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QUANTIFICATION OF CARBOHYDRATES, PROTEINS AND ANTIOXIDANT ENZYMES IN WITHANIA COAGULANS FROM BALOCHISTAN

Misbah Javed[1], Ayesha Mushtaq[2]*, Farida Behlil[1], Farukh Bashir[1], Musarrat Riaz[1], Shagufta Fahmid[1], Fazeela Mandokhail[1], Abdul Haq[3]

[1]**Department of Chemistry,** Sardar Bahadur Khan Women's University (SBKWU), Quetta, Pakistan
[2]**Department of Bio-Chemistry,** Sardar Bahadur Khan Women's University (SBKWU), Quetta, Pakistan
[3]**Directorate of Water Management (HEIS),** Agriculture Research Institute (ARI), Sariab Road, Quetta, Pakistan
***Corresponding Author:** Ayesha Mushtaq. E. mail: ayeshamushtaq2000@yahoo.com

Abstract
Withania coagulans is getting very popular due to its medicinal properties. The present work was intended to quantify the level of carbohydrates, proteins and antioxidant enzymes including Superoxide dismutase, Catalase and Peroxidase in herbal medicinal plant Withania coagulans collected from different areas of Balochistan. To check the presence of carbohydrates, seeds of Withania coagulans were treated with Fehling Reagent which confirmed the presence of reducing sugars. The quantification of carbohydrates was done by using Anthrone method and finally absorbance was checked at 630 nm. Quantification of proteins was conducted by exercising Bradford Method and absorbance was measured at 595 nm by using spectrophotometer. Enzymatic extraction from seeds and leaves was accomplished by using phosphate buffer and centrifugation at 1500 rpm for twenty minutes. Superoxide dismutase level was assessed by utilizing Giannopolitis & Ries method whereas catalase and peroxidase activities were determined by employing strategy of Chance & Maehly with certain modifications. The results disclosed mean carbohydrate level of 6.27 mg/kg which signifies its hypoglycemic properties. The mean protein contents for seeds and leaves are 265.67 mg/kg and 186.33 mg/kg respectively. Superoxide dismutase contents are found to be the highest amongst all three antioxidant enzymes. Hence, it can be concluded that Withania coagulans due to presence of different phytochemical constituents has several protective and medicinal uses.
Key words: *Catalase, Extraction, Peroxidase, Superoxide dismutase, Withania coagulans*

INTRODUCTION

Markets are loaded with variety of synthetic medicines having significantly higher costs, genuine side effects on living tissues along-with adverse effects on the environment and biodiversity whereas, therapeutic plants and the medications acquired from herbal plants are lower in prices, have little or no side effects, environment friendly and are well recognized amongst people (1). Almost 75-80% of the entire population is dependent on medicinal flora (2) as the same is extensively utilized for the cure and prevention of certain ailments (3-5). Pakistan is bestowed with rich biodiversity and favorable geographical environment besides important curative plants reserves (6), yet its flora is neither explored properly from curative prospects nor for food and dietetic esteem (7); regardless of the fact that medicinal plants are deliberated as most precious supplements in treating different ailments (8). Herbal medicinal plants assume a vibrant role in the manufacturing of modern medicines (9).

Withania coagulans commonly known as Paneer bandh is considered as an essential medicinal plant (10), whose taxonomy reflects that the plant relates to Family: *Solonaceae,* Genus: *Withania,* Tribe: *Physaleae* and Subfamily: *Solanoideae* (11). The botanical features of *Withania coagulans* enlightened that it's a rigid grayish-white tiny herb, 30-90cm height, leaves 2.5-7.5 centimeters, occasionally sub-divided quadrangular, classically ellipsoid, thickheaded and tapered at the nethermost and really small peduncle. Its leaves are thickly lined with tiny, grayish, stellated tomentum while flowers are seven to twelve metrics linear, yellow and dioeciously polygamous. They are in auxiliary determinate groups, seeds are ear fashioned, hard calyx and in brown hue, glabrous with brown pulp and have fruit like scent (12). The flower blooming period of

the plant is November - April and berries developed during January-May. No distinction is made in the berries sold in the market attained from *W. coagulans* and *W soninifera*. Microscopic examination showed that both species are indistinguishable including smooth and whitish surface and short and bland crack (13).

Withania coagulans includes diversified phytochemicals viz. alkaloids, steroids, esterases, phenolic mixes, saponins, tannins, proteins, starches, free amino acids, greasy oils, fatty oils, basic oils, essential oils and natural acids. Pharmacological examination elucidated the relationship of these constituents with the specific steroidal lactones named as "Withanolides" found in *Withania coagulans*. The aforementioned phytochemicals found in *W. coagulans* makes it magnificent for its consumption by accustomed practioners (14). Almost all plant parts including fruits, seeds, leaves, roots, stem, bark and berries are consumable entities (11). The notable phytochemical constituents in it are “Withanolides” primarily found in leaves and roots which may range 0.001-0.5% in concentration on dry weight basis (15). The plant is used in dried form and autumn is an appropriate time for its harvesting (16). Warm weather conditions like in tropics (Indian Subcontinent: Nepal, Bharat and Pakistan) and Western Asia including Afghanistan are the native sites of *Withania coagulans* (17).

Withania coagulans is renowned to treat dropsy, upset stomach, ulcers, viscose infections, rheumatism, and other infectious diseases (18). Fruit of aforementioned plant shows anti-hyperglycemic activity against diabetes mellitus (19-21). Liquefied extract of *Withania coagulans* fruit exhibits antidyslipidemic and antihyperglycemic activity in animals (22). Some other useful attributes of this plant are anti-inflammatory (23), cardio toxicity (24), wound healing activity (19), antifungal (25), hypolipidemic (26), and hepatoprotective (27). The fruit being distinctive in its properties is widely consumed as a “blood purifier” besides recommendation of the same against different respiratory disorders (19).

Balochistan due to its dry and warm weather conditions serves as favorable site for the growth of *Withania coagulans* which possess different curative and preventive attributes like antidyslipidemic, antihyperglycemic, antibacterial, antifungal etc. The present work is intended to elucidate the defense mechanism of *Withania coagulans* which includes antioxidant enzymatic assay like Catalases, Superoxide Dismutases, & Peroxidases obtained from (Khuzdar, Mach, Kharan, Musakhail, Sibi and Harnai) regions from Balochistan province of Pakistan. Currently, people are not well aware of its pharmaceutical importance in Balochistan, therefore this study is deliberated to quantify the carbohydrates, proteins and antioxidant enzymes; Peroxidases, Catalases & Superoxide Dismutases levels in *Withania coagulans* and to investigate its significance as herbal medication against various ailments.

MATERIALS AND METHODS

SAMPLE COLLECTION

Withania coagulans plant samples (leaves and seeds) were collected from different areas of Balochistan (Khuzdar, Mach, Kharan, Musakhail, Sibi and Harnai). Plant sample was identified from department of Botany Sardar Bahadur Khan Women’s University Quetta Balochistan. After shade drying the plant, samples (leaves and seeds) were crushed into powder and stored into airtight containers for further analysis.

PREPARATION OF EXTRACT

For extract preparation 1000g of plant samples were macerated with methanol for one week. Then these filtered and by using rotary evaporator at 35°C filtrate was evaporated to gain crude extract for various quantitative and quantitative analysis.

QUALITATIVE EXAMINATION FOR CARBOHYDRATES

Mathur, Agrawal & Shrivastava, approach was employed to determine the occurrence of carbohydrates wherein, minute volume of the test plant seed (methanolic extract) filtrate was placed into the test tube which follows an addition of Fehling’s Reagent (2 ml) in the same test tube and afterwards placement in the water bath at 60°C (28). Subsequently, the same test tube encompassing filtrate and

Fehling's Reagent was supplemented with 2 ml of concentrated H_2SO_4 which caused the development of brick red shading that confirmed the existence of reducing sugars.

QUANTITATIVE EXAMINATION FOR CARBOHYDRATES

Carbohydrate quantification was materialized by utilizing the methodology of Hedge and Hofreiter (29) in which hydrolysis of complex sugars into simpler ones was accomplished by consuming dilute hydrochloric acid. Later on, carbohydrates were permitted to dry out to hydro methyl furfural in warm acidic medium. The biochemical thus acquired arranges with a green shaded element anthrone; that shows absorption mostly at 630 nm. Plant sample was measured upto 100 mg and hydrolyzed by placing in water bath with 5 ml of 2.5 N Hydrochloric acid (HCl) for three hours and permitted to cool at 25-30℃. The arrangement was treated with sodium carbonate (Na_2CO_3) until the effervesce stopped, enhancing the capacity to 100 ml and then centrifuged it which triggered the formation of supernatant. One millilitre of the supernatant was taken for evaluation and 1 ml of the working preparation was utilized to plan principles. A quantity of 1 ml was used from all the test tubes consuming refined water and 4 ml of anthrone reagent was incorporated in this preparation and subsequently allowed to warm in a bubbling water bath for 8 minutes. This arrangement is permitted to cool swiftly and read the green to dull green absorbance at 630 nm.

QUALITATIVE EXAMINATION FOR PROTEINS

To determine the existence of proteins, Mathur, Agrawal and Shrivastava procedure (28) was employed in which a test tube having 2 ml of filtrate with 2 ml of 10% sodium hydroxide preparation was taken and afterwards allowed to warm for 10 minutes. Minute volume of 7% copper sulfate solution was added in the above blend which resulted in the development of purplish violet concealing and hence, it affirmed the presence of proteins.

PROTEIN QUANTIFICATION

Bradford strategy (30) was employed to enumerate protein contents in seed and leave samples of *W. coagulans* wherein; Bovine Serum Albumin (BSA) is utilized as a standard. The Bradford reagent was formulated in a way that (0.1g) of "Coo massive blue" was degenerated in 50 ml refined water and subjected to stirring for 10 minutes. Afterwards, it was diluted with refined water up to 200 millilitres and filtration was done. 10 ml of conc. Bradford reagent was further diluted 1:5 proportion to make working solution. For standard curve, dilution of BSA 0.8, 0.6, 0.4, 0.2 and 1 ml preparation was applied. The test tubes were labelled for leaf extract and one for blank solution separately. The reagent containing test tubes comprises of 100 µl of protein sample & 5 ml Bradford reagent whereas the blank ones encompass 100 µl of distilled H_2O & 0.1 ml BSA. The solutions incubated in black for 20 minutes & absorbance was noted at 595nm by means of spectrophotometer. Standard curve was plotted for different dilutions.

ENZYME EXTRACTION

For antioxidant enzymatic extraction, weighing of seeds & leaves of *Withania coagulans* on a digital balance was accomplished. Half gram of both leaves and seed samples was taken and then crumpled in pestle & mortar utilizing 50 milli-molar chilled phosphate buffer solution (PH 7.8), pursued by filtration via cloth stuff. Filtrate thus obtained was permitted to centrifuge at 1500 rpm for twenty minutes at 4^0C & the supernatant was consumed for enzymatic extraction.

SUPEROXIDE DISMUTASE (SOD) ASSAY

Giannopolitis and Ries method (31) was consumed for accomplishment of Superoxide Dismutase (SOD) assay and obstruction of photochemical reduction of Nitro blue tetrazolium was detected at 560nm by UV/VIS spectrophotometer. For SOD Assay, solution encompassing 50 µl of the enzymatic abstract and 50 µl of Nitro Blue Tetrazolium (NBT) deteriorated in Ethanol was utilized. Around 1.3 µl riboflavin, 13 mM of Methionine, 75 mM of Ethylene Diamine Tetra Acetate (EDTA) and phosphate buffer (7.8 pH) was supplemented. Five test tubes were booked comprising various quantities of enzyme abstract of leaves of *W. coagulans* as 0.8, 0.6, 0.4, 0.2, & 1 ml adulterated with distil water to create volume of 1ml and addition of

above solution was made in all test tubes. Another additional test tube containing all other reagents and having no plant extract was taken. Identical treatment was exercised with seed extracts of *W. coagulans*. This preparation was permitted to remain under the fluorescent light (30 W) for five minutes to attain a standard curve. Absorbance was tested after five minutes with UV/VIS spectrophotometer at 560nm by comparing the enzymatic solution with plant specimen.

CATALASE ASSAY

Activity of catalase was determined by applying the methodology of Chance & Maehly (32) with bit modifications. A preparation comprising enzymes in various dilutions as (0.2, 0.4, 0.6, 0.8, and 1 ml), 50 mM potassium phosphate buffer with pH 7.0 (2 ml) and 5.9 mM hydrogen peroxide (900 µl) was taken. The reaction commenced soon after placement of enzymatic abstract. Diminution in the absorbance of the reaction solution was witnessed at 240 nm after every 30 seconds utilizing UV/VIS spectrophotometer.

PEROXIDASE ASSAY

Chance & Maehly Method (32) with certain changes was applied for the determination of Peroxidase activity as was used in Catalase Assay. An arrangement was taken encompassing enzymes in various dilutions as (0.2, 0.4, 0.6, 0.8 and 1 ml) with 50 mM potassium phosphate buffer with pH 7.0 (0.9 ml), 20 mM hydrogen peroxide (1 ml) and Guaiacol (1 ml). The reaction got initiated after placement of enzymatic abstract which resulted in an upsurge in the absorbance of the reaction solution witnessed at 470 nm noted after every 20 seconds by UV-Vis spectroscopy.

RESULTS

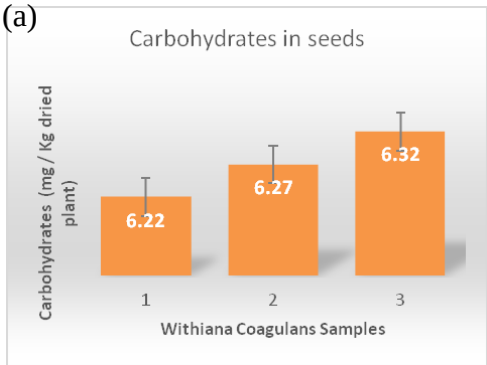
The results of the current study showed that *Withania coagulans* seeds contain slightly variable concentration of carbohydrates with an average value of 6.27±0.05 mg/kg of the dried plant (Fig. 1a). For protein quantification, it was noted that absorbance got amplified with the rise in concentration of protein. Amplification in absorbance was noted by utilizing UV/VIS spectrophotometer wherein, a standard curve was strategized for an upsurge in absorbance. The linear regression equation was applied for evaluating protein contents in the leave samples of *Withania Coagulans*. Mean Protein concentration recorded in leaves is 186.33±5.51 mg/kg (Fig. 1b). Protein quantification in three different seed samples of *Withania coagulans* showed a highest value of 270 mg/kg with a lowest protein concentration of 260 mg/kg. The mean protein concentration noted in seeds was 265.670 mg/kg (Fig. 1c).

This study also unveiled that absorbance also amplified with the upsurge in the concentration of enzyme extract at 590 nm using UV-Vis Spectrophotometer. The Superoxide Dismutase (SOD) contents in leave specimens were substantiated and the mean value observed was 66.37± 0.92 units/mg of proteins in seeds and 19.19±0.75units/mg in seeds respectively (Figs. 1d & ie). Contrary to SOD assay, for determination of Catalase activity, diminution in the absorbance with the rise in the quantity of enzyme extract was observed at 300 nm UV-Vis Spectrophotometer. Enzyme quantification in plant samples was done by applying linear regression equation. Mean values for Catalase enzyme thus obtained in seed and leave samples are 4.62±0.09units/mg and 13.46±0.71 units/mg respectively (Figs. 1f & 1g).

Peroxidase assay also showed increased absorbance with the increase in the concentration of enzyme extract at 470nm. The highest Peroxidase activity recorded in seeds was 5.69units/mg while lowest being noted is 3.99units/mg with a mean value of 4.86±0.85units/mg of proteins. Similarly, mean Peroxidase enzyme value noted in leave samples of *Withania coagulans* was 6.37±0.58 units/mg of proteins (Figs. 1h & 1i).

The overall comparison of all biochemical constituents showed that proteins are the most abundant biomolecules in the seeds of *Withania coagulans* with a mean value of 265.670±5.13 mg/kg of a dried plant sample (Fig. 1j). The Antioxidants enzymatic comparison unveiled that Superoxide Dismutase is the most abundant enzyme in seeds of *Withania coagulans* with a mean value of 66.37±0.92 units/mg while Catalase enzyme concentration was found to be the lowest amongst all the three enzymes and the mean value recorded was 4.62±0.09units/mg of protein contents. Similarly, Superoxide Dismutase concentration was

(a)



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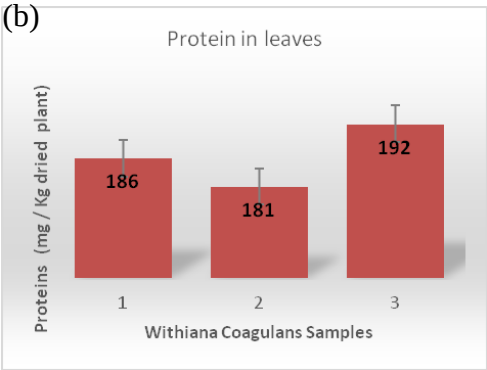

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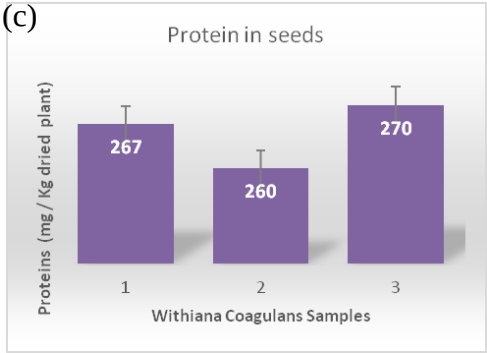

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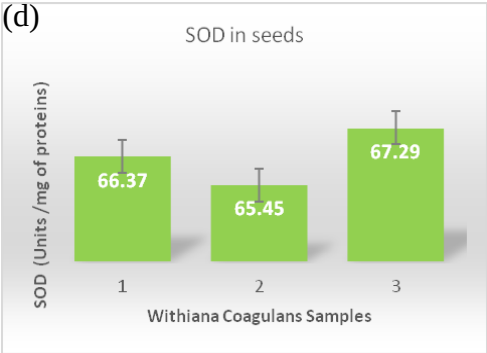

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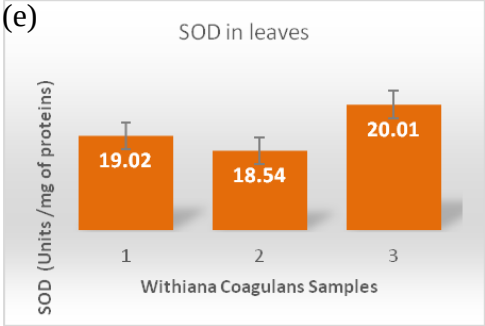
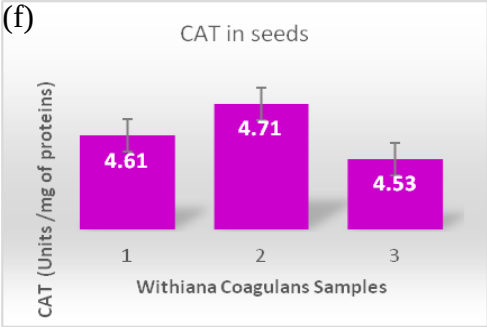
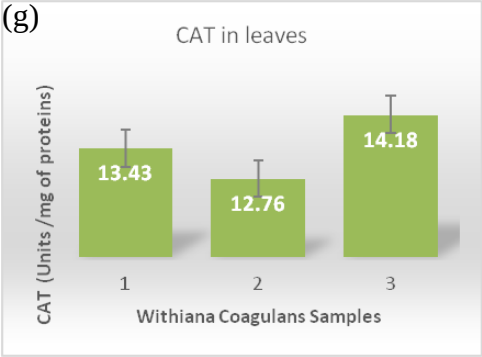


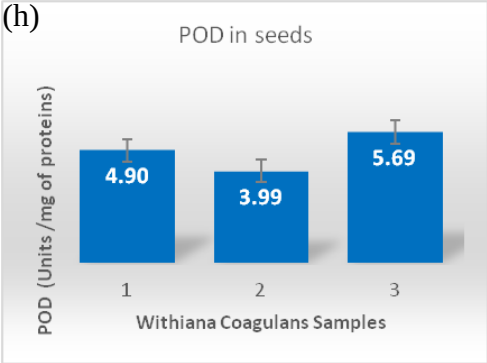
(f)



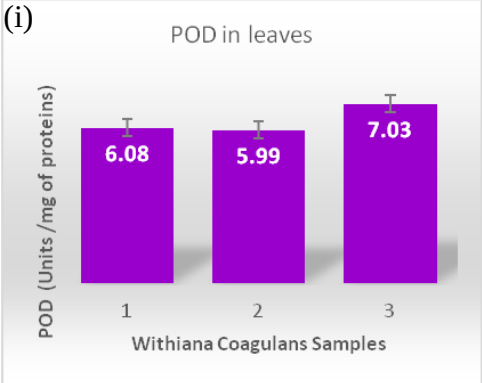
(g)



(h)



(i)



(j)

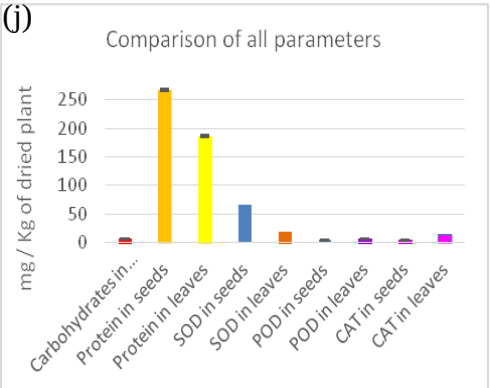


Fig. 1(a). Carbohydrate conc in seeds of *W. Coagulans;* **(b).** Protein in leaves of *W. Coagulans;* **(c).** Protein in seeds of *W. Coagulans*; **(d).** SOD contents in seeds of *W. Coagulans*; **(e).** SOD concentration in leaves of *W. Coagulans*; **(f).** Catalase conc in seeds of *W. Coagulans*; **(g).** Catalase conc in leaves of *W. Coagulan*; **(h).** Peroxidase conc in seeds of *W. Coagulans*; **(i).** Peroxidase conc in leaves of *W. Coagulan*; **(j).** Comparison of all parameters in *W. Coagulans*

found to be the highest in leave samples with a mean value of 19.19±0.75 units/mg while the lowest concentration was recorded for the Peroxidase enzyme whose mean value was 6.37±0.58 units/mg of Protein. The results revealed that Superoxide Dismutase (SOD) was found to be the highest in both leave and seed samples of *Withania coagulans* amongst all the three antioxidant enzymes. All the values of analyzed parameters are presented in Table I. Three samples *Withania coagulans were analyzed* for each parameter; carbohydrates, proteins, Antioxidant enzymes (SOD, CAT, POD) in seeds and Leaves. Statistical analysis was performed, and Mean values, Standard Deviation and standard Errors are presented in Table I.

Table I. Mean Values of different parameters of *Withania Coagulans*

Plant Part Used (leaves/Seeds)	Name of Bio-molecules	Plant Samples			Mean Value	St. Deviation	St. Error
		Sample 1	Sample 2	Sample 3			
Seeds	Carbohydrates	6.22	6.27	6.32	6.27	0.05	0.03
	Proteins	267	260	270	265.67	5.13	2.96
	Superoxide Dismutase	66.37	65.45	67.29	66.37	0.92	0.53
	Peroxidase	4.90	3.99	5.69	4.86	0.85	0.49
	Catalase	4.61	4.71	4.53	4.62	0.09	0.05
Leaves	Protein	186	181	192	186.33	5.51	3.18
	Superoxide Dismutase	19.02	18.54	20.01	19.19	0.75	0.43
	Peroxidase	6.08	5.99	7.03	6.37	0.58	0.33
	Catalase	13.43	12.76	14.18	13.46	0.71	0.41

*Values of different parameters carbohydrates, proteins, Antioxidant enzymes (SOD, CAT, POD) in seeds and Leaves of *Withania Coagulans* are presented as means of three values along with their Standard Deviation and Standard Error

DISCUSSION

A variety of plants are known to have significant medicinal importance and are being used traditionally in homes and currently in Pharmaceutical industry for the cure and prevention of different infectious and metabolic ailments. The consumption of herbal medication is universally increasing because of fewer or no side effects, cost effectiveness and are readily available as compared to the synthetic and inorganic medications (22). The hypoglycaemic plants have been utilized in most parts of the world as their characteristic features comprises of both inorganic and organic constituents. The inorganic ingredients of therapeutic herbal plants essentially contain minerals which assumes an instrumental job in promoting hypoglycaemic activity (28, 29). Presently, *Withania Coagulans* the herbal medicinal plant is used to study its Phytochemical constituents as the plant is known to have various therapeutic and curative attributes which include anti-inflammatory, antimicrobial, antidyslipidemic, antihyperglycemic, Central Nervous System depressant, anti-cancerous, wound healing, antifungal, hypolipidemic, hepatoprotective, cytotoxic activities and is also utilized as a blood purifier. This plant due to its geographic suitability and favourable ecological niche is found generally in hot weather conditions typically in tropical parts of Pakistan.

In the current research work, seeds and leaves of *Withania coagulans* were utilized to enumerate phyto-constituents like proteins, carbohydrates and antioxidant enzymes. The study revealed that *Withania coagulans* contain significant amount of carbohydrates, proteins and antioxidant enzymes, proteins being the most abundant phytochemical constituents. Similarly, Superoxide Dismutase was found to be the most abundant antioxidant enzyme amongst all the three antioxidant enzymes. Antioxidants are fortified for settling or neutralizing free radicals prior to their attack on cells and are entirely rudimentary for maintaining ideal cell functionality and play a primary role in the well-being and prosperity of living tissues. Peroxidase takes an attention in outstanding number of physiological processes like biosynthesis of lignin and ethylene, defends against microbes and injuries, digests auxins and provides relief during stress

conditions. Peroxidase is also acknowledged as one of the best heat stable catalyst in plants and this heat shielding effect has been supported by several other agents. During dry spell conditions, the movement of anti-cancerous agents has also been witnessed as altered. In such cases, peroxidase encompasses as the key defense line against reactive oxygen species (ROS) and triggers alteration in its mobility and consequently renowned as an emblem of a redox status changer in homoiohydric plants.

In addition to antioxidants, W. coagulans is also found supplemented with steroidal lactones, well-known as Withanolides (naturally occurring polyhydroxy C-28 steroidal lactones). The various pharmacological properties of this plant are believed to be due to the presence of adequate quantities of antioxidants, withanolides and other phytochemicals and almost our body synthesizes its own antioxidant defense system; besides, antioxidants and poly-nutrients also exist in organic food that makes our body healthy (30). The anti-oxidant enzymes like Catalase, Peroxidase and Superoxide Dismutase are valued for body' defense framework as they encourage wound healing and eliminate ROS. Different kinds of environmental stress (dry & hot weather, chilling) seemed to cause an enhancement in free-radicals and lipid hydroperoxides due to diminution in the activity of catalase. Flexibility of plants to circumstances triggering harm might be due to their greater ability to remove ROS through ROS detoxifying biochemicals like Superoxide dismutase, Catalase and Ascorbate peroxidase, thereby, assumes shielding task to the natural compounds from oxidative damage.

The present study not only confirmed the presence of antioxidants in *Withania coagulans* but also quantified the amount of antioxidant enzymes along-with proteins and carbohydrates. The findings of this study are found in consonance with the aforementioned studies. Different pharmacological properties are found associated with *Withania coagulans* which are attributable because of the diversity of phytochemicals which may have different modes of action but collectively they have a sympathetic and helping role in the normal functionality of cells. In this study, the abundance of the proteins, presence of adequate quantities of antioxidant enzymes confirmed the hypoglycemic, anti-cancerous, anti-inflammatory, wound healing, blood purifying and antifungal properties of *W. coagulans*.

CONCLUSION

The present study quantified carbohydrates, proteins and antioxidant enzymatic machinery which include Peroxidase, Catalase and Superoxide dismutase. Proteins are found to be the most abundant biomolecules amongst all the tested biomolecules while superoxide dismutase is in higher concentration amongst all the three antioxidant enzymes. The abundance of proteins and an existence of adequate quantities of antioxidant enzymes confirmed the pharmacological properties of *Withania coagulans* which makes it a good herbal plant. Hence, it can be concluded that seeds and leaves of *Withania coagulans* being an herbal plant can be used as a curative and preventive measure against different ailments.

Conflict of interest:

Authors have no conflict of interest.

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