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In Vitro Evaluation of Withania Coagulans for its Thrombolytic, Anti-Oxidant, Anti-Bacterial and Cytotoxic Potential

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Abstract

This research aimed to find antibacterial, thrombolytic, cytotoxic and antioxidant potential of different extracts (ethanol, methanol, ethyl acetate) of Withania coagulans seeds. Anti-bacterial activity was carried out through well diffusion method. Thrombolytic activity was determined by the percentage of clot lysis. Cytotoxic activity was performed through hemolytic method. Antioxidant activity of extracts was determined by using 2, 2-diphenyl- 1-picryllhydrazyl radical scavenging assay, peroxidase (POD) assay, superoxide dismutase (SOD) assay and catalase (CAT) assay. Total phenolic and flavonoid contents were determined. Ethanol extract exhibited maximum antibacterial effects (p>0.001) against both strains as compared to methanol and ethyl acetate extracts. All the extract showed concentration dependent thrombolytic activity. Ethanolic extract of showed maximum %age of clot lysis followed by methanol and then ethyl acetate extract. Cytotoxic activity when performed indicated that among all extracts Methanol extract of seeds showed maximum cytotoxic activity. The ethanol extract of seeds showed maximum total phenolic contents and total flavonoids contents. When DPPH free radical scavenging assay was carried ethanol extract of seeds of W. coagulans plant showed maximum %age inhibition followed by methanol extract and then Ethyl acetate extract. Similarly, ethanol extract showed maximum Peroxidase assay, superoxide dismutase, Catalase activities. Thus, results of study showed that different extracts ethanol, methanol and ethyl acetate of Withania coagulans seeds exhibited antimicrobial, cytotoxic and antioxidant potential, however, among the extracts ethanol possess the maximum therapeutic potential against all activities.

Keywords: *Thrombolytic activity, anti-bacterial activity, cytotoxic potential, plant extract.*

Introduction

About two-thirds of the world's population, including many countries and communities, rely on plant medicines for primary health care. They are widely used around the world because they have increased multicultural acceptance, improved compatibility with the human body, and improved adaptation to human bodies. (Srivastava 2018). These medicinal plants are either "wild species" that grow naturally in self-reliant biological systems and may thrive irrespective of unconventional "Domesticated species"; that emerged through technological processes like selection or breeding and depend on monitoring for

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the sake of their existence (Jamshidi-Kia, Lorigooini, and Amini-Khoei 2017).

Recent research evidence has extensively shown that conventional medicinal plants play an important role in preventing and controlling various metabolic diseases such as diabetes, heart disease and certain types of cancer. Various therapeutic products are commonly produced using individual or combination medicinal plants. In some cases, medicinal plants are the main source of raw materials for other drugs. (Sofowora, Ogunbodede, and Onayade 2013). Due to the significance of herbs in medicine, in-depth studies have been conducted in recent years to isolate and define the active components of medicinal plant.

Withania coagulans (WC) is a member of the Solanaceae family, is one of the key ayurvedic medicinal herbs used for thousands of years in Pakistan and India. It is also known as vegetable rennet, Indian cheesemaker, Indian rennet, Paneer ke phool, Paneer band, or Paneer dodis. It is typically found in the Eastern Mediterranean and approaches South Asia. It flourishes in arid areas like the Thal and Cholistan deserts in Pakistan (Gupta and Keshari 2013). Because the fruits and leaves of W. coagulans have the ability to coagulate milk, they are prevalently referred to as paneer. The pulp and husk of berries, which contain an enzyme with milk coagulating activity, are thought to be responsible for the fruit's milk coagulating effects (Salehi et al. 2017). Diabetes can be treated through its berries as it exhibits hypoglycemic properties. Sleeplessness, excessive salivation, and impotence are all conditions they aid with along with digestive tract infections, dyspepsia, and chronic liver diseases. The infusion of berries is known to have anthelmintic properties (Hanif et al. 2021). It is known to have anti-inflammatory, diuretic, anti-bacterial, cardioprotective, anti-fungal, hypoglycemic, hepatoprotective, anti-oxidative, and anti-mutagenic properties due to its presence of an active compound, a cyclic enzyme. In addition to withanoids, W. coagulans also contains many phytochemicals such as tannins, flavonoids, and -sterols. Some studies indicate that W. coagulans and their active ingredients have many pharmacological and therapeutic properties and can therefore be considered as new drugs to treat various diseases. The aims and objectives of this research was to assess the thrombolytic activity, anti-bacterial activity and cytotoxic potential of W. coagulans fruit extract.

Chemicals and Reagents

Ethanol (Merck, Germany), Sodium hydroxide (Merck, Germany), Hydrochloric acid (Merck, Germany), n-hexane (Merck, Germany), Ciprofloxacin (Sigma-Aldrich), Methanol (Merck, Germany), Hydrogen peroxide (Merck, Germany), 2,2-diphenyl-1-hydrazyl (DPPH) reagent (Sigma-Aldrich) , TritonX-100 (Merck, Germany), Nutrient agar (Oxide, UK), di potassium hydrogen phosphate (Merck, Germany), Sodium chloride (Merck, Germany), Sodium hydrogen phosphate (Merck, Germany), Folin ciocalteu reagent (Merck, Germany) Potassium chloride (Merck, Germany).

Selection and Identification of Plant Material

Therapeutic plant for the current study was obtained from local market of Mianwali-Pakistan. Fruit and other parts are taxonomically recognized and confirmed from Department of Botany, Government College University, Faisalabad - Pakistan. Seeds of selected medicinal plant i.e.; Withania coagulans are used to prepare extracts.

Extract Preparation

The fruit of selected plant were crushed to fine powder in an electric grinder. 250g of the powdered sample was taken and allowed to macerate in 500ml of each solvent ethanol, methanol and ethyl acetate separately. The samples were stirred daily for a week. After a week filtration of the soaked plant material was done, with Whatman filter paper No.3. The filtered

material was subjected to evaporate on rotary evaporator Laborota 4000 efficient (Heidolph) at 40-degree Celsius. After evaporation, the crude ethanolic, methanolic and ethyl acetate extracts were collected in autoclaved falcon tubes and stored in laboratory at the temperature of 20°C. In order to calculate the percentage yield of the concentrated extracts (g/100 g of dry plant), the concentrated extracts were weighed. The percentage yield was ethanol extract (13%), methanol extract (11.5%) and ethyl acetate extract (10.9%)

$$\%\text{age yeild} = \frac{\text{Weight of dried extract}}{\text{Weight of dried plant material}} \times 100$$

Screening for Anti-Bacterial Activity

Staphylococcus aureus (S. aureus); is the gram-positive bacteria and Escherichia coli (E. coli); the gram-negative bacteria bacterial strains were screened. In Nutrient agar from Oxoid (UK), bacterial strains were cultivated for a whole night at 37°C. By well diffusion method, the compounds' antibacterial activity was identified. 107 colony-forming units (CFU)/mL of bacteria cells in a suspension of verified microorganisms in 100 µL on nutritional agar medium. The antibacterial activity of the samples was examined through the well diffusion technique. As a positive control / standard anti-bacterial drug, ciprofloxacin was used to compare the sensitivity of the strain in the tested microbial species. Plates were incubated at 4°C for 2 hours to identify bacterium strains, then at 37°C for 18 hours. By measuring the organism's growth inhibition zones in millimeters, using a zone reader and comparing the results to the standard antibacterial activity was assessed (Parveen et al. 2023).

Screening for Thrombolytic Activity

The thrombolytic activity of the extract was determined by a method designed by (Oatman et al. 2021) with minor adjustments and modifications. Blood samples were collected from healthy participants who had never used oral contraceptives or anticoagulants. To conduct this study, healthy volunteers (n = 3) supplied 7 ml of blood from the venous system and then transferred it to a separate, pre-weighed, sterilized microcentrifuge tube (1 ml/tube). Then, the microcentrifuge tube was incubated for 45 minutes at 37 °C. Once the clot has formed, the serum is completely removed from the tube, and each clot is weighed again to calculate the weight of the clot (the weight of the clot is the weight of the tube containing the clot – the weight of the tube is separate). Clot-containing microcentrifuge tubes were clearly labelled. The material is thoroughly mixed with a vortex mixer after a thorough dissolved solvent. Subsequently, the test samples were produced at different concentrations of 2, 4, 6, 8, and 10 mg/ml, respectively. The soluble supernatant is removed from suspension by placing it in a tight container, mixing it, and filtering through a 0.22-micron needle filter. After filtration, each label tube adds 100 l of solvent extraction (2–10 mg/ml) to each label tube. As standard thrombolytics/positive/negative control, streptocysteine and distilled water were mixed into tubes, each with 100l of clot. All tubes are then further incubated at 37°C for 90 minutes, monitoring the clot lysis. The difference in weight measured before and after clotlysis was finally quantified as a percentage of clotting lysis (Parameswaran et al. 2021).

Screening for Cytotoxic Potential of Seeds by Haemolytic Activity

The hemolytic activity of selected seeds was evaluated according to the method used by (Zohra and Fawzia 2014). Three mL freshly collected heparinized bovine blood was taken. The blood was centrifuged for five minutes at 1,000xg to separate plasma. The cells were washed three

times in five milliliters of chilled (4°C) sterile Phosphate-Buffered-Saline (PBS), pH 7.4. In each experiment, 108 erythrocytes per millilitre were maintained. Separately, the drug extracts of 100 microliters (L) were combined with human cells (108 cells/mL). After 10 minutes of stirring, the sample was incubated for 35 minutes at 37 degrees C. After incubation, the sample is immediately centrifuged for 5 minutes at 1000 g and then cooled for 5 minutes. The supernatant of each tube was removed and diluted with a cooling (4°C) PBS with 100 L. Triton X-100 (0.1% v/v) is used as the positive control, and Phosphate buffer saline (PBS) is the negative control. Both substances are processed exactly in the same way. Using Quant (Biotek, USA), the absorption was measured at 576 nm. The percentage of RBC lysis for each sample was determined (Shahzadi, Zaib and Zabi, 2019).(Shahzadi et al. 2019).

%age Hemolysis = Sample Absorbance - Negative control absorbance / Positive control absorbance × 100

Determination of Total Phenolic Contents (TPC)

Folin-Ciocalteu technique, as reported by Naz et al. (2021), was used to determine the sample's total phenolic content. Different gallic acid concentrations were used to produce the calibration curve. Gallic acid solution 0.10 mg/mL in methanol at concentrations of 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, were combined with a ten-fold diluted Folin-Ciocalteu reagent, 5 mL, and 4 mL of Na_2CO_3 (sodium carbonate) (20%). After an hour, the absorbance was measured at 765 nm, and the calibration curve was created by plotting the absorbance as a function of concentration. After mixing the same reagent as before with 1mL of sample extract (0.001g/mL), the absorbance of the resulting blue color complex was measured at 765nm after an hour. Gallic acid was used as the standard for quantification (Sharif et al. 2018). The total amount of phenolic compounds present in plant extracts were calculated as gallic acid equivalents (GAE) using the formula below (Shahzadi et al. 2019).

T = C x V / M

Here,

T = total phenolic content concentration in mg GAE/g plant extract.
C = the gallic acid concentration evaluated from the calibration curve in mg/mL.
V = the extract's volume in milliliters.
M = the mass of plants extract in grams (gm).

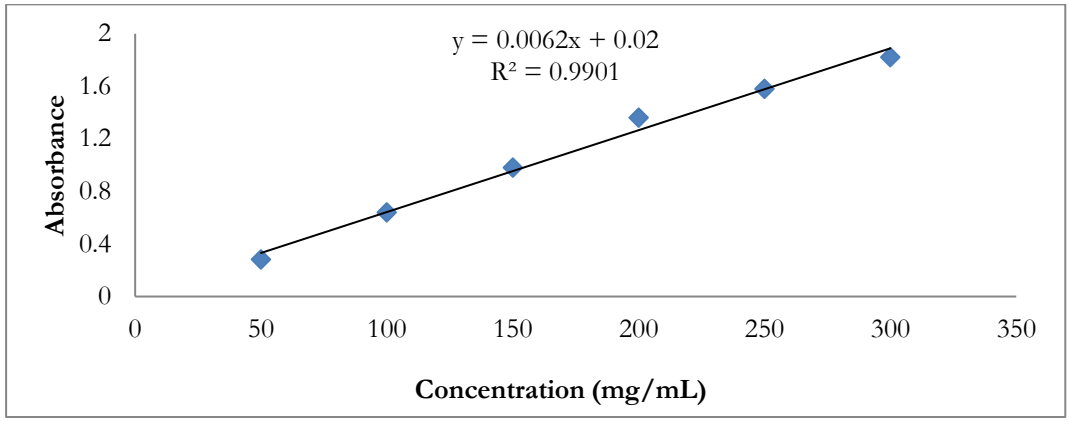

Fig. 19: Gallic Acid Standard Curve.

This graph shows the curve of gallic acid. For the quantification of total phenolic contents (TPC), gallic acid is employed as the reference. Numerous botanical components and therapeutic plants naturally contain gallic acid, which is a phenolic component. Therefore, when a plant's phenolic contents are determined, it is compared with standard curve of gallic acid (Genwali, Acharya, and Rajbhandari 2013).

Determination of Total flavonoids Contents (TFC)

Following the procedure outlined by (Genwali, Acharya, and Rajbhandari 2013), the sample's total flavonoid content was determined. In simple terms, the combination was incubated for 6 minutes after being combined with 0.5mL of the sample, 2mL of distilled water, and 0.15mL of a 5% sodium nitrate (NaNO2) solution. The mixture was then incubated for a further 6 minutes with 0.15 mL of 10% aluminium chloride ($AlCl_3$) solution before being treated with 4% sodium hydroxide (NaOH) solution. The amount of the reaction mixture was increased to 5mL by adding methanol and vigorously mixing it. The reaction mixture's absorbance was measured at 510 nm after 15 minutes of incubation. The flavonoid total contents of the extracts were expressed as g catechin equivalents per mL (g catechin eq/ml) of plant extract using the catechin linear regression curve.

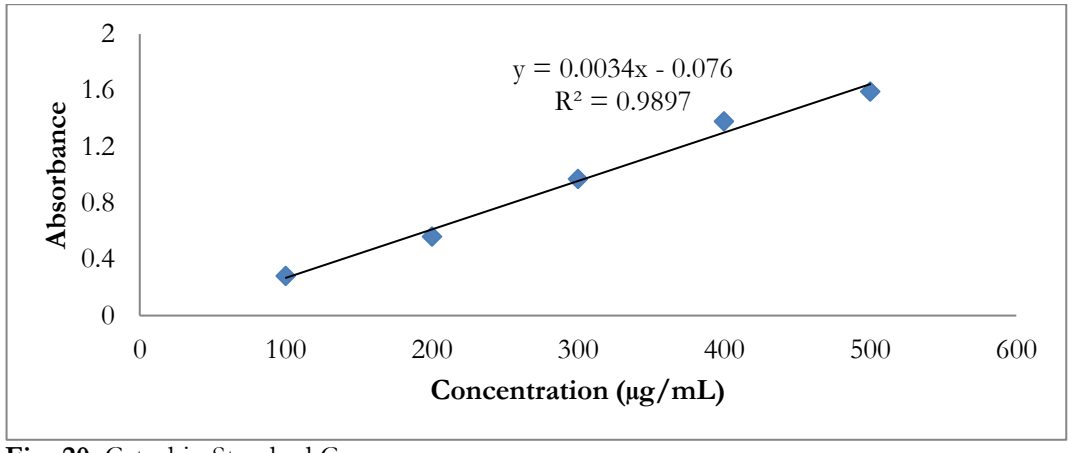

Fig. 20: Catechin Standard Curve.

DPPH Scavenging Activity

The antioxidant potential of the sample was assessed using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. This process involved adding 1mL of freshly prepared 0.004% DPPH in methanol solution to 3mL of sample and keeping the resultant solution in the darkness / shade for 30 minutes. The absorbance was then determined at 517 nm. High free radical scavenging efficiency is found in reaction mixtures with low absorbance. Moreover, the standard ascorbic acid's antioxidant activity was investigated. As a control, a solution without plant extract was utilized. All the tests were performed three times. The formula shown below was employed to determine the percentage inhibition of DPPH radical samples.

$$\%\text{age inhibition of DPPH} = \frac{\text{Absorbance of blank } (A_0) - \text{Absorbance of sample } (A_1)}{\text{Absorbance of blank } (A_0)} * 100$$

Where

A_1 = Sample absorbance.
A_0 = Blank absorbance.

The sample concentration (IC50) that delivers 50% inhibition was identified using a graph of the inhibition percentage versus sample concentration. Testing was carried out three times. Ascorbic acid served as a positive control.

Peroxidase (POD) Assay

The amount of enzyme needed for guaiacol oxidation was determined as peroxidase (POD) activity. By applying the same enzyme extract that was used to evaluate TSP, the peroxidase activity was evaluated. Reaction mixture with the IUPAC name 2-methoxyphenol contains 800 mL of K_3PO_4 (potassium phosphate) buffer (pH 5) together with 100 mL of H_2O_2 (40 mM) and 100 mL of guaiacol (20 mM). An ELISA plate was used to assess the absorbance at 470 nm after 100 mL of the mixture used for the reaction and 100 mL of the enzyme extract were added.

Superoxidase Dismutase (SOD) Assay

By measuring the inhibition rate in the photoreduction of nitrobluetetrazolium (NBT) by the mean of SOD enzymes, superoxide dismutase (SOD) activity was determined. The reaction mixture employed in this activity contains 50 mM NBT, 100 mL of crude extract, 0.1 mM ethylene diamine tetra acetic acid (EDTA), 50 mM Na_3PO_4 (sodium phosphate) buffer with pH 7.6, 12 mM L-methionine, 50 mM Na_2CO_3 (sodium carbonate), 50 mM NBTand 10 mL of riboflavin (vitamin B2). On the other hand, in the absence of crude extract, the control reaction was initiated. 15 minutes of ambient white light exposure to the following reaction combination was done in order to test the SOD activity. After 15 minutes of incubation, the absorbance at 560 nm was measured with a spectrophotometer.

The amount of superoxide dismutase enzyme that inhibited the photochemical reduction of (NBT) was utilized to evaluate SOD assay. The reaction mixture contained 100 litres of TSP-like enzyme extract i.e.; 200 L of triton X, 100 L of NBT, 200L of methionine, 800 L of distilled water and 500 litres of K_3PO_4 (potassium phosphate) buffer (pH 5). 15 minutes of exposure to UV rays, 100 mL of riboflavin, and an ELISA plate were used to evaluate the absorbance at 560 nm.

Catalase Activity (CAT)

The method recommended by (Sadeghian, Kojouri, and Mohebbi 2012) was used to measure the catalase activity. H_2O_2 known as hydrogen peroxide was used as a substrate in this method using an ultraviolet (UV) spectrophotometer (UV-1601, Shimadzu, Germany) and catalase enzymes. By quantifying the decline in absorbance for 5 minutes at 240 nm, H_2O_2 breakdown was measured. Consumed H_2O_2 per minute/mg of protein served as an estimate for this activity's results.

The amount of hydrogen peroxide (H_2O_2) that the enzyme converted into water (H_2O) and oxygen (O_2) was used to calculate catalase assay. Catalase activity was assessed using the same enzyme extract that was used to calculate TSP. 100 L of enzyme extract and 100 L of H_2O_2 (5.9 mM) were added to a well plate, and the absorbance at 240 nm on an ELISA plate was measured (Claiborne 2018).

Statistical Analysis

The statistical analysis is done by using SPSS and data is arranged using one way ANOVA.

Results and Discussion

Anti-Bacterial Activity

Against S. aureus the ethanolic extract of seeds of WC showed maximum zone of inhibition of 12mm, followed by methanolic and ethyl acetate Table 1. Against E coli the ethanol extract

showed maximum inhibition zone of 15mm, methanol extract showed 12mm and ethyl acetate showed 7mm. Among all extract the ethanol extract showed maximum zone of inhibition.

Table 1: Zone of Inhibition by Various Extracts of Seeds of WC Pant Against S. Aureus and E. Coli Bacteria.

Plant extracts	Zone of inhibition	
	Staphylococcus aureus	E. coli
Ethanolic extract of seeds of WC	12mm	15mm
Methanolic extract of seeds of WC	11mm	12mm
Ethyl acetate extract of seeds of WC	8mm	7mm
Ciprofloxacin Standard	38mm	36mm

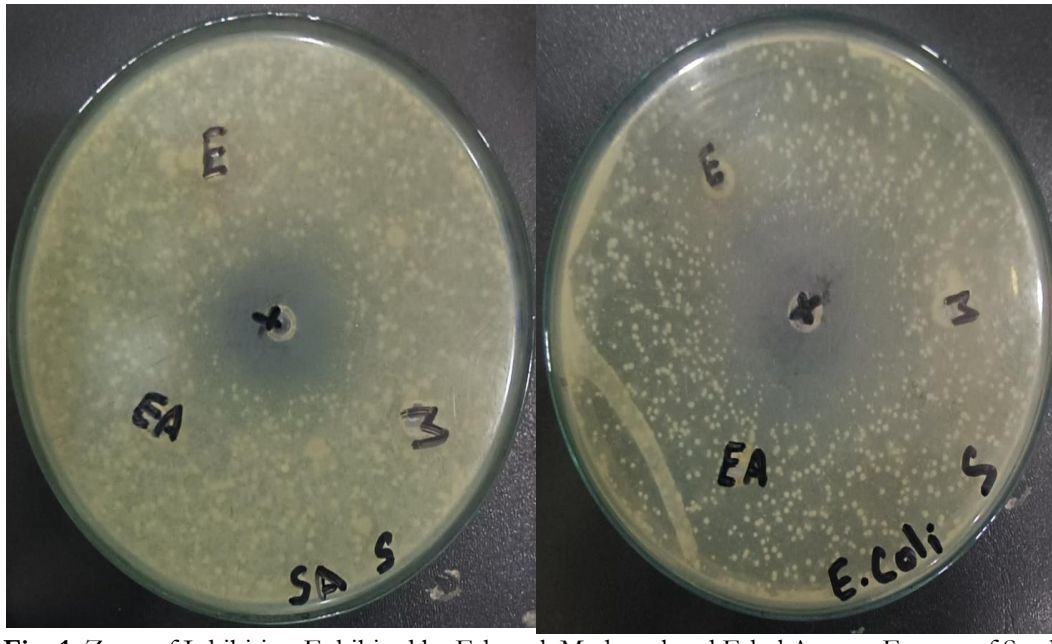

Fig. 1: Zone of Inhibition Exhibited by Ethanol, Methanol and Ethyl Acetate Extract of Seeds of W. Coagulans Plant Against S. Aureus and E. Coli Bacteria.

Thrombolytic Activity through Clot Lysis

Thrombosis is the formation of blood clot within blood vessels; which may completely or partialy blocks the blood flow and eventually leads towards mayocardial infarction. Therefore, this research activity is carried out to find thrombolytic potential of seeds of WC plant (Rashid et al. 2023).

For this purpose, three extracts i.e.; in ethanol, methanol and ethyl acetate solvents of seeds of WC plants have been prepared separately each with three different concentrations i.e.; T1 (ethanol 50mg/ml), T2 (ethanol 100mg/ml), T3 (ethanol 150mg/ml), T4 (methanol50mg/ml), T5 (methanol 100mg/ml), T6 (methanol 150mg/ml), T7 (ethyl acetate 50mg/ml), T8 (ethyl acetate 100mg/ml), and T9 (ethyl acetate 150mg/ml). Thrombolytic activity is determined by measuring the extent of lysis of blood clot. Ethanolic extracts of seeds of WC at concentrations of 50, 100 and 150 mg/ml showed 17.20%, 17.59% and 16.81% of clot lysis respectively. Methanolic extracts of seeds of selected plant at concentrations 50, 100 and 150 mg/ml showed clot lysis of 14.67%, 15.45% and 14.88% respectively. Blood clots when treated with ethyl

acetate extracts of seeds of selected plants at concentrations 50, 100 and 150 mg/ml showed 15.53%, 16.41% and 15.66% of clot lysis respectively. These results indicate the thrombolytic potential of seeds of WC.

Table: 2: Thrombolytic Potential of Various Extracts of Seeds of WC Plant.

Plant extracts	Concentration	Clot weight	wt of released clot	%age of clot lysis
Withania coagulans' seeds ethanol extract	50mg	471	81	17.20
	100mg	449	79	17.59
	150mg	452	76	16.81
Withania coagulans' seeds methanol extract	50mg	443	65	14.67
	100mg	466	72	15.45
	150mg	457	68	14.88
Withania coagulans' seeds ethyl acetate extract	50mg	470	73	15.53
	100mg	463	76	16.41
	150mg	447	70	15.66

These values indicate that seeds of W. coagulans plant have considerable thrombolytic potential and can be used to treat pathological conditions like thrombosis and atherosclerosis and can also be utilized as preventive therapy as well as supportive drug with other anti-coagulant drugs.

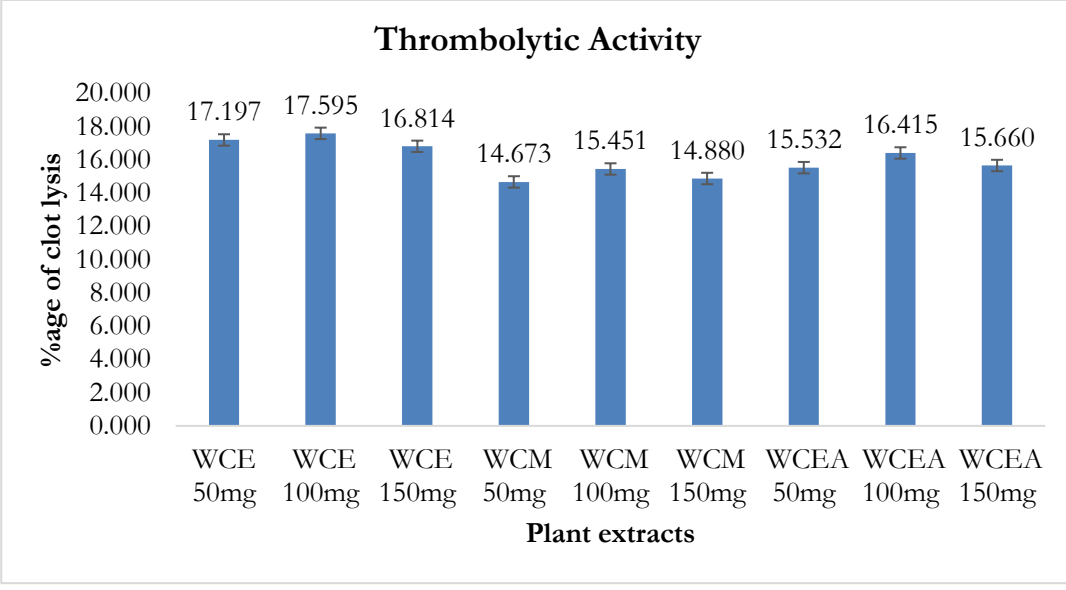

Fig. 2: Thrombolytic Potential of Various Extracts of Seeds of W. Coagulans Plant at Different Concentrations.

WCE (Withania coagulans ethanol extract), WCM (Withania coagulans methanol extract), WCEA (Withania coagulans ethyl acetate extract)

Cytotoxic Activity

"Cyto" word denotes a cell, while "Toxic" denotes a poison. The term "cytotoxic" refers to any substance or chemical that can harm or kill cells. The determination of new cytotoxic substances is important to find new chemotherapeutic agents which might help in killing the

benign tumors and to stop the metastasis of cancerous cells (Paudel, Chand, Pant, and Pant 2019).

To carry out this activity Triton X-100 (0.1% v/v) was taken as positive control and phosphate buffer saline (PBS) as negative control. This activity was performed on erythrocytes by haemolytic method. When treated with ethanolic extract of seeds of WC at concentration of 50, 100 and 150 mg/ml showed 12.985%, 12.216% and 13.153% of cytotxicity respectively. Methanolic extracts of seeds of selected plant at concentration 50, 100, and 150 mg/ml showed cytotoxicity of 13.922% 16.165% and 16.968% respectively. Upon treatment with extracts of ethyl acetate at concentrations of 50, 100 and 150 mg/ml showed cytotoxicity of 12.918%, 13.822% and 12.784% respectively.

Table 3: Cytotoxic Potentials of Various Extracts of Seeds of WC Plant at Different Concentrations.

Plant extracts	Concentration	Sample Absorbance	Negative Control	Positive Control Absorbance	Final cytotoxicity %age
Withania coagulans' seeds ethanol extract	50mg	0.471	0.083	2.988	12.985
	100mg	0.448	0.083	2.988	12.216
	150mg	0.476	0.083	2.988	13.153
Withania coagulans' seeds methanol extract	50mg	0.499	0.083	2.988	13.922
	100mg	0.566	0.083	2.988	16.165
	150mg	0.59	0.083	2.988	16.968
Withania coagulans' seeds ethyl acetate extract	50mg	0.469	0.083	2.988	12.918
	100mg	0.496	0.083	2.988	13.822
	150mg	0.465	0.083	2.988	12.784

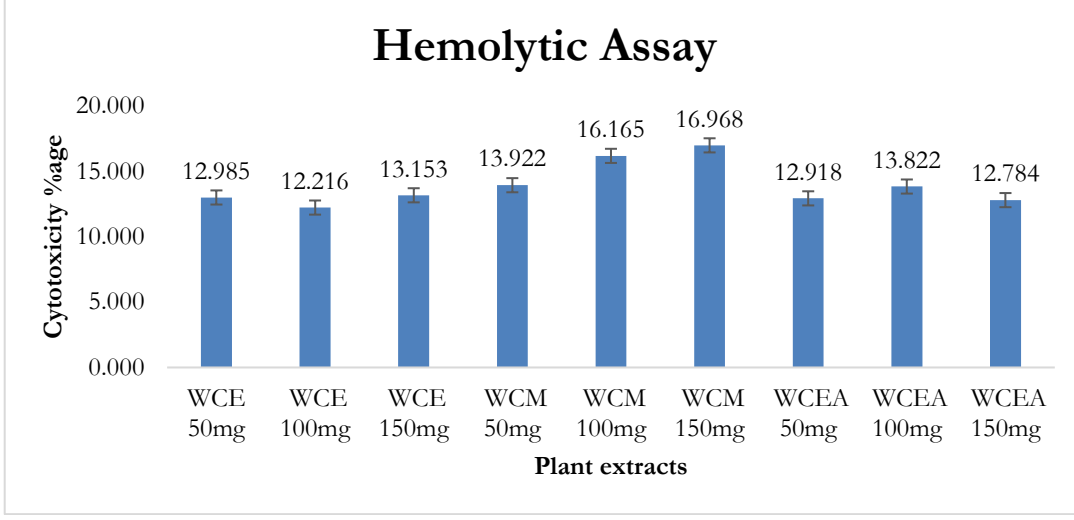

Fig. 3: Cytotoxicity in %Age Shown by Various Extracts of Seeds of W. Coagulans Plant at Various Concentrations.

WCE (Withania coagulans ethanol extract), WCM (Withania coagulans methanol extract), WCEA (Withania coagulans ethyl acetate extract)

Anti-Oxidant Activity

Free radicals or ROS (Reactive Oxygen Species) can be scavenged by the chemicals or agents referred to as antioxidants. The enhanced antioxidant capacity of a live organism is facilitated by natural antioxidants present in medicinal plants. Plants contain antioxidants as well. It has been demonstrated that antioxidants produced from medicinal plants are effective in healing wounds that are caused by the production of free radicals and oxidative stress (Tajner-Czopek et al. 2020; Farag, Abdel-Latif, Abd El Baky, and Tawfeek 2020).

Reactive oxygen species, which are the main culprits behind many illnesses including cancer, arthritis, and neurological degeneration, accelerate ageing and destroy human cells when their levels grow. These natural antioxidant substances actively counteract the harmful effects caused by an excess of reactive oxygen species. Due to the importance of natural antioxidants, we evaluated the antioxidant activity of the studied plant extracts produced in ethanolic solvent in the current study.

For the purpose to evaluate anti-oxidant activity both enzymatic and non-enzymatic anti-oxidant assays are used.

Total Phenolic Contents (TPC)

The hydroxyl groups in botanical extracts play a vital role in enabling scavenging of free radical, and phenolic compounds are significant plant components with redox characteristics that are responsible for antioxidant activity.

This activity is carried out through Folin-Ciocalteu method. Gallic acid is taken as standard in this case. Assay done with ethanolic extract of seeds of WC plant at concentration of 50, 100 and 150mg/ml showed phenolic content of 251.80mgGAE/g, 264.40mgGAE/g and 260.00mgGAE/g respectively. Upon treatment with methanolic extract of seeds of selected plant at concentrations of 50, 100 and 150mg/ml showed TPC of 75mgGAE/g, 194.80mgGAE/g and 223.00mgGAE/g respectively. Extracts of ethyl acetate at concentrations 50, 100 and 150mg/ml when evaluated showed TPC of 30.20mgGAE/g, 101.40mgGAE/g and 169.20mgGAE/g respectively. Hence ethanolic extract at concentration of 150mg/ml showed maximum phenolic content among all other prepared extracts.

Table 4: Phenolic Contents Present in Various Extracts of Seeds of WC Plant at Different Concentrations

Plant extracts	Concentration	Sample Absorbance	Blank Absorbance	mgGAE/g
Withania coagulans' seeds ethanol extract	50mg	2.678	1.419	251.80
	100mg	2.741	1.419	264.40
	150mg	2.719	1.419	260.00
Withania coagulans' seeds methanol extract	50mg	1.794	1.419	75.00
	100mg	2.393	1.419	194.80
	150mg	2.534	1.419	223.00
Withania coagulans' seeds ethyl acetate extract	50mg	1.57	1.419	30.20
	100mg	1.926	1.419	101.40
	150mg	2.265	1.419	169.20

Mggae/G- Milligrams of Gallic Acid Equivalents per Gram.

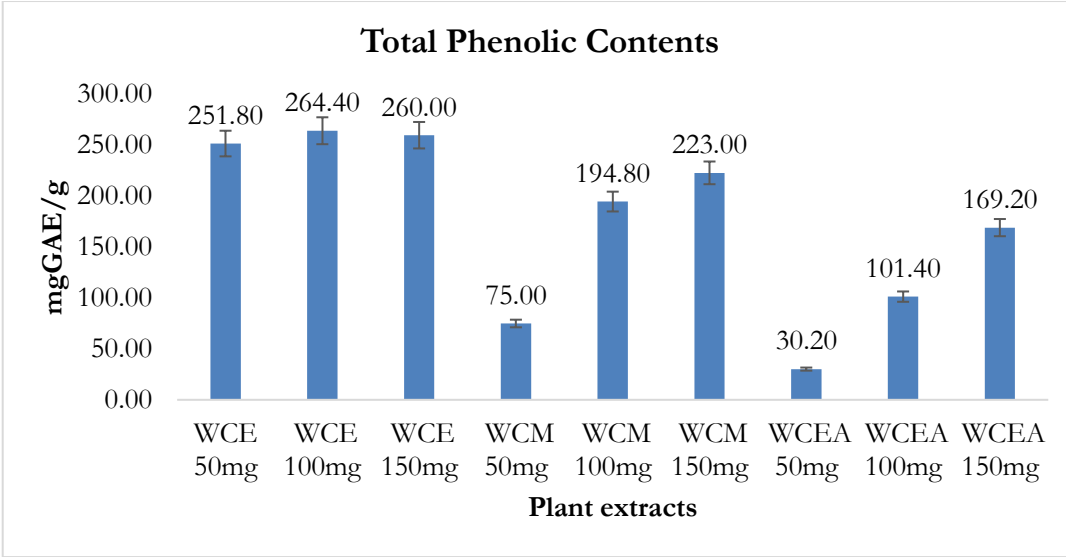

Fig. 4: Phenolic Contents Present in Various Extracts of Seeds of W. Coagulans Plant Prepared in Different Solvents at Different Concentrations.

Phenolic contents of seeds of Withania coagulans plant measured in milligrams of gallic acid equivalents per gram (mgGAE/g). WCE (Withania coagulans ethanol extract), WCM (Withania coagulans methanol extract), WCEA (Withania coagulans ethyl acetate extract)

Total Flavonoids Content (TFC)

Different plant components include flavonoids; which belongs to the class of polyphenolic substances, which exist both in their free form and as glycosides. Based on their capacity to donate hydrogen atoms to the free radicals, flavonoid molecules are significant antioxidant substances that are responsible for neutralizing free radicals. Additionally, for scavenging of free radicals they possess the appropriate structural qualities.

Table 5: Flavonoids Contents Present in Various Extracts of Seeds of WC Plant at Different Concentrations.

Plant extracts	Concentration	Absorbance	µg catechin equivalents per mL
Withania coagulans' seeds ethanol extract	50mg	0.637	156.316
	100mg	0.554	134.474
	150mg	0.575	140.000
Withania coagulans' seeds methanol extract	50mg	0.218	46.053
	100mg	0.143	26.316
	150mg	0.131	23.158
Withania coagulans' seeds ethyl acetate extract	50mg	0.525	126.842
	100mg	0.517	124.737
	150mg	0.582	141.842

Total Flavanoids Contents determined in ethanol, methanol and ethyl acetate extract of seeds of Withania coagulans plant each at 50, 100 and 150mg of concentration measured in microgram catechin equivalents per milliliters.

In this work sample activity is compared with standard curve of catechin. Flavanoids content are measured as µg catechin equivalents per mL. Ethanolic extracts of seeds of WC at concentrations of 50, 100 and 150mg/ml showed flavanoid contents of 156.316, 134.474 and 140.000 µg catechin equivalents per mL respectively. Seeds of WC when evaluated with methaolic extracts with concentrations 50, 100 and 150 mg/ml showed TFC of 46.053, 26.316 and 23.158 µg catechin equivalents per mL respectively. Extracts of seeds of WC are prepared in ethyl acetate solvent at concentrations 50, 100 and 150mg/ml showed total flavanoid contents of 126.842, 124.737 and 141.842 µg catechin equivalents per mL respectively.

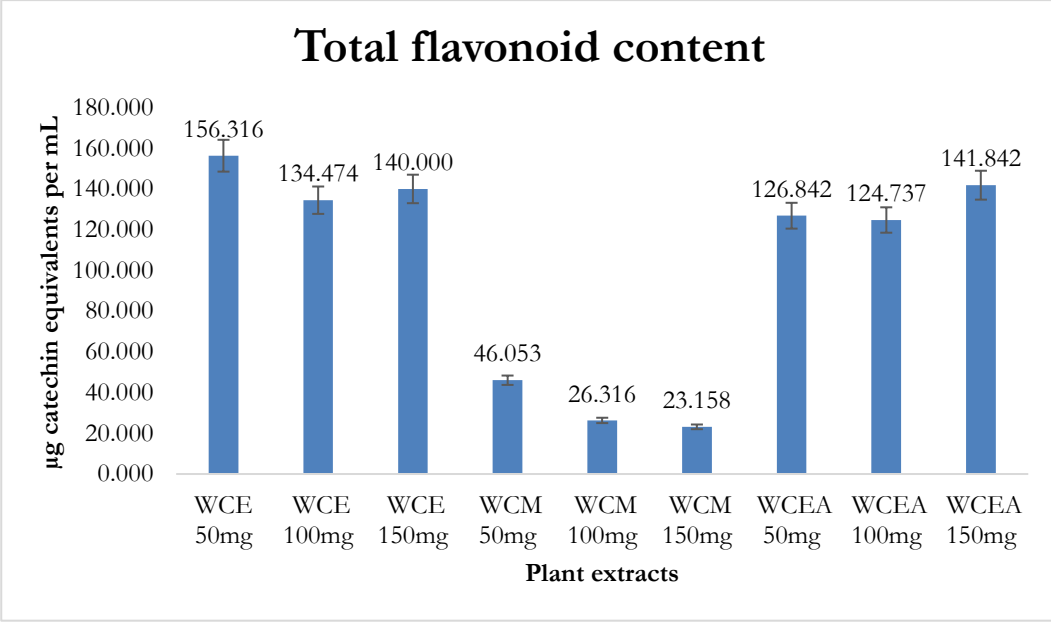

Figure 5: Flavanoid Contents Present in Various Extracts of Seeds of W. Coagulans Plant at Different Concentrations.

WCE (Withania coagulans ethanol extract), WCM (Withania coagulans methanol extract), WCEA (Withania coagulans ethyl acetate extract), µg- microgram, mL- milliliters.

DPPH Scavenging Assay

This test is considered more accurate and valid for a substance to find out its antioxidant potential. In this assay activity is performed against 2, 2-diphenyl-1-picrylhydrazyl which is a strong and stable free radical. Ascorbic acid is taken as standard and placebo as negative control in comparison to the sample. When ethanolic extracts of seeds of WC were tested at concentrations of 50, 100 and 150mg/ml showed 59.919, 75.483 and 76.602 % inhibition of DPPH free radicals respectively. Methanolic extracts of seeds of WC plant at concentrations50, 100 and 150mg/ml exhibit 72.330, 73.347 and 73.042 % inhibition of DPPH free radicals respectively. When tested against ethyl acetate extracts of seeds of WC plant at concentrations of 50, 100 and 150mg/ml showed 32.350, 34.283 and 34.588 % inhibition of DPPH of free radicals respectively. Among all the extracts ethanolic extract at 150mg/ml of concentration showed the maximum inhibition of DPPH•.

Table 6: DPPH Assay of Various Extracts of Seeds of Withania Coagulans Plant Each at Different Concentration.

Plant extracts	Concentration	Blank absorbance	Sample absorbance	% inhibition of DPPH
Withania coagulans' seeds ethanol extract	50mg	0.983	0.394	59.919
	100mg	0.983	0.241	75.483
	150mg	0.983	0.23	76.602
Withania coagulans' seeds methanol extract	50mg	0.983	0.272	72.330
	100mg	0.983	0.262	73.347
	150mg	0.983	0.265	73.042
Withania coagulans' seeds ethyl acetate extract	50mg	0.983	0.665	32.350
	100mg	0.983	0.646	34.283
	150mg	0.983	0.643	34.588

Inhibition of DPPH free radicals by ethanol, methanol and ethyl acetate extracts of seeds of Withania coagulans plant each at 50, 100 and 150mg of concentration

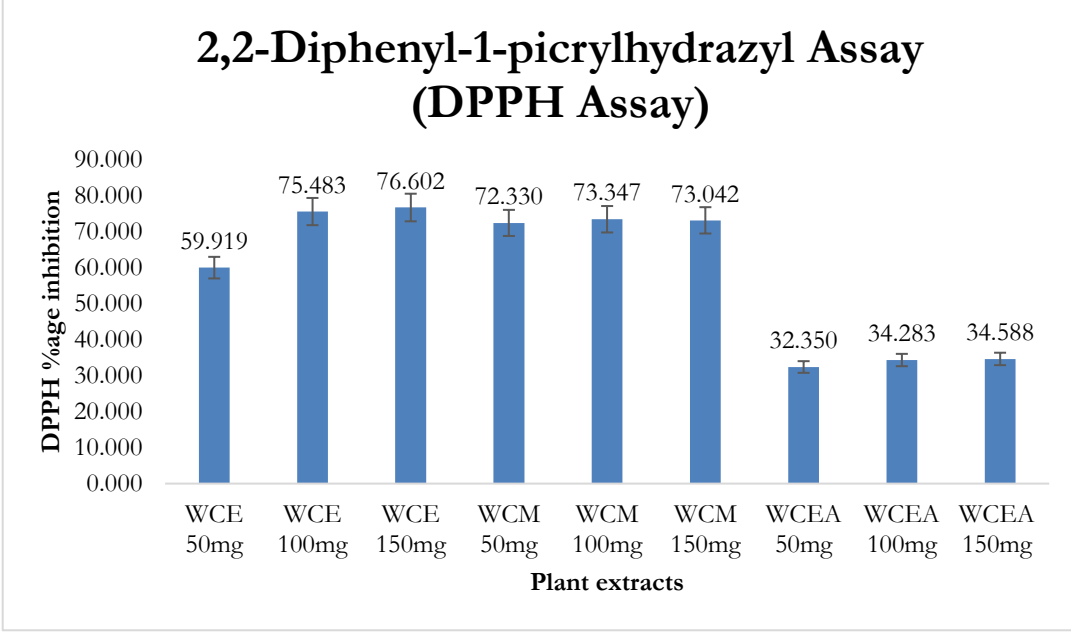

Fig 6: %Age Inhibition of DPPH Free Radical by Various Extracts of Seeds of W. Coagulans Plant at Different Concentrations.

WCE (Withania coagulans ethanol extract), WCM (Withania coagulans methanol extract), WCEA (Withania coagulans ethyl acetate extract), DPPH (diphenyl picryl hydrazyl)

Peroxidase (POD) Assay

In peroxidase assay the enzyme peroxidase reduces the hydrogen peroxide into water and oxygen. Ethanolic extracts of seeds of WC at 50, 100 and 150mg/ml concentration showed peroxidase acitivity of 6.124, 3.968 and 4.083 U/mg protein respectively. Activity when performed with methanolic extracts of seed of WC at concentration 50, 100 and 150mg/ml showed peroxidase activity of 4.725, 4.908 and 5.390 U/mg protein respectively. Upon treatment with ethyl acetate extracts of seeds of WC plant at concentrations of 50, 100 and

150mg/ml showed peroxidase activity of 5.826, 6.353 and 4.771 U/mg protein respectively. Among all the extracts the ethyl acetate extract of seeds of selected plant showed maximum peroxidase activity.

Table 7: Peroxidase Activity Exhibited by Various Extracts of Seeds of WC Plant at Various Concentrations.

Plant extracts	Concentration	Pod Absorbance	Final POD (U/mg protein)
Withania coagulans' seeds ethanol extract	50mg	0.267	6.124
	100mg	0.173	3.968
	150mg	0.178	4.083
Withania coagulans' seeds methanol extract	50mg	0.206	4.725
	100mg	0.214	4.908
	150mg	0.235	5.390
Withania coagulans' seeds ethyl acetate extract	50mg	0.254	5.826
	100mg	0.277	6.353
	150mg	0.208	4.771

POD (Peroxidase), U/Mg Protein (Unit Per Milligram of Protein).

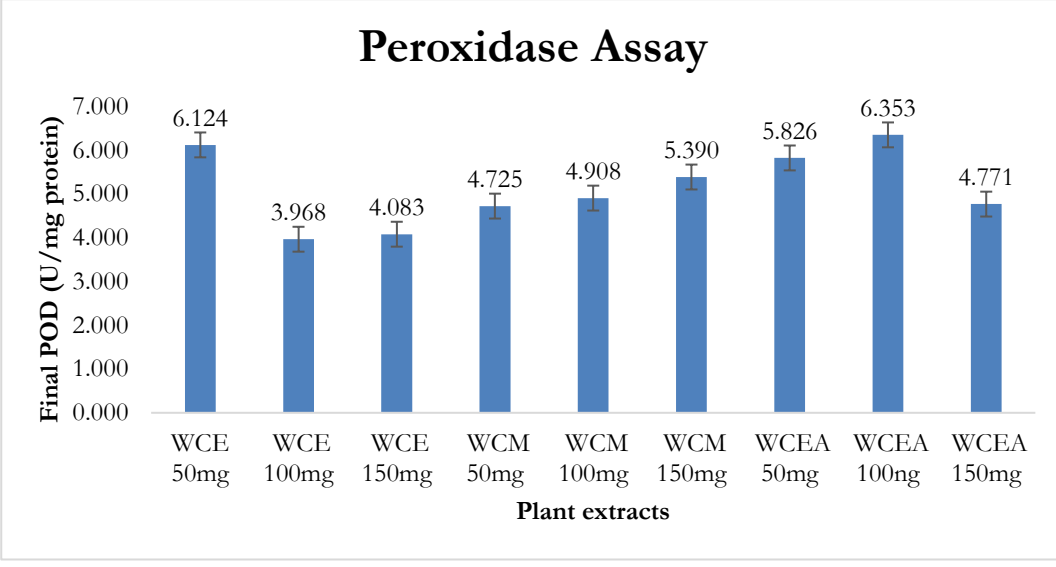

Fig. 7: Peroxidase Activity Measured in U/Mg Protein of Ethanol, Methanol and Ethyl Acetate Extracts of Seeds of W. Coagulans Plant Each with Three Different Concentrations.

WCE (Withania coagulans ethanol extract), WCM (Withania coagulans methanol extract), WCEA (Withania coagulans ethyl acetate extract), U/mg protein (unit per milligram of protein)

Superoxide Dismutase (SOD) Assay

An important defence mechanism against superoxide radical toxicity is provided by superoxide dismutase enzymes, which causes the reduction of nitro blue tetrazolium. In this activity placebo is taken as negative control in comparison to the sample extracts. Ethanolic extracts of seeds of WC plant at concentrations 50. 100 and 150mg/ml showed superoxide dismutase

activity of 8.772, 6.297 and 5.760 U/mg protein respectively. When treated with methanolic extracts of seeds of WC plant at concentrations of 50, 100 and 150mg/ml showed SOD activity of 6.349, 6.988 and 7.072 U/mg protein respectively. Activity when performed with ethyl acetate extracts of seeds of WC showed SOD results of 6.297, 5.780 and 5.092 U/mg protein respectively. Among all the extracts ethanolic extract of seeds at 50mg/ml concentration showed the maximum result.

Table 8: Superoxide Dismutase Activity Exhibited by Various Extracts of Seeds of WC Plant

Plant extracts	Concentration	SOD absorbance	Final SOD (U/mg protein)
Withania coagulans' seeds ethanol extract	50mg	1.14	8.772
	100mg	1.588	6.297
	150mg	1.736	5.760
Withania coagulans' seeds methanol extract	50mg	1.575	6.349
	100mg	1.431	6.988
	150mg	1.414	7.072
Withania coagulans' seeds ethyl acetate extract	50mg	1.588	6.297
	100mg	1.73	5.780
	150mg	1.964	5.092

Superoxide Dismutase activity of ethanol, methanol and ethyl acetate extracts of seeds of Withania coagulans plant each measured at 50, 100 and 150mg of concentration. SOD (superoxide dismutase), U/mg protein (unit per milligram of protein)

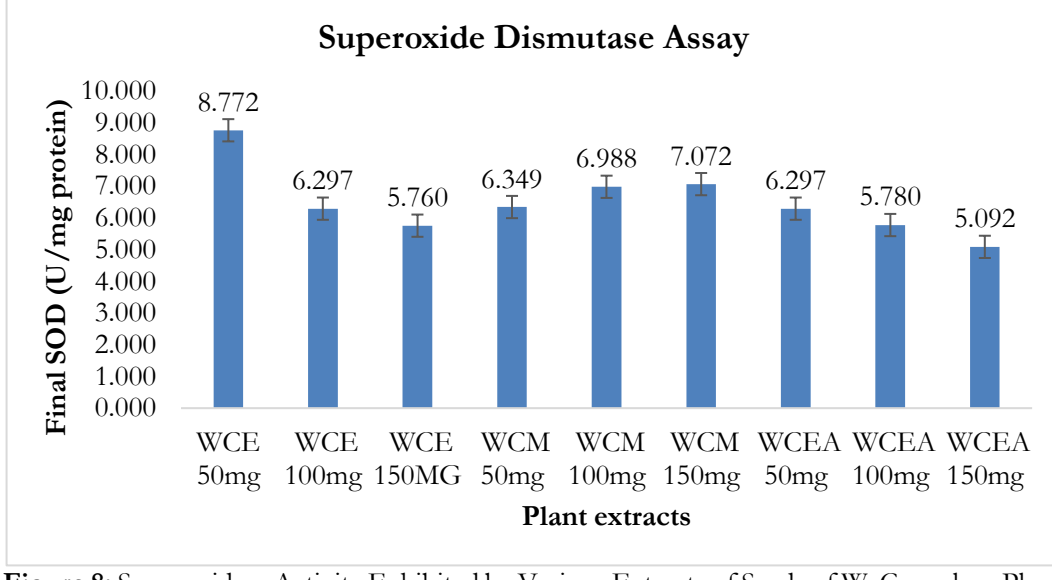

Figure 8: Superoxidase Activity Exhibited by Various Extracts of Seeds of W. Coagulans Plant.

WCE (Withania coagulans ethanol extract), WCM (Withania coagulans methanol extract), WCEA (Withania coagulans ethyl acetate extract), SOD (superoxide dismutase), U/mg protein (unit per milligram of protein)

Catalase (CAT) Assay

By monitoring the drop in H_2O_2 concentration, the degree of catalase activity in a extract / sample is identified. This enzyme prevents the cell from oxidative stress. Activity when

performed through ethanolic extracts of seeds of WC plant at concentrations 50, 100 and 150 mg/ml showed catalase activity of 30.688, 30.275 and 24.037 U/mg protein respectively. Upon treatment with methanolic extracts of seeds of WC plant at concentrations 50, 100 and 150mg/ml showed catalase activity of 23.601, 24.014 and 24.174 U/mg protein respectively. Ethyl acetate extracts of seeds of WC plant at concentrations 50, 200 and 150 mg/ml showed catalse activity of 25.000, 23.257 and 24.289 U/mg protein respectively.

Table: 9: Catalase Activity Exhibited by Various Extracts of Seeds of WC Plant at Various Concentrations.

Plant extracts	Concentration	CAT absorbance	Final CAT (U/mg protein)
Withania coagulans' seeds ethanol extract	50mg	1.338	30.688
	100mg	1.32	30.275
	150mg	1.048	24.037
Withania coagulans' seeds methanol extract	50mg	1.029	23.601
	100mg	1.047	24.014
	150mg	1.054	24.174
Withania coagulans' seeds ethyl acetate extract	50mg	1.09	25.000
	100mg	1.014	23.257
	150mg	1.059	24.289

Catalase assay of ethanol, methanol and ethyl acetate extracts of seeds of Withania coagulans plant each determined at 50, 100 and 150mg of concentration. CAT (catalase), U/mg protein (unit per milligram of protein)

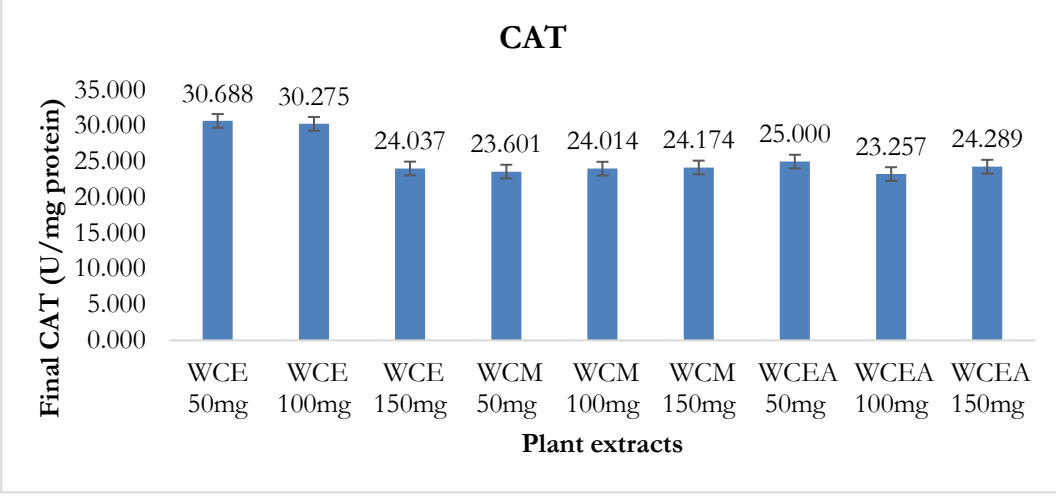

Figure 10: Catalase Activity of Various Extracts of Seeds of *W. Coagulans* Plant Each At Different Concentrations.

WCE (Withania coagulans ethanol extract), WCM (Withania coagulans methanol extract), WCEA (Withania coagulans ethyl acetate extract), CAT (catalase), U/mg protein (unit per milli gram of protein)

Future researchers will find a lot of worthwhile information and knowledge in this comprehensive research. But sill in-vivo evaluation and clinical trials has to be done yet in order to bring a novel herbal drug in the field of medicine with economical rates and lesser side-effects.

Conclusion

In this study, various activities performed in in-vitro environment indicate that this subshrub has antibacterial properties along with thrombolytic and cytotoxic potential. Presence of phytochemicals like phenolic and flavonoid contents proves its antioxidant properties. This study suggests the seeds of Withania coagulans plant as a new antibiotic medicine. For this purpose, more clinical practice and trials are needed. Furthermore, it is important to thoroughly research the toxicity and mode of action of these plant extracts against S. aureus and E. coli bacteria. To replace those drugs that are proving resistant to bacterial pathogens, it will prove itself to be new antibiotic produced from naturally existing herbs. Moreover, this medicinal flora has thrombolytic potential which could be used to prevent myocardial infarction. This research suggests the advance study in animal model to further explore the thrombolytic potential of this subshrub so that so that it may contribute towards new uni-herbal formulation which may help in the treatment of deep venous thrombosis, stroke and heart attack. The effectiveness of a traditionally used medicinal plant as a cytotoxic medicine should also be confirmed by studies on in vivo trials. Its commercial potential is also needed to be explored. For this purpose, industrial based research is needed by pharmaceutical companies. Additional research on this plant is essential in order to fully understand its impact on various diseases and its mode of action. It might be regarded as a great herbal medication in the future for the prevention and treatment of numerous illnesses.

List of Abbrevations

WHO World Health Organization
APIActive Pharmaceutical Ingredients
TOM Traditional Oriental Medicine
NPGS National Plant Germplasm System
LDL Low Density Lipoprotein
AST Aspartate Aminotransferase
ALP Alkaline Phosphatase
H2O2 Hydrogen Peroxide
MRI Magnetic resonance Imaging
TPA Tissue Plasmiogen Activator
TXA2 Thromboxane A2
BC Before Christ
MVA Mevalonate
MEP Methyl Erythritol Phosphate

Conflict of Interest

The authors have no conflict of interest**.**

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