International Journal of Phytopharmacology
Research Article

www.onlineijp.com

e- ISSN 0975 – 9328
Print ISSN 2229 – 7472

ANTIDIABETIC, ANTIOXIDANT, ANTIBACTERIAL ACTIVITIES AND GC-MS ANALYSIS OF *WITHANIA COAGULANS* DUNAL-(RISHYAGANDHA)

Sivaraj .C[*], Saraswathi .K[1], Jayashree .M[2], Srija Reddy .C[2], Arumugam .P[1]

[*,1]ARMATSBiotek Training and Research Institute, Chennai – 600 032, Tamil Nadu, India.
[2]Department of Biochemistry, Meenakshi College for Women, Kodambakkam, Chennai– 600 024, Tamil Nadu, India.

ABSTRACT

Withania coagulans (Indian rennet) is an important medicinal plant which belongs to family Solanaceae. Roots, leaves, stem, green berries, fruits, seed, bark of this plant is used as folk medicine, because of its medicinal properties like antifungal, antibacterial, antitumor, antidiabetic, antibiotic, antistress, antiasthmatic, anticancer, antioxidant, anti-inflammatory activities. The research studies were carried out to evaluate the antidiabetic, antioxidant, and antibacterial activities of dried fruit extract of *W. coagulans* and to identify the bioactive compounds by GC-MS analysis. Antioxidant activities such as DPPHradical, superoxide ($O_2^{\cdot}$-) radical scavenging activities, Fe^{3+} reducing power and phosphomolybdenum reduction activities were performed for dried fruit extract in order to reveal the efficacy of antioxidant molecules in neutralizing the free radicals. The maximum alpha amylase enzyme inhibition ofmethanol dried fruit extract of *W. coagulans* was 66.60±0.23% at 300 µg/mL concentration and the IC_{50} value was 174.91µg/mL concentration respectively. The maximum DPPH$^{\cdot}$ radical and superoxide ($O_2^{\cdot}$-) radical scavenging activities of methanol dried fruit extract of *W. coagulans* were 54.68±0.23% and 42.85±0.35% at 120 µg/mL concentration and the IC_{50} values were 118.53 µg/mL and 140.02 µg/mL concentration respectively. The maximum Fe^{3+} and Mo^{6+} reduction of methanol dried fruit extract of *W. coagulans* were 77.94±0.45% and 86.13±0.21% at 120 µg/mL concentration and the RC_{50} values were 40µg/mL and 17.07µg/mL concentration respectively. GC-MS analysis of methanol dried fruit extract of *W. coagulans* showed the presence of many active compounds such as a-Pinene, Longifolene-I2.Elaidic acid, isopropyl ester, etc. The antibacterial activity for the methanol dried fruit extract of *W. coagulans* confirmed maximum inhibitory property as 20 mm against tested bacterial pathogens at 375 µg/mL concentration.

Key words: *W. coagulans*, DPPH$^{\cdot}$ radical, superoxide ($O_2^{\cdot}$-) radical, diastase, GCMS.

Corresponding Author**C. Sivaraj** Email: shivaraj27@gmail.com

INTRODUCTION

The traditional medicines are increasingly solicited through the traditional practitioners and herbalists in the treatment of infectious diseases.

Medicinal plants play a vital role for the development of new drugs. The bioactive extract should be standardized on the basis of active compounds (Dymock *et al.*, 1972). *Withania coagulans* is commonly known as 'Indian cheese maker' or 'vegetable rennet' because fruits and leaves of this plant are used as a coagulant. This shrub is, common in, East India, Afghanistan and Nepal. In India it occurs in Rajasthan, Punjab, Simla, Garhwal and Kumaun. The milk coagulating property of the fruits is attributed to the pulp

Access this article online

DOI: http://onlineijp.com/

DOI: http://dx.doi.org/10.21276/ijp.2020.11.1.3

Quick Response code

Received:21.08.19	Revised:11.09.19	Accepted:18.09.19

and husk berries which contain an enzyme called Withanin, having milk-coagulating activity. In Pakistan, the berries of *W. coagulans* are commonly used to clot milk which is called, 'paneer'. The fruits are sweet and used to treat dyspepsia, flatulent colic and other intestinal infections.In some parts of Indian-Pak sub-continent, the berries areused as a blood purifier. The twigs are often chewed forteeth cleaning and the smoke of the plant is inhaled for pain relief in tooth ache. In addition, *W. coagulans*is used to treat impotence, nervous exhaustion, disability, insomnia, failure to thrive in children. Flowers of the plant are useful in the treatment ofdiabetes (Krishnamurthi, 1969; Bown, 1995)

The term "withanolide" is a structural term that has been used for "withan" from the genus Withania, and "olide" is chemical term for a lactone. Different withanolides, withacoagin and coagulan reported from *W. coagulans*. Withaferin A (Steroidal lactones of withanolide series) had been isolated from fruits of *W. coagulans*(Maurya and Akanksha, 2010).Anti proliferative, Pre-apoptotic, anti-invasive, anti-osteoclastogenic, antiangiogenic, anti-metastatic, radio sensitizing, antiarthritic and cardioprotective effects assigned to Withanolide may be mediated in part through the suppression of NF-EB and NF-EB regulated gene products (Amit vaibhav *et al.,* 2013). The research studies were carried out to evaluate the antidiabetic, antioxidant, and antibacterial activities of dried fruit extract of *W. coagulans* and to identify the bioactive compounds by GC-MS analysis.

Taxonomic Classification of *Withania coagulans*

Domain: Eukaryota
Kingdom: Plantae
Division: Magnoliophyta
Class: Magnolipsida
Order: Solanales
Family: Solanaceae
Genus: *Withania*
Species: *coagulans*
Binomial name: *Withania coagulans*

MATERIALS AND METHODS

Collection of Plant material and extract preparation

The fruits of *Withania coagulans* were collected from the market at Maduvinkarai, Guindy,Chennai, India. The fruits were dried in shade, cut into small pieces and about 10 grams of dried fruits of *Withania coagulans* were soaked in methanol for 72 hours (Trease and Evans, 1983). Then the supernatant was filtered and condensed in a hot plate at 50°C, to yield gummy extract for further studies.

Qualitative phytochemical analysis

The methanol dried fruit extract of *W. coagulans* was subjected to preliminary phytochemical screening using standard methods and screened for different classes of phytoconstituents using specific reagents (Harborne, 1973; Raaman, 2006).

Determination of total phenols

Folin-Ciocalteau reagent method was used to determine the total phenolic compounds with slight modifications. One hundred µL of methanol dried fruit extract (1 mg/mL) of *W. coagulans* was mixed with 900 µL of methanol and 1 mL of Folin Ciocalteau reagent (1:10 (v/v) diluted with distilled water). After 5 min, 1 mL of 20% Na_2CO_3 (w/v) solution was added. The mixture was then allowed to stand for 30 min incubation in dark at room temperature. The absorbance was measured using UV-Vis Spectrophotometer at 765 nm (Spanos and Wrosltad, 1990) and the total phenolic content was expressed in terms of gallic acid equivalent (µg/mg of extract), which is a common reference compound.

Determination of total flavonoids

The total flavonoid content of methanol dried fruit extract of *W. coagulans*was determined using aluminium chloride colorimetric method with slight modifications. One mL of methanol dried fruit extract (1 mg/mL) of *W. coagulans* was mixed with 0.5 mL of 5% (w/v) sodium nitrite solution and incubated for 5 min at room temperature. Then, 0.5 mL 10% (w/v) aluminium chloride solution was added and incubated for further 5 min at room temperature followed by the addition of 1 mL of 1 M NaOH solution. The absorbance was measured using UV-Vis Spectrophotometer at 510 nm (Liu *et al.*, 2007) and the total flavonoid content was expressed in terms of quercetin equivalent (µg/mg of extract), which is a common reference compound.

Antidiabetic activity by Starch-iodine method

α- amylase enzyme inhibition activity was carried out based on the starch-iodine test. The total mixture was composed of various concentrations (50-300 µg/mL) of methanol dried fruit extract of *W. coagulans*, 10 µL of alpha amylase enzyme prepared in 0.02 M sodium phosphate buffer (pH 6.9 containing 6 mM sodium chloride) and was incubated at 37°C for 10 min. Then soluble starch (1%, w/v) was added to each reaction set and incubated at 37°C for 60 min. One hundred µL of 1 M Hydrochloric acid was added to stop the enzymatic reaction and followed by 200 µL of iodine reagent (5 mM Iodine and 5 mM Potassium Iodide) was added. The colour change was noted and the absorbance was measured using UV-Vis Spectrophotometer at 595 nm (Hossan*et al.,* 2009) and the IC_{50} was calculated. The control reaction representing 100% enzyme activity did not contain any dried fruit extract. A dark-blue colourindicates the presence of starch; a yellow colour indicates the absence of starch, while a brownish colour

indicates partially degraded starch in the reaction mixture. In the presence of inhibitors from the extract, the starch added to the enzyme assay mixture is not degraded and gives a dark blue colour complex, whereas no colour complex is developed in the absence of the inhibitor, indicating that starch is completely hydrolyzed by α-amylase. Acarbose was used as the standard reference. The percentage inhibition of alpha amylase enzyme was calculated as:

$$\% \text{ inhibition of alpha amylase enzyme} = \left[\frac{\text{Sample} - \text{Control}}{\text{Sample}}\right] \times 100$$

Antioxidant activities

DPPH˙ radical scavenging activity

The antioxidant activity of methanol dried fruit extractof *W. coagulans*was measured on the basis of the scavenging activity of the stable 1, 1- diphenyl 2-picrylhydrazyl (DPPH) free radical. One mL of 0.1 mM DPPH solution was mixed with 1 mL of various concentrations (20-120 µg/mL) of dried fruit extractof *W. coagulans*. The mixture was then allowed to stand for 30 min incubation in dark condition. One mL of methanol and one mL of DPPH solution was used as the control. The decrease in absorbance was measured using UV-Vis Spectrophotometer at 517 nm (Khalaf*et al.,* 2008) and the IC_{50} was calculated. Ascorbic acid was used as the standard reference. The percentage of inhibition was calculated as:

$$\% \text{ of DPPH}^{\cdot} \text{ radical inhibition} = \left[\frac{\text{Control} - \text{Sample}}{\text{Control}}\right] \times 100$$

Superoxide ($O_2^{\cdot}$-) radical scavenging activity

The Superoxide ($O_2^{\cdot}$-)radical scavenging activity was carried out by the modified method (Lokesh Deb *et al.,* 2009). The reaction mixture contains different concentrations (20-120 µg/mL)of methanol dried fruit extract of *W. coagulans* with 50 mM of phosphate buffer (pH 7.6), 1.5 mM of riboflavin, 12mM of EDTA and 50 mM of NBT, added in that sequence. The reaction was started by illuminating the reaction mixture for 15 min. Immediately after illumination, the absorbance was measured using UV-Vis spectrophotometer at 590 nm and the IC_{50} was calculated. Ascorbic acid was used as the standard reference. The percentage of inhibition was calculated as:

$$\% \text{ of Superoxide } (O_2^{\cdot}\text{-}) \text{ radical inhibition} = \left[\frac{\text{Control} - \text{Sample}}{\text{Control}}\right] \times 100$$

Ferric (Fe^{3+}) reducing power activity

The reducing power of methanol dried fruit extract of *W. coagulans*determined by slightly modified method (Oyaizu, 1986). One mL of methanol dried fruit extract of *W. coagulans*of different concentrations (20-120 µg/mL) was mixed with phosphate buffer (1 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (1 mL, 1 % w/v). The mixtures were then incubated at 50°C in water bath for 30 min. 0.5 mL of trichloroacetic acid (10 % w/v) was added to all test tubes. Then to the mixture 0.1 mL of ferric chloride (0.01%, w/v) was added and the absorbance was measured using UV-Vis Spectrophotometer at 700 nm and the RC_{50} was calculated. Ascorbic acid was used as the standard reference. The percentage of Fe^{3+}reduction was calculated as:

$$\% \text{ of } Fe^{3+} \text{ reduction} = \left[\frac{\text{Sample} - \text{Control}}{\text{Sample}}\right] \times 100$$

Phosphomolybdenum reduction activity

The antioxidant capacity of dried fruit extract of *W. coagulans* was assessed as described (Prieto *et al.,* 1999). The methanol dried fruit extract of *W. coagulans* with concentrations (20-120 µg/mL) was combined with reagent solution containing ammonium molybdate (4 mM), sodium phosphate (28 mM) and sulphuric acid (600 mM). The reaction mixture was incubated in water bath at 90°C for 90 min. The absorbance of the coloured complex was measured using UV-Vis Spectrophotometer at 695 nm and the RC_{50} was calculated. Ascorbic acid was used as the standard reference. The percentage of Mo^{6+} reduction was calculated as:

$$\% \text{ of } Mo^{6+} \text{ reduction} = \left[\frac{\text{Sample} - \text{Control}}{\text{Sample}}\right] \times 100$$

Thin layer chromatography

Thin layer chromatography (TLC) was carried out for the methanol dried fruit extract of *W. coagulans* in Merck TLC aluminium sheets, silica gel 60 F254 (20 x 20 cm), preloaded plates . The dried fruit extract was spotted at 0.3 mm above from the bottom of the TLC plate. The chromatogram was developed in a mixture of suitable solvent system. The spots were visualized under UV light at 254 nm (Stahl, 2005). The ratio in which distinct bands appeared was optimized and R_f values were calculated.

Calculation of $R_f$value:

$$R_f \text{ value} = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}}$$

Antibacterial activity

Microbial strains used for antibacterial assessment

The microorganisms of Gram negative strains such as *Escherichia coli, Klebsiella pneumonia* and*Shigellaflexneri*as well as Gram positive strains such as *Staphylococcus aureus, Bacillus subtilis* and *Micrococcus luteus*were used for the evaluation of antibacterial activity. Various concentrations of methanol dried fruit extract of *W. coagulans*(125 µg, 250 µg and 375 µg) were added into the wells made using sterile cork borer. Tetracycline (20 µg) was used as a positive control. The plates were then incubated at 37°C for 24 hours. The

antibacterial activity for the dried fruit extract was assessed by measuring the diameter of the inhibition zone formed around the well made in nutrient agar medium (Kubo *et al.,* 2002).

Gas chromatography–Mass Spectrometry (GC–MS) analysis

For GC-MS analysis, the methanol dried fruit extract of *W. coagulans* were injected into a HP-5 column (30 m X 0.25 mm i.d with 0.25 µm film thickness), Agilent technologies 6890 N JEOL GC Mate II GC-MS model. Following chromatographic conditions were used: Helium as carrier gas, flow rate of 1 mL/min; and the injector was operated at 200°C and column oven temperature was programmed as 50-250°C at a rate of 10°C/min injection mode. Following MS conditions were used: ionization voltage of 70 eV; ion source temperature of 250°C; interface temperature of 250°C; mass range of 50-600 mass units (Saraswathi*et al.,* 2019).

Identification of components

The database of National Institute Standard and Technology (NIST) having more than 62,000 patterns was used for the interpretation on mass spectrum of GC-MS. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library.

RESULTS AND DISCUSSION

Phytochemical analysis

The phytochemical analysis of methanol dried fruit extract of *W. coagulans*showed the presence of alkaloids, terpenoids, phenolic compounds, flavonoids, glycosides and saponins (Table 1) indicating various therapeutic activities.

Total phenols and flavonoids determination

The phenol and flavonoid compounds quantified in the methanol dried fruit extract of*W. coagulans* seemed to be responsible for the antioxidant activity. The total phenol content was 254.25±0.17 µg/mg of GAE and the total flavonoid content was 28.20±0.38 µg/mg of QE in the extract(Table 2). These results provide a comprehensive profile of the antioxidant activity of methanol dried fruit extract of *W. coagulans*with respect to their phenols and flavonoids content.

The difference in amounts of phenols is probably related to geographical and enviromental factors, processing methods and other intrinsic factors (genetic, extracting solvent) and extrinsic (environmental, handling and development stage) which may play role in such a large variation (FratiaFratianni*et al.,* 2007). Also, the phenol content of a plant depends on a number of as well as the Folin-Ciocalteau assay gives a crude estimate of the total phenolic compounds present in an extract/fraction. It is not specific to polyphenols, but many interfering compounds may react with the reagent, giving elevated apparent phenolic concentrations (Prior *et al.,* 2005). Oxidative stress is considered to be substantial, if not crucial, in the initiation and development of many current conditions and diseases, including: inflammation, autoimmune diseases, cataract, cancer, parkinson's disease, arteriosclerosis and aging (Lukyanova*et al.,* 2007).

(1) Scavenging radical species such as ROS/ reactive nitrogen species (RNS)
(2) Suppressing ROS/RNS formation by inhibiting some enzymes or chelating trace metals involved in free radical production;
(3) Up regulating or protecting antioxidant defense (Cotelle, 2001).
The reduction activity of phenolic and flavonoid compounds depends on the number of free hydroxyl groups in the molecular structure, which would be strengthened by steric hindrance (Rice-Evans *et al.,* 1996).

Antidiabetic activity by Starch-iodine colour method

Diabetes mellitus (DM) is a chronic disease characterized by a deficiency in insulin production and its action or both. That leads to prolonged hyperglycemia with disturbances in most metabolic processes inside the human body (Bastaki, 2005). The most popular methods for diagnosis of diabetes includes the measuring of fasting plasma glucose level (FPG), which is done in the early morning. Patients with FPG below 100 mg/dl are considered normal; those between 100 and 125 mg/dl indicate pre-diabetic while those individuals with glucose levels above 125 mg/dl are considered diabetic (Peters *et al.,* 1996). The alpha-glucosidase inhibitors "starch blockers" inhibit certain enzymes responsible for the breakdown of carbohydrates in the small intestine. They act mainly by decreasing the rate of carbohydrate absorption in the body. Moreover, acarbose, an important example in this class, reversibly inhibits both pancreatic α-amylase and α-glucosidase enzymes by binding to the carbohydrate-binding region and interfering with their hydrolysis into mono-saccharides. This results in a slower absorption together with a reduction in postprandial blood-sugar levels (DeFronzo, 1999; Lebovitz, 1997).

The ability of for α-amylase enzyme inhibition was assessed by starch-iodine method. The maximum α-amylase enzyme inhibition of methanol dried fruit extract of *W. coagulans* was 66.60±0.23% at 300 µg/mL concentration (Table 3 and figure 2)and the IC_{50} value was 174.91 µg/mL concentration respectively. It was compared with the standard Acarbose (IC_{50} = 10.47 µg/mL concentration).

Higher concentrations of Magnesium and lower concentrations of Potassium play a vital role in diabetes

management (Lopez-Ridaura*et al.,* 2004; Fox *et al.*, 2001). Hence the significant antidiabetic potential of *Withaniacoagulans* could be due to the high concentration of Magnesium along with Calcium. The Ca^{2+} ion activates insulin gene expression via CREB (Calcium Responsive Element Binding Protein) and is responsible for exocytose of stored insulin (Veiga*et al.,* 2006).

Antioxidant activities

DPPH˙ radical scavenging activity

DPPH˙ radical scavenging method is a decolorization assay that will measure the capacity of antioxidants to directly scavenge DPPH˙radicals by monitoring its absorbance using spectrophotometer at wavelength of 517 nm (Awika*et al.,* 2003). The DPPH method provided rapid and an easy way to evaluate the antioxidant activity of most of the plant extracts. The methanol dried fruit extract of *W. coagulans* to scavenge free radicals was assessed by using DPPH˙ radical as the substrate, which measures the hydrogen or electron donating ability of dried fruit extract. The methanol dried fruit extract of *W. coagulans* reducing the stable purple colour DPPH (1,1-diphenyl-2- picrylhydrazyl) free radical to the yellow coloured 1,1-diphenyl-2-picryl hydrazine and the reduction capacity increases with increasing concentration of the extract. The maximum DPPH˙ radical scavenging activity of methanol dried fruit extract of *W. coagulans* was 54.68±0.23% at 120 µg/mL concentration(Table 4 and Figure 3). The IC_{50} value was 118.53 µg/mL concentration respectively and was compared with standard ascorbic acid (IC_{50} = 8.56 µg/mL concentration).

Superoxide ($O_2^{\cdot-}$)radical scavenging activity

Superoxide anion is also very harmful to cellular components and their effects can be magnified because it produces other kinds of free radicals and oxidizing agents. Flavonoids are effective antioxidants, mainly because they scavenge superoxide anions. Superoxide anions derived from dissolved oxygen by the riboflavin-light-NBT system will reduce NBT in this system. In this method, superoxide anion reduces the yellow dye (NBT^{2+}) to blue formazan, which is measured at 590 nm in UV-Vis spectrophotometer. Antioxidants are able to inhibit the blue NBT formation and the decrease of absorbance with antioxidants indicates the consumption of superoxide anion in the reaction mixture (Wickens, 2001). The maximum superoxide ($O_2^{\cdot-}$) radical scavenging activity of methanol dried fruit extract of *W. coagulans*was 42.85±0.35% at 120 µg/mL concentration (Table 4 and Figure 3) and the IC_{50}value was 140.02 µg/mL concentration respectively. It was compared with the standard of ascorbic acid (IC_{50} = 10.72 µg/mL concentration).

Ferric (Fe^{3+}) reducing power activity

The reducing power assay was carried out by the reduction of Fe^{3+} to Fe^{2+} by the methanol dried fruit extract of *W. coagulans* and the subsequent formation of ferro-ferric complex. Fe (III) reduction is often used as an indicator of electron donating activity, which is an important mechanism of phenolic antioxidant action (Yildirim*et al.,* 2001). The reducing ability of a compound generally depends on the presence of reductones (antioxidants), which exert the antioxidant activity by breaking the free radical chain by donating a hydrogen atom or by neutralizing the free radicals by donating an electron and become lone pair of electrons instead of odd electron. The reduction ability increases with increase in concentration of the extract. The maximum Fe^{3+} reduction of methanol dried fruit extract of *W. coagulans* was 77.94±0.45% at 120 µg/mL concentration (Table 5 and Figure 4) with the RC_{50}value of 40 µg/mL concentration respectively and was compared with the standard ascorbic acid (RC_{50} = 8.94 µg/mL).

Phosphomolybdenum reduction activity

Metal-Catalyzed Oxidation (MCO) systems catalyze the reduction reaction, which alters the nature of proteins at the metal-binding site and cause DNA and protein damage (Stadtman, 1990). The total antioxidant activity of methanol dried fruit extract of *W. coagulans* was measured by phophomolybdenum reduction method which is based on the reduction of Mo (VI) to Mo (V) by the formation of green phosphate/Mo (V) complex at acidic pH, with a maximum absorption at 695 nm. The maximum phosphomolybdenum reduction of methanol dried fruit extract of *W. coagulans*was 86.13±0.21% at 120 µg/mL concentration (Table 5 and Figure 4) with the RC_{50}value of 17.07 µg/mL concentration respectively. It was compared with the standard ascorbic acid (RC_{50} = 7.62 µg/mL).

Thin Layer Chromatography

Thin layer chromatography analysis for methanol dried fruit extract of *W. coagulans*was carried out in the solvent system of Toluene:Ethanol in the ratio of 0.2:1.8 (Figure 5) and retention factor was calculated based on the solvent front movement. Different R_f values of the compounds provides an idea about their polarity that may also help in selecting a particular solvent system for further isolation of any compound from the plant extracts using chromatographic and spectroscopic techniques (Biradar and Rachetti, 2013). Compounds showing high R_f value in less polar solvent system have low polarity while those with a low $R_f$value have high polarity (Talukdar*et al.,* 2010).

Antibacterial activity

The methanol dried fruit extract of *W. coagulans* were investigated for *in vitro* antibacterial activity against

microorganism including Gram-positive bacteria (*Bacillus subtilis, Micrococcus luteus,Staphylococcus aureus*) and Gram-negative bacteria *(Escherichia coli, Shigella flexneri, Klebsiella pneumoniae*). The antibacterial sensitivity of the crude extract and their potency were assessed quantitatively by measuring the diameter of clear zone in cultures in petriplates. The maximum inhibitory effect for methanol dried fruit extract of *W. coagulans* against tested bacterial pathogens such as *Bacillus subtilis* and *Staphylococcus aureus* were 20 mm at 375 µg/mL concentration respectively (Table 6 and Figure 6). The antibacterial activity of the dried fruit extract could be correlated as due to the presence of secondary metabolites such as flavonoids, phenolic compounds, terpenoids, tannins and alkaloids that adversely affect the growth and metabolism of microbes.

Tannins bind to proline rich proteins and interfere with the protein synthesis (Shimada, 2006). Flavonoids are hydroxylated phenolic substance known to be synthesized by plants in response to microbial infection and it should not be surprising that they have been found in vitro to be effective antimicrobial substances against a wide array of microorganisms. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls (Marjorie, 1999). Antimicrobial property of saponins is due to its ability to cause leakage of proteins and certain enzymes from the cell (Zablotowicz *et al.,* 1996). Steroids have been reported to have antibacterial properties, the correlation between membrane lipids and sensitivity for steroidal compound indicates the mechanism in which steroids specifically associate with membrane lipid and exerts its action by causing leakages from liposomes (Raquel F. Epand, 2007).

Gas chromatography–Mass Spectrometry (GC–MS) analysis

GC-MS studies have been increasingly applied for the analysis of medicinal plants as this technique has proved to be a valuable method for the analysis of nonpolar components and volatile essential oil, fatty acids, lipids and alkaloids. GC-MS analysis was carried out for the methanol dried fruit extract of *W. coagulans* and the eluted compounds were showed in Table 7. The identification of the phytoactive compounds was confirmed based on the peak area, retention time and molecular formula along with the pharmacological activities (Figure 7 and Table 8).

Table 1.Qualitative analysis of methanol dried fruit extract of *W. coagulans*

S.No	Phytochemicals	Tests	Results
1	Alkaloids	Wagner's reagent	+
2	Terpenoids	$CHCl_3$ + conc. H_2SO_4	+
3	Steroids	Liebermann–Burchard test (acetic anhydride+ Con. H_2SO_4)	+
4	Flavonoids	NaOH solution	+
5	Phenols	$FeCl_3$ solution	+
6	Tannins	Lead acetate solution	+
7	Glycosides	Sodium nitroprusside solution + Con. H_2SO_4	+
8	Saponins	Foam test	+

Table 2. Quantitative estimations of methanol dried fruit extract of *W. coagulans*

S.No	Phytochemicals	Value (µg/mg)
1	Phenols	254.25±0.17 GAE
2	Flavonoids	28.20±0.38 QE

Table 3. Starch-iodine colour activity of methanol fruit extract of *W. coagulans*

S.No	Concentration(µg/mL)	Percentage of inhibition*
1	50	11.31±0.42
2	100	20.37±0.38
3	150	38.04±0.17
4	200	57.17±0.19
5	250	62.93±0.40
6	300	66.60±0.23

(*Average value of 3 replicates)

Table 4. DPPH˙ radical and Superoxide ($O_2^{\cdot-}$) radical scavenging activities of methanol dried fruit extract of *W. coagulans*

S.No	Concentration (µg/mL)	Percentage of inhibition*	
		DPPH˙ radical	Superoxide ($O_2^{\cdot-}$) radical
1	20	9.37±0.25	8.16±0.16
2	40	18.75±0.31	12.24±0.32
3	60	22.50±0.17	17.95±0.28
4	80	36.87±0.48	24.48±0.10
5	100	42.18±0.11	36.73±0.41
6	120	54.68±0.23	42.85±0.35

(*Average value of 3 replicates)

Table 5. Phosphomolybdenum and Fe^{3+} reduction activities of methanol dried fruit extract of *W. coagulans*

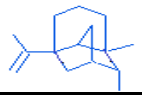
S.No	Concentration (µg/mL)	Percentage of reduction*	
		Fe^{3+} reduction	Mo^{6+} reduction
1	20	25.00±0.36	58.56±0.39
2	40	50.00±0.29	61.48±0.30
3	60	63.41±0.43	65.21±0.26
4	80	69.38±0.22	77.04±0.39
5	100	73.68±0.15	80.00±0.16
6	120	77.94±0.45	86.13±0.21

(*Average value of 3 replicates)

Table 6. Antibacterial activity of methanol dried fruit extract of *W. coagulans*

S.No	Bacterial pathogens	Zone of inhibition (mm)			
		Standard Tetracycline – 20 µg	125 µg	250 µg	375 µg
1	*Bacillus subtilis*	24	17	19	20
2	*Micrococcus luteus*	19	12	15	18
3	*Staphylococcus aureus*	21	14	16	20
4	*Escherichia coli*	21	15	17	18
5	*Shigellaflexneri*	33	12	13	19
6	*Klebsiellapneumoniae*	22	16	17	19

Table 7. GCMS analysis of methanol dried fruit extract of *W. coagulans*

S.No	RT	Compound Name	Compound Structure	Mol. Wt (g/mol)	Mol. Formula
1	11.67	`a – Pinene		136.234	$C_{10}H_{16}$
2	13.08	Undecane		156.308	$C_{11}H_{24}$
3	14.60	Longifolene-I2		204.351	$C_{15}H_{24}$
4	16.00	Dodecanoic acid, 10-methyl; methyl ester		228.371	$C_{14}H_{28}O_2$
5	16.55	n-Hexadecenoic acid		256.424	$C_{16}H_{32}O_2$

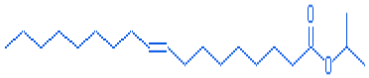
6	17.53	10-Octadecenoic acid, methyl ester		296.488	$C_{19}H_{36}O_2$
7	18.12	9-Hexadecenoic acid		254.408	$C_{16}H_{30}O_2$
8	21.53	Elaidic acid, isopropyl ester		324.541	$C_{21}H_{40}O_2$
9	25.8	Methoxy acetic acid, octadecyl ester		342.556	$C_{21}H_{42}O_3$

Table 8. Pharmacological activities of methanol dried fruit extract of *W. coagulans*

S.No	Compound Name	Pharmacological activity*
1	n-Hexadecenoic acid	Antioxidant activity Nematicide Pesticide Lubricant Antiandrogenic activity Flavor enhancer Hemolytic agent
2	10-Octadecenoic acid, methyl ester	Antioxidant and antimicrobial activities
3	9-Hexadecenoic acid	Antioxidant and anticancer activities
4	Elaidic acid, isopropyl ester	Anti-inflammatory activity Hypocholesterolemic activity Cancer preventive agent Hepatoprotective activity Anticoronary activity Antieczemic activity Insectifuge activity
5	Methoxy acetic acid, octadecyl ester	Antimicrobial activity

***-**(Dr. Duke's: Phytochemical and Ethnobotanical Databases; Elaiyaraja and Chandramohan, 2016; Syeda*et al.*, 2011; Hema *et al.*, 2011).

Fig 1. Habitat of *Withania coagulans*

Fig 2. Starch-iodine colour activity of methanol fruit extract of *W. coagulans*

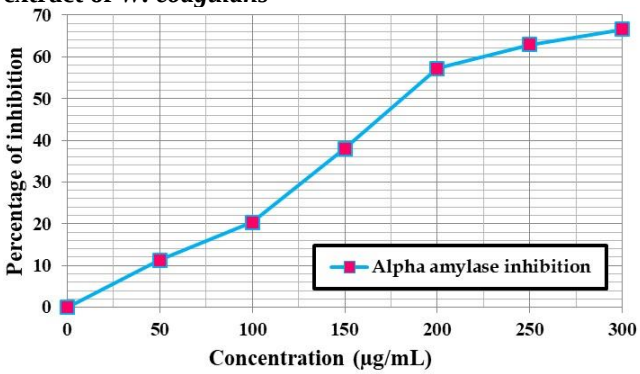


Fig 3. DPPH˙ radical and Superoxide ($O_2^{\cdot-}$) radical scavenging activities of methanol dried fruit extract of *W. coagulans*

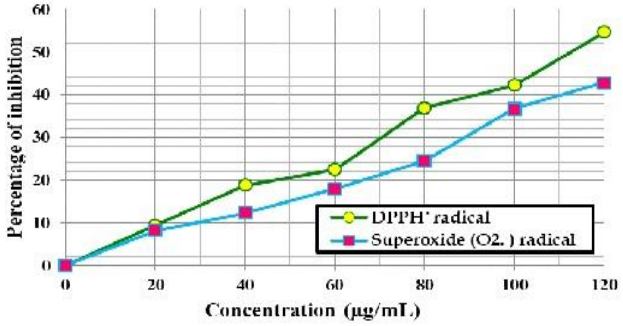


Fig 4. Phosphomolybdenum and Fe^{3+} reduction activities of methanol dried fruit extract of *W. coagulans*

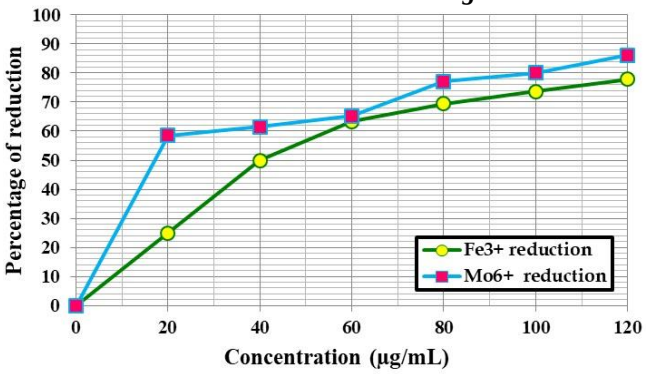


Fig 5. Separation of active compounds by Thin Layer Chromatography

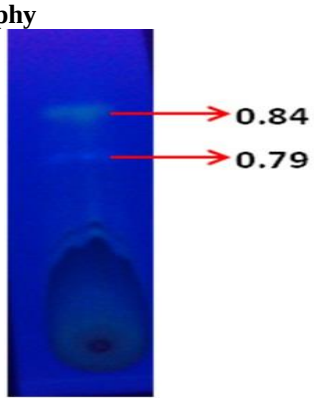


Fig 6. Antibacterial activity of methanol dried fruit extract of *W. coagulans*

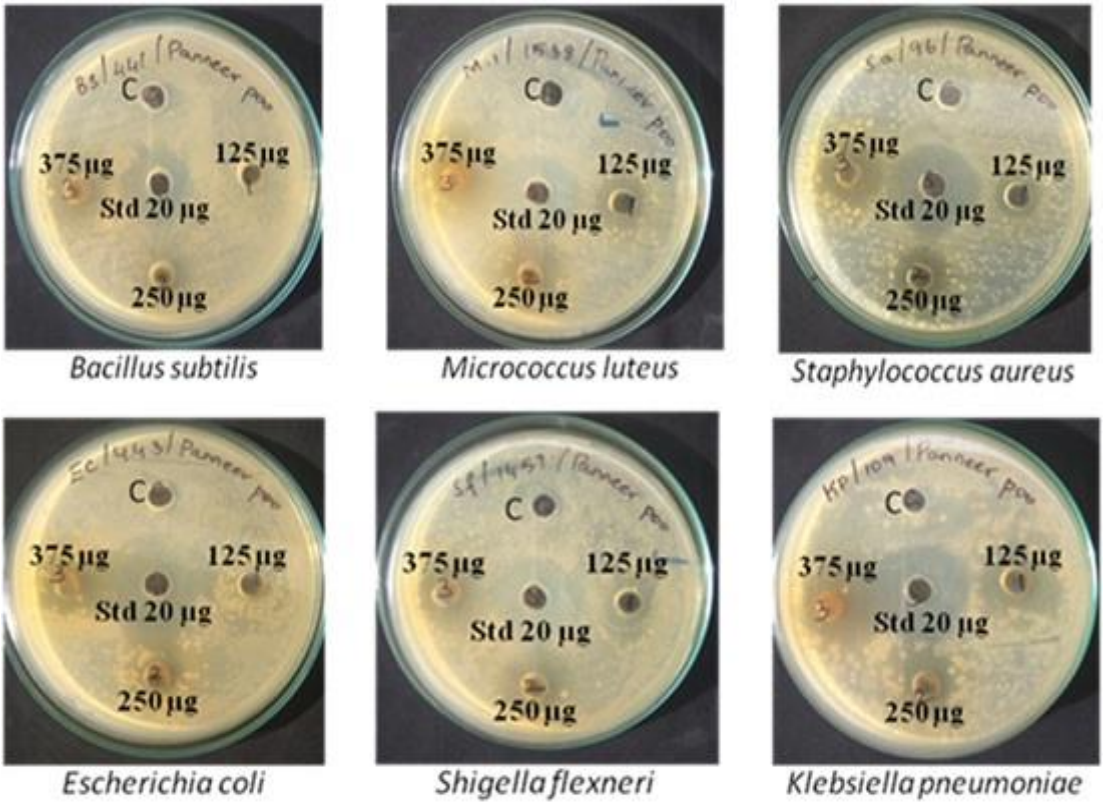
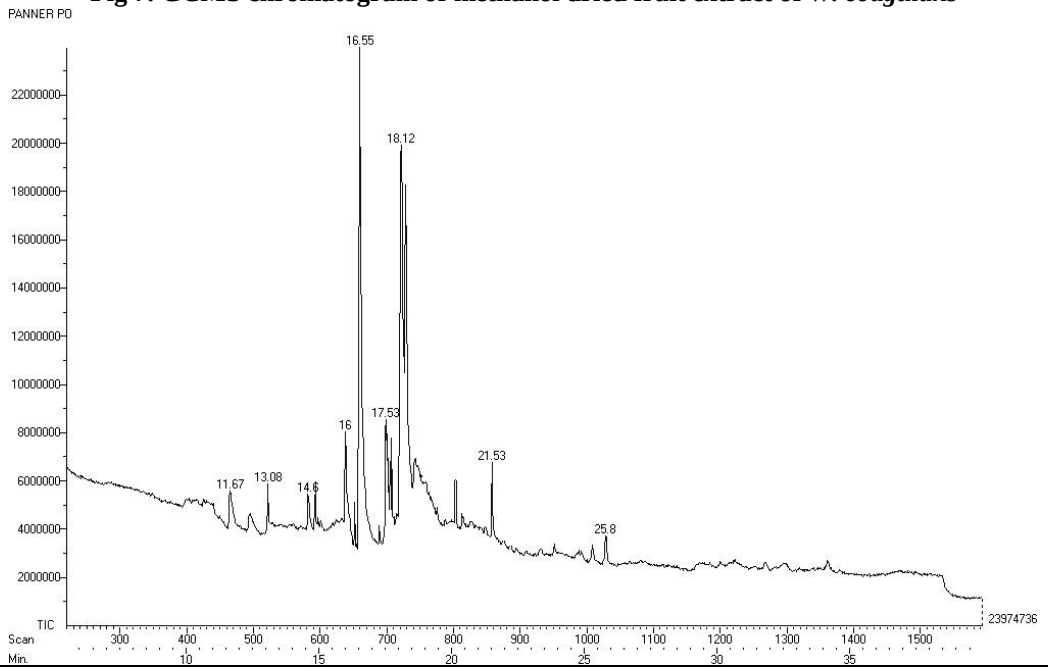


Fig 7. GCMS chromatogram of methanol dried fruit extract of *W. coagulans*



CONCLUSION

The results of the present study indicate that the methanol dried fruit extract of *W. coagulans*has significant antioxidant activities to reduce harmful effect of radicals and antidiabetic activities to reduce the blood glucose. Further molecular studies are required to find out the mechanism of action of bioactive compounds present in *W. coagulans* before it can be recommended for any practical widespread use of the plant. The results obtained in the present study provide promising guideline regarding the potential uses of *W. coagulans*as an antioxidant agent. An optimized antidiabetic screening method is of in very need for the discovery of potent herbal based antidiabetic drugs. Moreover, the mode of action and mechanism of the isolated active molecules should be studied in vivo to prove the stability and safety of the drug when being converted as capsules, tablets, etc.

ACKNOWLEDGEMENT

The authors are thankful to Armats Biotek Training and Research Institute for providing facilities to carry out research work.

CONFLICT OF INTEREST: None declared

REFERENCES

Amit V, Singh OP, Tiwari SK. *Withaniacoagulans* – An overview with special reference to Diabetes mellitus. *Indian journal of research*, 7, 2013, 1-6.

Awika M, Rooney LW, Wu X, Prior RL. *Cisneros Zevallos* L. Screening methods to measure antioxidant activity of Sorghum (*Sorghum ialmatei*) and Sorghum product. *Journal of Agricultural and Food Chemistry*, 51, 2003, 6657-6662.

Bastaki A. Diabetes mellitus and its treatment,*Int J Diabetes Metabolism*, 13, 2005, 111-134.

Biradar RS, Rachetti DB. Extraction of some secondary metabolites & thin layer chromatography from different parts of *Centellaasiatica* L. *American Journal of Life Sciences*, 1(6), 2013, 243-247.

Bown D. Encyclopedia of Herbs and their uses, Dorling Kindersley, London, 1995, 500.

Cotelle N. Role of flavonoids in oxidative stress. *Curr. Topics Med. Chem*, 1, 2001, 569-590.

DeFronzo RA, Pharmacologic therapy for type 2 diabetes mellitus, *Anna Inter Med*, 131, 1999, 281-303.

Dr. Duke's: Phytochemical and Ethnobotanical Databases.

Dymock W, Waden CJH, Hopper D, PharmacographiaIndica', reprinted by institute of health and Tibbi research, Karachi, 1972, 306.

Elaiyaraja A, Chandramohan G, Comparative phytochemical profile of *Indoneesiellaechioides* (L.) Nees leaves using GC-MS, *Journal of Pharmacognosy and Phytochemistry*, 5(6), 2016, 158-171.

Fox C, Ramsoomair D, Carte C, Magnesium its proven and potential clinical significance, *Southern Med J*, 94(12), 2001, 1195-1201.

FratiaFratianni F, Tucci M, De-Palma M, Pepe R, Nazzaro F, Polyphenolic composition in different parts of some cultivars of globe artichoke (*Cynaracardunculus* L. var. scolymus (L.) Fiori), *Food Chem*, 104, 2007, 1282–1286.

Harborne JB, Phytochemical Methods, Chapman and Hall Ltd., London, 1973, 49- 188.

Hema R, Kumaravel S, Alagusundaram J. *Am. Sci*, 7 (27), 2011.

Hossan SJ, El-Sayed M, Aoshima H. Antioxidative and anti α-amylase activities of four wild plants consumed by nomads in Egypt, *Orient Pharm Exp Med*, 9(3), 2009, 217-224.

Khalaf NA, Shakya AK, Al-othman A, El-agbar Z, Farah H, Antioxidant activity of some common plant, *Turk J Biol*, 32, 2008, 51-55.

Krishnamurthi A. The Wealth of India, Vol VIII, Publication Information Directorate, CSIR, New Delhi, 1969, 582.

Kubo I, Muroi H, Himejima M. Antimicrobial activity of green tea flavour components and their combination effects. *Journal of Agric Food Chem*, 40, 2002, 245-248.

Lebovitz HE. Alpha-glucosidase inhibitors.*EndocrinolMetabolClin North Amer*, 26, 1997, 539-551.

Liu X, Dong M, Chen X, Jiang M, Lv X, Yan G. Antioxidant activity and phenolics of endophytic*Xylaria sp.* from *Ginkgo biloba. Food Chemistry*,105, 2007, 548-554.

Lokesh Deb SK, Dubey, Avijeet Jain, Amit Kumar Jain, Pandian GS. Free radical scavenging activity of aqueous n- butanol fraction of *PrunusPersica* aqueous extract. *Journal of Natural Remedies*, 9(2), 2009, 152-158.

Lopez-Ridaura R, Willett WC, Rim EB, Liu S, Stampfer MJ, Manson JE, Hu FB. Magnesium intake and risk of type 2 diabetes in men and women. *Diabetes Care*, 27(1), 2004, 134-140.

Lukyanova LD, Storozheva ZI, Proshin AT. Corrective effect of flavonoid containing preparation extralife on the development of Parkinson's syndrome. *Bull. Exp. Biol. Med*, 144, 2007, 42-45.

Marjorie C. Plant Products as Antimicrobial Agents. *Clinical Microbiology Reviews*, 12, 1999, 564-582.

Maurya J, Akanksha J. Chemistry and pharmacology of *Withaniacoagulans*: an Ayurvedic remedy, *PharmaPharmacol*, 62, 2010, 153-160. Oyaizu M. Studies on products of browning reaction: antioxidative activities of products of browning reaction prepared from glucosamine. *Jpn. J. Nutr*, 44, 1986, 307-315.

Peters AL, Davidson MB, Schriger DL. A clinical approach for the diagnosis of diabetes mellitus. *J Am Med Ass*, 276, 1996, 1246-1252.

Prieto P, Pineda M, Aguilar M. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. *Analytical Biochemistry*, 269, 1999, 337-341.

Prior RL, Wu X, Schaich K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal Agriculture and Food Chemistry*, 55, 2005, 2698A-J.

Raaman N. Phytochemical techniques. New India Publishing Agency, New Delhi: 2006, 306.

Raquel F. Epand, Bacterial lipid composition and the antimicrobial efficacy of cationic steroid compounds. *BiochimicaetBiophysicaActa*, 2007, 2500–2509.

Rice-Evans CA, Miller NJ, Paganga G. Structure antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic. Biol. Med*, 1996, 20: 933-956.

Saraswathi K, Sivaraj C, Arumugam P. Antioxidant Activities, Thin Layer Chromatographic Analysis and GCMS Analysis of *Capsicum annuum* L.: A Comparison of Green and Red Chilli. *J. Biol. Chem. Research*, 36(1), 2019, 184-197.

Shimada T. Salivary proteins as a defense against dietary tannins. *J. Chem. Ecol*, 32(6), 2006, 1149-1163.

Spanos GA, Wrosltad RE. Influence of processing and storage on the phenolic composition of Thompson seedless grape juice, *Journal of Agricultural & Food Chemistry*, 38, 1990, 1565-1571.

Stadtman ER. Metal ion-catalyzed oxidation of proteins: Biochemical mechanism and biological consequences. *Free Radical Biology and Medicine*, 9, 1990, 315-325.

Stahl E. Thin Layer Chromatography, 2nd ed., Springer Pvt. Ltd., New Delhi, 2005, 85.

Syeda FA, Habib-ur-Rahman AM Khan, Choudahry MI, Atta-Ur-Rahman. *Inter. J. Genetics Mol. Biol*, 3(95), 2011.

Talukdar AD, Choudhury MD, Chakraborty M, Dutta BK, Phytochemical screening and TLC profiling of plant extracts of *Cyatheagigantea* (Wall. Ex. Hook.) Haltt. and*Cyatheabrunoniana*. Wall. ex. Hook. (Cl. &Bak.), *Assam University Journal of Science & Technology: Biological and Environmental Sciences*, 2010, 5(1): 70-74.

Trease GE, Evans WC. Textbook of Pharmacognosy, 12th ed., BallieseTindall and Company Publisher, London, 1983, 343-383.

Veiga S, Leonelli E, Beelke M, Gareia-Segura VM, Melcangi RC. Neuroactive steroids prevent peripheral myelin alterations induced by diabetes.*NeurosciLett*, 402(1- 2), 2006, 150-53.

Wickens AP, Aging and the free radical theory. *Respiratory Physiology*, 128, 2001, 379-391.

Yildirim A, Mavi A, Kara AA, Determination of antioxidant and antimicrobial activities of *Rumexcrispus* L. extracts. *J. Agric. Food Chem*, 49, 2001, 4083-4089.

Zablotowicz RM, Hoagland RE, Wagner SC, Effect of saponins on the growth and activity of rhizosphere bacteria. *AdvExp Med Biol*, 405, 1996, 83-95.