

Research Article (Open access)

Effect of Chloroform Fraction of *Withania coagulans* Bud on the Regulation of GLUT4 and PPAR γ -Expressions Levels in Diabetic L6 Myotubes

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ABSTRACT- The present research article was described about the hypotriglycerdemic activity of *Withania coagulans* bud extract. *Withania coagulans* Dunal belonging to the family Solanaceae is a small bush which is widely spread in South Asia. The biological activity of withanolides from *Withania coagulans* has antihyperglycaemic activity and the plant is commonly called as Indian cheese maker due to the milk coagulation characteristics of the bud. The present study was to investigate preliminary studies shows satisfactory result. The chromatographic studies like TLC, HPTLC and HPLC show good spot. HPTLC shows maximum height and area of 18.83%. HPLC shows maximum peak at 1.867 minutes having area coverage of 87.4%. The free radical scavenging activity of chloroform fraction (CF) of a crude drug shows 510 $\mu\text{g/ml}$ of scavenging activity. The IC₅₀ value for MTT assay was found to be 84.7 $\mu\text{g/ml}$. The GLUT4 study shows significant uptake of glucose. PPAR gamma activity regulation of glucose disposal and insulin sensitivity in the skeletal muscles shows concentration dependence response using standard Pioglitazone. The bud of *Withania coagulans* will be a promising medicine for more ailments.

Key-words- *Withania coagulans*, Hypotriglycerdemic, HPLC, HPTLC, GLUT-4, MTT assay

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INTRODUCTION

Withania coagulans (*W. coagulans*) Dual belongs to family Solanaceae and is one of the important medicinal plants. It is distributed in the east of the Mediterranean region and extends to South Asia¹. There are two species of *Withania*, viz., *W. somnifera* and *W. coagulans*, which are distributed in east of the Mediterranean region extending to South Asia. *W. coagulans* Dunal is a rigid, gray-whitish small shrub, about 60-120 cm tall. The leaves are about 2.5-7.5 cm long and 1.5 cm broad, usually lanceolate oblong. The flowers are about 7-12 mm across, yellowish, and are dioecious and polygamous in nature. The berries are about 7-12 mm in diameter, red, smooth and enclosed in leathery calyx.

The seeds are dark brown, ear shaped, glabrous with sharp fruity smell.² Aqueous and chloroform extracts prepared from *W. coagulans* buds showed pharmacological effects on blood glucose, lipid profile and body weight.

Type 2 diabetic is an islet paracrinopathy in which the reciprocal relationship between the glucagon-secreting alpha cell and the insulin-secreting beta cell is lost, leading to hyperglucagonemia and hence the consequent hyperglycemia³. *W. coagulans* Dunal, is commonly known as 'Indian cheese maker' or 'vegetable rennet' because fruits and leaves of this plant are used as a coagulant. The fruits of the plant are sweet and are reported to be sedative, emetic, alterative and diuretic. Flowers of the plant are useful in the treatment of Diabetes⁴. They are also used in dyspepsia, flatulent colic and other intestinal infections. In addition; *W. coagulans* 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 biliousness⁵. This plant has been reported to possess antimicrobial, anti-inflammatory, antitumor, hepatoprotective, anti-hyperglycemic, cardiovascular, immuno-suppressive, free radical

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scavenging and central nervous system depressant activities.⁶

MATERIALS AND METHODS

This study was carried out in Department of Pharmaceutics at Vels University, Chennai, India during the period of September 2012 – September 2013.

Collection: The plant specimen (whole plant) for the proposed study was purchased from the commercial shops in Paris, Chennai, TamilNadu. It was identified and authenticated by Dr. P. Jayaraman, Director Plant Anatomy Research Centre (PARC), Tambaram, Chennai. A voucher specimen No. PARC/2012/1279 has been deposited in the herbarium of the Department of Pharmacognosy, School of Pharmaceutical Sciences, Vels University for further reference.

Extraction procedure: The bud was crushed and powdered. 200gms of the powdered drug was macerated with 500 ml of chloroform for 72 hours. After 48 hours it was filtered using a muslin cloth. The filtrate was defatted with n-hexane⁷. The defatted chloroform extract was concentrated by evaporation at 45° C. The concentrated mass was stored in well labeled and closed container in cool conditions. This extract was used for all the studies.

EVALUATION STUDY

Phytochemical screening study: The preliminary phytochemical screening of the chloroform fraction was carried out as per the standard procedures⁸. The fraction was subjected to various qualitative tests and the chemical constituents of the sample were identified.

Physicochemical analysis⁹: Ash value (total ash, water soluble ash, acid insoluble ash), Extractive value, Loss on drying and Swelling index were carried out as per the procedure given in standard book. The result was shown in Table 1 of results and discussion.

CHROMATOGRAPHY STUDY

Thin layer chromatography: Thin layer chromatography (TLC) is an important technique for identification and separation of mixtures of organic compounds. In TLC, components of the mixture are partitioned between an adsorbent and a solvent which flows through the adsorbent¹⁰. The result was shown in Figure 1 of results and discussion portion.

Solvent system: Ethyl acetate – Toluene (11: 3), Sample solution: Chloroform extract of *Withania coagulans* bud and detecting agent: Anisaldehyde reagent.

HPTLC: Preparation of sample solution: Accurately weighed 250 mg of the chloroform fraction of *Withania coagulans* was transferred to a 100mL volumetric flask dissolving in 80 mL of chloroform.

Instrumentation and chromatographic conditions: HPTLC was performed on 10 cm × 10 cm aluminum backed plates coated with silica gel 60F254 (Merck,

Mumbai, India). Sample solution was applied to the plates as bands 8.0 mm wide, 30.0 mm apart, and 10.0 mm from the bottom edge of the chromatographic plate by use of a Camag (Muttenez, Switzerland) Linomat V sample applicator equipped with a 100-μL Hamilton (USA) syringe. Ascending development to a distance of 80 mm was performed at room temperature (28 ± 2°C), with Toluene: Ethyl acetate (9.8 : 0.2) (v/v), as mobile phase, in a Camag glass twin-trough chamber previously saturated with mobile phase vapors for 20 min. After development, the plates were dried with a hair dryer and then scanned at 254 nm with a Camag TLC Scanner with WINCAT software, using the deuterium lamp. The method was validated according to the ICH guidelines. The result was shown in Figure 2 of results and discussion portion.

HPLC: Sample Preparation: The chloroform extract of the *Withania coagulans* bud was filtered through a 0.45 μm Millipore membrane prior to HPLC analysis.

Chromatographic condition: HPLC is a technique used to separate a mixture of compounds. The active component of the column, the sorbent, is typically a granular material made of solid particles 2-50 micrometers in size. The components of the sample mixture are separated from each other due to their different degrees of interaction with the sorbent particles¹¹. The result was shown in Figure 3 of results and discussion portion.

Chromatographic Conditions

Sample injection volume: 20μL, Column temperature: 25 °C, Flow rate: 1.0mL/min

Mobile phase: Acetonitrile and 0.1% aqueous phosphoric acid (v/v).

DPPH radical scavenging activity: About 0.1M solution of DPPH in methanol was prepared and 1 ml of this solution was added to 3 ml of the different concentration (25-800 μg/ml) of chloroform fraction (CF) and control in different test tubes. The mixture was shaken and allowed to stand at room temperature for 30 min and the absorbance was measured at 517 nm using a spectrophotometer.

$$\% \text{ Inhibition} = (\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Sample}}) \times 100 / \text{Abs}_{\text{Control}}$$

Where Abs_{Control} is absorbance of control at time = 0 and Abs_{Sample} is absorbance of test sample. The IC₅₀ Value for extracts was also calculated. The results are mentioned in Figure 4 of results and discussion portion.

CYTOTOXICITY STUDIES –MTT ASSAY: MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) were cleaved by mitochondria dehydrogenase in viable cells there by yield a measurable purple product Formosan. This Formosan production is proportionate to the viable cell number and inversely proportional to the degree of cytotoxicity.

Preparation of medium: L6 Rat skeletal muscle Cells were cultured in Dulbecco's Modified eagle Medium (DMEM) at 37°C in a humidified atmosphere of 5% CO₂ in

air. The tissue culture bottle was observed for growth. Then TPVG was added over the cells of a medium and incubate at 37°C for 5 minutes for Disaggregation and the cells become individual and its present as suspension. Take one ml of the suspension and pour it into 96 well plates then incubate for 2 days.

Assay procedure: After incubation, remove the medium from the wells for MTT assay. And add 200µl of MTT concentration of (5mg/ml and incubate for 6-7hrs in 5% CO₂ incubator. After incubation 1ml of DMSO was added in each well and mix by pipette and leave for 45seconds and it shows the purple color formation. The suspension is transferred in to the cuvette of spectrophotometer and O.D values are read at 595nm and % of cell viability was calculated using the formula.

Graph was plotted using the % of cell viability at Y-axis and concentration of the sample in X-axis. The result was shown in Figure 5 and 6 of results and discussion portion.

$$(\text{OD of sample} / \text{OD of cell control}) \times 100 = \% \text{ cell viability}$$

in vitro glucose uptake activity-

Preparation of cell culture: Monolayer of L-6 cells was maintained in Dulbecco's Modified eagle Medium (DMEM) with 4.5 g/l glucose, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum. Cells were incubating at 37°C with ambient oxygen and 5% CO₂ on continuous passage of TPVG solution.

Glucose uptake assay: When semi confluent monolayer was formed, the culture was renewed with serum free DMEM containing 0.2% BSA and incubated for 18 h at 37°C in the CO₂ incubator. After 18 h, the media was discarded and cells were washed with KRP buffer once. The cells were treated with Insulin, standard drug and plant extract and added glucose (1M) and incubated for half an hour. The supernatant was collected for glucose estimation by god- pod method. The result was shown in Figure 7 of results and discussion portion.

$$\text{Glucose concentration } \text{mmol/l} = \frac{A_{\text{sample}}}{A_{\text{standard}}} \times 55.5 \text{mg/dl} = \frac{A_{\text{sample}}}{A_{\text{standard}}} \times 100$$

PPAR Gamma Agonist Activity: Make TF binding mix (25ul 2X TF binding buffer X Nuclear extract (2-10ug). Add 10ul competition oligo to prevent the TF binding to the immobilized oligo on the well. Add the mix on a well and incubate for 30 minutes without shaking. Discard the contents and wash by adding 200µl of 1X Assay wash buffer (3 time)¹⁶. After the last wash, remove any remaining liquid by inverting the plate against clean paper towels. Add 100µl of diluted antibody against PPAR gamma to well and incubate for 1 hour at room temperature with gentle shaking. Add 100 µl of diluted HRP conjugate secondary antibody to well and incubate for 45 min at room temperature with gentle shaking. Add 100µl of substrate to well and incubate for 5-10 minutes. Add 50µl of stop solution to well. The color in the wells should change from

blue to yellow. Determine the optical density of each well with a microplate reader at 450 nm within 30 minutes. The result was shown in Figure 8 and 9 of results and discussion portion.

RESULTS AND DISCUSSION

Phytochemical screening tests for the chloroform fraction of the buds of *Withania coagulans* were carried out to identify the phyto-constituents present in the extract. The results of these tests indicated the presence of presence of Glycosides, Alkaloids, Tannins, Flavanoids, Steroids, Resins, Saponins, Gums and Mucilage, and Amino Acids in the fraction. The ash value of the bud of *Withania coagulans* was found to be 11.9% of this 1.20% was found to be water soluble and 0.75% was found to be acid insoluble the aqueous soluble extractive value, alcohol soluble extractive value and the chloroform extractive value were found to be 0.148%, 0.045% and 0.782% respectively. The loss on drying was evaluated and was found to be 0.6%. The results were tabulated in Table 1.

Table 1. Physiochemical analysis

Parameters	Results
Swelling index	1.0
Extractive value	
Aqueous soluble extractive	0.148%
Alcohol soluble extractive	0.045%
Chloroform soluble extractive	0.782%
Ash value	
Total ash	11.9% w/w
Aqueous soluble ash	1.20% w/w
Acid insoluble ash	0.75% w/w
Loss on drying	0.6%

Chromatographic studies

TLC was carried out by using a solvent system and 10 spots were identified with R_f values ranging from 0.092 – 0.803. This was compared with diosgenin as standard which has a R_f value of 0.370. The result was shown in Figure 1.



Fig1: TLC of chloroform fraction

Based on the number of peaks expressed in the HPTLC fingerprinting of the chloroform fraction of *Withania coagulans* bud was selected and used for the forthcoming studies. The peak obtained in this is the 13th peak having a maximum height of 333.2 and area of 18.83%. The result was shown in Figure 2.

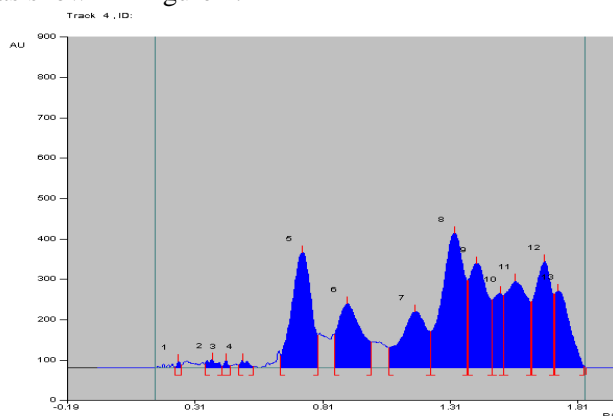


Fig 2: HPTLC fingerprinting of chloroform fraction (CF)

The HPLC studies showed that most of the compounds in the chloroform fraction of *Withania coagulans* got eluted within 8.363 minutes. The concentration of acetonitrile used during this retention time is 2-10%. The maximum peak obtained at 1.867 minutes having area coverage of 87.4%. The result was shown in Figure 3.

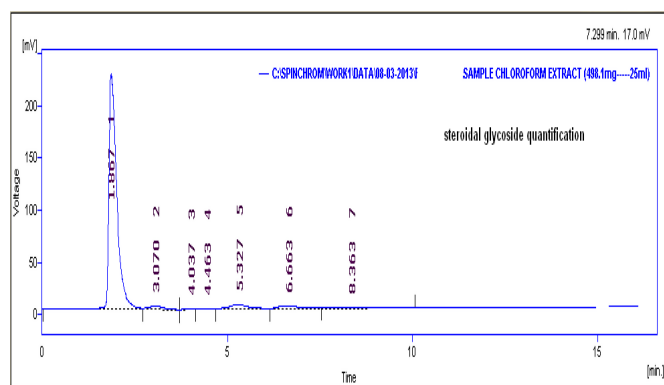


Fig 3: HPLC of chloroform fraction (CF)

The DPPH released free radical scavenging activity was determined and 50% of free radical inhibition was found to be 510 µg/ml for chloroform fraction of *Withania coagulans* bud and ascorbic acid respectively. The result was shown in Figure 4.

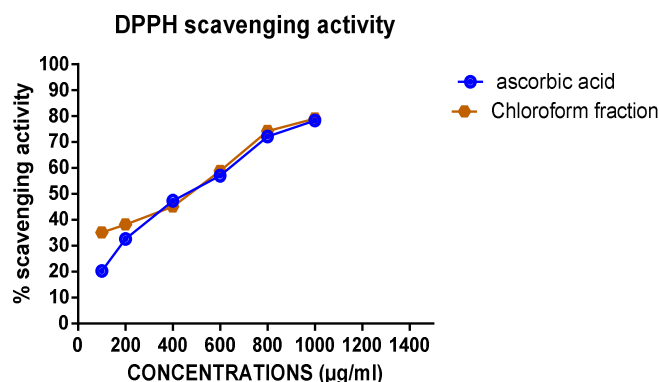


Fig. 4 DPPH scavenging activity of chloroform fraction (CF)

The Cytotoxicity studies showed that the fraction has concentration dependent increase in toxicity. The IC₅₀ value was found to be 84.7 µg/ml. The result was shown in Figure 5 and 6.

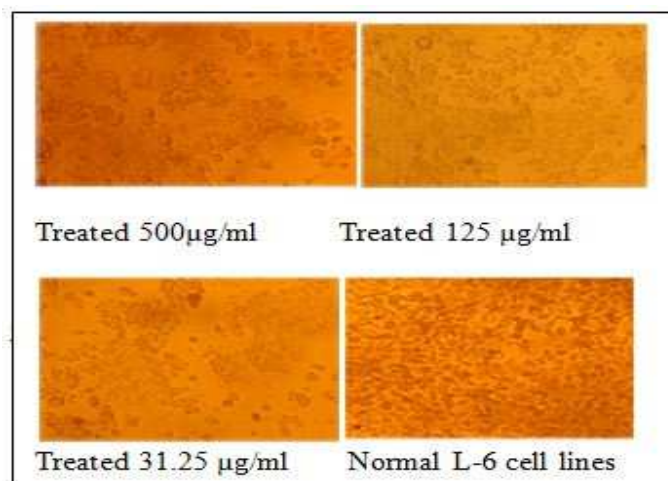


Fig 5: Cytotoxicity effect on skeletal muscle cells

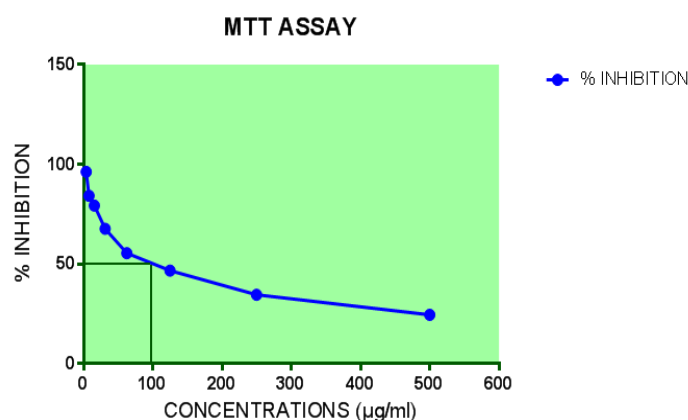


Fig 6: MTT assay on skeletal cell

The GLUT4 studies showed, when insulin is low, GLUT4 is sequestered in intracellular vesicles in muscle and fat cells. Insulin induces a rapid increase in the uptake of glucose by inducing the translocation of GLUT4 from these

vesicles to the plasma membrane. Glut 4 transporter uptake was measured using L6 cell lines with chloroform extract shows significant increase in the glucose uptake in GLUT 4 study. The result was shown in Figure 7.

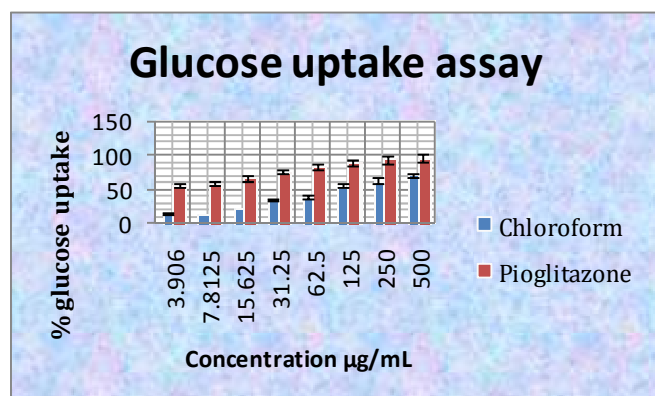


Fig 7: Glucose uptake assay

PPAR gamma expressed is measured in terms of optical density and is compared with relative intensity of β -actin. It was found that has there is a concentration dependent response of the chloroform fraction of *Withania coagulans* bud. Both GLUT 4 and PPARG studies β actin is used as standard and the test fraction shows good agreement with standard. The result was shown in Figure 8 and 9.

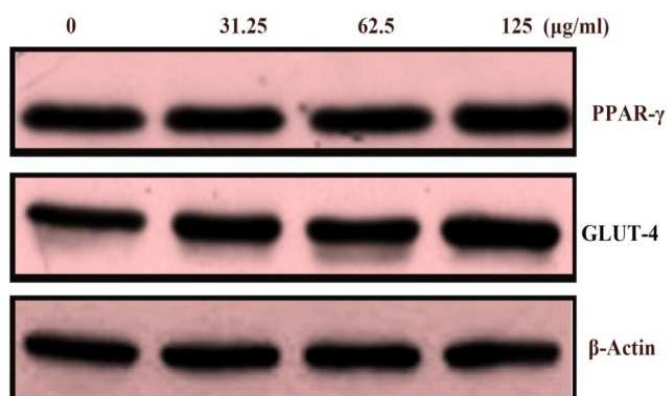


Fig8: GLUT-4, PPAR- γ , β -actin Fingerprinting

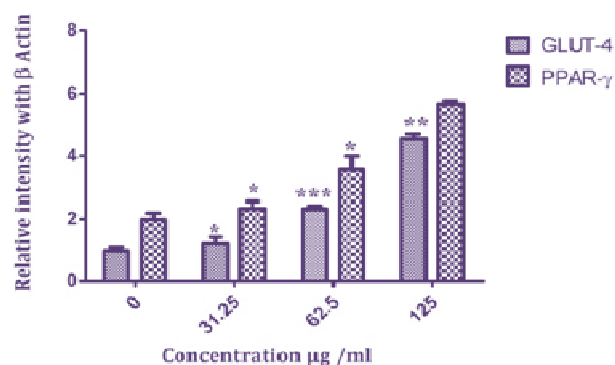


Fig 9: Comparison of GLUT-4, PPAR γ expressed with β -actin

CONCLUSION

From this study we can come to a conclusion that the chloroform fraction of *Withania coagulans* bud has hypoglycemic activity. This can be used for the treatment of insulin resistant diabetes mellitus by enhanced expression of ligand gated form of PPAR γ present in the skeletal muscles. The activity of the drug may be due to PPAR γ induction by the insulin mimicking activity of the chloroform fraction which contains steroidal glycosides and other chemical compounds.

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