajid *et al.,* 2012). For identification and quantification, 11 standard phenolic acids were used as reference compounds. Overall, seven phenolic were detected in *W. somnifera* fruit extracts tested from Pakistan. The level of gallic acid was observed to be highest among others with value as high as 2,288.48 mg/kg. The lowest quantity of phenolics was noted for water extract which indicates that water mostly extracted non-phenolic compounds, or it extracted compounds which are not biologically active.

The highest level of gallic acid was detected in *n*-hexane fraction/extract at retention time of 10.11 min. Four other phenolic acids such as vanillic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid (*p*-HBA), and sinapic acid were also detected. Due to the presence of high concentration of phenolics, this fraction was found to be more effective as per assays performed. A study performed on hepatocellular carcinoma cell lines revealed that phenolic compounds significantly induce apoptosis in SMMC-7721 cells *in vitro* (Sun *et al.,* 2016). Another phenolic agent, sinapic acid, is well known for having anticancer properties against colon cancer cell and prostate cell line PC3 and LNCaP; so, it is assumed that anticancer property of this plant may be due to the presence of notable levels of these phenolic compounds (Eroğlu *et al.,* 2018). Higher concentration of gallic acid also supports antioxidant properties of the tested extracts. Further studies are required to isolate bioactive phenolics from the tested plant extracts and to check their mechanism of action and properties for specific applications.

Table 4. Phenolics composition (mg/kg) of different solvent extracts from *Withania somnifera* fruit.

Compound (µg/mL)	Retention time (min)	Methanol	*n*-hexane	Ethanol	Ethyl acetate	water	Total (mg/kg)
Gallic acid	10.11		2,288.48				2,288.48
Protocatechuic acid	14.01		--	-		--	00
Sinapic acid	19.51	49.07	94.07		13.05	5.07	161.26
2,4 DHBA	24.01	--	--	26.51	30	--	56.51
Vanillic acid	28.45	10.62	17.96	13.51		16.96	59.05
Caffeic acid	30.51	--	--	--	-	--	00
p-HBA	32.12	13.27	9.05	12.14		5.05	39.51
p-coumaric acid	36.86	0.7-	15	7.14	32		54.84
Ferulic acid	48.78		--	--	51		51
m-coumaric acid	53.61	--	--	--			00
o-coumaric acid	57.63	--	--	--			00

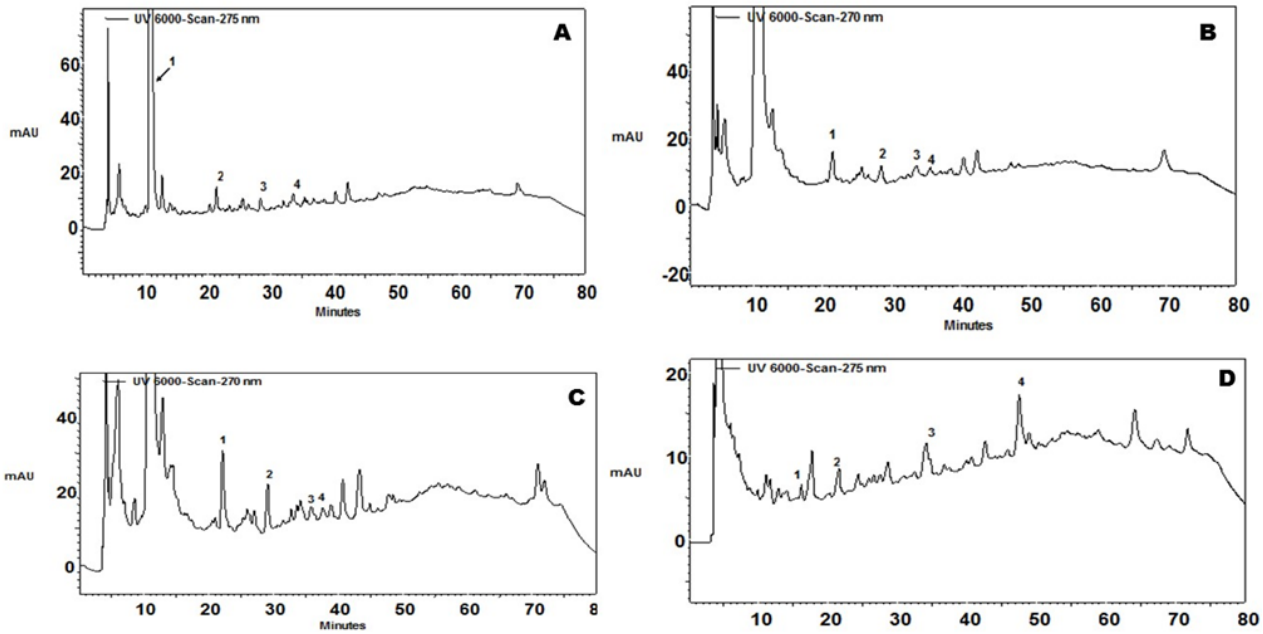

Figure 1. A) *n*-hexane fraction phenolic analysis (1: gallic acid, 2: sinapic acid, 3: vanillic acid, 4: *p*-HBA, and 5: *p*-coumaric acid); B) methanolic fraction (1: sinapic acid, 2: vanillic acid, 3: *p*-HBA, and 4: *p*-coumaric acid); C) ethanolic extract (1: gallic acid, 2: 2,4 DHBA, 3: vanillic acid, 4: p-HBA, and 5: *p*-coumaric acid); and D) ethyl acetate fraction (1: gallic acid, 2: protocatechuic acid, 3: vanillic acid, 4: coumaric acid, and 5: ferulic acid).

Conclusion

Overall, methanol and *n*-hexane extracts from *W. somnifera* fruit were found to be more potent among others. The findings support that bioactive present in the *W. somnifera* fruit exhibited potential as anticancer, antibacterial, and antifungal agents. To check the individual phenolic in each extract, HPLC analysis was performed against 11 standard phenolics. Gallic acid was observed to be the principal phenolic acid detected in the tested extracts, and its higher concentration can be linked to strong antioxidant effects/properties of this plant. As compared to other extracts, *n*-hexane fraction/extract was found to be phenolic rich. Further studies are recommended to be carried out to elucidate the mechanism of actions of these compounds against specific diseases such as cancer, especially in the case of *n*-hexane fraction.

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