ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF VARIOUS EXTRACT OF FRUITS OF *WITHANIA COAGULANCE DUNAL* WITH CHEMICAL CHARACTERIZATION

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Summary

In the present investigations, attempts were made to study detail phytochemical parameters and pharmacological activities, particularly antioxidant and antimicrobial activity of fruits of *Withania coagulance dunal* belonging to family Solanaceae.

Recently, as numbers of herbal products are being introduced in the market it has become imperative to scrutinize herbal products for evaluating their acclaimed properties; this priority is necessary and important as a large section of Indian population utilizes such products without having access to the scientific data.

The selected plant *Withania coagulance* have multiple therapeutic properties like of antifungal, larvicidal, emetic, antibacterial, sedative, antiasthematic, antinflammatory, cardiovascular, diuretic, and hepatoprotective action but its antioxidant and antimicrobial activity of active constituent and its structural elucidation is still not reported. Plant from other species of the same family i.e. *Withania somnifera* is reported to possess all these activities [1-8]. The berries of selected plant *Withania coagulance* in folkloric is used for similar purpose but the scientific data of *Withania coagulance* targeting these activities till date is not reported. Hence, it was thought worthwhile to screen *Withania coagulance* for its antioxidant and antimicrobial activity and isolate the molecule responsible for respective activity.

Keywords: Withania coagulance, Antioxidant, Antimicrobial.

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1. Introduction

Withania coagulance dunal, known as paneer in Marathi, is employed in ancient Indian medicine for variety of antifungal, larvicidal, emetic, antibacterial, sedative and hepatoprotective action. Ayurvedic physicians use the fruit and stem of Withania coagulance as antioxidant and antimicrobial in tribal areas of India. Despite its use in India in above mentioned processes, it's antioxidant and antimicrobial effect has not been so far studied scientifically. Thus, it is worthwhile to screen extracts for its antioxidant and antimicrobial activity and isolate the molecule responsible for antioxidant and antimicrobial activity.

2. Materials and Methods

2.1 Drugs and chemicals

All the solvents used for the extraction process are of Laboratory grade. Triton x –100 (SMAR Chemicals, Nagpur).

2.2 Plant Extract

The powdered material of fruits of *Withania coagulance* was extracted in a Soxhlet apparatus by charging (500g each batch) and successive hot continuous extraction was carried out using petroleum ether (60-80°C), benzene, chloroform, acetone, rectified spirit and hydroalcohol (50:50). The percentage extractive value of the hydroalcoholic extract was found to be highest i.e. 3%w/w.

3. Experimental

3.1 Preliminary Phytochemical tests

Preliminary phytochemical screening of the extracts was studied, which showed presence of Sterols, Alkaloids, Flavonoids, Saponins, Sugars, Coumarins and Aminoacids.

3.2 Successive hot continuous extraction

Results of the extractives were obtained by successive hot continuous extraction of the dried coarse powdered fruits of *Withania coagulance* with different solvents of ascending polarity. Their colour, consistency and percentage extractive values are given in table No.1.

Table No. 1: Successive hot continuous extraction of fruits of *Withania coagulance*

Sr. no.	Solvent	Color and Consistency	% Extractive Value w/w
1	Petroleum ether (60-80^{0}C)	Dark yellowish, Oily	1.16
2	Benzene	Brown, Sticky, oily	0.54
3	Chloroform	Brown, Sticky, solid	0.447
4	Acetone	Dark Brown, Solid	0.970
5	Rectified spirit	Dark Shiny Brown, Solid	2.930
6	Hydroalcohol	Dark Shiny Brown, Solid	3.0

The percentage extractive value of the hydroalcoholic extract was found to be highest i.e. 3%w/w.

3.3 Antioxidant activity

The *In vitro* antioxidant activity of extracts was determined by DPPH free radicals. To 1.0 ml of DPPH solution of and 1.0 mL of hydro-alcoholic solution and 0.95 ml of 0.05 M Tris-Hcl buffer was added. To this solution 50 µl of each extract of specific strength was added and absorbance was recorded at 517 nm. Percent scavenging of DPPH radical was calculated by comparing absorbance between the test and diluent control mixture. The percentage peroxide value/ DPPH scavenging activity of extracts is shown in table No. 2. An antioxidant activity is expressed as IC_{50} *Value*. IC_{50} *Value* is defined as the concentration in µg/ml of extract required for inhibiting the formation of DPPH radical by 50%. Hydroalcholic extract at the concentration of 1000 µg/ml inhibitited the formation of DPPH radical by 50.45%. This was the lowest when compared to other extracts which required higher concentrations.

3.4 Antimicrobial activity

The antimicrobial activity was determined using filter paper disc method. The extracts were completely dried at normal conditions and were dissolved in DMSO. The stock solutions of each extract were prepared by diluting it with purified water (500 µg/ml). The filter paper discs impregnated with plant extracts were aseptically and carefully placed on the nutrient agar plates. The discs soaked with antibiotic solutions were also placed on nutrient agar plates as standards. All the nutrient agar plates were incubated at 37^{o}C for 24 hours after which the plates were observed for clear zone of inhibition. The results are shown in table No. 3.

Table No. 2: Percentage peroxide value/ DPPH scavenging of extracts

		Pet. Ether Extract		Benzene Extract		Chloroform Extract	
Sr. no.	**Concentration** μg **/mL**	**Absorbance**	**DPPH scavenging activity %**	**Absorbance**	**DPPH scavenging activity %**	**Absorbance**	**DPPH scavenging activity**
1.	Control	0.2200	--	0.2200	--	0.2200	--
2.	200	0.1987	9.68	0.1923	12.59	0.1912	13.09
3.	400	0.1866	15.15	0.1803	18.04	0.1753	20.31
4.	600	0.1645	25.22	0.1676	23.81	0.1602	27.18
5.	800	0.1519	30.95	0.1400	36.36	0.1451	34.04
6.	1000	0.1401	36.31	0.1305	40.68	0.1301	40.86
7.	1200	0.1284	41.72	0.1150	47.72	0.1154	47.54
8.	1400	0.1162	47.18	0.1043	52.59	0.1009	54.13
9.	1600	0.1042	52.63	--	--	--	--

		Acetone Extract		Rectified spirit Extract		Hydroalcoholic extract	
Sr. no.	**Concentration** μg **/mL**	**Absorbance**	**DPPH scavenging activity %**	**Absorbance**	**DPPH scavenging activity**	**Absorbance**	**DPPH scavenging activity %**
1.	Control	0.2200	--	0.2200	--	0.2200	--
2.	200	0.2056	6.54	0.1809	17.77	0.1822	17.18 %
3.	400	0.1901	13.59	0.1650	25.00	0.1632	25.81 %
4.	600	0.1751	20.40	0.1501	31.77	0.1462	33.54 %
5.	800	0.1501	31.77	0.1353	38.50	0.1200	45.45 %
6.	1000	0.1356	38.36	0.1201	45.40	0.1090	50.45 %
7.	1200	0.1200	45.45	0.1045	52.50	--	--
8.	1400	0.1053	52.13	--	--	--	--

Hydroalcholic extract showed the antimicrobial activity against *Staphylococcus aureus* (+) and *Escherichia coli* (–). The other extracts did not show any antimicrobial activity.

All the observations regarding various activities carried out showed that, only hydroalcholic extract of *Withania coagulance* possessed antioxidant and antimicrobial activity, hence were chosen for further study.

Table No. 3: Antimicrobial activity of different extracts

Sr. No	Test Drug	*Staphylococcus aureus* (+)	*Bacillus subtilis* (+)	*Escherichia coli* (–)	*Proteus vulgaris* (–)
1.	Streptomycin std.			+++	+++
2.	Amoxycilin std.	+++	+++		
3.	Ether extract	–	–	–	–
4.	Benzene extract	–	–	–	–
5.	Chloroform extract	–	–	–	–
6.	Acetone extract	–	–	–	–
7.	Rectified spirit	–	–	–	–
8.	Hydroalcoholic extract	+++	–	+++	-

3.5 Thin layer chromatography of hydroalcoholic extract

Thin layer chromatography of the hydroalcoholic extract was carried out by using Silica gel G as an adsorbent and numbers of solvent systems were tried. The solvent system, which gave best resolution, was considered valid and useful. The hydroalcoholic extract showed 3 spots, which were confirmed by HPTLC using same solvent system [methanol: ethyl acetate: toluene (2: 2: 5)]. The results are given in table No. 4.

Table No. 4: Thin layer chromatography of Hydroalcoholic extract

Sr. no.	Solvent system	No. of spots in iodine vapours	Rf value
1.	Methanol : Ethylacetate : Toluene (2 : 2 : 5)	1	0.5625
2.		2	0.6875
3.		3	0.8125

3.6 Column chromatography of hydroalcoholic extract

The Column chromatography was carried out using gradient elution technique to isolate the number of chemical constituents present in the hydroalcoholic extract. The elution of the column yielded 3 compounds. The results are given in table No.5.

Table No. 5: Column chromatography of hydroalcoholic extract

Solvent	Fractions (5 ml)	Constituents	Detection
Petroleum ether	1-12	Solvent front	Detection on preparative TLC Methanol: Ethylacetate: Toluene (2 : 2 : 5)
Petroleum ether: Benzene			
4 : 1	13-24	-	
3 :2	25-36	-	
2 :3	37-48	-	
1 : 4	49-60	-	
Benzene	61-72	-	
Benzene: Acetone			
4 : 1	73-84	-	
3 :2	85-96	-	
2 :3	97-108	-	
1 : 4	109-120	-	
Acetone	121-132	-	
Acetone: Methanol			
4 : 1	133-144	-	
3 :2	145-156	III	
2 :3	157-168	III	
1 : 4	169-180	III, II	
Methanol	181-192	II	
Methanol : Water			
4 : 1	193-204	II, I	
3 :2	205-216	I	
2 :3	217-228	I	
1 : 4	229-230	-	

All three separated constituents of hydroalcoholic extract were subjected to evaluation of antioxidant activity and antimicrobial activity. The procedure followed was same as mentioned earlier for respective activities. Purification of the constituents obtained was done by preparative TLC.

4. Result and Discussion

4.1 Antioxidant activity

Table No. 6: Antioxidant activity of isolated constituents

		Constituent 1		Constituent 2		Constituent 3	
Sr. no.	**Concentration µg /mL**	**Absorbance**	**DPPH scavenging activity %**	**Absorbance**	**DPPH scavenging activity %**	**Absorbance**	**DPPH scavenging activity %**
1.	Control	0.2200	--	0.2200	--	0.2200	--
2.	200	0.2105	4.31	0.215	2.22	0.2051	6.77
3.	400	0.1981	9.95	0.1975	10.22	0.1951	11.31
4.	600	0.1854	15.72	0.1903	13.5	0.1739	20.95
5.	800	0.1729	21.4	0.1828	16.90	0.1542	29.90
6.	1000	0.1607	26.95	0.1751	20.40	0.1352	38.54
7.	1200	0.1483	32.59	0.1676	23.81	0.1152	47.63
8.	1400	0.1359	38.22	0.1501	31.77	0.0954	56.63
9.	1600	0.1232	44.00	0.1429	35.04	--	--
10.	1800	0.1102	50.00	0.1351	38.59	--	--
11.	2000	--	--	0.1275	42.04	--	--
12.	2200	--	--	0.12	45.45	--	--
13.	2400	--	--	0.1123	48.95	--	--
14.	2600	--	--	0.105	52.27	--	--

The concentration (µg **/mL**) required to inihibit the formation of DPPH radical by 50.00%. was too high when compared to Hydroalcholic extract (active) in all the three separated constituents.

4.2 Antimicrobial activity

Table No. 7: Antimicrobial activity of isolated constituents

Sr. No	**Test Drug**	*Staphylococcus aureus* (+)	*Bacillus subtilis* (+)	*Escherichia coli* (–)	*Proteus vulgaris* (–)
1.	**Streptomycin std.**			+++	+++
2.	**Amoxycilin std.**	+++	+++		
3.	**Constituent no 1**	++	–	++	–
4.	**Constituent no 2**	–	–	–	–
5.	**Constituent no 3**	–	–	–	–

The constituent No. 1 showed the antimicrobial activity against *Staphylococcus aureus* (+) and *Escherichia coli* (–).antimicrobial activity with other constituents was not visible when compared to standard. Observations regarding various activities carried out on isolated constituents showed that only constituent No. 1 showed antimicrobial activity. The constituent No. 1 showed the antimicrobial activity against *Staphylococcus aureus* (+) and *Escherichia coli* (–). Hence constituent no 1 was chosen for physical and chemical characterization.

Table No. 8: Physical characterization of constituent No. 1

S.No	Physical parameters	Constituent No. 1
1.	**Colour**	Shiny brown
2.	**Odour**	Fruity
3.	**Solubility**	Soluble in methanol / chloroform, partly soluble in benzene / ether
4.	**Melting point**	220 - 222 ^{o}C
5.	**Rf value in solvent system**	0.56 Methanol: Ethylacetate : Toluene (2: 2: 5)

Table No. 9: Chemical characterization of constituent No. 1

S.No.	Chemical parameters	Constituent No. 1
1.	**λmax in nm**	214
2.	**IR spectroscopy (KBr) max cm^{-1}**	3455{OH stretching}, 2940, 2869{alkyl C – H stretching}, 1740 (C = O stretching), 1698 (Saturated lactone ring stretching), 1469.
3.	**H-NMR spectroscopy (δ values)**	5.28 ppm, 7.46 ppm, 2.17 ppm, 3.67 ppm, 4.23 ppm, 1.53 ppm, 0.71 ppm, 1.18 ppm, 2.15 ppm, 4.21 ppm, 2.28 ppm, and 0.81 ppm.
4.	**Mass spectroscopy**	Showed 2 prominent peaks (M - 1) $^{+}$ at m/z 483 and (M - 2) $^{+}$ at m/z 484. The other peaks obtained at 464, 441, 377, 365, 338, 280, 279, 247, 242, 241, 239, 210 and 202.
5.	**Elemental analysis (C, H %)**	Carbon = 74.73 %, Hydrogen = 9.045 %

4.3 Physical Characterization

The constituent No.1 obtained was shiny brown crystalline compound soluble in methanol / chloroform, partly soluble in benzene / ether with melting point ranging between 220.-222 ^{o}C. The purity of the constituent was determined by thin layer chromatography, which exhibited single spot with Rf value 0.6521

4.4 Chemical Characterization

The constituent No. 1 showed absorption maxima at 214nm. The IR showed strong absorption peaks at 3455 cm^{-1}, 2940 cm^{-1}, 2869 cm^{-1}, 1740 cm^{-1}, 1698 cm^{-1} and 1469 cm^{-1}. H-NMR of the constituent No. 1 gave δH values at 5.28 ppm, 7.46 ppm, 2.17 ppm, 3.67 ppm, 4.23 ppm, 1.53 ppm, 0.71 ppm, 1.18 ppm, 2.15 ppm, 4.21 ppm, 2.28 ppm, and 0.81 ppm. The mass spectra of the constituent No. 1 showed prominent peaks $(M - 1)^{+}$ at m/z 483 and $(M - 2)^{+}$ at m/z 484. The mass fragments of the constituent No. 1 were at 464, 441, 377, 365, 338, 280, 279, 247, 242, 241, 239, 210 and 202. The elemental analysis of the constituent No. 1 indicated 74.73 % of carbon and 9.045 of hydrogen.

4.5 Interpretation of Physical and Chemical Parameters:

The UV absorption of the constituent No. 1 (UV λmax = 214nm) points towards the presence of only one αβ unsaturated carbonyl system in the molecule, it can be therefore concluded that 6 membered ring lactone is saturated. Supporting evidence was found in the IR spectrum which shows one band C = O at 1698 cm -1for a saturated 6 membered ring lactone. IR spectra also exhibited a strong absorption bands at 3455 cm -1 which indicated the presence of OH group might be due to presence of moisture. The H-NMR spectrum of the constituent No. 1 exhibited signals attributed to the proton at C-22 (22H) δ 4.23 ppm which is characteristics of Withanoloids and signals for olifinic protons at C- 2, C- 3 (2H & 3H) at δ5.28ppm & δ 7.46ppm respectively. Signals were also obtained at δ 1.53, δ0.71 and δ 1.8 for methyl group at C – 19, C – 18 and C – 21(19H, 18H & 21H) respectively and δ 2.15, δ 4.21, δ 2.28, δ0.81(28H, 27H, 29H & 30H) respectively. The mass spectra of the constituent No. 1 showed prominent peaks (M - 1)+ at m/z 483 and (M - 2)+ at m/z 484, hence molecular weight of the constituent No. 1 is 484. The results of spectral studies suggest that the molecular formula for the constituent No. 1 is C30H44O5. The literature survey reveled that the observed physical and chemical characteristics of constituent NO. 1 corresponds to the data of Withanoloide. The attention can be drawn towards signal of the 22H, which is the characteristics and useful for identification of side chain in all withanoloids.

The *In vitro* antioxidant activity of extracts was determined by DPPH free radicals. Percent scavenging of DPPH radical was calculated by comparing absorbance between the test and diluent control mixture. Hydroalcholic extract at the concentration of 1000 µg/ml inhibitited the formation of DPPH radial by 50.45%. This was the lowest when compared to other extracts which required higher concentrations.

The antimicrobial activity was determined using filter paper disc method. The filter paper discs impregnated with plant extracts and antibiotic solutions respectively were aseptically and carefully placed on the nutrient agar plates. Hydroalcoholic extract showed the antimicrobial activity against *Staphylococcus aureus* (+) and *Escherichia coli* (–). The other extracts did not show any antimicrobial activity.

All the observations regarding various activities carried out showed that, only hydroalcoholic extract of *Withania coagulance* possessed antioxidant and antimicrobial activity, hence were chosen for further study.

Thin layer chromatography of the active extract was carried out by using Silica gel G as an adsorbent and numbers of solvent systems were tried. The solvent system, which gave best resolution, was considered valid and useful. The hydroalcoholic extract showed 3 spots, which were confirmed by HPTLC using same solvent system [Methanol: Ethylacetate: Toluene (2: 2: 5)].After preliminary phytochemical screening and thin layer chromatography, compounds were isolated and separated in pure form, from the active extracts with the help of column chromatography using gradient elution technique. The elution of the column yielded 3 compounds.

The chromatographic pattern was carefully studied and the fractions which showed the identical RF value were mixed. After mixing the number of fractions, at the end, only 3 fractions remained. TLC results of these 3 fractions showed single spot. All the three fractions were concentrated to a small volume. Compounds were further purified by means of preparative thin layer chromatography using Methanol: Ethylacetate: Toluene (2: 2: 5) as solvent system. The three dried separated constituents were stored in an airtight container. All three separated constituents were subjected to evaluation of antioxidant and antimicrobial activities. Observations regarding various activities carried out showed that only constituent No. 1 showed antimicrobial activity. The constituent No. 1 showed the antimicrobial activity against *Staphylococcus aureus* (+) and *Escherichia coli* (–).

Physical characterization & Chemical Characterization of the separated active constituent was carried out. The results of active constituent No.1 showed that it is brown crystalline compound soluble in methanol / chloroform, partly soluble in benzene / ether with melting point ranging between 220.-222^{o}C. The UV absorption constituent No. 1 (UV λmax =214nm) points towards the presence of only one αβ unsaturated carbonyl group , functional groups in IR spectrum shows one band C = O at 1698 cm -1for a saturated 6 membered ring lactone, prominent peaks (M - 1)+ at m/z 483 and (M - 2)+ at m/z 484 in Mass chromatography and the H-NMR spectrum of the constituent No. 1 attributed to the proton at C-22 (22H) § 4.23 ppm which is characteristics of Withanoloids The results of spectral studies suggest that the molecular weight and molecular formula of constituent No. 1 is 484 and $C_{30}H_{44}O_5$ respectively. The literature survey revels that the observed physical and chemical characteristics of constituent NO. 1 corresponds to the known data of Withanoloid. The attention can be drawn towards signal of the 22H, which is the characteristics and useful for identification of side chain in all withanoloids.

The work presented here is a small attempt to evaluate the herbal drugs. This is a small contribution of scientifically utilizing these natural products. It is hoped that such type of studies will reclaim our faith in herbal products and traditional medicines so that it will add novel drugs in our armory to fight various diseases. The WHO dream of providing health for all would only be achieved by a systematic knowledge and study of traditional medicines and tribal medicines.

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