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Preliminary Phytochemical Screening, *in vitro* Antimicrobial and Antioxidant Evaluation of *Withania somnifera* Dunal

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Abstract: *Withania somnifera* is commonly used as a folk medicine for the treatment of various diseases. The present investigation intended with various phytochemical screening, antimicrobial and antioxidant studies were carried out on the fruits of the *Withania somnifera.* Preliminary phytochemical evaluation of the crude extract and various fractions revealed the presence of alkaloids, steroids, saponins, tannins, reducing sugars, cardiac glycosides and coumarins. The *in vivo* antimicrobial activity of crude extract and fractions of *W. somnifera* were investigated. The crude extract and fractions exhibited antimicrobial activity against selected bacterial strain, with zone of inhabitation (14, 12 and 10 mm) respectively at the tested concentration of (22 mg/ml). All fractions were also evaluated for antioxidant activity in the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay. Among them, ethyl acetate fraction and methanolic extract showed significance scavenging activity. The present studies so far indicated that *W. somnifera* could prove to be a good natural source of a potent antimicrobial and antioxidant agent.

Key words: *Withania somnifera* · Phytochemical profiling · Antimicrobial activity · Antioxidant activity

INTRODUCTION

Genus *withania* belong to plant family solanaceae comprises of 84 genera and more than 3000 species distributed throughout the world. Berry of *W. somnifera* (ashwagandha) is ingredient of many ayurveda's and produces successfully developed medicines for tumors, tubercular glands, carbuncles and ulcers [1]. Phytochemical studies showed that ashwagandha possesses anti-inflammatory, antitumor, anti-stress, antioxidant, immunomodulatory, hemopoetic and rejuvenating properties. It also appears to exert a positive influence on the endocrine, cardiopulmonary and central nervous system [2]. The fruits of the plant have a milk coagulation property attributed to the pulp and husk of the berry which has been used in the preparation of vegetable rennet ferment for cheese [3]. The fruits are also reported to be sedative, emetic and stomachic, a blood-purifier and febrifuge and alternative, diuretic and bitter tonic in dyspepsia as well as growth promoter in infants [4]. The extensively used of *W. somnifera* as a folk medicine for the treatment of various diseases and observed biological activities were due to secondary metabolites found in the methanolic extract and subfractions.

MATERIALS AND METHODS

Plant Material: *W. somnifera* fruits were collected from Peshawar, Khyber Pakhtunkhwa Pakistan, in December 2011. The plant was identified by taxonomist, Prof. Dr. A. Rashid Department of Botany; University of Peshawar, Pakistan and voucher specimen was preserved at the Department of Botany, University of Peshawar, Peshawar.

Extraction and Fractionation: Shade dried and powdered fruits of *W. somnifera* was exhaustively filled in the Soxhlet extractor flask and extracted successively with methanol for 48 h. The solvent extract was filtered and concentrated using rotary evaporator at 40°C which was suspended in water and successively partitioned with *n*-hexane, chloroform, ethyl acetate and methanolic fraction.

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Micro-Organism Collection and Preservation: Three selected bacterial strains; *Klebsiella pneumonia*, *Staphylococcus aureus* and *Bacillus subtilis* used were obtained from stock culture of the Center of Phytomedicine and Medicinal Organic Chemistry, University of Peshawar and stored in Muller-Hinton agar at low temperature (4°C) prior to subculture.

Antimicrobial assay of the different fractions were performed using standard procedure [5] against particular bacterial strains. Modified agar well diffusion method was implemented to test the antimicrobial potential of the fractions by well diffusion methods with the use of Muller-Hinton agar as medium. The culture was prepared in triplicate incubated at 37°C temperature for a period of 24 to 72 hours. About 0.6 ml of the broth culture of the test organism was put in sterile petri dish and added 20 ml of the sterile molten MHA. Wells were bored into the medium using 0.2 ml of the fraction using Streptomycin (2 mg/ml) as a standard of antimicrobial agent. Inoculation was done for 1 h to ensure the diffusion of the antimicrobial agent into the medium. The inoculated plates were incubated for 24 h at 37°C. DMSO served as negative control. Diameters of the inhibition zone of microbial growth were measured in millimeter (mm).

Antioxidant Assay: The hydrogen atom or electron donation abilities of the corresponding extract/fractions were measured from the bleaching of the purple-colored methanol solution of 1, 1-diphenyl-1-picrylhydrazyl (DPPH) along with standard querceitin. Experiments were carried out according to the method of Blois [6] with a slight modification. Briefly, a 1 mM solution of DPPH radical solution in methanol was prepared and 1 ml of this solution was mixed with 3 ml of sample solutions in methanol (containing 20-100 µg) and control (without sample). The solution was stand for 30 min in dark and then absorbance was measured at 517 nm. Decreasing of the DPPH solution absorbance indicates an increase of the DPPH radical-scavenging activity. Scavenging of free radicals by DPPH as percent radical scavenging activities (% RSA) was calculated as follows.

$$\% \text{ DPPH} = \frac{\text{Control absorbance-extract absorbance x 100}}{\text{Control absorbance}}$$

Phytochemical Profiling of Fruits: The chemical tests were performed on the hexane, chloroform, ethyl acetate and methanolic extracts of *W. somnifera* using standard procedure [7-10] to recognize the bioactive secondary metabolite.

Test for Alkaloids: About 0.2 g of each of fraction was warm with 2% of H_2SO_4 for two minutes. The reaction mixture was filter and added a few drops of Dragendroff's reagent to each filtrate. Orange red precipitate indicates the presence of alkaloids moiety.

Test for Tannins: A small quantity of each extract was mixed with water and heated on water bath and filtered. A few drops of ferric chloride were added to each filtrate. A dark green solution indicates the presence of tannins.

Test for Anthraquinone: About 0.5 g of each extract was boiled with 10% HCl for few minutes on water bath. The reaction mixture was filtered and allows cooling. Equal volume of $CHCl_3$ was added to each filtrate. Few drops of 10% ammonia was added to each mixture and heated. Rose-pink color formation indicates the presence of anthraquinone.

Test for Glycosides: Each extract was hydrolyzed with HCl and neutralized with NaOH solution. A Few drops of Fehling's solution A and B were added to each mixture. Formation of red precipitate indicates the presence of glycosides.

Test for Reducing Sugar: Each extract was shaken with distilled water and filtered. The filtrates were boiled with few drops of Fehling's Solution A and B for few minutes. An orange red precipitate indicates the presence of reducing sugars.

Test for Saponins: 0.2 g of each extract was shaken with 5 ml of distilled water and heated to boiling. Frothing (appearance of creamy miss of small bubbles) shows the presence of saponins.

Test for Flavonoids: About 0.2 g of each extract was dissolved in diluted NaOH (0.1 N) and few drops of HCl were added. A yellow solution that turn colorless indicates the presence of flavonoids.

Test for Phlobatanins: About 0.5 g of each extract was dissolved in distilled water and filtered. The filtrate was boiled with 2% HCl solution. Red precipitate shows the presence of phlobatanins.

Test for Steroids: About 2 ml of acetic anhydride was added to the mixture of 0.5 g of each extract and conc. H_2SO_4 (2 ml). The color change from violet to blue or green in some samples indicates the presence of steroids.

Test for Terpenoids: About 0.2 g of each extract was mixed with 2 ml of chloroform and conc. H_2SO_4 (3 ml) was carefully added to form a layer. The formation of a reddish brown coloration at the interface indicates positive results for presence of terpenoids.

Test for Cardiac Glycosides: About 2 ml of the plant extract, 1 ml of glacial acetic acid and 5% ferric chloride was added. Then few drops of conc. H_2SO_4 were added. Presence of greenish blue colour indicates the presence of cardiac glycosides.

Test for Coumarins: A 3 ml of 10% NaOH was added to 2 ml of aqueous extract formation of yellow colour indicates the presence of coumarin.

Test for Emodins: A 2 ml of NH_4OH and 3 ml of benzene was added to extract. Appearance of red color indicates the presence of emodins.

Test for Fixed Oil and Fats: A small quantity of extract was pressed between two filter papers; oil stain on the filter paper indicates the presence of fixed oil.

Test for Anthocyanin and Betacyanin: To 2 ml of plant extract, 1ml of NaOH (2N) was added and heated for 5 minutes at 100°C. Formation of bluish green color indicates the presence of anthocyanin and formation of yellow color indicates the presence of betacyanin.

RESULTS AND DISCUSSION

The weight percentage yield of the fruits is shown in Table 1. The results of antimicrobial and antioxidant activities of crude extracts along with various fractions are shown in Table 2 and 3. While preliminary phytochemical profiling of the crude extract and fractions of *W. somnifera* are listed in Table 4.

Table 1: Extractive values of the crude extract and fractions of the fruits of *W. somnifera*

Solvent	Weight of crude extract (g)	Percentage yield
n-Hexane extract	19.0	9.40
Chloroform extract	24.5	12.1
Ethyl acetate extract	8.0	3.96
Methanol extract	35	17.3
Residue remaining	40	19.8
Crude extract	202	6.7

Table 2: Antimicrobial assay of the crude extract and fractions of the fruits of *W. somnifera*

Crude extract/Fraction	K.P	B.S	S.A
F1	10	12	12
F2	x	x	x
F3	10	12	14
F4	10	x	10
F5	10	12	14
DMSO	x	x	x
Imipenem	30	30	28

Key words: well size 6 mm, F1= *n*-hexane, F2= chloroform, F3= EtOAc, F4=4 MeOH and F5= methanolic crude extract; B.C = *Bacillus subtilis*, K.P = *Klebsiella pneumoniae*, S.A = *Staphylococcus aureus*.

Table 3: Phytochemical profiling of the crude extract and fractions of the fruits of *W. somnifera*

Chemical constituents	*n*-Hexane (fraction)	Chloroform (fraction)	Ethyl acetate (fraction)	Methanol (fraction)	Methanolic crude extract
Tannins	-	-	+	+	+
Alkaloids	-	+	+	+	+
Anthraquinones	-	-	-	-	-
Glycosides	-	-	-	-	+
Reducing sugar	-	-	-	+	+
Saponins	-	-	+	+	+
Flavonoids	-	-	-	-	-
Phlobatanins	-	-	-	-	+
Steroids	-	-	-	-	+
Terpenoids	+	+	+	+	+
Cardiac glycoside	+	+	+	+	+
Coumarin	-	-	+	+	+
Emodins	-	-	-	-	-
Oil and fats	+	+	+	+	+
Anthocyanin	-	-	-	-	-
Betacyanin	-	+	-	-	+

Key words: (-) Absent, (+) Present.

Table 4: DPPH radical scavenging activities of the crude extract and fractions of *W. somnifera*

n-Hexane Fraction		Chloroform fraction		EtOAc Fraction		Methanol fraction		Methanolic crude extract	
Conc. (μg/ml)	%DPPH	Conc. (μg/ml)	%DPPH	Conc. (μg/ml)	%DPPH	Conc. (μg/ml)	%DPPH	Conc. (μg/ml)	%DPPH
20	2.64	20	2.35	20	1.68	20	2.94	20	4.88
40	4.13	40	12.3	40	13.97	40	4.79	40	7.40
60	84.17	60	19.02	60	16.7	60	11.7	60	15.55
80	11.3	80	20.4	80	20.2	80	15.06	80	18.55
100	12.9	100	21.29	100	26.9	100	17.08	100	25.44
250	16.7	250	47.05	250	49.9	250	42.8	250	50.5
500	21.9	500	72.3	500	81.9	500	71.7	500	85.1

The preliminary phytochemical screening of the fruits of *W. somnifera* revealed the presence of bioactive secondary metabolite *viz.,* alkaloids, saponins, glycosides, steroids, terpenoids, tannins and coumarins and reducing sugar. These bioactive secondary metabolites showed the medicinal value of *W. somnifera* which is used as anti-spasmodic, anti-ulcerative and arthopathies relaxant [4].

The extracts and fractions were furthers evaluated for their antibacterial potential against selected bacterial strain such as *Staphylococcus aureus*, *Strap Epidermis*, *Bacillus subtilis*, *Escherichia coli* and *Klebsiella pneumonia* (Table 2). Generally *n*-hexane and ethyl acetate were fractions were found active against selected bacterial strain and thus display high inhibitory zone of (14 mm) at the tested concentration of (22 mg/ml). The antioxidant activity was performed by DPPH radical scavenging assay. Different fractions showed activity at different level (Table 4). The low anti radical activity was found in methanol crude extract and ethyl acetate which is almost near to the activity shown by standard querceitin. Chloroform and *n*-hexane fraction also showed moderate activity while *n*-hexane fraction was the least active fraction among entire fractions. The present finding showed that the fruit of *Withania somnifera* can be taken in good quantity in order to reduce the risk of various types of diseases causes due to free radicals. Free radical damage of nervous tissue may contribute to neuronal loss in cerebral ischemia and may be involved in normal aging neurodegenerative diseases, e.g. especially, schizophrenia, Parkinson's, Alzheimer's and other diseases [2]. The pharmacological activity of *W. somnifera* was confirmed from the antimicrobial and antioxidant assay of crude extract various fractions. The *n*-hexane and ethyl acetate were active against selected bacterial strain (Table 2) and thus display high inhibitory zone of (14 mm) at the tested concentration of (22 mg/ml).

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