

RRST-Phytochemistry

Phytochemical Screening and Determination of Antioxidant Potential of Fruits Extracts of *Withania coagulans*

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Article Info	Abstract
Article History Received : 10-03-2011 Revised : 24-03-2011 Accepted : 27-04-2011	In the present study, the phytochemical and antioxidant properties of methanolic and aqueous extracts of fruits of <i>Withania coagulans</i> were compared and investigated to find out the number of different phytoconstituents present in the plant which makes it remarkable for its use by traditional practitioners. Phytochemical screening of both the extracts showed the presence of alkaloids, steroids, phenolic compounds, tannins, saponin, carbohydrates, proteins, amino acids and organic acids. Further study showed high in vitro antioxidant activity in both of the extracts when compared to standard ascorbic acid but aqueous extracts showed higher antioxidant potential in comparison to that of methanolic extract.
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Introduction

Medicinal plants play a vital role for the development of new drugs. Plants are important sources of medicines and presently about 25% of pharmaceutical prescriptions in the United States contain at least one plant-derived ingredient [1]. *Withania coagulans* Dunal is common throughout Pakistan. It is also found in North-West India and Afghanistan. The plant is known by different names in different local languages, such as 'Akri' or 'Puni-ke-bij' in hindi, 'Tukhme- Kaknaje-hindi' in Persian. Spicebajja in Afghan, 'Khamjira' in Punjabi and 'Punir band' or 'Punir-ja-fota' in Sindhi [2]. *Withania coagulans* Dunal belongs to family Solanaceae. *Withania* is a small genus of shrubs, which are distributed in the East of the Mediterranean region and extend to South Asia. The berries of the shrub are used for milk coagulation. It is popularly known as Indian cheese maker. In Punjab, the fruit of *W. coagulans* are used as the source of coagulating enzyme for clotting the milk which is called 'paneer'. They are also used in dyspepsia, flatulent colic and other intestinal infections. In some parts of Pak-Indian sub-continent, the berries are used as a blood purifier. The twigs are chewed for cleaning of teeth and the smoke of the plant is inhaled for relief in toothache [2, 3]. *Withania coagulans* (Stocks) Dunal is used to treat nervous exhaustion, disability, insomnia, wasting diseases, failure to thrive in children, impotence. Its fruits are used for liver complaints, asthma and biliousness. Flowers of *Coagulans* (Stocks) Dunal are used in the treatment of diabetes [4]. The root is harvested in autumn and dried for later use [5]. Some caution is advised in the use of these plants since it is toxic [6]. Antimicrobial, anti-inflammatory, antitumor, hepatoprotective, antihyperglycemic, cardiovascular, immunosuppressive, free radical scavenging

and central nervous system depressant activities of the plant have been reported [7].

Materials and Methods

All the materials and reagents used for the study were from CDH, Ranchem and Hi Media Ltd., India.

Preliminary Phytochemical Screening

Standard screening tests of methanolic and aqueous extracts were carried out for various plant constituents. The crude extracts were screened for the presence or absence of secondary metabolites such as alkaloids, steroidal compounds, phenolic compounds, flavanoids, saponin, tannins using standard procedures [8, 9].

Tests for Carbohydrates: A small quantity of the extracts was dissolved separately in 4 ml of distilled water and filtered. The filtrate was subjected to Molisch's test to detect the presence of carbohydrates and further addition of Fehling's reagent if showed the brick red colour confirmed the presence of reducing sugar.

Detection of Glycosides: Another portion of the extract was hydrolysed with hydrochloric acid for few hours on a water bath and the hydrolysate was subjected to Legal's and Borntrager's test to detect the presence of different glycosides. Ammonia layer acquires pink color, showing the presence of glycosides.

Test for Proteins: The 2 ml of filtrate was treated with 2 ml of 10% sodium hydroxide solution in a test tube and heated for 10 minutes. A drop of 7% copper sulphate solution was added in the above mixture. Formation of purplish violet color indicates the presence of proteins.

Test for Alkaloids: A 100mg of an extract was dissolved in dilute hydrochloric acid. Solution was clarified by filtration. Filtrate was tested with Dragendorff's and Mayer's reagents. The treated solution was observed for any precipitation.

Confirmatory Test: Five grams of the extract was treated with 40% Calcium hydroxide solution until the extract was distinctly alkaline to litmus paper, and then extracted twice with 10 ml portions of chloroform. Chloroform extracts were combined and concentrated in vacuo to about 5ml. Chloroform extract was then spotted on thin layer plates. Solvent system (n-hexane-ethyl acetate, 4:1) was used to develop chromatogram and detected by spraying the chromatograms with freshly prepared Dragendorff's spray reagent. An orange or dark colored spots against a pale yellow background was confirmatory evidence for the presence of alkaloids.

Test for Phytosterol: 0.5 gm of extract was treated with 10 ml chloroform and filtered. The filtrate was used to test the presence of phytosterols and triterpenoids. The extract was refluxed with solution of alcoholic potassium hydroxide till complete saponification has taken place. The mixture was diluted and extracted with ether. The ether layer was evaporated and the residue was tested for the presence of phytosterol. The residue was dissolved in few drops of dilute acetic acid, 3 ml of acetic anhydride followed by few drops of concentrated sulphuric acid. Appearance of bluish green color shows the presence of phytosterol.

Tests for steroidal compounds

a) **Salkowski's Test** - 0.5g extracts were dissolved in 2ml chloroform in a test tube. Concentrated sulfuric acid was added on the wall of the test tube to form a lower layer. A reddish brown colour at the interface indicated the presence of steroid ring (i.e., the aglycone portion of the glycoside).

b) **Lieberman's Test** - 0.5 g extracts were dissolved in 2ml of acetic anhydride and cooled in an ice-bath. Concentrated sulfuric acid was then carefully added. A colour change from purple to blue- green indicated the presence of a steroid nucleus, i.e., aglycone portion of the cardiac glycosides.

Tests for Flavanoids

a) **Tests for free flavanoids**- 5mm of ethyl acetate was added to a solution of 0.5g of the extract in water. The mixture was shaken, allowed to settle, and inspected for the production of yellow colour in the organic layer, which is taken as positive for free flavanoids.

b) **Lead acetate test**- To a solution of 0.5 g extract in water, about 1ml of 10% lead acetate solution was added. Production of yellow precipitate is considered as positive for flavanoids.

c) **Reaction with Sodium hydroxide**- Dilute sodium hydroxide solution was added to a solution of 0.5g of the extract in water. The mixture was inspected for the production of yellow colour which considered as positive test for flavanoids.

Tests for Saponin

Froth Test: 0.5g extracts were dissolved in 10ml of distilled water for about 30 seconds. The test tube was stoppered and shaken vigorously for about 30 seconds. The test tube was allowed to stand in a vertical position and

observed over 30 minutes period of time. If a "honey comb" froth above the surface of liquid persists after 30 minutes the sample is suspected to contain saponin.

Test for Tannins

a) **Ferric chloride Test**- A portion of the extracts were dissolved in water. The solution was clarified by filtration; 10% ferric chloride solution was added to the clear filtrate. This was observed for a change in colour to bluish black.

b) **Formaldehyde Test**- To a solution of about 0.5g extract in 5ml water, three drops of formaldehyde and six drops of dilute hydrochloric acid were added. The resulting mixture was heated to boiling for 1min and cooled. The precipitate formed (if any) was washed with hot water, warm alcohol, and warm 5% potassium hydroxide successively. A bulky precipitate, which leaves a coloured residue after washing, indicated the presence of phlobatannins.

c) **Test for Phlobatanins**- Deposition of a red precipitate when an aqueous extract of the plant part was boiled with 1% aqueous hydrochloric acid was taken as evidence for the presence of phlobatannins.

d) **Modified iron complex Test**- To a solution of 0.5g of the plant extract in 5ml of water a drop of 33% acetic acid and 1g sodium potassium tartarate was added. The mixture was warmed and filtered to remove any precipitate. A 0.25% solution of ferric ammonium citrate was added to the filtrate until no further intensification of colour is obtained and then boiled. Purple or blackish precipitates, which are insoluble dilute ammonia, denotes the presence of in hot water, alcohol, or dilute ammonia, denotes the presence of pyrogallol tannin.

Tests for fixed oils

Spot test: Small quantities of various extracts were separately pressed between two filter papers. Appearance of oil stain on the paper indicates the presence of fixed oil. Few drops of 0.5 N alcoholic potassium hydroxide were added to a small quantity of various extracts along with a drop of phenolphthalein. The mixture was heated on a water bath for 1-2 hours. Formation of soap or partial neutralization of alkali indicates the presence of fixed oils and fats.

Determination of in vitro antioxidant activity

Fenton's reaction [10] was used for determination of in vitro antioxidant activity. The hydroxyl radical attached deoxyribose and initiated a series of reaction that eventually resulted in the formation of thiobarbituric acid reaction substance (TBARS). the measurement of TBARS thus gives an index of free radical scavenging activity. The reaction mixture consisted of a deoxyribose (3 mM, 100 μ l), ferric chloride (Fe^{3+} 0.2 mM 50 μ l), EDTA (0.1mM 50 μ l), ascorbic acid (0.1 mM 100 μ l), stock solution of aqueous and methanolic extracts at 10 mg/ml were prepared from which 100-1000 μ l were added in reaction mixture, the final volume was made up to 1ml by adding adequate quantity of phosphate buffer saline (pH, 7.4) and incubated for 1 hour at 37°C. The reaction was stopped by adding 0.5 ml of 5% TCA and 0.5 ml of 1% TBA the mixture was than incubated for 20 minutes in a boiling water bath. The absorbance was measured at 532 nm. Ascorbic acid was used as positive control. The results are expressed as the percentage inhibition of TBARS.

Results and Discussion

Phytochemical screening of the extracts

The results confirmed the presence of alkaloids, glycosides, steroids, saponin and oils in both the extracts of the fruit of the plant. Some of the constituents were observed

in one or the other extracts. These phytochemical constituents are good source of antimicrobial and antioxidant activity [7]. The results of phytochemical screening are reported in Table 1.

Table 1: Phytochemical Screening of solvent extracts of *Withania coagulans* fruits

TESTS	TESTS / REAGENTS	A.E	M.E
1. Tests for Carbohydrates	Fehling's Test	+	+
	Molisch Test	-	+
2. Tests for Glycosides	Bontrager's Test	+	+
3. Tests for alkaloids	Dragendroff's Reagent	+	+
	Mayer's Reagent	+	+
4. Test for Phytosterol	Chloroform	-	-
	Liebermann Burchard Test	-	+
5. Tests for steroidal compounds	Salkowski's Test	+	+
	Lieberman's Test	+	+
6. Tests for flavanoids	Tests for free flavanoids	-	-
	Lead Acetate Test	-	+
	Sodium Hydroxide	+	+
7. Tests for Saponins	Froth Test	+	+
8. Tests for Tannins	Ferric chloride Test	-	-
	Formaldehyde Test	-	-
	Test for Phlobatanins	-	-
	Modified iron complex Test	-	-
9. Tests for fixed oils	Spot test	+	+

(Here, +: indicates the presence; - : absence; A.E, Aqueous extract; M.E., Methanolic extract)

In vitro antioxidant activity of the extracts

The extract of *Withania coagulans* fruit had showed good antioxidant property in Fenton reaction model, the test drug(s) were compared with a low concentration of ascorbic acid (10 mg/ml). The aqueous extracts showed potent activity in comparison to methanolic extracts. The aqueous extracts were

found to have maximum % inhibition of TBARS in comparison to that of standard antioxidant and methanol extracts. The results are reported in Table 2 and Figure 2. Thus the present investigation revealed that aqueous extracts have potent antioxidant activity in comparison to methanol extract and standard antioxidant used.

Table 2: Percent TBARS inhibition of different Extracts of *Withania coagulans* and Ascorbic Acid

S. No.	Conc. of Ascorbic acid (10mg/ml)	% inhibition of TBARS	Conc. of Aqueous extract (10mg/ml)	% of TBARS	Concentration of Methanolic extract (10mg/ml)	% inhibition of TBARS
1.	0.047	31.5	0.081	54.3	0.051	34.2
2.	0.050	33.5	0.098	65.7	0.068	45.6
3.	0.069	46.3	0.177	118.7	0.057	38.2
4.	0.075	50.3	0.245	164.4	0.098	65.7
5.	0.142	95.2	0.384	257.7	0.127	85.2
6.	0.165	110.7	0.395	265.1	0.115	77.1
7.	0.182	122.1	0.571	383.2	0.222	148.1
8.	0.208	139.5	0.641	430.2	0.280	187.9
9.	0.198	132.8	0.655	439.5	0.289	193.9
10.	0.212	142.2	0.845	567.1	0.345	231.5

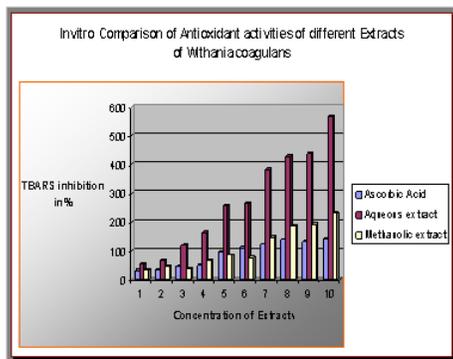


Figure 2: Percent TBARS inhibition of different Extracts of *Withania coagulans* and Ascorbic Acid

Conclusion

In the present investigation it is revealed that there are several phytochemical constituents present in the methanol and aqueous extracts of *Withania coagulans*. These phytochemical constituents are responsible for antioxidant activity of the plant. The results are in accordance with the results of *in vitro* antioxidant activity as revealed by Fenton's reaction. Further studies on isolation and characterization of the specific constituent (s) are needed to validate our results. The study thus can be further utilized to formulate the natural antioxidant which can be used as a dietary supplement to fight against several diseases such as ageing, arteriosclerosis etc. which caused due to Reactive Oxygen Species (ROS).

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