PHARMACOGNOSTIC AND PHYTOCHEMICAL EVALUATION OF FLAVONOID RICH ETHYL ACETATE FRACTION OF *WITHANIA COAGULANS* DUNAL

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ABSTRACT: -

Withania coagulans (common name: Paneer Dodi) extracts have traditionally been used in the coagulation of milk to make Indian cheese and have also been used in the traditional Indian system of medicine due to its numerous therapeutic properties. The consumption of the raw aqueous extract is traditionally practiced by immersing the fruit overnight in water. The validation of these extracts against diabetes therefore warrants a rigorous and thorough investigation through scientific approaches.

Based on the above discourse, it was planned to screen various extracts of *W. coagulans* berries for the possible presence of glucose-lowering components. The phytochemical screening of aqueous, hydroalcoholic, ethanolic and methanolic extract revealed the rich presence of flavonoids in the aqueous extract which was further fractionated with ethyl acetate to recover flavonoid rich fraction. The presence of flavonoids was confirmed by chemical test and TLC. Further, quantitative estimation revealed the total flavonoid content of aqueous and ethyl acetate fraction to be 55.93 µg/ml and 57.44 µg/ml respectively. Thus it can be claimed that antidiabetic potential of aqueous extract of *Withania coagulans,* is attributed to the presence of flavonoids. The TLC studies also suggested that the major component amongst the present flavonoids could be Quercetin. However to support this finding further spectroscopic studies are planned.

INTRODUCTION:

Withania is a minor genus of shrubs in the Solanaceae family (which has about 2000–3000 species divided into around 90 families) [1]. *Withania* species may be found all across the world, from the East Mediterranean region to South Asia. *Withania coagulans* is a flowering plant of the Solanaceae family with roughly 23 species occurring as native in parts of North Africa, the Middle East, and the Canary Islands [2,3]and in various sections of Afghanistan, Pakistan, India, and Nepal. In India, *W. coagulans* may be found in Punjab, Rajasthan, Simla, Kumaun, and Garhwal. [4]

This plant is recognozed by different local names in different languages, such as Akri, Punir bandh, Paneer Dodi, or Puni-ke-Bij (Hindi), Khamjira (Punjabi), Tukhmekaknaje-Hindi (Persian), Spiubajja

(Afghanistan), Punirband or Punir-ja-fota (Sindhi), Indian Cheesemaker, Indian Rennet, Vegetable Rennet (English), Asvagandha (Urdu) [5]. *Withania coagulans* The name, Paneerdodi or Paneerdoda

came from the berries' property to coagulate milk to manufacture Indian cheese, and hence *coagulans* [6,7,8].

Withania coagulans (Paneer dodi) in aqueous extract form is popularly known in the old Indian system of medicine for the treatment of diabetes[9]. While the raw extract is typically consumed by soaking the fruit in water overnight, its bitter taste and strong odour dissuade ingestion of sufficient amounts to provide a health-beneficial effect [10,11]. To get around this, organically produced extracts can be encapsulated into concentrated versions that can be used as nutraceuticals [12].

Although, the literature suggests that the aqueous extract of *Withania coagulans* is an effective remedy for diabetes, there is no scientific data available for the antidiabetic active components there in. Therefore the efforts are made in this study to screen the fruits for the presence of potential components that can be further claimed for its antidiabetic potential.

Plant Profile:

Taxonomical Classification [12]:

Kingdom: Plantae

Division: Magnoliophyta

Order: Solanales

Family: Solanaceae

Genus: *Withania*

Figure 1 *Withania coagulans* plant

Chemical Constituents:

Withania coagulans berries contain an enzyme that causes milk to coagulate, two esterases, free amino acids, fatty oil, an essential oil, and alkaloids. Proline, hydroxyproline, valine, tyrosine, aspartic acid, glycine asparagine, cysteine, and glutamic acid are the amino acids that are present. The alcoholic fruit extract has yielded fourteen alkaloidal components [13]
Steroids, alkaloids, phenolic compounds, tannins, saponins, carbohydrates, proteins, amino acids, and organic acids are few more phytoconstituents found in this plant. Pharmacological research has linked these properties to the Withanolides, a steroidal lactone found in *Withania*. The principal Withanolides found in *Withania somnifera* and *Withania coagulans* include Withaferin A, Withanolide A, Withanone etc. The flavonoids isolated from berries showed significant antidiabetic activity [14].

1. Materials and Methods:

1.1 Plant Collection and identification:

Dried fruits of *Withania coagulans* were purchased from the local market in the month of October, 2021 and identified and authenticated by Alirsan Pharmaceuticals, Mumbai. The hulls from the fruits were removed and dried for 2-3 days. After thorough drying, the fruits were crushed into coarse powder by electrical grinder and stored in clean, sterilized airtight container for further use.

1.2 Morphological

The standardization of *Withania coagulans* fruit was carried out by examining its organoleptic characters. (Table 1 and Figure 4).

Microscopical evaluation of various parts of the plant was also carried out and compared with the official records (Figure 5,6,7).

1.3 Determination of Physicochemical Parameters

Proximate analysis:

Physicochemical constants such as moisture content, ash value, extractive value and foreign organic matter were studied using standard methods to determine its purity and stability [15].

a) Determination of foreign organic matter:

5 gm of air dried coarsely powdered drug was spread in a thin layer. The sample was inspected with the unaided eye. The foreign organic matter was separated manually as completely as possible. Sample was weighed and percentage of foreign organic matter was determined from the weight of the drug taken [15].

b) Determination of loss on drying:

2 gm of sample was transferred to accurately weighed glass-stopper, shallow weighing bottle and sample was distributed evenly and poured to a depth not exceeding 10 mm. Then loaded bottle was kept in an oven for one hour. The sample was dried to constant weight. After drying it was stored at room temperature in a glass desiccator. The loss on drying was calculated in terms of percent w/w [15].

c) Determination of ash value:

1. Ash value -

The ash value is used to determine the quality and purity of powdered crude medicines. Total ash, acid insoluble ash, and other ash values were estimated using fine powdered fruit [15].

a) Determination of Total Ash -

About 2 gm of the powdered drug was accurately weighed in silica crucible, which was previously ignited and weighed. On the bottom of the crucible, the powdered medication was placed in a thin, equal layer. The crucible was gradually burnt at a higher temperature until it was no longer red and carbon-free. After cooling, the crucible was weighed. The total ash was determined as a percentage of the total ash [15].

b) Determination of Acid Insoluble Ash -

The total ash was determined by boiling 25 ml of 2N hydrochloric acid for five minutes and filtering the resulting ash. The ash less filter paper was used to capture the insoluble ash, which was then washed in hot water. The insoluble ash was transferred to a pre-weighed silica crucible that had been fired, cremated, cooled, and weighed before to use. To keep the weight consistent, the technique was repeated. Using the air-dried medication as a reference, the percentage of acid insoluble ash was calculated [15].

d) Determination of extractive value:

1. Extractive Value –
   These are useful for determining the ingredients of crude drugs that can't be determined any other way. It also specifies the kind of ingredient found in the medicine. The Indian Pharmacopoeia, Indian Herbal Pharmacopoeia, and British Herbal Pharmacopoeia all have distinct sorts of extractive values. The selected plant was subjected for following extractive values [16]**.**

a) Alcohol Soluble Extractive Value - About 5 gm of powdered components were macerated for 24 hours in a stoppered conical flask with 100 ml of 90% ethanol, with intermittent shaking during the first 6 hours, and the first 5 ml was discarded. The filtrate was then evaporated on a tarred evaporating dish for 25 mL, and the residue was dried at 105 °C until it reached a consistent weight. In comparison to the air-dried sample, the percentage of alcohol soluble extractive was determined [16].

b) Water Soluble Extractive Value - In a stoppered conical flask, around 5 gm of powdered material was macerated with 100 ml of chloroform water for 24 hours, with intermittent shaking during the first 6 hours and the first 5 ml discarded. The filtrate was evaporated in 25 ml on a tarred evaporating dish, and the residue was dried at 105 °C until it reached a consistent weight. In comparison to the air-dried material, the proportion of water-soluble extractive was determined [16].

1.4 Extraction:

For extraction standard procedure recommended in Harborne et al,1973 was adapted Air-dried *W. coagulans* fruit (1kg) was crushed in mechanical grinder and the coarse powder was used for the extraction [17,20].

A) Aqueous Extract:

1. Cold maceration- Cold maceration was achieved by dissolving 100g coarse powder in 300ml distilled water and leaving it for 24 hours. The next day, the extract was recovered by filtering off the coarse powder with a Whatman filter paper.

2. Hot decoction- For a hot decoction, 100g coarse powder was boiled with 300ml distilled water in a multi-station water bath for 1-1.5 hours, followed by filtration and concentration.

B) Hydro-alcoholic Extract:

1. Cold maceration- For cold maceration of hydroalcoholic extract, 100g coarse powder was dissolved in 300ml of 1:1 water: ethanol solvent ratio and allowed to macerate for 24 hours.

The extract was recovered the next day by filtering off the coarse powder using Whatman filter paper.

C) Ethanolic Extract:

1. Cold maceration- For obtaining ethanolic extract by cold maceration, 100g coarse powder was dissolved in 300ml of 90 percent ethanol and allowed to macerate for 24 hours The extract was recovered the next day by filtering off the coarse powder using Whatman filter paper.

D) Methanolic Extract:

1. Soxhlet method- The dried fruits of *Withania Coagulans Dunal* were coarsely crushed and exhaustively extracted in a Soxhlet apparatus, using methanol as a solvent.
2. Cold maceration- Same procedure as mentioned in B and C was adopted.

2.5 Phytochemical Screening of *Withania coagulans* Dunal Extracts:

The fruit extracts prepared above were subjected to preliminary phytochemical screening for the detection of various constituents present. The term qualitative analysis refers to the establishing and proving the identity of a substance. The active ingredients, after isolation, can be incorporated into the modern medicine system for the development of newer formulation for therapeutic ailments [18].

2.6 Thin Layer Chromatography:

On a 10x10 cm and 0.25 mm thick plate, TLC was carried out according to conventional protocol. In a 1:2 ratio, a slurry of Silica Gel G was made (1part Silica and 2 parts water). It was applied on a grease-free glass plate. The coat was 0.25 mm thick. For 10-20 minutes, the plate was left to air dry at room temperature. After that, the plate was activated in a hot air oven for 1 hour at 100-120 °C. Meanwhile, the plates are being activated, and the mobile phase is being prepared and stored in a TLC solvent chamber for saturation. Various extracts (aqueous, methanolic, ethanolic, hydroalcoholic) were spotted onto the plate using a capillary after the silica plate had been activated. The chromatoplate was developed by placing it in a saturated TLC solvent chamber. The diverse solvent systems utilised for various extracts for various compounds. Before the solvent front reached the plate's top edge, the development was stopped. After removing the chromatoplate, it was left to dry for 10 minutes [19].

2.7 Quantitative Analysis:

The estimation of total contents of Flavonoids in Aq. extract of *W. coagulans* fruit was carried using standard protocols described as follows.

a) Total Flavonoid Estimation:

The modified spectrometric technique was used to quantify the total flavonoid content of the aqueous extract. Several concentrations of quercetin (standard) were prepared from a 1 mg/ml stock solution, including 0.2 mg/ml, 0.4 mg/ml, 0.6 mg/ml, 0.8 mg/ml, and 1 mg/ml. In order

to calculate the extract's flavonoid concentration, 0.5 ml of extract was made from several stock solutions containing 1 mg/ml flavonoids. 0.15 mL of a 5% sodium nitrate solution was added after diluting the solution to 2.5 mL, and it was then incubated for 6 minutes at room temperature. At room temperature, 0.15 mL of 10 percent aluminium chloride was added and incubated for 6 minutes. 2 mL of sodium hydroxide at 4% was added and well mixed. After mixing, the solution was diluted to 5 mL with distilled water and allowed to sit at room temperature for 15 minutes. The absorbance was calculated at 415 nm. The extract's total flavonoid concentration was calculated as mg of flavonoid / g of extract [20].

3. Fractionation of crude extract:

100 ml of cold aqueous extract was treated with 50 ml 2M HCL for hydrolysis (boil for 45min at 100^0c), The extract then was filtered and was mixed with equal volumes of ethyl acetate in a separating funnel. Ethyl acetate layer was then separated and tested chemically for the presence of flavonoid aglycones. (Figure 3) [18].

Figure 3: Ethyl Acetate Fraction

3.1 Phytochemical Screening of Flavonoid Rich Ethyl Acetate Fraction:

The Flavonoid Rich Ethyl Acetate Fraction were subjected to preliminary phytochemical screening for the detection of various constituents present especially for the confirmation of flavonoid content. [18.21].

3.2 Thin Layer Chromatography for Flavonoid Rich Ethyl Acetate Fraction:

Solvent systems were developed for establishing the TLC patterns for the ethyl acetate fraction of the *Withania coagulans*. Various visualization techniques were used to come up with the best TLC fingerprint, like UV 254, UV 366. The developed plates were dried in air, visualized in UV at wavelengths 254 and 366 nm and photographed [19,20,21].

3.3 Quantitative Analysis:

a) Total Flavonoid Estimation:

The total flavonoid content in flavonoid rich ethyl acetate fraction was determined by modified spectrometric method [20].

4. Result and Discussion:

a) Macroscopic description:

Leaves: 2.5-5.7 by 1-2.2 cm., lanceolate-oblong, obtuse, entire, clothed with a persistent not easily detachable greyish tomentum, of a uniform colour on both sides, thick, more or less rugose, base acute, running down into an often-obscure petiole; petiole 6 mm. long but often indistinct.

Flowers: Dioecious, in axillary clusters; pedicles 0-6mm. long, deflexed, slender

Fruits: Berry 6-8 mm. diam., globose, smooth, closely girt by the enlarged membranous calyx which is scurfy pubescent outside.

Seeds: 2.5-3mm diameter, dark brown, ear-shaped, glabrous; Flowering period: from January to April and berries ripen during January to May. The natural regeneration is from seed.

Figure 4 Fruit Macroscopy

b) Microscopical description:

The microscopical powdered characteristics of *Withania coagulans*. fruit were studied. The results of powdered characteristics are presented in (Figure 2,3,4)

Fruit was transversely sectioned for anatomical identification, and the section revealed that the epidermis is uniseriate and made up of thin walled, or non-cuticularized tubular cells, and that certain epidermal cells' outer walls are extended outward to create unicellular structures. Vascular bundles in the parenchyma are radial and comprised of bigger, polyhedral cells with noticeable intercellular gaps. Powdered tissue displayed pitted and lignified parenchyma under a microscope. Two distinct parenchyma cell types, bigger polyhedral cells with intercellular space and smaller cells without intercellular gaps, were identified. Fruit characteristics that can be seen up close can be used as diagnostic indicators. Microscopic examination of the transverse slice revealed the presence of scalariform thickening in the arteries and pitted, lignified parenchyma cells, which are traits of the Solanaceae family.

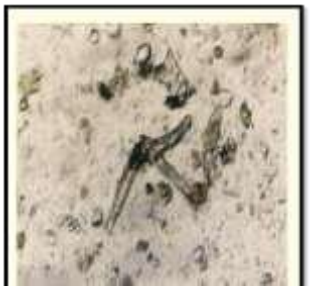

Figure 5
Trichomes

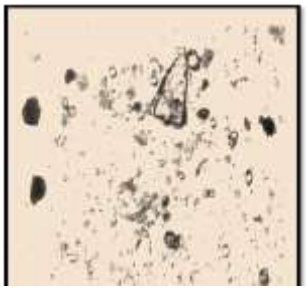

Figure 6
Calcium oxalate crystals

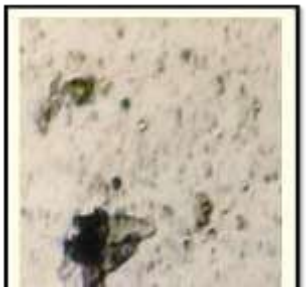

Figure 7
Parenchyma cells

c) Physicochemical standards:

Various physicochemical standards such as total ash, acid insoluble ash, water soluble extractive and alcohol soluble extractive values are reported in (table 1).

Table 1: Physicochemical Evaluations:

Physicochemical Standards	**%W/W**
Foreign Matter	2.90%
Moisture Content	1.2%
Loss on Drying	5%
Total Ash	4%
Acid Insoluble Ash	0.5%
Water Soluble Extractive	21.44%
Alcohol Soluble Extractive	5.27%

Figure 8 Foreign matter

Figure 9 Total ash

d) Preliminary phytochemical screening:

Phytochemical screening of cold aqueous extract and hot aqueous extract showed the presence of alkaloids, carbohydrate, amino acid, flavonoids whereas hydroalcoholic extract, ethanolic extract and methanolic extract showed the presence of mainly carbohydrate, amino acid, protein, steroids and flavonoids (Table 2).

Table 2: Preliminary phytochemical screening

Sr No	Phytoconstituents	Aqueous extract	Hot Aqueous extract	Hydro alcoholic extract	Methanolic extract	Ethanolic extract
1	**Test For Alkaloids**					
	Dragendroff's Test	-	-	-	++	+
	Mayer's Test	-	-	-	++	+
	Wagner's Test	-	-	-	++	+
	Hager's Test	-	-	++	++	+
2	**Test For Carbohydrates**					
	Feheling's Test	++	++	++	++	++
3	**Test For Amino Acid**					
	Xanthoprotein Test	++	++	++	++	++
4	**Test For Protein**					
	Millions Test	-	-	++	++	++
5	**Test For Steroids**					
	Salkowski Test	-	-	++	++	++
6	**Test For Flavonoids**					
	Shinoda Test	++	++	++	++	++
	Alkaline Reagent Test	++	++	++	++	++
	Lead Acetate Test	++	++	++	++	++

e) Thin Layer Chromatography:
Thin Layer Chromatography of cold aqueous extract showed the presence of phenolics and flavonoid compounds (table 3,4).

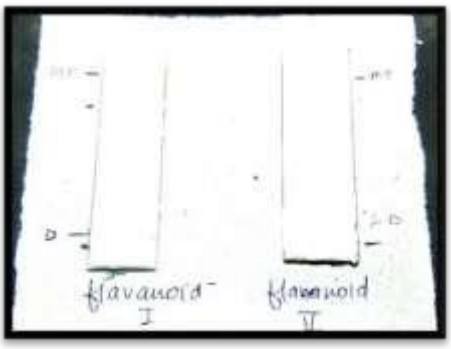

Figure 10 TLC for Flavonoids

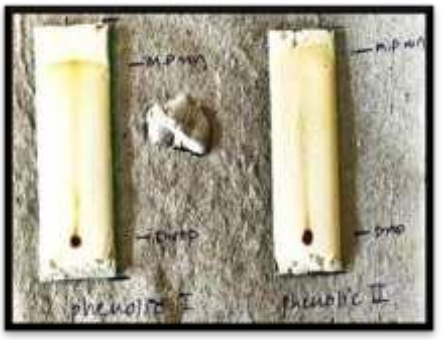

Figure 11 TLC for Phenolics

Table 3: Thin Layer Chromatography:

Sr. No.	**Phytoconstituent**	**Solvent System And Ratio**	**Rf Value**
1	Alkaloids	1) Toluene: Ethyl Acetate: Diethyl Amine (14:4:2 V/V/V) 2) Chloroform: Methanol (17:3 V/V)	1) -- 2) --
2	Phenolics	1) Toluene: Ethyl Acetate (18.6:1.4 V/V) 2) Toluene: Acetone: Formic Acid (9.2:9.2:0.7 V/V/V) 3) Benzene: Methanol: Acetic Acid (15:2.8:1.4 V/V/V) 4) Ethyl Acetate: Benzene (9:11 V/V)	1) -- 2) -- 3) 0.58 4) 0.32
3	Steroids	1) Toluene: Ethyl Acetate (18.6:1.4 V/V)	1) --
4	Carbohydrates	1) Methanol: Water (18:2 V/V)	1) --
5	Flavonoids	1) Acetic Acid: Conc. HCL: Water (13.8:1.38:4.6 V/V/V) 2) Chloroform: Ethyl Acetate (12:8 V/V)	1) 0.84 2) --
6	Tannins	1) Chloroform: Water (12:8 V/V)	1) --

Table 4: Thin Layer Chromatography:

Sr. No.	**Compounds**	**Solvent System**	**Rf Value**
1	Quercetin, Kaempferol, and Related Compounds	1) Hexane: Chloroform (10:10 v/v) 2) Methanol: Chloroform: Acetic acid (10:10:1v/v/v)	1) 0.85 2) 0.64
2	Flavanone, Chalcone	1) Methanol: Water (15:5 v/v)	1) 0.90
3	Flavonoids	1) Methanol: Water: Acetic acid (6:12:2 v/v/v)	1) 0.77

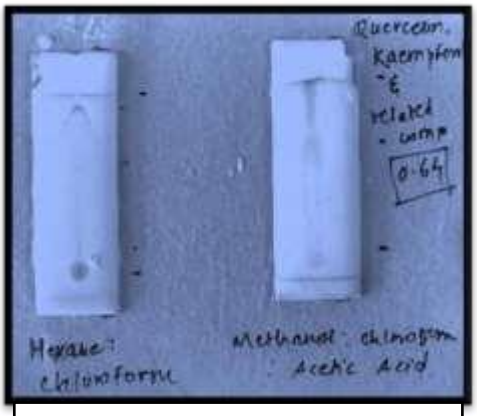

Figure 12 TLC for Quercetin, Kaempferol, and Related Compounds

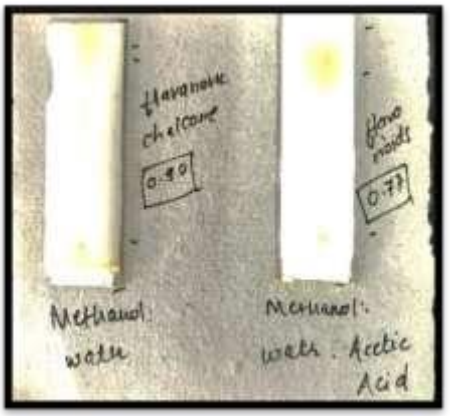

Figure 13 TLC for Flavanone, Chalcone and Flavonoids

Quantitative Analysis:

The quantitative analysis of the extract in order to estimate the concentration of Flavonoids was performed and following results were obtained. (table 5).

Table 5: Total Flavonoid Estimation:

Sr.No.	Extract	Flavonoids
1	Aqueous extract	55.93 µg/ml
2	Flavonoid Rich Ethyl Acetate Fraction	57.44 µg/ml

Phytochemical Screening of Flavonoid Rich Ethyl Acetate Fraction:

Phytochemical screening of Flavonoid Rich Ethyl Acetate Fraction shows the presence of flavonoid group (table 6).

Table 6: Phytochemical Screening of Flavonoid Rich Ethyl Acetate Fraction:

Sr. No.	Phytoconstituents	Ethyl acetate fraction	Water fraction
1.Test For Alkaloids	Dragendroff's test	-	-
	Mayer's test	-	-
	Wagner's test	-	-
	Hager's test	-	-
2.Test for Carbohydrates	Feheling's test	-	-
3.Test For Amino acid	Xanthoprotein test	-	-
4. Test for Proteins	Millions test	-	-
5.Test for Steroids	Salkowski test	-	-
6. **Test for Flavonoids**	Shinoda test	+++	+++
	Alkaline Reagent Test	+++	+++
	Lead acetate test	+++	+++

f) Thin Layer Chromatography Flavonoid Rich Ethyl Acetate Fraction:

Thin Layer Chromatography of cold aqueous extract confirmed the presence of quercetin compound (table 7).

Table 7: Thin Layer Chromatography Flavonoid Rich Ethyl Acetate Fraction

Sr. No.	Compound	Solvent System	RF Value (Standard Quercetin)	RF Value (Ethyl Acetate Fraction)
1	Quercetin	1) Acetic acid: Con. HCL: Water (13.8:1.38:4.6 v/v)	0.90	0.89
		2) Methanol: Chloroform: Acetic acid (10:10:1 v/v)	0.89	0.86
		3) Toluene: Methanol: Ethyl acetate (8:8:2 v/v)	0.86	0.84

Figure 14 TLC for Quercetin

Figure 15 TLC for Quercetin

5. Conclusion:

W. coagulans has been reported to exhibit several pharmacological activities. As mentioned in Kirtikar & Basu 1999, *Withania coagulans* berries are used for the treatment of asthma, biliousness, strangury, wounds, dyspepsia, flatulent colic, chronic liver complaints and intestinal infections in the indigenous system of medicine. In some parts of Indian sub- continent, the berries are used to treat diabetic patients. The ethanopharmacology of the plant states that the aqueous extract of *W. coagulans* berries *when taken empty stomach is* very effective in lowering the raised blood sugar levels. Steroidal lactones that are reported in the plant are not detected in aqueous extract, which indicates that the antidiabetic activity may not be due to steroidal lactones present. That raises the question as to what sugar lowering components could be present in the aqueous extract. This scientific question was addressed by performing the phytochemical screening of various extracts of the berries. The cold aqueous extract which showed rich presence of flavonoids was further fractionated with ethyl acetate to recover flavonoid rich fraction. The qualitative and quantitative estimation indicated the rich presence of quercetin which will further be confirmed by spectroscopic evidences.

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