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Research Article

Isolation of Active Fractions and Evaluting its Antidiabetic Property By Estimating Enzyme Inhibitory Activity of Withanolides from *Withania coagulans*

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ABSTRACT

In herbal medicinal systems, *Withania coagulans* (*W. coagulans*) has a well-established reputation for having significant biological potential. Different parts of the plant are used to treat different problems such as insomnia, liver problems, asthma, biliousness, and other conditions in addition to being reported to be sedative, emetic, diuretic, antidiabetic, antimicrobial, anti-inflammatory, antitumor, hepatoprotective, anti-hyperglycemic, cardiovascular, immuno-suppressive, and central nervous system depressant. The withanolides found in *Withania coagulans* have garnered attention in the scientific community because of the various medicinal uses that they have. The basic diabetes principle is the increased blood glucose in which starch is hydrolyzed into maltose by alpha amylase enzyme and maltose in glucose by alpha-glucosidase enzyme. Similarly, sucrose hydrolyses into glucose and fructose, these sugar units cause hyperglycemia when present in blood. The current work examines the chemical and biological evaluation of the *W. coagulans* acetone and n-hexane fractions and determine the anti-diabetic potential of *Withania coagulans* by calculating its enzyme inhibitory activity based on its antidiabetic and antiglycation potential.

As a result, this research provides a basis to extract the diabetic properties of W.coagulans in its pure form and provides the data that increasing concentration of inhibitors (*Withania coagulans*), the enzyme activity decreases.

Keywords: Antidiabetic, Antiglycation potential, Enzyme inhibitory activity, *Withania coagulans*

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INTRODUCTION

Many plants have been claimed to be useful in the treatment of diabetes mellitus in the natural system of medicine. In India, there are two species of *Withania*, a small genus of shrubs located in the east of the Mediterranean region and extending to South Asia. The plant *Withania coagulans* Dunal (Family: Solanaceae), often known as Indian cheese producer, was chosen for this investigation (Jaiswal D, *et al*., 2009). Solanaceae family of shrubs, which includes about 2000-3000 species spread across 90 genera, includes the tiny genus *Withania. Withania* species can be found across the world, from South Asia to the East Mediterranean (Datta A, *et al*., 2013; Maher S, *et al*., 2020). *Withania coagulans* has hypoglycemic action, making it an effective and safe alternative therapy for diabetes. In streptozotocin-induced rats, *Withania coagulans* demonstrated hypoglycemic action. Significant improvements in symptoms and indications were noted, and euglycemia was achieved in type 2 diabetes (Gupta V and Keshari BB, 2013; Hemalatha S, *et al*., 2004).

Withania coagulans is widely known for having a wide range of biological potential. This literature claims that various parts of the plant are used to treat a variety of conditions, including impotence, wasting illnesses, children's underdevelopment, insomnia, and nervous exhaustion (Mathur D and Agrawal RC, 2011). Its seeds' active ingredient, which also causes milk to coagulate, is utilized in traditional treatments. It has also been demonstrated that this plant's fruit is effective in treating biliousness, asthma, and liver problems, as well as being emetic, diuretic, and sedative. Diabetes can be treated with the flowers of *W. coagulans* (Atta-ur-Rahman, *et al*., 1998; Peerzade N, *et al*., 2018). In addition to its ethnobotanical uses, this plant has been linked to several reported therapeutic benefits, such as antihyperglycemic, anti-inflammatory, antitumor, antimicrobial, hepatoprotective, cardiovascular, immuno-suppressive, free radical scavenging, and central nervous system depressant properties. Moreover, this plant has been reported to have leishmanicidal, antidiabetic, and antimutagenic properties. Withanolides are lactone steroids that have been identified as the main bioactive phytoconstituents from *Withania coagulans* (Abbas S, *et al*., 1993; Atta-ur-Rahman, *et al*., 1998; Verma PK, *et al*., 2010; Peerzade N, *et al*., 2018 and Yousaf Z, *et al*., 2008).

Withania coagulans have certain anti-diabetic properties which inhibits enzymes like alpha-amylase and alpha-glucosidase. These enzymes are responsible for the hydrolysis of maltose into glucose and sucrose to glucose and fructose, and further these sugar units cause hyperglycemia when present in blood (Tiwari V, 2020).

Based on the anti-diabetic and antiglycation potential of *Withania coagulans*, current study focuses on the extraction and purification of withanolides and provides information that activity of the enzyme is inversely proportional to the concentration of the inhibitors; with increasing concentration of inhibitors (*Withania coagulans*), the enzyme activity decreases (Maher S, *et al*., 2020; Tiwari V, 2020).

MATERIALS AND METHODS

Plant material

The plant material was collected from a local ayurvedic shop in NIBM Pune, India.

Purification method

Column chromatography of the extracted *Withania coagulans*

was done in four fractions. Each fraction was subjected to Thin-Layer Chromatography (TLC). TLC was carried out on pre-coated silica gel plates and the R_f value was calculated using the following formula-

R_f (Retention factor) value=Distance travelled by the solute/Distance travelled by the solvent

Extraction and isolation

Withania coagulans was dried completely in air before being macerated in methanol for 24 hours and filtered. The filtered solvent was then evaporated in the water bath at 70°C to 100°C until a powder like substance was visible. The extract was then extracted with hexane to remove nonpolar constituents for 18 h, this was achieved by Soxhlet method of extraction, The residual aqueous phase was then filtered and subjected to column chromatography using n-hexane gradient elute resulting in four fractions. The fraction (F) F1, F2, F3, F4 were then subjected to column chromatography using silica gel and acetone/hexane solvent system for 10 minutes (Maher S, *et al*., 2020; Ali A, *et al*., 2015; Meena AK, *et al*., 2021). The purification of withanolides was confirmed as single spots were discovered on silica gel plate (*Figure 1*). The Optical Density (OD) of all four factions were taken at 580 nm and photometric readings were taken at 240 nm, for further confirmation (Maher S, *et al*., 2020; Shukla K, *et al*., 2012).

Figure 1: Silica gel plate chromatography; (A) Fraction 0 (F0) the very first elute of the extract which was subjected to column chromatography, (B) Fraction 1 (F1) second elute of the extract which was subjected to column chromatography, (C) Fraction 2 (F2) third elute of the extract which was subjected to column chromatography, (D) Fraction 3 (F3) third elute of the extract which was subjected to column chromatography

RESULTS AND DISCUSSION

Enzyme inhibition analysis

The enzyme activity of the fraction F4 was determined after column chromatography, seven test tubes-one blank, one without inhibitor, and the other five with the inhibitor. In blank test tube enzyme, buffer and DNSA (3,5-Dinitrosalicylic acid) were added; in without inhibitor test tube enzyme, buffer, starch, and DNSA were added. In with inhibitor test tube enzyme, buffer, DNSA and inhibitor were added and incubated at room temperature for 10 minutes. After incubation starch was added and incubated at room temperature for 20 minutes, later DNSA was added and incubated in water bath for 15 min until the color changes. Spectrophotometric readings were taken at 545 nm and were calculated. The test which didn't contained any inhibitor (test without inhibitor) turned into red colour and with inhibitor test turned into yellow color (*Figure 2*).

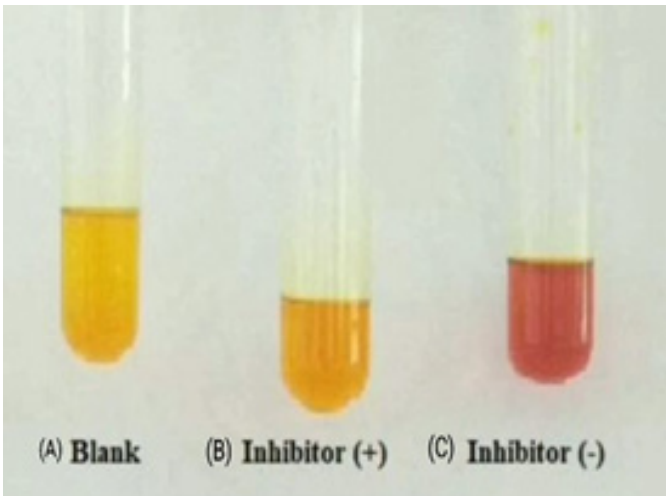

Figure 2: Enzyme activity of fraction F5; (A) Blank (Yellow colour), contains enzyme, buffer and DNSA, (B) With inhibitor (Yellow Color), contains enzyme, buffer, DNSA and inhibitor, (C) Without inhibitor (Red Color), contains enzyme, buffer, starch, and DNSA

Starch complex $\xrightarrow{\alpha-amylase}$ Maltose unit $\xrightarrow{DNSA-reagent}$ Red color complex (545 nm)

Enzyme inhibition assay

Fractions F1, F2, F3, F4 were subjected to spectrophotometry at 240 nm and OD at 580 nm (Maher S, *et al*., 2020; Meena AK, *et al*., 2021). Fraction 4 (F4) was then subjected to column chromatography to assess the enzyme's inhibitory activity. Ethylenediaminetetraacetic acid (EDTA) was employed as the enzyme inhibitor. Seven test tubes-one blank, one devoid of the inhibitor, and the other five-were run through a spectrophotometer at 545 nm (Verma PK, *et al*., 2010; Ali A, *et al*., 2015; Meena AK, *et al*., 2021; Asghar A, *et al*., 2021). The enzyme activity was calculated using the formula-

Enzyme activity (µmol/min)=µg of maltose released × 1000/Molecular weight of maltose × incubation time

Where,

µg of maltose released=Absorbance/slope (Slope: -0.00096)

Molecular weight of maltose=342

Incubation time=10 minutes

Characterization of the withanolides

As per the studies explained by Atta-ur-Rahman, *et al*., 1998; Tiwari V, 2020; Shukla K, *et al*., 2012; Naz A and Choudhary MI, 2003, the absorbance readings indicated that antidiabetic Withanolide A in its pure form was successfully extracted and isolated (*Table 1*). The results were confirmed by silica gel paper chromatography (*Table 2*), while the Rf value of Withanolide-A is 0.410 in formulation (Ali A, *et al*., 2015) and reference standards were found comparable under UV light at 240 nm with total absorbance 960 nm (*Table 2*). These values were further matched with the already provided data of pure form Withanolide-A (Verma PK, *et al*., 2010; Ali A, *et al*., 2015; Meena AK, *et al*., 2021).

Table 1: Retention values of different fractions

Fractions (F)	R_f values
F1	0.41
F2	0.41
F3	0.41

F4	0.41
F5	0.41
Note: The standard R_f value of Withaferin A is between 0.35 to 0.45 and Withanolide A is 0.45 (Meena AK, *et al*., 2021)	

Table 2: Absorbance of fraction (F1-F4) with respect to blank i.e., Distilled Water (DW) using shimazdzu UV spectrophotometry at 240 nm wavelength

Sample number	Fraction (F)	Absorbance	Absorbance (k) (240.00 nm)
1	Blank (DW)	4.000 A	960
2	F1	4.000 A	960
3	F2	4.000 A	960
4	F3	4.000 A	960
5	F4	4.000 A	960

Enzyme inhibitory activity

Enzyme activity reading proves presence of antidiabetic potential of *Withania coagulans* in the test without inhibitor which is 8.32 (*Table 3*). Similarly, enzyme activity with inhibitor shows that five tests are in decreasing order which is 5.483, 4.873, 4.568, 3.350, 0.6091 for test 1,2,3,4,5 respectively (*Tables 4 and 5*). The values were further compared with the data which was already provided (Meena AK, *et al*., 2021).

Table 3: Enzyme activity readings

Test		Enzyme activity (µmol/min)
Without inhibitor		8.832
With inhibitor	Test (Inhibitor concentration)	
	100 µg/ml	5.482
	200 µg/ml	4.873
	300 µg/ml	4.568
	400 µg/ml	3.35
	500 µg/ml	0.6091
Note: Slope=0.00096, molecular weight=342, Absorbance=0.029, 0.018, 0.016, 0.015, 0.011, 0.002 respectively		

Table 4: Absorbance of enzyme's inhibitory activity at 545 nm (0.206 A) in UV spectrophotometer

Sample number	Test	Absorbance	Absorbance (k) (545.0 nm)
1	Blank	0.111	60.44
2	Without inhibitor	0.029	15.86
3	With inhibitor	300 µg/ml	300 µg/ml
I	0.2 µg/ml	0.018	9.5375
II	0.4 µg/ml	0.016	8.8835
III	0.6 µg/ml	0.015	8.1205
IV	0.8 µg/ml	0.011	5.777
V	1 µg/ml	0.002	1.199

Table 5: Observation of enzyme inhibition assay

Test	Enzyme 0.5% (ml)	PBS (ml)	Inhibitor concentration (ml) (EDTA=(500 µg/ml)	Starch 1% (ml)	DNSA (ml)	OD (545 nm)
Blank	0.5	1	-	-	1	0
Without inhibitor	0.5	1	-	1	1	0.029
Test with inhibitor	0.5	0.8	0.2	1	1	5.482
	0.5	0.6	0.4	1	1	4.873
	0.5	0.4	0.6	1	1	4.568
	0.5	0.2	0.8	1	1	3.35
	0.5	-	1	1	1	0.609

Withanolides are lactone steroids that have been identified as the main bioactive phytoconstituents from *Withania coagulans*. These withanolides were extracted using n-hexane method and the rf values of the fractions were measured and compared to already available data (Meena AK, *et al*., 2021; Kumar S, *et al*., 2018). The standard photometric reading was also a match. There were fluctuations in F0, F2 and F3 readings while F1 remained constant which was +4.00 nm (close to 5 ± 1) (Ali A, *et al*., 2015; Meena AK, *et al*., 2021). The results indicated the withanolides A were extracted and isolated in its pure form. It is known that with increasing concentration of inhibitor, which is *Withania coagulans*, the enzyme activity decreased successively as compared to the one without Inhibitor showing maximum enzyme activity (Tiwari V, 2020; Shukla K, *et al*., 2012). Alpha amylase activity was measured *in vitro* by hydrolysis of starch in presence of alpha amylase enzyme. This process was quantified using DNA reagent, which gave us which gave red color on reaction with reducing sugar maltose. The intensity of red color indicated that the enzyme induced hydrolysis of starch into maltose. The extract processed alpha-amylase inhibitory activity as the intensity of the color was less in test without inhibitors (Atta-ur-Rahman, *et al*., 1998; Shukla K, *et al*., 2012). In other words, the intensity of red color is inversely proportional to alpha amylase inhibitor activity. When the calculated results were compared with the data provided, it showed presence of antidiabetic potential of *Withania coagulans* (Tiwari V, 2020; Atta-ur-Rahman, *et al*., 1998; Shukla K, *et al*., 2012).

CONCLUSION

Extraction of Withanolide-A was carried out on Soxhlet with n-hexane to remove non-polar constituents, then subjected to TLC for further purification. The fractions of *Withania coagulans* extract were subjected to silica gel plate chromatography and R_f values of fraction were compared with already provided data online to check the presence of pure form anti-diabetic compounds from *Withania coagulans*. Anti-diabetic potential was further determined by estimating enzyme inhibitory activity. The enzyme activity decreased with increase in concentration while maximum enzyme activity was seen in the test without inhibitor. The results revealed that enzyme activity is inversely proportional to the amount of inhibitor. Therefore, it can be concluded that maximum enzyme activity was observed in the absence of inhibitor which indicates the presence of antidiabetic properties. This study provides a scientific foundation for extraction and isolation of pure form anti-diabetic compounds from *Withania coagulans* and determines anti-diabetic potential by estimating enzyme inhibitory activity.

REFERENCES

1. Jaiswal D, Rai PK, Watal G. Antidiabetic effect of *Withania coagulans* in experimental rats. Indian J Clin Biochem. 2009; 24: 88-93.
2. Datta A, Bagchi C, Das S, Mitra A, de Pati A, Tripathi SK. Antidiabetic and antihyperlipidemic activity of hydroalcoholic extract of *Withania coagulans* Dunal dried fruit in experimental rat models. J Ayurveda Integr Med. 2013; 4(2): 99.
3. Maher S, Choudhary MI, Saleem F, Rasheed S, Waheed I, Halim SA, *et al*. Isolation of antidiabetic withanolides from *Withania coagulans* Dunal and their *in vitro* and *in silico* validation. Biology. 2020; 9(8): 197.
4. Gupta V, Keshari BB. *Withania coagulans* Dunal (paneer doda): A review. Int J Ayurvedic Herb Med. 2013; 3(5): 1330-1336.
5. Hemalatha S, Wahi AK, Singh PN, Chansouria JP. Hypoglycemic activity of *Withania coagulans* Dunal in streptozotocin induced diabetic rats. J Ethnopharmacol. 2004; 93(2-3): 261-264.
6. Mathur D, Agrawal RC. *Withania coagulans*: A review on the morphological and pharmacological properties of the shrub. World J Sci Technol. 2011; 1(10): 30-37.
7. Atta-ur-Rahman, Choudhary MI, Qureshi S, Gul W, Yousaf M. Two new ergostane-type steroidal lactones from *Withania coagulans*. J Nat Prod. 1998; 61(6): 812-814.
8. Peerzade N, Sayed N, Das N. Antimicrobial and phytochemical screening of methanolic fruit extract of *Withania coagulans* L. Dunal for evaluating the antidiabetic activity. Pharma Innov J. 2018; 7: 197-204.
9. Abbas S, Jamal SA, Choudhary MI. New withanolides from *Withania* sp. J Nat Prod. 1993; 56(7): 1000-1006.
10. Verma PK, Rajurkar S, Gaikwad NJ, Kamboj V. The isolation and structure elucidation of new Withanaoloides from *Withania cogulans* with antidiabetic activity. Acta Pharm Sci. 2010; 52(2).
11. Yousaf Z, Masood S, Shinwari ZK, Khan MA, Rabani A. Evaluation of taxonomic status of medicinal species of the genus *Hyoscyamous, Withania, Atropa* and *Datura* based on poly acrylamide gel electrophoresis. Pak J Bot. 2008; 40(6): 2289-2297.
12. Tiwari V. Determine anti-diabetic potential of plants in lab/ prevention of hyperglycemia. Learn Life Sciences. 2020.
13. Ali A, Maher S, Khan SA, Chaudhary MI, Musharraf SG. Sensitive quantification of six steroidal lactones in *Withania coagulans* extract by UHPLC electrospray tandem mass spectrometry. Steroids. 2015; 104: 176-181.
14. Meena AK, Rekha P, Perumal A, Gokul M, Swathi KN, Ilavarasan R. Estimation of Withaferin-A by HPLC and standardization of the Ashwagandhadi lehyam formulation. Heliyon. 2021; 7(2).
15. Shukla K, Dikshit P, Shukla R, Gambhir JK. The aqueous extract of *Withania coagulans* fruit partially reverses nicotinamide/ streptozotocin-induced diabetes mellitus in rats. J Med Food. 2012; 15(8): 718-725.
16. Asghar A, Aamir MN, Shah MA, Syed SK, Munir R. Development, characterization and evaluation of *in vitro* anti-inflammatory activity of *Withania coagulans* extract and extract loaded microemulsion. Pak J Pharm Sci. 2021; 34.
17. Naz A, Choudhary MI. Withanolides from *Withania coagulans*. Phytochemistry. 2003; 63(4): 387-390.
18. Kumar S, Singh R, Gajbhiye N, Dhanani T. Extraction optimization for phenolic-and withanolide-rich fractions from *Withania somnifera* roots: Identification and quantification of withaferin A, 12-deoxywithastromonolide, and withanolide A in plant materials and marketed formulations using a reversed-phase HPLC-photodiode array detection method. J AOAC Int. 2018; 101(6): 1773-1780.