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Research Article

Effects of ethanolic and aqueous extracts of *Withania coagulans* in prevention of oxidation and glycation mediated protein, DNA and lipid damage

E. R. Banerjee[1*], A. Marik[1], R. Dutta[1] and S. Biswas[1]

[1]Immunology and Regenerative Medicine Research Unit, Department of Zoology, University of Calcutta, Kolkata-700019, West Bengal, India

Abstract

Protective dietary components derived from plants have been the subject of increased attention in human nutrition research in recent years, giving rise to the concepts of functional foods and nutraceuticals. Compared to the production of chemical medications, which can involve large-scale industrial procedures and result in waste, harvesting and growing medicinal plants can be a more sustainable, eco-friendly and holistic approach. Traditional remedies from their local settings, which make them accessible and economical, especially in rural places, are undoubtedly a better option for long-term environment-friendly development. *Withania coagulants*, also known as Rishwagandha- is significant from an economic and medicinal standpoint. They are cultivated and used in numerous regions, and they are crucial to Southeast Asian traditional medicine. The study highlights the importance of *Withania coagulans* extract as a functional dietary supplement in combating oxidative stress-related diseases, particularly those linked to modern dietary habits. The findings reveal that the water extract consistently exhibits the highest potency across a range of assays, including BSA oxidation, insulin oxidation, DNA oxidation, lipid peroxidation, AGE formation, alpha-amylase, alpha-glucosidase, and lipase inhibition. The water extract demonstrates not only the highest average inhibition percentages but also the lowest IC50 values, indicating superior antioxidant and enzyme inhibitory properties compared to the ethanolic extracts. The water extract's ability to inhibit lipid peroxidation and enzymatic activity associated with postprandial hyperglycemia underscores its potential to mitigate the health risks associated with unhealthy diets, making it a promising eco-friendly, sustainable dietary supplement in modern nutrition.

Keywords: Biochemical assays, Extract, Health benefits, *Withania coagulans*

Highlights

- *Withania coagulans* holds both economic and medicinal value, crucial in traditional medicine.
- *Withania coagulans* extract as functional dietary supplement, for oxidative stress-related diseases.
- Extracts tested are: water extract (W), 70% ethanol extract (E70), and 100% ethanol extract (E100).
- Water extract showed the highest potency in various biochemical assays.
- Water extract had the lowest IC50 values (superior antioxidant and enzyme inhibitory properties).

INTRODUCTION

Plants, like most other organisms, allocate a significant amount of resources to enhance their own chance of survival by adapting to various stressors and changing environments. These large plethora compounds obtained from plants often retain their functions ex-planta or outside the plant and are extensively exploited by humans for the betterment of our own lives. Majority of food additives, fragrances, flavorings substances, agrochemicals, pesticides and most important medicines originate from plants. Nearly 40-45% of all pharmaceuticals used in allopathic medicine are natural chemicals or sourced from natural sources; according to WHO, among all the available drugs, the top 20 drugs are derived from plants. Nearly 75% of all infectious diseases and 60% of cancers can be prevented by natural products (Weststrate *et al*., 2002). Herbal remedies have long been used to treat and prevent a wide range of illnesses, and plants continue to be the sole sustainable natural source of various secondary metabolites with potential medical applications because complex natural chemicals are usually difficult to synthesize otherwise.

Protective dietary components originating from plants have become a more significant focus of human nutrition research in recent years from a health perspective, leading to the concept of functional foods and nutraceuticals. International Food Information

*Corresponding Author, E-mail: erb@caluniv.ac.in; enaraybanerjee@gmail.com

Council defines functional foods as foods that may provide health benefits beyond basic nutrition. A wide range of chemical compounds produced by plants that do not directly support a plant's growth and development are referred to as secondary metabolites (Mandal *et al*., 2021). Plants produce secondary metabolites like terpenoids, alkaloids, and phenylpropanoids, which are essential to their survival under specific ecological conditions including biotic and abiotic stresses. Secondary metabolites are distributed across a smaller area than primary metabolites. A certain secondary metabolite is often associated with a specific taxonomic group or species of plant; one such family of plants is Solanaceae.

The Solanaceae family of plants is significant and has long been used in both conventional medicine and human nourishment. Solanaceae members are abundant in bioactive compounds and have long been used by various cultures across the world (Afroz *et al*., 2020; Kova *et al*., 2021). As a member of the Solanaceae family, *Withania coagulans* is sometimes referred to as Indian cheese producer, paneer dodi, or vegetable rennet (Fig. 1). This plant's fruit is used in the dairy industry to coagulate milk. The 84 genera that make up the Solanaceae family contain approximately 3,000 species. Of these, only two species- *Withania somnifera*, also known as Ashwagandha, and *Withania coagulans*, also known as Rishwagandha- are significant from an economic and medicinal standpoint. The primary pathophysiological abnormalities of type 2 diabetes (T2DM)- insulin resistance and inadequate insulin secretion- as well as the ensuing diabetic complications are the main focus of the potential pharmacological treatment of this condition. The majority of the secondary health issues occur due to the formation of advanced glycation end products that further lead to an unending cycle of free radical generation and oxidative damage. Anti-age therapy is also a recent attribute of diabetes management, and many of the upcoming drugs are in preclinical testing; hence, natural substances and their molecular structures are currently important places to start looking for new drugs. In humans, medication options include thiazolidinediones, statins, ALT-711 (alagebrium), angiotensin converting enzyme inhibitors, benfotiamine, pyridoxamine, and aminoguanidine. Alagebrium, thiazolidinediones, and statins are the most current and promising anti-AGEs medications. Although some of the research supports the effectiveness of these pharmacological strategies, other reports yielded contradictory results showing toxicity (Nenna *et al*., 2015; Song *et al*., 2021). Previous studies have demonstrated that a wide range of natural substances, including vitamins, alkaloids, terpenoids, polyphenols, polysaccharides, and polysaccharides, are promising candidates for the development of novel medications that block the production of AGEs. By scavenging free radicals, chelating metal ions, ensnaring

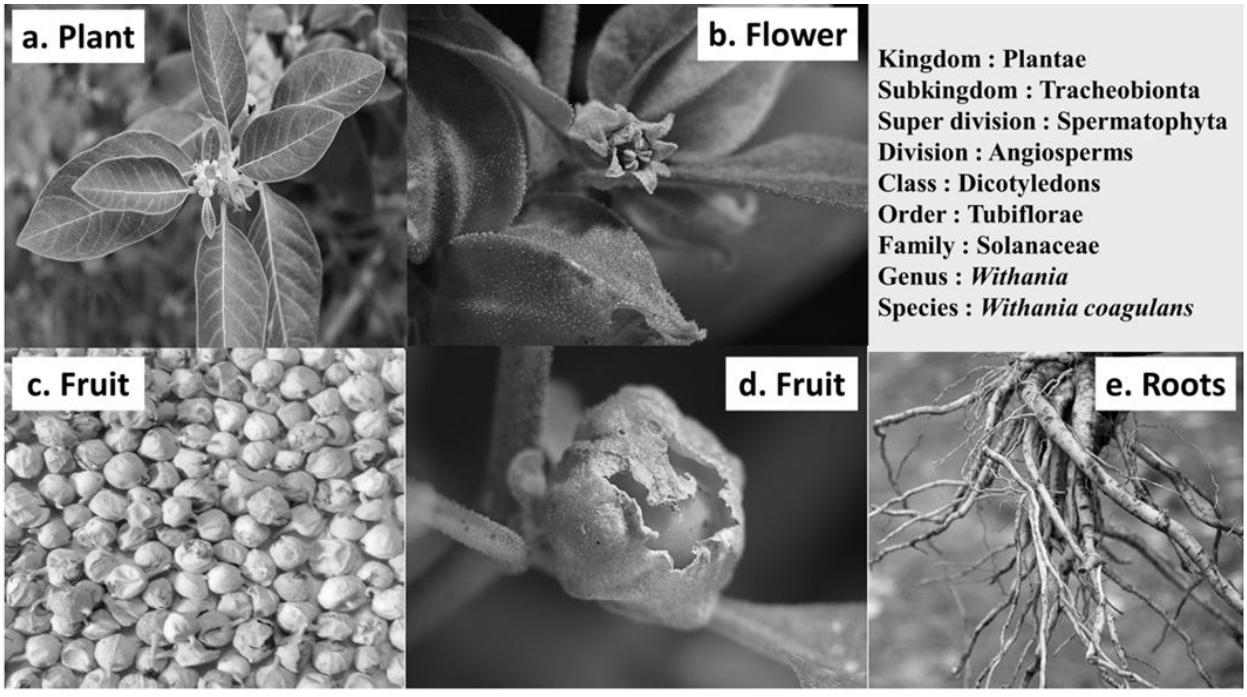


Fig. 1. Parts of *Withania coagulans* plant that have been used in traditional medicine.

active carbonyl compounds, covering the glycation sites of proteins, and reducing blood glucose levels, these substances prevent the formation of AGE as *Withania coagulans* has been documented to possess antidiabetic, antimicrobial, anti-inflammatory, anticancer, hepatoprotective, antihyperglycemic, cardiovascular, immunosuppressive, free radical scavenging, and central nervous system depressive properties. As a result, active compounds isolated from plants with a long history of use in Ayurvedic and indigenous medicine (Dhar *et al*., 2015) are likely to be safer than those obtained from plant species with no history of human use, making *W. coagulans* (Rishwagangha) ideal for drug discovery studies (Khan *et al*., 2021).

MATERIALS AND METHODS

Formation of extract from fruits of *Withania coagulans*

The process began with the collection of dried fruits from *Withania coagulans*. These fruits were then mechanically crushed using a pestle and mortar to produce a coarse powder. The powder obtained was weighed, and a portion was soaked in a 1:10 (W/V) mixture of water and ethanol (70% and 100%) for 48 hours, with the mixture kept on a rocker during working hours. After the incubation period, the extract was filtered using Whatman filter paper (90 mm) to separate the coarse powder. The water extract was immediately stored at -20°C, while the alcoholic extract was subjected to evaporation in a water bath at 60°C before being stored at the same temperature. Extracts were then lyophilized. Following this process, the lyophilized extracts were stored in a cold room at 4°C for further experimentation. For the water extract, the incubation period was limited to 24 hours to prevent contamination.

Effect of *Withania coagulans* extract in prevention of oxidative damage

Protein-oxidation inhibition assay: The pathophysiology of diabetes and its related consequences has been linked to oxidative stress, which is caused by an imbalance between the body's antioxidant defense systems' capacity to neutralize free radicals and their production (Collins *et al*., 1998; Miwa *et al*., 2000). The build-up of oxidative stress products, especially reactive oxygen species, can cause damage to a variety of biological macromolecules, such as proteins, lipids, and DNA, in people with poorly managed diabetes (Ighodaro, 2018). ROS generation is elevated in chronic hyperglycemia. Numerous proteins, including those essential for insulin signaling, can be harmed by these ROS (Bloch-Damti and Bashan, 2005). An important side effect of the elevated oxidative stress seen in diabetes is protein oxidation. Diabetes has been linked to elevated levels of protein carbonyls, a measure of protein oxidative degradation, in affected persons. These levels have also been linked to the severity of complications associated with diabetes. To check for the protein effect of *Withania coagulans* on protein oxidation, 40 µL of plant extract was added to 100 mL of 50 mM potassium phosphate buffer containing NaF. 50 µL of 10 mM $FeSO_4$ and 10 mM 50 µL of EDTA were also added in the presence of 2 µL of 30% hydrogen peroxide (H_2O_2) solution. The mixture was kept for 5 minutes for incubation. After that, 40 µL of 50 mg/mL bovine serum albumin or 40 µL of 1 IU of insulin (56 mM) was added and incubated for 30 minutes. Then, 50 µL of trichloro acetic acid (TCA) was added to stop the reaction and centrifuged at 4000 rpm for 10 minutes. The supernatant was discarded, and the pellet was dissolved in the prepared buffer solution and analyzed the intrinsic fluorescence in spectrophotometer at excitation wavelength of 350 nm and emission at 450 nm for BSA and at excitation of 275 nm and emission of 305 nm for insulin (Njayou *et al*., 2008; Xu *et al*., 2008; Guedes *et al*., 2009; Talha *et al*., 2021). Green tea (T) was used as a positive control.

DNA-oxidation inhibition assay: Just like proteins, DNA oxidation and damage is also one of the main ways that oxidative stress may contribute to the pathogenesis of diabetes (Adel *et al*., 2018). DNA damage caused by oxidative stress can include base alterations, single-strand breaks, and double-strand breaks. This damage can impede cellular function, cause cellular malfunction, and eventually result in the development of diabetes complications (Krapfenbauer *et al*., 1998; Zhang *et al*., 2005). For determining the effect of *Withania coagulans* on the oxidation of DNA, DNA was extracted from goat liver and then in 200 µL of potassium phosphate buffer, 0.5 µL of 10 mM $CuCl_2$, 30% of H_2O_2, 5 µL of 1 mM acetic acid, 10 µL of plant extract was added and kept for 5 minutes for incubation. After that, 20 µL of extracted DNA was added and incubated for 10 minutes. EDTA was added as a chelating agent, and the DNA was measured by its absorbance at 260 nm, adding EtBr in a spectrophotometer. Ascorbic acid was used as the positive control (Lodovici *et al*., 2001; Adinortey *et al*., 2018). Green tea (T) was used as a positive control.

Lipid peroxidation inhibition assay: Lipids also undergo oxidation ROS, such as hydroxyl radicals, which initiate lipid peroxidation by removing allyl hydrogen from lipid molecules, creating lipid-free

radicals with a carbon nucleus. Lipid-free radicals combine with oxygen to form lipid peroxy radicals. These radicals then chain-react, continuously generating new lipid free radicals and lipid hydrogen peroxide by extracting hydrogen from other lipid molecules. For lipid peroxidation assay, 250 µL of 10% egg homogenate made in distilled water, V/V, was added to 50 µL of extract. Then, the volume was made up to 500 µL by adding dH_2O. Next, 25 µL of $FeSO_4$ (0.07 mM) was added to it. After that, this mixture was incubated for 30 minutes. Then 750 µL of 20% acetic acid (pH 3.6) was added with it. 750 µL of 0.8% TBA (w/v) (prepared in 1% sodium dodecyl sulphate) was added to the mixture. After that, 25 µL of 20% TCA was added with it. Then, it was vortexed and heated in boiling water for 60 minutes. Next, it was cooled. After cooling, 3 mL of 1-butanol was added with it and centrifuged at 3000 rpm for 10 minutes. The organic upper layer was collected, and OD was measured at 532 nm (Chang and Kim, 2018). Ascorbic Acid (AA) was used as a positive control.

Effect of *Withania coagulans* extract in prevention of Advance Glycation End-product formation

Protein-Advance Glycation End-product formation inhibition assay: Glycation is a non-enzymatic reaction between biomolecules (protein, lipids and nucleic acids) and reducing sugars like glucose, fructose and ribose. After a long period of time, this forms a heterogeneous group of chemical moieties (Amadori products) known as Advanced Glycation End-products (AGEs). The process of AGE formation is spontaneous. In the case of diabetes mellitus type II, a persistent hyperglycemic state in the blood glucose leads to the formation of these advanced glycation end-products (AGEs). The AGE has its chief cellular receptor RAGE. Upon engagement, it activates a lot of signaling pathways like MAPK, TGF-β, JNK, etc., generating an increased level of oxidative stress, protein denaturation, and thus inflammation. For Protein-Advance Glycation End product formation inhibition assay 40 µL of Bovine serum albumin of conc. 50 mg/mL or 40 µL of 1 IU of insulin (56 mM) was taken in 100 µL of 50 mM K phosphate buffer in PBS (pH-7.4). Then, 40 µL of 200 mM glucose in PBS (pH-10) was added and mixed. The plant extract was added, and the mixture was incubated at 50°C for 48 hours and kept blank at 4°C. After the incubation, it was mixed with 40 µL of 100% TCA (Trichloro Acetic Acid). Following that, it was centrifuged for 4 minutes at 15000 RPM and 4°C. The supernatant was discarded. Then the pellet was dissolved in 200 µL of PBS (pH-7.4) and 200 µL of PBS (pH-10). Next, the fluorescence spectrophotometry analysis was done in a spectrophotometer at excitation 350 nm - emission 450 nm for BSA and at excitation 275 nm - emission 305 nm for insulin (Julius and Hopper, 2017; Premakumara and Abeysekera, 2023). Green tea (T) was used as a positive control.

DNA-Advance Glycation End product formation inhibition assay: DNA can cross-link with several chemicals, including nucleotide AGEs such as N2-carboxymethyl oxyguanosine and 5-glycolyl deoxycytidine. Glycation of DNA begins with the creation of Amadori products and eventually leads to DNA AGEs which can cause mutations, DNA strand breakage, cytotoxicity, and inflammation. Accumulated products are found in disorders such as diabetes, uremia, atherosclerosis, asthma, arthritis, myocardial infarction, nephropathy, retinopathy, periodontitis, neuropathy, and others that cause inflammation (Al-Hindawi *et al*., 1992; Khalid *et al*., 2022). DNA was extracted from goat liver using Invitrogen DNA isolation kit. After getting pure DNA of concentration 481 µg/mL, the DNA was treated with a reducing sugar (glucose), the same as protein, to produce DNA-AGEs and added plant extract to check the inhibitory effect of the formation of DNA-AGEs. EtBr binds to the minor groove of the double helix structure of the DNA and gives fluorescence. By using this characteristic, we compared the total pure DNA content, the damaged DNA content and the inhibited damaged DNA content using the spectrophotometer at 510 excitation 590 emission (Ashraf *et al*., 2012; Bagherzadeh-Yazdi *et al*., 2020). Green tea (T) was used as a positive control.

Effect of *Withania coagulans* extract on metabolic enzymes

Reducing the rise in blood glucose that is dependent on diet is a well-known treatment strategy for the management and prevention of diabetes. This can be accomplished by inhibiting digestive enzymes like alpha-amylase and alpha-glucosidase in the gut, which blunts the body's natural ability to break down carbohydrates in food.

Alpha amylase inhibition assay: Alpha-amylase, which is primarily found in saliva and the pancreas (Stiefel and Keller, 1973), hydrolyses the glycosidic bonds in starch and related substrate polysaccharides to produce oligosaccharides and simple absorbable sugars. This aids in initiating the chemical process of breaking down carbohydrates, thereby increasing postprandial hyperglycaemia. Then about 50 µL of plant extract was added in a micro-centrifuge tube together with 50 µL of

0.02M sodium phosphate buffer of pH 6.9 containing α-amylase solution (0.5 mg/mL). Next, pre-incubation was done at 25°C for about 10 minutes. After that, 50 µL of 1% starch solution in 0.02M sodium phosphate buffer of pH 6.9 was added at timed intervals. The solution was incubated at 25°C for 10 minutes. In order to end the reaction, 100 µL of the dinitrosalicyclic acid (DNS) reagent was added with it. Next, the tubes were incubated in boiling water for 5 minutes, followed by cooling down the solution at room temperature. One mL of distilled water was added to the reaction mixture prior to measuring the absorbance using the spectrophotometer at 540 nm (Poovitha and Parani, 2016). Acarbose (A) is used as a positive control.

Alpha glucosidase inhibition assay: Glucosidases are enzymes that hydrolyze glycosidic bonds in carbohydrates, leading to the release of glucose. These enzymes break down the complex carbohydrates (such as starch and glycogen) and disaccharides into simple sugars like glucose. Alpha-glucosidase hydrolyzes the terminal alpha-1,4-glycosidic linkages of oligosaccharides, releasing single glucose units. Alfa-glucosidase hydrolyzes alfa-glycosidic bonds in alfa-linked polysaccharides. Increased glucosidase activity has been seen in many diabetes complications, suggesting a possible relationship between this enzyme and the development of this disease (Ofosu *et al*., 2020). For the alpha-glucosidase inhibition assay, 20 µL of 1U/mL alpha-glucosidase enzyme was taken in a potassium phosphate buffer solution, and 40 µL of different concentrations of plant extract was added to it. Incubated for 15 minutes at 37ºC temperature. After that, 40 µL of PNPG substrate was added and incubated for 40 minutes at room temperature. Then, we stopped the reaction using 100 µL of 0.1M Na_2CO_3. The whole mixture was cooled down, and the PNPG content was measured using a spectrophotometer at absorbance spectra of 540 nm. (Bhatia *et al*., 2019). Green tea (T) is used as a positive control.

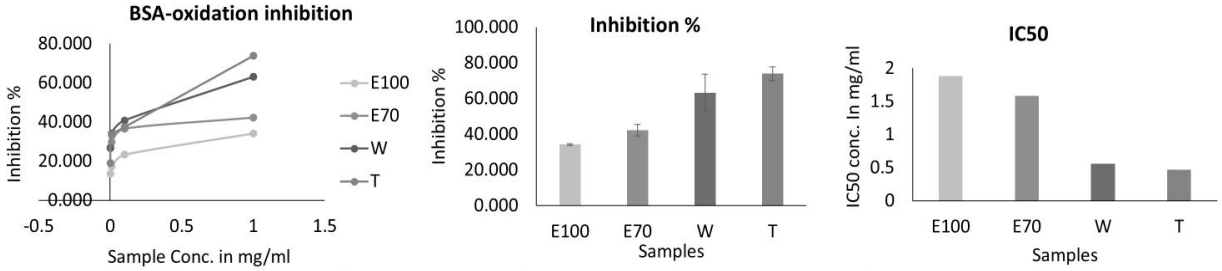
Lipase inhibition assay: Lipase is an enzyme primarily involved in the digestion, transport, and processing of dietary lipids (fats, oils, and triglycerides). It catalyzes the hydrolysis of triglycerides into free fatty acids and glycerol. This process is essential for the absorption of dietary fats in the intestine. Elevated free fatty acid levels have been linked to insulin resistance, as they decrease glucose transport and insulin signaling in skeletal muscle. For the Lipase inhibition assay, at first, PNP substrate was made by 5 mµ in 2 propanol 1 mg/mL and enzyme stock was prepared 1 mg/mL by taking 40 units in dH_2O. Working concentration was made by 25 µL adding in 975 µL dH_2O. Now, the sample was taken 10 µL, 200 µL dH_2O and 20 µL enzyme and incubated for 5 minutes at 30ºC. Then 50 µL of 5 mM PNP-A substrate was added and incubated at 30°C for 15 minutes. OD value was checked using a spectrophotometer at absorbance spectra 540 nm Jaradat *et al*., 2017. Green tea (T) is used as a positive control.

Calculations

The IC50 was calculated in a x-y plot with the data fitting in straight line (linear regression). IC50 value was then estimated using the fitted line, i.e., Y = a * X + b, IC50 = (0.5 - b)/a. where y = % scavenging and x = concentration. Y = 50 is used to identify the concentration. Sample t-test was used to compare each sample to the blank value to find whether the extract made any significant difference. To compare the different test groups, One-way ANOVA was done, followed by Tukey's test for post HOC analysis. Tukey's Honestly Significant Difference (HSD) test indicates if a difference between groups is statistically significant. A p-value less than 0.05 indicates that the differences between groups are actually significant. All the statistical analyses were done using Microsoft Excel and the following software (https://astatsa.com/OneWay_Anova_with_TukeyHSD/).

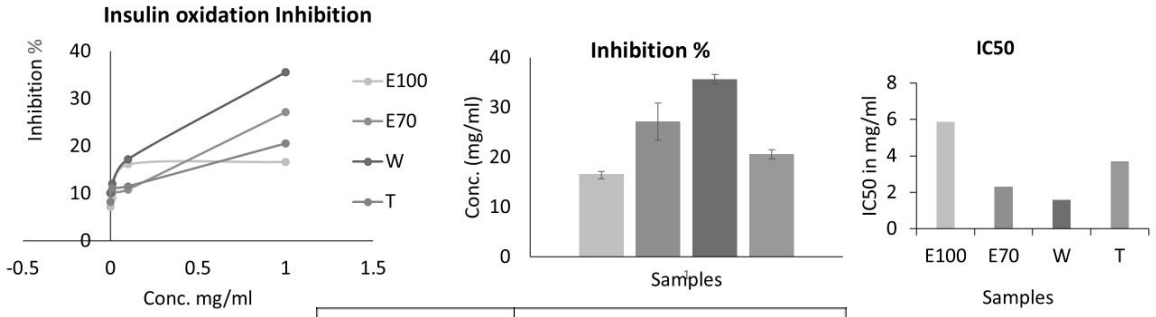
RESULTS

Protein-oxidation inhibition assay: In the BSA oxidation inhibition assay, the average inhibition percentages were 42.36% for E70, 34.228% for E100, and 63.162% for W. The IC50 values, representing the concentration required to achieve 50% inhibition, were 0.560 mg/mL for W, 1.586 mg/mL for E70, and 1.884 mg/mL for E100 (Fig. 2). These values indicate that W is the most effective at inhibiting BSA oxidation, followed by E70 and E100. Therefore, W exhibits the highest inhibition of BSA oxidation, with E70 and E100 showing progressively lower levels of inhibition. A one-way ANOVA was done to compare the different test groups, followed by Tukey's test for post-HC analysis. Tukey's Honestly significant difference (HSD) test indicated a statistically significant difference of $p<0.05$ between positive control green tea extract and ethanolic extracts of *Withania coagulans* and between water extracts of *Withania coagulans* and ethanolic extracts of *Withania coagulans*. There was no significant difference between the activity of ethanolic extracts of *Withania coagulans*. Similarly, there was no significant difference between the activities of the positive control green tea extract and water extract of *Withania*



Treatment Pairs	Tukeys HSD P- value inference
T vs E70	**P<0.01
T vs E100	**P<0.01
Tvs W	Insignificant
E70 vs E100	Insignificant
E70 vs W	*P<0.05
E100 vs W	**P<0.01

Fig. 2. Bovine serum albumin oxidation inhibition assay. a. Average inhibitory percentage of *Withania coagulans* extracts; b. Inhibition curve; c. IC50 activity of *Withania coagulans*, BSA: Bovine Serum Albumin d. Tukey's post Hoc analysis, E70: 70% ethanolic extract of *Withania coagulans*, E100: 100% ethanolic extract of *Withania coagulans*, W: Water extract of *Withania coagulans*, T: Green tea extract, IC50: Half-maximal inhibitory concentration



Treatment Pairs	Tukeys HSD P- value inference
T vs E70	*P<0.05
T vs E100	Insignificant
T vs W	**P<0.01
E70 vs E100	**P<0.01
E70 vs W	**P<0.01
E100 vs W	**P<0.01

Fig. 3. Insulin oxidation inhibition assay. a. Average inhibitory percentage of *Withania coagulans* extracts; b. Inhibition curve; c. IC50 activity of *Withania coagulans*; d. Tukey's post Hoc analysis, E70: 70% ethanolic extract of *Withania coagulans*, E100: 100% ethanolic extract of *Withania coagulans*, W: Water extract of *Withania coagulans*, T: Green tea extract, IC50: Half-maximal inhibitory concentration

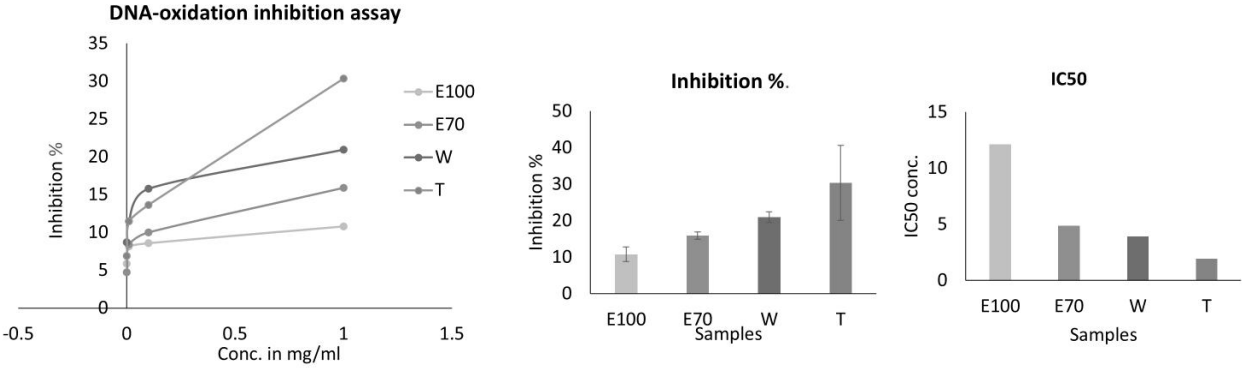
coagulans, suggesting the efficacy of water extract of *Withania coagulans* is as potent as the positive control for BSA oxidation inhibition.

In the insulin oxidation inhibition assay, the average inhibition percentages were 27.177% for E70, 16.656% for E100, and 35.620% for W. The IC50 values, which indicate the concentration needed for 50% inhibition, were 5.871 mg/mL for W, 1.595 mg/mL for E70, and 2.317 mg/mL for E100 (Fig. 3). These results show that W has the highest average inhibition percentage, but it requires a higher concentration to achieve 50% inhibition compared to E70 and E100. In terms of effectiveness, E70 and E100 show better performance in inhibiting insulin oxidation based on their IC50 values,

with E70 being the most effective, followed by E100. The overall order of inhibition effectiveness is W > E70 > E100. To compare the different test groups, One-way ANOVA was done followed by Tukey's test for post Hoc analysis. Tukey's Honestly significant difference (HSD) test indicated statistically significant difference of p<0.05 between Water extracts of *Withania caogulans* and positive control green tea extract and also with both the ethanolic extracts of *Withania coagulans*. Both Water extract and ethanolic extract of *Withania coagulans* showed significantly (Tukey's HSD p value <0.05) better activity when compared with the positive control green tea extract for insulin oxidation inhibition.

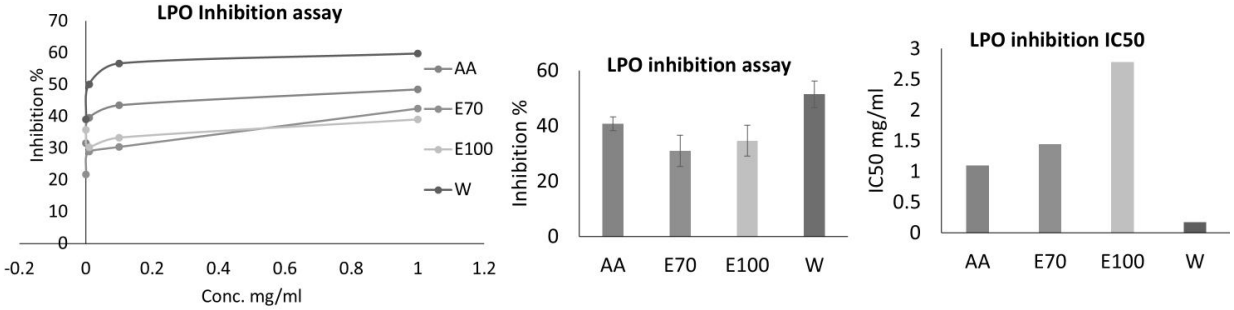
DNA-Oxidation inhibition assay: In the DNA oxidation inhibition assay, the average inhibition percentages were 15.921% for E70, 10.802% for E100, and 20.964% for W. The IC50 values, indicating the concentration needed to achieve 50% inhibition, were 12.141 mg/mL for W, 3.935 mg/mL for E70, and 4.865 mg/mL for E100 (Fig. 4). Despite W showing the highest average inhibition percentage, it requires a higher concentration for 50% inhibition compared to E70 and E100. This suggests that E70 is more effective at inhibiting DNA oxidation than E100, while W, despite its higher inhibition percentage, is less effective in terms of IC50 values. The effectiveness in inhibiting DNA oxidation follows the order W > E70 > E100. To compare the different test groups, One-way ANOVA was done, followed by Tukey's test for post Hoc analysis. Tukey's Honestly significant difference (HSD) test indicated a statistically significant difference of p<0.05 between positive control green tea extract and ethanolic extracts of *Withania coagulans* and between water extracts of *Withania coagulans* and ethanolic extracts of *Withania coagulans*. There was no significant difference between the activities of the positive control green tea extract and water extract of *Withania coagulans* suggesting the efficacy of water extract of *Withania coagulans*is as potent as the positive control for DNA oxidation inhibition.

Lipid peroxidation Inhibition: In a lipid peroxidation inhibition assay, the average inhibition percentages for the samples E70, E100, and W were 30.903%, 34.572%, and 51.361%, respectively. This data shows that sample W exhibits the highest inhibition of lipid peroxidation, followed by E100 and then E70 (Fig. 5). The IC50 values, which indicate the concentration required to inhibit 50% of lipid peroxidation, were 1.440 mg/mL for W, 2.779 mg/mL for E70, and 0.1701 mg/mL for E100. A lower IC50 value suggests greater potency, so W is the most potent inhibitor, followed by E70, and finally E100. The overall trend for lipid peroxidation inhibition is summarized as W > E70 > E100, indicating that W is the most effective inhibitor among the three samples. To compare the different test groups, One-way ANOVA was done, followed by Tukey's test for post



Treatment Pairs	Tukeys HSD P- value inference
T vs E70	**P<0.01
T vs E100	**P<0.01
T vs W	Insignificant
E70 vs E100	**P<0.01
E70 vs W	**P<0.01
E100 vs W	**P<0.01

Fig. 4. DNA-Oxidation inhibition assay. a. Average inhibitory percentage of *Withania coagulans* extracts; b. Inhibition curve; c. IC50 activity of *Withania coagulans*; d. Tukey's post Hoc analysis, E70: 70% ethanolic extract of *Withania coagulans*, E100: 100% ethanolic extract of *Withania coagulans*, W: Water extract of *Withania coagulans*, T: Green tea extract, IC50: Half-maximal inhibitory concentration



Treatment Pairs	Tukeys HSD P-value inference
AA vs E70	Insignificant
AA vs E100	*P<0.05
AA vs W	*P<0.05
E70 vs E100	Insignificant
E70 vs W	** P<0.01
E100 vs W	** P<0.01

Fig. 5. LPO inhibition by *Withania coagulans* sample. a. Average inhibitory percentage of *Withania coagulans* extracts; b. Inhibition curve; c. IC50 activity of *Withania coagulans*, PO: Lipid peroxidation; d. Tukey's post Hoc analysis, E70: 70% ethanolic extract of *Withania coagulans*, E100: 100% ethanolic extract of *Withania coagulans*, W: Water extract of *Withania coagulans*, AA: ascorbic acid; IC50: Half-maximal inhibitory concentration

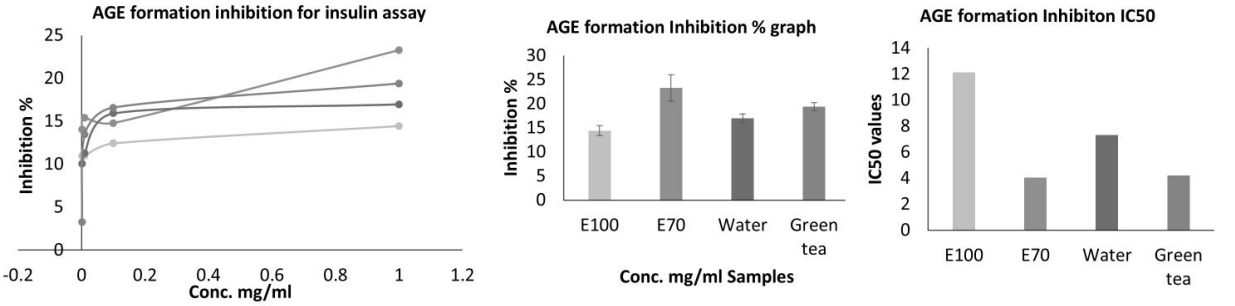


Fig. 6. AGE inhibition for insulin by *Withania coagulans*. a. Average inhibitory percentage of *Withania coagulans* extracts; b. Inhibition curve; c. IC50 activity of *Withania coagulans*; d. Tukey's post Hoc analysis, AGE: Advance glycation end product, E70: 70% ethanolic extract of *Withania coagulans*, E100: 100% ethanolic extract of *Withania coagulans*, W: Water extract of *Withania coagulans*, T: Green tea extract, IC50: Half-maximal inhibitory concentration

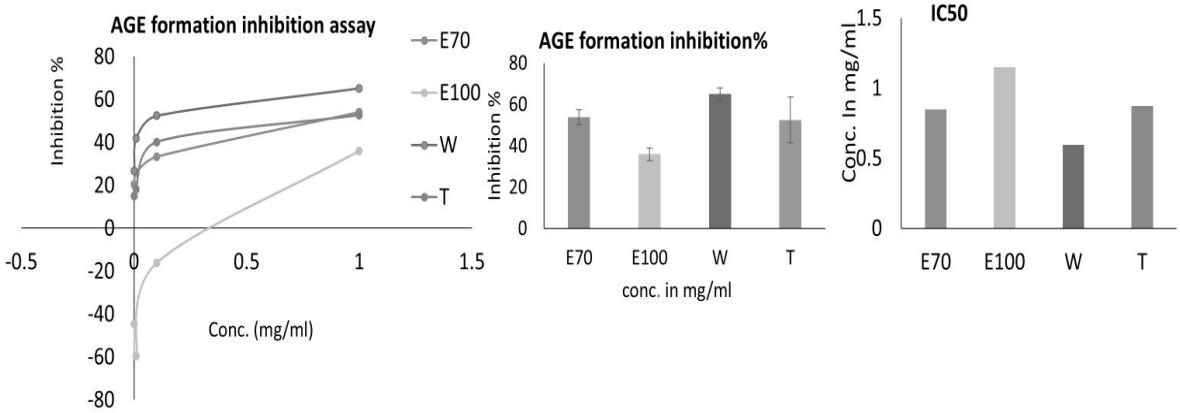
hoc analysis. Tukey's Honestly Significant Difference (HSD) test indicated a statistically significant difference of $p<0.05$ between the Water extract of *Withania coagulans* and ethanolic extracts of *Withania coagulans*. There was no significant difference between the activity of 70% and 100% ethanolic extracts of *Withania coagulans*. Similarly, there was no significant difference between the activities of the positive Ascorbic acid and 70% ethanolic extract of *Withania coagulans,* suggesting the efficacy of both extracts to be the same. On the other hand, water extract of *Withania coagulans* showed significantly better results compared to the positive control green tea extract with a $p<0.05$, thereby suggesting better efficiency of water extracts in prevention of lipid peroxidation.

Protein-Advance Glycation End product formation inhibition assay: In the Advanced Glycation End-products (AGE) formation inhibition assay involving insulin, the average inhibition percentages for the samples E100, E70, and W were 14.425%, 23.29%, and 16.991%, respectively. This suggests that E70 has the highest inhibitory effect on AGE formation, followed by W and then E100 (Fig. 6). The IC50 values, which indicate the concentration needed to inhibit 50% of AGE formation, were 12.138 mg/mL for E100, 4.043 mg/mL

for E70, and 7.310 mg/mL for W. A lower IC50 value denotes greater potency, meaning E70 is the most potent inhibitor in this assay, followed by W, and then E100. The inhibition trend for AGE formation is summarized as E70 > T > W > E100, indicating that E70 is the most effective inhibitor among the samples tested. In comparison between the different test groups, One-way ANOVA was done, followed by Tukey's test for post Hoc analysis. Tukey's Honestly significant difference (HSD) test indicated no statistically significant difference between the activity of the 70% ethanolic extract and Water extract of *Withania coagulans* with the positive control green tea extract suggesting both the extract showed similar inhibitory activity on insulin glycation with respect to the positive control.

In the Advanced Glycation End-products (AGE) formation inhibition assay, the average inhibition percentages for the samples E70, E100, and W were 53.972%, 36.018%, and 65.162%, respectively. This indicates that sample W exhibits the highest inhibition of AGE formation, followed by E70 and then E100. The IC50 values, which represent the concentration required to inhibit 50% of AGE formation, were 7.3107 mg/mL for W, 40.0438 mg/mL for E70, and 12.1383 mg/mL for E100 (Fig. 7). Typically, a lower IC50 value signifies greater potency, which suggests that sample W is the most potent inhibitor in this context, followed by E100, and then E70. However, the inhibition trend provided is summarized as E70 > T > W > E100, which suggests that, overall, E70 is considered the most effective inhibitor, followed by W and then E100. This discrepancy might be due to other factors not reflected solely in the IC50 values, possibly including the overall inhibition percentages or other assay-specific considerations. In comparison between the different test groups, One-way ANOVA was done, followed by Tukey's test for post Hoc analysis. Tukey's Honestly significant difference (HSD) test indicated no statistically significant difference between the activity of the 70% ethanolic extract and Water extract of *Withania coagulans* with the positive control green tea extract suggesting both the extract showed similar inhibitory activity on BSA glycation with respect to the positive control.

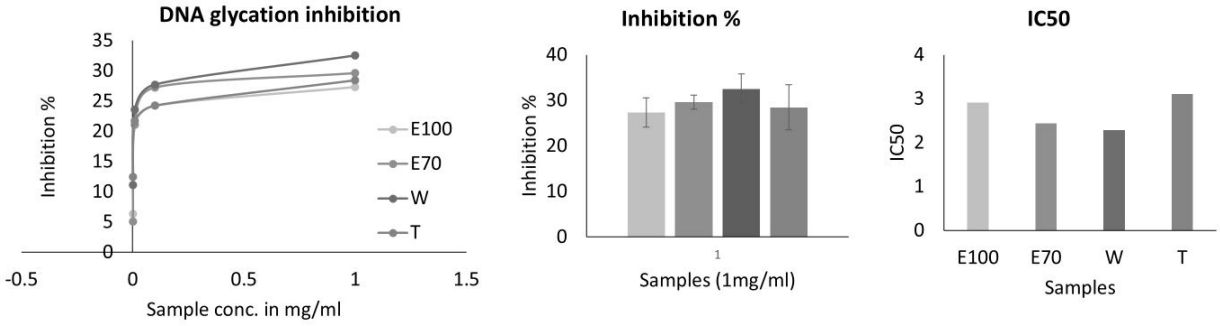
DNA-Advance Glycation End product formation inhibition assay: In the DNA glycation inhibition assay, at a concentration of 1 mg/mL, the inhibition percentages were



Treatment Pairs	Tukeys HSD P- value inference
T vs E70	Insignificant
T vs E100	**P<0.01
T vs W	Insignificant
E70 vs E100	**P<0.01
E70 vs W	**P<0.01
E100 vs W	**P<0.01

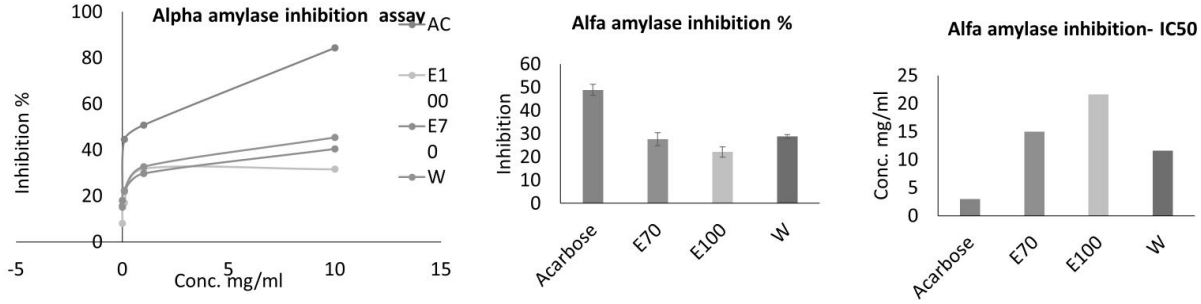
Fig. 7. AGE inhibition for BSA by *Withania coagulans*. a. Average inhibitory percentage of *Withania coagulans* extracts; b. Inhibition curve; c. IC50 activity of *Withania coagulans*; d. Tukey's post Hoc analysis, AGE: Advance glycation end product, E70: 70% ethanolic extract of *Withania coagulans*, E100: 100% ethanolic extract of *Withania coagulans*, W: Water extract of *Withania coagulans*, T: Green tea extract, IC50: Half-maximal inhibitory concentration

29.623% for E70, 27.339% for E100, and 32.529% for W. The IC50 values, representing the concentration required for 50% inhibition, were 2.438 mg/mL for E70, 2.917 mg/mL for E100, and 2.288 mg/mL for W (Fig. 8). None of the extracts showed significant differences in One-way ANOVA and Tukey's test for post Hoc analysis suggesting there was no significant difference between the activities different extract of *Withania coagulans* on DNA glycation.



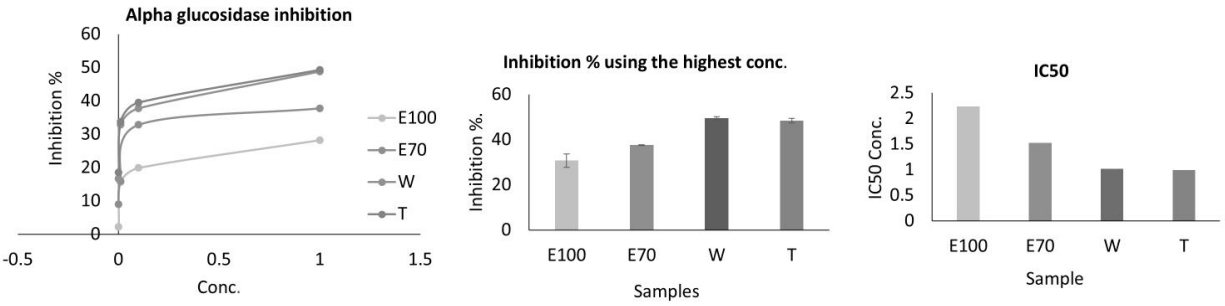
Treatment Pairs	Tukeys HSD P- value inference
T vs E70	Insignificant
T vs E100	Insignificant
T vs W	Insignificant
E70 vs E100	Insignificant
E70 vs W	Insignificant
E100 vs W	Insignificant

Fig. 8. DNA glycation inhibition assay. a. Average inhibitory percentage of *Withania coagulans* extracts; b. Inhibition curve; c. IC50 activity of *Withania coagulans*; d. Tukey's post Hoc analysis, E70: 70% ethanolic extract of *Withania coagulans*, E100: 100% ethanolic extract of *Withania coagulans*, W: Water extract of *Withania coagulans*, T: Green tea extract, IC50: Half-maximal inhibitory concentration



Treatment Pairs	Tukeys HSD P- value inference
A vs E70	**P<0.01
A vs E100	**P<0.01
A vs W	**P<0.01
E70 vs E100	*P<0.05
E70 vs W	Insignificant
E100 vs W	**P<0.01

Fig. 9. Alpha amylase inhibition assay of *Withania coagulans*. a. Average inhibitory percentage of *Withania coagulans* extracts; b. Inhibition curve; c. IC50 activity of *Withania coagulans*; d. Tukey's post Hoc analysis, E70: 70% ethanolic extract of *Withania coagulans*, E100: 100% ethanolic extract of *Withania coagulans*, W: Water extract of *Withania coagulans*, A: acarbose, IC50: Half-maximal inhibitory concentration



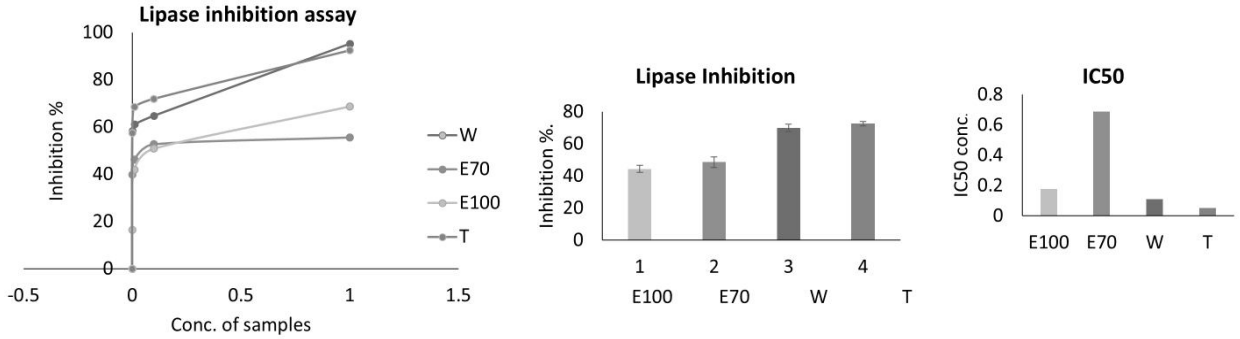
Treatment Pairs	Tukeys HSD P- value inference
T vs E70	**P<0.01
T vs E100	**P<0.01
T vs W	Insignificant
E70 vs E100	**P<0.01
E70 vs W	**P<0.01
E100 vs W	**P<0.01

Fig. 10. Alfa glucosidase inhibition by *Withania coagulans* sample. a. Average inhibitory percentage of *Withania coagulans* extracts; b. Inhibition curve; c. IC50 activity of *Withania coagulans*; d. Tukey's post Hoc analysis, E70: 70% ethanolic extract of *Withania coagulans*, E100: 100% ethanolic extract of *Withania coagulans*, W: Water extract of *Withania coagulans*, T: Green tea extract, IC50: Half-maximal inhibitory concentration

Alpha amylase inhibition assay: The IC_{50} value alpha amylase inhibition of the water extract, 70% ethanolic extract and 100% ethanolic extract of *W. coagulans* were 11.593 (mg/mL), 14.969 (mg/mL) and 21.651 (mg/mL), respectively; hence water extract of *W. coagulans* showed higher antioxidant activity compared to others. The Acarbose Equivalent Anti-diabatic Capacity (AEAC) values were 0.258, 1.014, 0.138 for Water, 70% ethanol, and 100% ethanol extracts, respectively (Fig. 9). To compare between the different test groups, One-way ANOVA was done followed by Tukey's test for post Hoc analysis. Tukey's Honestly significant difference (HSD) test indicated a statistically significant difference of $p<0.05$ between positive control acarbose and all three extracts of *Withania coagulans*, suggesting that acarbose showed better activity in inhibiting alfa amylase enzyme compared to the tested samples of *Withania coagulans*. Similarly, according to HSD, the difference between the activity of the water extract of *Withania coagulans* and the 70% ethanolic extract of *Withania cogulans* was not significant.

Alpha glucosidase inhibition assay: The inhibition assay of alpha-glucosidase, an enzyme involved in the breakdown of carbohydrates was done by using three different samples labeled E70, E100, and W. The average percentages of inhibition of the enzyme of these samples were 30.712% for E70, 37.592% for E100, and 49.508% for W. This suggests that sample W has the highest inhibitory effect, followed by E100, and then E70. The IC50 values, which represent the concentration of the substance required to inhibit 50% of the enzyme's activity, were 1.525 mg/mL for E70, 2.236 mg/mL for E100, and 1.019 mg/mL for W (Fig. 10). The lower the IC50 value, the more potent the inhibitor, indicating that sample W is the most potent inhibitor among the three, followed by E70, and then E100. The order of potency is confirmed with the notation "T = W > E70 > E100," showing that sample W is the most effective in inhibiting alpha-glucosidase, followed by E70 and then E100. To compare the different test groups, One-way ANOVA was done, followed by Tukey's test for post Hoc analysis. Tukey's Honestly significant difference (HSD) test indicated a statistically significant difference of $p<0.05$ between positive control green tea extract and ethanolic extracts of *Withania coagulans* and between water extracts of *Withania coagulans* and ethanolic extracts of *Withania coagulans*. There was no significant difference between the activities of the positive control green tea extract and water extract of *Withania coagulans*, suggesting the efficacy of water extract of *Withania coagulans* is as potent as the positive control for inhibition of alfa glucosidase.

Lipase inhibition: The result of lipase inhibition assay shows the average percentage of inhibition for the samples E100, E70, and W, were reported as 44.429%, 48.577%, and 69.877%, respectively. This indicates that sample W has the highest lipase inhibition capacity, followed by E70, and then E100. The IC50 values, which



Treatment Pairs	Tukeys HSD P- value inference
T vs E70	**P<0.01
T vs E100	**P<0.01
T vs W	Insignificant
E70 vs E100	**P<0.01
E70 vs W	**P<0.01
E100 vs W	**P<0.01

Fig. 11. Lipase inhibition by *Withania coagulans* sample. a. Average inhibitory percentage of *Withania coagulans* extracts; b. Inhibition curve; c. IC50 activity of *Withania coagulans*; d. Tukey's post Hoc analysis, E70: 70% ethanolic extract of *Withania coagulans*, E100: 100% ethanolic extract of *Withania coagulans*, W: Water extract of *Withania coagulans*, T: Green tea extract, IC50: Half-maximal inhibitory concentration

represent the concentration needed to inhibit 50% of the enzyme's activity, were 0.688 mg/mL for E70, 0.178 mg/mL for E100, and 0.110 mg/mL for W. The lower the IC50 value, the more potent the inhibitor (Fig. 11). Thus, sample W is the most potent lipase inhibitor among the three, followed by E100 and then E70. To compare the different test groups, One-way ANOVA was done, followed by Tukey's test for post Hoc analysis. Tukey's Honestly significant difference (HSD) test indicates statistically significant differences ($p<0.05$) between positive control green tea extract and ethanolic extracts of *Withania coagulans*, and between water extracts of *Withania coagulans* and ethanolic extracts of *Withania coagulans*. There was no significant difference between the activities of the positive control green tea extract and water extract of *Withania coagulans,* suggesting the efficacy of water extract of *Withania coagulans* is as potent as the positive control for inhibition of lipase enzyme.

The water extract of *Withania coagulans* inhibited 63.162 percent bovine serum albumin oxidation while the ethanolic extracts E70 and E100 showed inhibition percentage of 42.36 and 34.22, respectively. The trend was similar for inhibition of insulin oxidation, with water extract showing 35.620% inhibition, while the ethanolic extracts showed comparatively lesser inhibition of insulin oxidation with values of 27.177% and 16.656% for E70 and E100, respectively. Unlike protein oxidation inhibition, the results of DNA oxidation inhibition results were not impressive in all three extracts, accounting for only 15.921% and 10.802% for ethanolic 70 and 100, and 20.964% for Water extract. In a lipid peroxidation inhibition assay, the average inhibition percentages for the samples E70, E100, and W were 30.903%, 34.572%, and 51.361%, respectively. This data follows the same trend of water extract showing better inhibition of lipid peroxidation compared to ethanolic extracts. In the Advanced Glycation End-products (AGE) formation inhibition assay, the average inhibition percentages of bovine serum albumin (BSA) for the samples E70, E100, and W were 53.972%, 36.018%, and 65.162%, respectively and for inhibition of DNA glycation the percentages were 29.623% for E70, 27.339% for E100, and 32.529% for water extract. The IC_{50} value alpha amylase inhibition of the water extract, 70% ethanolic extract and 100% ethanolic extract of *W. coagulans* were 11.593 (mg/mL), 14.969 (mg/mL) and 21.651 (mg/mL), respectively. Hence, the water extract of *W. coagulans* showed higher inhibition than that of others. Similarly, the IC50 values, which represent the concentration of the substance required to inhibit 50% of alfa glucosidase activity, were 1.525 mg/mL for E70, 2.236 mg/mL for E100, and 1.019 mg/mL for W. The lower the IC50 value, the more potent the inhibitor, indicating that sample W or the Water extract of *Withania coagulans* is the most potent inhibitor among the three, followed by E70, and then E100. The IC50 values for enzyme lipase activity were 1.525 mg/mL for E70, 2.236 mg/mL for E100, and 1.019 mg/mL for Water extract of *Withania coagulans*. In summary, the water extract (W)

of *Withania coagulans* is consistently the most effective inhibitor across the various assays, followed by E70 and then E100, highlighting the superior antioxidant and enzyme inhibitory properties.

DISCUSSION

Optimal nutrition is imperative to human health. According to WHO 2022, an unhealthy diet is one of the major risk factors for a range of chronic diseases, including diabetes, cancer, cardiovascular diseases, and other conditions linked to obesity. Modern-day diets containing Westernized and overprocessed food often lack the beneficial components of their natural counterparts; hence, additional nutritional supplements become necessary where the prospects of functional foods can be very promising. Functional foods are foods that may provide health benefits beyond basic nutrition. Thus, from all the above-mentioned experiments *Withania coagulans* shows major potential to be a functional food, thereby reducing the risk of diseases in the long run. Further preclinical *in vivo* studies are required to further investigate and justify the activities of the extract in living systems.

The build-up of oxidative stress products, especially reactive oxygen species, can cause damage to a variety of biological macromolecules, such as proteins, lipids, and DNA, in people with poorly managed diabetes (Ighodaro, 2018). ROS generation is elevated in chronic hyperglycemia. Numerous proteins, including those essential for insulin signaling, can be harmed by these ROS (Bloch-Damti and Bashan, 2005). An important side effect of the elevated oxidative stress seen in diabetes is protein oxidation. Proteins can be structurally and functionally compromised by oxidative alteration, which can have a variety of negative consequences such as changed enzyme activity, interference with cellular signaling networks, and heightened vulnerability to proteolytic breakdown (De *et al*., 2013). Therefore, *Withania coagulans* extract shows promising potential to support overall health and well-being by preventing oxidation of major macromolecules protein, DNA and lipids. Further investigation of these areas in *in vivo* preclinical models and clinical trials are required to confirm the findings shown in *in vitro* studies. The majority of the secondary health issues occur due to the formation of advanced glycation end products that further lead to an unending cycle of free radical generation and oxidative damage. Moreover, since anti-AGE therapy is a relatively new feature of diabetes treatment and many of the impending medications are still in preclinical development, natural chemicals and their molecular structures are currently crucial starting points for drug discovery. Inhibition of metabolic enzymes is also a way of dealing with metabolic conditions like diabetes and obesity.

Inhibition of salivary and pancreatic alfa amylase and alfa glucosidase breaks down complex sugars into simple sugars; hence, increasing postprandial glucose levels is often used as a therapeutic measure. Hence, inhibitory actions of *Withania coagulans* on these enzymes show potential to reduce postprandial sugar spikes from high sucrose or high fructose containing overly processed foods, and also inhibition of lipase may reduce the free fatty acids levels in circulation due to slower breakdown of fats in the intestine from high-fat diets. Hence, supplementation of *Withania coagulans* extracts as a functional food shows potential in preventing the increasing health risks due to an unhealthy diet and sedentary lifestyle in modern civilization.

Both water and ethanol are green and polar protic solvents, but unlike ethanol, water is a non-selective universal solvent, and it also has two hydrogen atoms that make it more polar. Hence, it can dissolve a wide range of phytochemicals that would otherwise be immiscible in selective solvents like ethanol. Hence, the water extract of *Withania coagulans* is likely to contain all the hydrophilic substances such as saponins, phenolics, polysaccharides, etc, while the ethanolic extract of *Withania coagulans* is mostly selective for polyphenol and triterpenes. Hence, there is a high probability that a larger plethora of bioactive compounds present in the water extract is giving significantly better results in multiple assays (Kumar *et al*., 2023). Hence, water extract of *Withania coagulans* shows promising *in vitro* results; further investigations are required for using *Withania coagulans* for better management of diseases.

Conflict of interest: Authors have no conflict of interest in this study.

Author's contribution: AM: Involved in investigation, data generation, preparing original draft, manuscript writing statistical analyses, methodology and editing; RD: Extraction, manuscript writing and editing; SB: Involved in standardization of alfa amylase, lipid peroxidation, manuscript writing and editing; ERB: Engaged in conceptualization, data curation, supervision and final editing.

Data availability statement: All the data supporting the research findings have been presented in this paper. The corresponding author is willing to provide the raw data upon reasonable request.

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